Dynamic regulatory interaction between cytomegalovirus major tegument protein pp65 and protein kinase pUL97 in intracellular compartments, dense bodies and virions

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Abstract

Human cytomegalovirus (HCMV), a ubiquitous pathogen of considerable clinical importance. Understanding the processes that are important for viral replication is essential for the development of therapeutic strategies against HCMV infection. The HCMV-encoded protein kinase pUL97 is an important multifunctional regulator of viral replication. Several viral and cellular proteins are phosphorylated by pUL97. The phosphoprotein pp65 is one important substrate of pUL97. It is the most abundant tegument protein of HCMV virions, mediating the upload of other virion constituents and contributing to particle integrity. Further to that, it interferes with host innate immune defences, thereby enabling efficient viral replication. By applying different approaches, we characterized the pp65–pUL97 interaction in various compartments. Specifically, the pUL97 interaction domain of pp65 was defined (282–415). A putative cyclin bridge that enhances pUL97–pp65 interaction was identified. The impact of pUL97 mutation on virion and dense body morphogenesis was addressed using pUL97 mutant viruses. Alterations in the proteome of viral particles were seen, especially with mutant viruses expressing cytoplasmic variants of pUL97. On the basis of these data we postulate a so far poorly recognized functional relationship between pp65 and pUL97, and present a refined model of pp65–pUL97 interaction.

INTRODUCTION

Human cytomegalovirus (HCMV), a member of the beta-herpesvirinae, is a pathogenic agent of considerable clinical importance [1]. The question of how viral proteins interact with each other in order to provide optimal conditions for genome replication, capsid assembly, nucleo-cytoplasmic trafficking, tegumentation and envelopment is a major subject of recent research in the field. Viral capsid assembly and genome packaging is accomplished in the nucleus of infected cells [2]. The nucleo-cytoplasmic exit of capsids is mediated by their interaction with the nuclear egress complex (NEC), followed by an envelopment/de-envelopment process through the perinuclear space [3–6]. The question of whether some of the tegument or other regulatory proteins are already attached to the capsid surface prior to nuclear egress remains controversial at this point [7]. Following release into the cytoplasm, the capsids are directed to specialized perinuclear sites, also termed cytoplasmic viral assembly compartments (cVACs), which contain reorganized cellular membranes [8]. Tegumentation and envelopment occurs at these sites [9–11]. Subviral dense bodies (DBs) are also formed and enveloped in close proximity to cVACs [11]. Several viral proteins have been identified that are targeted to cVACs [8, 12]. Although there is evidence

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Abbreviations: AIDA, advanced image data analyser; ARPE-19, retinal pigment epithelial cells; CDKs, cyclin-dependent protein kinases; CoIP, coimmunoprecipitation; C-terminus, carboxyterminus; cVACs, cytoplasmic viral assembly compartments; DAPI, 4’,6-Diamidin-2-phenylindol; DBs, dense bodies; 3D, three-dimensional; ER, endoplasmic reticulum; GmbH, Gesellschaft mit beschränkter Haftung; HA, haemagglutinin; HCMV, human cytomegalovirus; HFF, human foreskin fibroblasts; HRP, horseradish peroxidase; H2O, water; IFI16, interferon-inducible protein 16; IVKA, in vitro kinase assay; LMB, leptomycin B; mAb, monoclonal antibody; MBV, maribavir; MCP, major capsid protein; NLS, nuclear localization signal; N-terminus, aminoterminus; pAb, polyclonal antibody; p.i., post-infection; PMSF, phenylmethylsulfonylfluorid; Pp65, phosphoprotein 65; p.p.m., parts per million; RB, retinoblastoma-associated protein; r.p.m., rounds per minute.

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One supplementary table and six supplementary figures are available with the online Supplementary Material.
that certain tegument proteins, such as pUL71, pUL94 and pUL99, are instrumental in the envelopment of HCMV virions, the mechanisms through which they participate in this process remain enigmatic [10, 13–15].

Viral enzymes are of particular interest for understanding the regulation of viral replication and morphogenesis, as they may interact at multiple levels with the viral replication cycle. Herpesviral protein kinases act as regulatory factors of virus-host interaction and display crucial functions during HCMV replication [16, 17]. The HCMV-encoded kinase pUL97 has been characterized as a viral orthologue of cellular cyclin-dependent protein kinases (CDKs [18]). This nuclear serine/threonine-type protein kinase is a multi-functional determinant of the efficiency of viral replication [16, 19–23]. pUL97 activity is directed to a number of viral and cellular phosphorylated substrate proteins (reviewed in [24]) and is required for efficient viral replication. The kinase exerts multiple functions at early and late stages of viral replication, such as viral DNA synthesis, transcription and, in particular, nucleo-cytoplasmic capsid egress [4, 7, 25]. For this reason, deletion of the ORF UL197 from the viral genome or pharmacological inhibition of pUL97 activity, albeit not fully incompatible with viral replication, results in a rigorous reduction of viral replication efficiency [20, 26–28]. Inhibitors targeting pUL97, such as maribavir (MBV, benzimidazole riboside), G06976 (indolocarbazole) or Axs396, VI7392 and VI7435 (quinazolines) showed strong antiviral activities both in vitro and in vivo [24, 29, 30]. MBV has been investigated in a number of phase III clinical trials with unfavourable outcomes, but such trials have recently been reinitiated using different dosing regimens (Shire Pharmaceuticals, UK).

The phosphoprotein 65 (pp65; pUL83) is the most abundant component of the HCMV virion tegument [31–33]. Although it is non-essential for viral replication in fibroblast cultures [34], its removal may impair replication in monocyte-derived macrophages [35]. Several functions have been assigned to pp65, including interference with the innate and adaptive immune defence mechanisms of the host [36–39], although discussions concerning some of these results have been controversial. It has also been suggested that pp65 may play a role in viral gene expression, and this may be mediated by virtue of the interaction of the tegument protein with the cellular restriction factor interferon-inducible protein 16 (IFN-16 [40, 41]). pp65 has also been shown to interact with several other viral proteins, including pUL97, pUL69, pUL84 [35, 42, 43] and pUL44 (Plachter et al., unpublished), while it may also interact with itself, leading to multimerization [44]. The interaction of pp65 with pUL97 appears to be of particular interest. Both proteins are integral components of the viral tegument. pp65 is a substrate of pUL97-mediated phosphorylation [20, 42, 45, 46]. Packaging of pp65 is impaired in the absence of pp65 [35, 47]. In turn, lack of pUL97 kinase activity results in aberrant accumulation of pp65 in large structures within infected cells, which also contain other viral proteins [48]. We were recently able to show that pUL97 inhibition leads to nuclear retention of pp65 [45], apparently disturbing its nucleo-cytoplasmic shuttling activity [49]. Interestingly, using a 9 amino acid insertion mutant of pp65 in a recombinant virus, we were also able to detect aberrant large globular structures in the nucleus of infected fibroblasts [45]. As with pUL97 inhibition, this pp65 mutant showed severe defects in the production of infectious virus, although viral genome replication remained unaffected. Taken together, these results led us to hypothesize that pp65–pUL97 interaction is a crucial step in the HCMV replication cycle. Disturbing this interaction may lead to a deleterious dysregulation of the viral protein interactome, resulting in severe impairment of viral productivity.

**RESULTS**

**Intracellular localization of pp65, pUL97 and pUL44 in the course of HCMV infection**

To approach the functional role of pUL97–pp65 interaction, the subcellular distribution of the two proteins in the course of HCMV infection was addressed in a first set of experiments. For a control, the localization of pUL44 (the processivity factor of the viral DNA polymerase) was assessed in parallel, since both pUL44 and pp65 represent phosphorylated substrates of pUL97 [50]. HFFs were infected with HCMV AD169-GFP at an m.o.i. of 1.0, fixed at 24, 48, 72 as well as 120 h post-infection (p.i.), and used for indirect immunofluorescence analyses (Fig. 1). At 24 h p.i., pp65 was localized exclusively in the nucleus. The protein was evenly distributed, lacking any notable pattern of sublocalization (Fig. 1a; panels 2, 7, 12). At 48 h p.i., pp65 was still mostly nuclear, with some additional staining in the cytoplasm (Fig. 1a, panels 3, 8, 13). From 72 h p.i. to later time points, pp65 was mainly found in the cytoplasm, partly localized in the cVACs (indicated by white arrows, Fig. 1a, panels 4, 5, 9, 10, 14, 15), as also reported by Sanchez and coworkers [8]. Staining for pUL97 displayed dot-like structures at 24 h p.i. (Fig. 1b, panels 17, 22, 27). At 48 h p.i., pUL97 was found in larger subnuclear structures, known to comprise viral replication compartments (Fig. 1b, panels 18, 23, 28). The nature of these replication compartments was verified by staining for pUL44, which is a component of the viral polymerase complex (Fig. 1c, panels 33, 38, 43; see also [51]). From 72 h p.i., localization of pUL97 became increasingly diffuse within the nucleus (Fig. 1b, panels 19, 24, 29). At 120 h p.i., a faint cytoplasmic staining became detectable (Fig. 1b, panels 20, 25, 30). pUL44 showed an initial, largely diffuse, intranuclear distribution at 24 h p.i. (Fig. 1c, panels 32, 37, 42), followed by a gradual targeting of the protein to viral replication compartments at 48 h p.i. (Fig. 1c, panels 33, 38, 43). At 72 h p.i., the replication compartments covered almost the entire nucleus, whereas pUL44 was mainly localized at the rim of these structures and in additional dot-like structures within the nucleus (Fig. 1c, panels 34, 39, 44). Such structures were most prominent at 120 h p.i. (Fig. 1c, panels 35, 40, 45). The results showed that both pp65 and pUL97 undergo considerable changes in their...
subcellular distribution as the viral replication cycle progresses. They also indicated that remarkably little colocalization between the two proteins was detectable, which was surprising in view of the efficiency with which they interact with each other on the biochemical level. This finding was confirmed when we addressed this question in detail by performing costaining of pp65 with pUL97 at the late time point of 72 h p.i. (Fig. 1d). Although various kinds of localization could be detected for pp65 in individual HCMV-positive cells, very little or no marked colocalization with pUL97 was noted (i.e. patterns of pp65 localization were labelled in the respective insets with 1, 2 and 3). These signal patterns, obtained in cells infected with HCMV strain AD169, were very similar to those obtained with strain TB40 (Fig. S1, available in the online Supplementary Material). Here, an additional evaluation of putative colocalization was performed by recording a series of Z-stacks, which were finally overlaid to a maximum projection (Fig. S1, right panels, Z-stack). Further, in this evaluation, only a minor fraction of yellow dot-structured signals in the cytoplasm provided an indication of pp65–pUL97 colocalization. The unexpected finding that the intracellular localization behaviours of the two interacting proteins differed substantially might be due to the fact that both pp65 and pUL97 undergo several different protein interactions and are able to engage in a number of activities linked with somewhat different sites of action. Thus, they do not show a single site of specific colocalization. This issue was additionally illustrated by an experiment, in which the nuclear export was blocked by treatment with leptomycin B (LMB). Under these conditions, when nucleo-cytoplasmic export of pp65 was blocked and both proteins were retained in the nucleus, even during late times of HCMV infection, confocal immunofluorescence imaging was performed using HFFs or epithelial ARPE-19 cells infected with HCMV strains AD169 (Fig. S2a) or TB40 (Fig. S2b), respectively. Interestingly, pp65 and pUL97, which showed a localization that was entirely restricted to the nucleus under LMB blocking, again did not express specific fine structures of colocalization, but were either smoothly localized over the nucleoplasm (pUL97) or accumulated along the rim of the nuclear envelope (pp65) (a feature that may correspond to our previous report on an association of pp65 with the viral nuclear egress complex [4]). Some yellow-stained merge signals of colocalization could be detected at the nuclear rim, but only to a very limited extent (Fig. S2a, panel 12, inset 3). A very similar result was obtained in the parallel setting using HCMV strain TB40 (Fig. S2b, panel 27, inset 6). Notably, three different patterns of intracellular localization were recorded for the two proteins under the conditions with or without LMB (solvent control MeOH), i.e. localization in nuclear areas (insets 3 and 6), in the cVAC (insets 2 and 5) or in cytoplasmic fine-speckled aggregates (insets 1 and 4). While only a very limited degree of colocalization between pp65 and pUL97 was noted in general, a more pronounced degree of colocalization was found in the third pattern, i.e. in the cytoplasmic speckles (Fig. S2a, panels 5–8, inset 1, and Fig. S2b, panels 20–23, inset 4). This finding underlines our statement that the two viral proteins do not show a marked colocalization, possibly as a result of the dynamic, short-lived characteristics of pp65–pUL97 interaction.

![Fig. 1. Subcellular localization of pp65, pUL97 and pUL44 in the course of HCMV infection. HFFs were infected with HCMV AD169-GFP (m.o.i.=1.0), fixed at indicated time points (24, 48, 72 and 120 h p.i.) and used for indirect immunofluorescence analysis. Viral proteins were immunostained with mAb-pp65, pAb-UL97 or mAb-UL44 and are shown in red. Cell nuclei were counterstained with DAPI as shown in blue. (a) Localization of pp65; cVACs are indicated by white arrows (panels 9, 10). (b) Localization of pUL97. (c) Localization of pUL44. (d) Costaining of pUL97 and pp65 at 72 h p.i. (see the three different patterns of pp65 localization, labelled 1, 2 and 3, in the enlarged insets).](image-url)
Fig. 2. Determination of the pUL97 interaction site in pp65 and confirmation of pp65 phosphorylation by pUL97 using Phos-taglabeling. (a) Expression constructs for full-length and truncated versions of pp65. Truncations of pp65 were generated by shortening the ORF of pp65 in quarters from the C- and N-termini, respectively. The expressed amino acid regions are indicated in parentheses. All proteins were fused to a C-terminal HA tag. The full-length pp65 is illustrated on top. The putative pUL97 interaction site, as identified by the results presented below, is highlighted in dark blue. Note that the constructs that proved to be expressed in valuable quantities are indicated by framed bars, while those expressed poorly are unframed. (b) Phos-tag labeling used to demonstrate the phosphorylation of pp65 by pUL97. 293 T cells were cotransfected with wild-type pp65-HA and pUL97-FLAG or the kinase-inactive mutant pUL97 (K355M)-FLAG, lysed 3 days post-transfection (p.t.) and used for Phos-tag analysis. The Phos-tag gel contained 6% acrylamide, 10 µM Phos-tag and 20 µM MnCl₂. Cotransfection of pp65 with an empty vector (lane 1) or the kinase-inactive pUL97 mutant (pUL97(K355M)-
Identification of the sequential domain in pp65 responsible for pUL97 interaction

The interaction of pp65 with pUL97 has been described before [42, 45]. In order to delimit the amino acid region of pp65 responsible for the interaction with pUL97, serial truncations of pp65 were generated (Fig. 2a) and used in coimmunoprecipitation (CoIP) experiments with pUL97. 293 T cells were cotransfected with expression plasmids for pUL97 and truncated versions of pp65. Cells were lysed and subjected to CoIP analysis (Fig. 2c). Precipitation of full-length pp65 [pp65(1–561)] with a tag-specific antibody resulted in coprecipitation of pUL97, confirming previous results (Fig. 2c, lane 2). The expression controls illustrated the reliable expression of most pp65 truncations in comparable amounts [only pp65(1–281)-HA, pp65(1–141)-HA and pp65(142–281)-HA were barely detectable, so these constructs were not included in further analysis]. All of the expressed versions were immunoprecipitated with the HA-specific antibody, as expected (precipitation controls). Three truncations, namely pp65(142–561)-HA, pp65(282–561)-HA and pp65(282–415)-HA, communoprecipitated pUL97-FLAG (Fig. 2c, lanes 10–11; Fig. 2d, lanes 10–11, 14). These constructs share amino acids 282–415 of pp65. Thus the results show that the region spanning amino acids 282–415 is necessary and sufficient to mediate pp65–pUL97 interaction. It should be stressed, however, that this does not exclude the possibility of additional indirect bridging mechanisms, such as an indirect interaction between pp65 and pUL97 (especially bridged via cellular cyclins, see below).

Confirmation of pp65 phosphorylation by pUL97 using Phos-tag labelling

The major tegument protein pp65 is one of the most abundant substrates of pUL97, as shown by in vitro kinase assays (IVKAs) [20, 45]. Instead of using a radioactive isotope of phosphate ($^{33}$P), we attempted to utilize the recently described non-radioactive Phos-tag method. This is a technique for differential electrophoretic separation of phosphorylated and non-phosphorylated proteins. To confirm the phosphorylation of pp65 by pUL97 using this technique, 293 T cells were cotransfected with expression constructs for wild-type pp65-HA and pUL97-FLAG or the catalytically inactive mutant pUL97(K355M)-FLAG (carrying an amino acid substitution of the essential lysine within the ATP-binding site). Cells were lysed 3 days after transfection and analysed by the Phos-tag method. The results demonstrate the pUL97-conferred phosphorylation of pp65 (Fig. 2b). The pp65-HA-specific band was split into two bands when wild-type pUL97-FLAG was present (Fig. 2b, lane 2). As phosphorylated proteins migrate more slowly in gels containing Phos-tag, the upper band is likely the phosphorylated variety of pp65-HA.

Impact of the mutation of predicted cyclin docking motifs of pp65 on interaction with pUL97

HCMV pUL97 is a CDK orthologue that associates with cellular cyclins [18, 52–54]. A sequence analysis of pp65 suggested the presence of three putative cyclin docking motifs spanning residues 119–123, 140–144 and 494–498, respectively. A possible cyclin-bridged interaction between pUL97 and pp65 was further investigated by substituting two amino acids within each of the three predicted cyclin docking motifs using site-directed mutagenesis. 293 T cells were cotransfected with mutated pp65-HA as well as wild-type pUL97-FLAG and used for CoIP analysis (Fig. 3a). In order to compare the communoprecipitated amounts of pUL97-FLAG, the band intensities were quantified with an advanced image data analyser (AIDA; Fig. 3b). The mutation of the two predicted cyclin docking motifs in the first half of pp65 had no measurable impact on the coimmunoprecipitation of pUL97-FLAG (Fig. 3, iii and iv), whereas the mutation of the cyclin docking motif in the C-terminal region (amino acids 494–498 RNLVP) showed a severe effect (Fig. 3, v). The substitution of Asn495 and Leu496 with Ala led to an almost sevenfold decrease in the quantity of communoprecipitated pUL97-FLAG. These results suggest that cyclins may indirectly reinforce or stabilize the pUL97–pp65 interaction. A schematic model of the respective ternary complexes is shown in Fig. S4 (please refer to the Methods section for details of the modelling procedure).

A pattern search for cyclin-binding motifs in known pUL97 substrates revealed a large number of candidate interaction sites (Table S1). However, it has to be kept in mind that the [RK]-x-1-x(0,1)-[FYLIVMP] pattern should be considered as fuzzy and thus may likely produce a significant portion of false positive hits (without conferring a biologically relevant activity in cyclin binding). For this reason, it appears to be...
necessary to include additional criteria to identify those motifs, which have a high probability of being functional, such as their evolutionary conservation and their location in non-globular, disordered parts of the protein. As an example, the KKLRF motif of the retinoblastoma-associated protein (Rb), which has been experimentally proven to be functional [55], is characterized by a high conservation score, combined with its localization outside of globular domains. The existence of functional motifs in pp65 and the Rb protein suggest that at least some of the pUL97 substrate proteins might use cyclin-docking motifs as a concept for protein interaction. The computational predictions of conservation and globularity are compiled in Table S1 as a basis for future experimental assessment of their biological functionality.

**Protein levels of pUL97 in virions and DBs of different HCMV strains and pUL97 mutants**

The viral kinase pUL97 is uploaded into the virion tegument and into DBs during morphogenesis [32, 33]. Deletion of the pp65 gene UL83 results in a subtle, but reproducible, reduction of pUL97 packaging [33, 47]. To test whether there were differences in the amount of pUL97 in virions and DBs of different HCMV strains, label-free quantitative mass spectrometry was performed [33, 56]. Purified virions and DBs of four different strains were analysed. The relative quantitative values (parts per million, p.p.m.) of pUL97 were compared (Fig. 4a). Statistical analysis revealed that most differences were of only moderate significance. Only the virions and DBs of HCMV R5 appeared to contain more pUL97, compared to the particles of the other viruses. In a second round of analyses, the impact of pUL97 mutation or truncation on its upload into viral particles was tested using individual mutant viruses (Fig. 4b). Mutant UL97(157-707) expressed the smallest pUL97 isoform [20], whereas mutant UL97(DNLS1/DNLS2) expressed a pUL97 isoform that shows cytoplasmic retention [20, 57, 58]. Virions of the parental strain HB15 contained significantly lower amounts of pUL97, compared to the particles of the other viruses. In a second round of analyses, the impact of pUL97 mutation or truncation on its upload into viral particles was tested using individual mutant viruses (Fig. 4b).
Abrogation of the catalytic activity of pUL97 leads to alterations in the outer tegument of HCMV virions

pp65 and other tegument and tegument-associated proteins are phosphorylated by pUL97 [33]. Although it has not been proven at this point, the level of tegument protein phosphorylation is likely involved in the regulation of HCMV particle assembly. We thus addressed the question of whether the abrogation of pUL97 kinase activity had an impact on the composition of the tegument. Quantitative mass spectrometry was performed on purified virions of the mutant UL97(DK355). This virus expresses a kinase-negative version of pUL97 [20, 59]. DBs could not be analysed, since the infection of HFFs with UL97(DK355) does not result in the release of detectable amounts of these subviral particles (data not shown). The relative amounts (p.p.m.) of outer tegument proteins in UL97(ΔK355) virions were compared to the results obtained using purified UL97(Mx4)-F virions. This recombinant virus expresses the largest pUL97 isoform. The results were normalized to the amount of the major capsid protein (MCP), set as 100% (Fig. 5). Statistical analysis revealed that pUL25, pp65, pUL35 and pUL45 were detected in larger amounts in the kinase-negative mutant. It remains unclear at this point whether this was due to the reduced packaging of other tegument proteins, or if pUL97 kinase activity restricted the upload of these proteins into virions. Protein kinase activity appeared to be dispensable for pUL97 upload, since the protein lacking kinase activity was packaged in similar amounts compared to the kinase-positive variant of UL97(Mx4)-F. Taken together, these data...
show that the loss of pUL97 kinase activity leads to subtle alterations in the composition of the outer tegument of HCMV virions.

**Cytoplasmic retention of pUL97 results in subtle alterations in the outer virion tegument**

Immunofluorescence analyses revealed that pUL97 was primarily located in the nucleus of infected cells at late times of infection, a time point at which virion morphogenesis is orchestrated in the cytoplasm. This was also confirmed in fractionation experiments, in which pUL97 was found to be primarily localized in the nucleus (Fig. S5). We still observed some colocalization of pUL97 and pp65 in dot-like structures in the cytoplasm at late times post-infection (see Fig. S1). To address the role of cytoplasmic pUL97 in HCMV morphogenesis, we investigated whether cytoplasmic accumulation of pUL97 affects the virion proteome. To address this, mass spectrometry was performed on virions of the mutants UL97(Mx4)-F and UL97ΔK355. Displayed are the data (p.p.m. values; means of five technical replicates) normalized to the amount of the major capsid protein MCP, set as 100 %. ANOVA analysis: *, P-value < 1×10^-3; **, P-value < 1×10^-4; ***, P-value < 1×10^-5. The bars represent deviations of the mean. Note the different scales in (a) and (b).

**DISCUSSION**

The interaction between pp65 and pUL97 has been known for many years [42, 45]. IVKAs in addition revealed that pp65 is one of the most abundant substrates of pUL97 [20, 45]. Here we started to approach the functional role of pp65–pUL97 interaction by studying the subcellular trafficking of both proteins, by defining the interaction domains of pp65 and by investigating the role of pUL97 in the packaging of pp65 and other viral proteins into particles. Based on the findings, we state the following: (i) pp65 and pUL97 transiently colocalize in intracellular compartments; (ii) a strongly detectable and tight mode of pp65–pUL97 interaction and pp65 phosphorylation through pUL97 has been verified using several different approaches; (iii) the pUL97 interaction region is determined by amino acids 282–415 of pp65; and (iv) the lack of...
pUL97 catalytic activity or changes in the subcellular localization of the kinase have some impact on the composition of the outer virion tegument.

Both pp65 and pUL97 displayed a nuclear phenotype in the early stages of infection, with pp65 and a fraction of pUL97 being evenly distributed in the nucleoplasm. However, pUL97 also appeared to be targeted to subnuclear structures, representing viral replication compartments as the infection progressed. At late time points of infection, the localization of pp65 changed from the nuclear to the well-known cytoplasmic phenotype. pUL97 started to become more diffusely located in the nucleoplasm, with only a faint staining in the cytoplasm at the same time. Consequently, the localization of both pUL97 and pp65 appeared to be dynamic in the course of infection. However, a defined colocalization of the two proteins was found only rarely at various time points after infection. This argues in favour of a transient enzyme/substrate rather than a structural interaction of pUL97 with pp65.

The amino acid region of pp65 responsible for the interaction with pUL97 was narrowed down to amino acids 282–415 by CoIP experiments, using transiently expressed truncated versions of pp65. However, some quantitative variation was seen between individual constructs of pp65 capable of pUL97 interaction. Since the detailed structural properties of pp65 are still unknown, it is possible that the N-terminus was highly disordered in this approach and thereby interfered with the correct folding of the interaction site. This notion is supported by the finding that the expression levels of the remaining proteins that included the N-terminus (except the full-length protein) were low, due to a presumably rapid degradation by cellular proteases.

HCMV pUL97 is considered to be a CDK orthologue that interacts with cellular cyclins [18, 52, 53]. Since the interaction between CDKs and cyclins is known to potentially increase the affinity to substrates [60], the possibility of a cyclin-bridged interaction was addressed. Based on a pattern-based search, three putative cyclin docking motifs within the amino acid sequence of pp65 were identified. The substitution of two amino acids within the cyclin docking motif in the C-terminal region (amino acids 494–498) resulted in a drastically reduced amount of coimmunoprecipitated pUL97. Interestingly, this motif was not located within the interaction site of pp65. This may indicate that the interaction between pp65 and pUL97 is indirectly reinforced or stabilized by cyclins. This effect and the interaction mediated by the determined interaction site may both contribute to an efficient interaction with pUL97. Nevertheless, further experiments are necessary to analyse the impact of cellular cyclins on the interaction between pp65 and pUL97 in functional terms. Interestingly, an additional CoIP experiment, performed under conditions of cyclin depletion prior to pp65–pUL97 CoIP, provided further indirect evidence for the potential supportive role of cyclins in the pUL97/substrate interaction. In this setting, the depletion of cyclins H, B1 and T1 was achieved by immunoprecipitation-based cyclin clearance using total lysates from HCMV AD169-infected HFFs (Western blot control stainings confirmed a very efficient depletion of the three cyclins, in the absence of a major effect on pp65 levels). Notably, when these cyclin-depleted protein lysates were used for CoIP of the pp65–pUL97 complex, using mAb-UL97 as the CoIP antibody, the levels of coimmunoprecipitated pp65 were reduced to 78% compared to 100% of pp65 in parallel samples without cyclin depletion (data not shown; densitometric quantitation was normalized to immunoprecipitated pUL97). This preliminary finding underlines our statement that the pUL97-interacting cyclins may serve as a bridge for an intensified binding of pp65 to pUL97, a bridging mechanism that may possibly also include further pUL97 interactors, in particular its phosphorylated substrates.
The presence of protein kinase activity in the different forms of HCMV particles was first described a long time ago [61, 62]. Later, pUL97 was identified as the viral kinase associated with virions [63]. The relevance of pUL97 kinase packaging into virions remains enigmatic at this point. This packaging, however, appears to be at least in part dependent on pp65. Deletion of the pp65 gene from the HCMV genome results in a reduced in vitro kinase activity of particle preparations and in a subtle impairment of pUL97 upload [33–35]. In this work, we found that cytoplasmic retention of pUL97 supports pp65 packaging into virions (Fig. 6), which occurs in cVACs. Thus there appears to be an intricate interaction between pUL97 and pp65 in the upload of the two proteins into virions, which depends on pUL97 kinase activity. It remains unclear at this point if pUL97-mediated phosphorylation of pp65 or of other substrates is relevant for this process.

A remarkable finding was that the loss of pUL97 kinase activity seemed to support the upload of some of the tegument proteins. The reason for this is unclear at this point, as it would suggest that pUL97-mediated phosphorylation was inhibitory to the upload of these proteins. The presence of phosphatases in the HCMV virion, as shown by our laboratory and others, may support this notion [33, 64]. Along these lines, recent work by others showed that HCMV tegument proteins may be less intensively phosphorylated than expected [65]. It is tempting to speculate that some of the tegument proteins may assemble into the virion tegument in a hypophosphorylated form, but need to be phosphorylated by pUL97 for disassembly following penetration. Further work on the targets of pUL97 phosphorylation and their role in HCMV virion assembly is required to investigate this in more detail.

The aberrantly enhanced cytoplasmic expression of pUL97 following UL97(ΔNLS1/ΔNLS2) infection led to increased packaging of pp65 into virions (see Fig. 6). This, together with the data obtained with the kinase-negative mutant (see Fig. 5), shows that cytoplasmic pUL97-kinase activity supports pp65 virion upload. Some of the other tegument proteins also appeared to be enhanced in UL97(ΔNLS1/ΔNLS2) virions. This is concordant with our previous finding that pUL25, pUL45 and pUL71 packaging in particular are linked to pp65 packaging [33]. A puzzling result, in this respect, however, was the fact that in the kinase-negative mutant pUL25 and pUL45 were enhanced in their packaging. A model would be that a balanced level of kinase and phosphatase activities in the cytoplasm is required for proper outer-tegument protein assembly. Cytoplasmic over-expression of pUL97 may affect that balance, leading to a disturbed composition of the outer tegument of the HCMV virion. Although this was not tested in this work, it may lead, amongst other consequences, to an alteration in particle stability, as we have seen for pp65-negative virions [33]. In contrast to this, the protein composition of DBs was only moderately dependent on the localization of pUL97 (Fig. S6). DB synthesis appears to proceed independently of virion morphogenesis, since substances that block viral genome packaging also block virion morphogenesis, while leaving DB morphogenesis unaffected [66]. The tight association with pUL97 suggests that both the morphological and functional activities of pp65 are regulated by the kinase. Cristea and coworkers reported a stimulatory effect of pp65 on HCMV immediate early gene expression, which was mediated via its interaction with the interferon-inducible protein 16 (IFI16, 40). The same authors showed that the interaction of pp65 blocked IFI16-mediated induction of antiviral cytokines in dependence on the phosphorylation of the tegument protein [39]. IFI16 also acts as a cellular restriction factor for HCMV replication by silencing viral early promoters [67]. Interestingly, the latter authors were able to show that IFI16 was exported to the cytoplasm at late times of HCMV infection, thereby counteracting IFI16-mediated nuclear silencing. This export function was coregulated by pUL97-mediated phosphorylation and a possible role for pp65 in that export was suggested [67]. In a later paper, it was reported that pp65-mediated recruitment of IFI16 to the early viral UL54 promotor mediated transcriptional suppression. All of these findings point towards pUL97-regulated essential functions of pp65 in subverting innate cellular defence mechanisms. However, a full understanding of how pp65 acts in this respect and what the role of pUL97 is in these processes has not been achieved. It is suggestive that the tight pp65–pUL97 interaction during various stages of viral replication is an essential determinant of viral pathogenesis. The results obtained in this study provide a promising basis on which to further elucidate the functional relevance of pUL97–pp65 interaction for HCMV morphogenesis and replication.

METHODS
Cells and viruses
The primary clinically relevant strains HCMV R1 and HCMV R5 were obtained from the Diagnostic Section (repositories for clinical specimens) of the Institute for Clinical and Molecular Virology, Friedrich-Alexander University of Erlangen-Nürnberg, Germany [20, 28]. These strains have lost their epithelial cell tropism due to passaging on fibroblast cultures. The TB40/E strain was originally isolated from a bone marrow transplant recipient and was subsequently cloned into a bacterial artificial chromosome vector, providing strain TB40-BAC4 after reconstitution [68, 69]. BADwt was obtained from Thomas Shenk (Princeton University, Princeton, NJ, USA) [70]. Towne-BAC was cloned by Marchini et al. [71] and was provided by Edward Mocarski (Emory University, Atlanta, GA, USA). Mutant HCMVs expressing kinase-negative, cytoplasmic dyslocalized variants or individual isoforms of pUL97 have been described elsewhere [20]. HB5 and HB15 are BAC-derived variants of the HCMV strain AD169 (provided by Martin Messerle and Eva Borst, Hannover [72]; Gabriele Hahn, Ingolstadt and Thomas Stamminger, Erlangen, Germany [73]). RVA-d65 is a pp65-deletion mutant [34]. HCMV AD169 is a
laboratory strain of HCMV that was originally isolated by Rowe and colleagues [74]. HCMV AD169-GFP is an AD169-derived recombinant virus containing an expression cassette for GFP within a non-essential stretch of the unique short (US) region of the viral genome [75]. All viruses were grown on human foreskin fibroblasts (HFFs) and infection experiments were performed with HFFs or ARPE-19 epithelial cells. Purification of virus particles from the culture supernatants of infected HFFs was performed using glycerol/tartrate gradient ultracentrifugation [31, 33]. The particles were collected after the final centrifugation step in 100–250 µl PBS. Protein concentrations were determined using the Pierce BCA protein assay kit (Thermo Scientific, Bonn, Germany). Twenty µg of virions and DBs was subsequently diluted to result in 50–80 µl aliquots and frozen at −80 °C until mass spectrometry was performed.

Generation of expression plasmids
Plasmid constructs were generated by the PCR amplification of UL83 fragments using template DNA referring to the HCMV strain sequence AD169varUK and by subsequent insertion into vector pcDNA3 (Invitrogen). The oligonucleotide primers used for PCR cloning were:

- 5'UL83-BamHI: CGAT GTAC GCG CCC ATG GCC GGC GCT CG
- 5'UL83(1)-BamHI: TGA GTAC GCG CCC ATG GCC GGC GCT CG
- 5'UL83(142)-BamHI: TGA GTAC GCG CCC ATG GCC GGC GCT CG
- 5'UL83(282)-BamHI: TGA GTAC GCG CCC ATG GCC GGC GCT CG
- 5'UL83(316)-BamHI: TGA GTAC GCG CCC ATG GCC GGC GCT CG
- 5'UL83(314)-HA-XhoI: TGA GTAC GCG CCC ATG GCC GGC GCT CG
- 3'UL83(264)-HA-XhoI: TGA GTAC GCG CCC ATG GCC GGC GCT CG
- 5'UL83(314)-HA-XhoI: TGA GTAC GCG CCC ATG GCC GGC GCT CG
- 3'UL83(314)-HA-XhoI: TGA GTAC GCG CCC ATG GCC GGC GCT CG

Site-directed mutagenesis
The genes of interest were amplified using Vent DNA polymerase and subsequently purified by gel extraction. After elution of the samples with distilled H2O, the PCR products were digested with 20 U of DpnI at 37 °C overnight to ensure the removal of methylated template DNA. Electrocompetent DH10B were transformed with the DpnI-treated samples and plated on LB agar plates containing appropriate antibiotics for the selection of positive clones, which were incubated overnight at 37 °C. Individual clones harbouring the desired constructs were verified by minipreparation and subsequent nucleotide sequencing. Alternatively, the GENEART site-directed mutagenesis system (Life technologies/Invitrogen) was used according to the manufacturer’s protocol. The oligonucleotide primers used for site-directed mutagenesis were:

- 5'UL83(M120A/L121A): TGA GTAC GCG CCC ATG GCC GGC GCT CG
- 5'UL83(H141A/L142A): TGA GTAC GCG CCC ATG GCC GGC GCT CG
- 5'UL83(N495A/L496A): TGA GTAC GCG CCC ATG GCC GGC GCT CG
- 5'UL83(M120A/L121A): TGA GTAC GCG CCC ATG GCC GGC GCT CG
- 5'UL83(N495A/L496A): TGA GTAC GCG CCC ATG GCC GGC GCT CG

Indirect immunofluorescence analysis using confocal laser-scanning microscopy
HFFs or ARPE-19 cells were grown on coverslips and infected with the respective HCMV strain. The cells were subsequently fixed, permeabilized and blocked according to Graf et al. [52]. Proteins were detected by incubation with the corresponding primary antibody for 90 min at 37 °C and subsequent incubation with a dye-conjugated secondary antibody for 45 min at 37 °C after washing 3 × with PBS. Then cells were washed another 3 × with PBS and mounted with Vectashield mounting medium containing DAPI and analysed using a DMi6000 B microscope and a 63× HCX PL APO CS oil immersion objective lens (Leica Microsystems, Mannheim, Germany). Confocal laser scanning microscopy was performed with a TCS SP5 microscope (Leica Microsystems). The images were processed using LAS AF software (version 1.8.2, build 1465; Leica Microsystems) and Adobe Photoshop (version 8.0.1, Adobe Systems, Inc.).

Coimmunoprecipitation analysis (CoIP)
The day prior to CoIP, 100 µl protein A-Sepharose beads (25 mg ml−1) in buffer A were washed 2 × with CoIP buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM PMSF, 2 µg aprotinin ml−1, 2 µg leupeptin ml−1 and 2 µg pepstatin ml−1; centrifugation at 10 000 rounds per minute (r.p.m.), 4 °C, 2 min) and subsequently mixed with 500 µl CoIP buffer and incubated with the respective antibody under rotation at 4 °C overnight. The next day, beads were washed 2 × with 1 ml CoIP buffer (centrifugation: 10 000 r.p.m., 4 °C, 2 min) to remove unbound antibody and leftover ice. PEI-transfected 293 T cells were washed with PBS, detached from the surface with a cell scraper, collected by centrifugation (2000 r.p.m., 4 °C, 5 min) and washed again with PBS. The cells were lysed with incubation with 500 µl CoIP buffer (including protease inhibitors) on ice for 20 min followed by centrifugation (14 000 r.p.m., 4 °C, 10 min) in order to separate the lysate
from insoluble cell debris. Fifty μl of each lysate was taken as an expression control, mixed with 50 μl protein loading buffer (2×) and boiled at 95 °C for 10 min. Unspecific binding of proteins was reduced by incubating the remaining lysate with uncoated protein A-Sepharose beads under rotation for 30 min at 4 °C. The pre-adsorbed lysates were then added to the antibody-coated beads and rotated for 2.5–4 h at 4 °C. Unbound proteins were removed by washing the beads 5× with CoIP buffer (including protease inhibitors; centrifugation: 10 000 r.p.m., 4 °C, 2 min). Bound proteins were eluted by the addition of 40 μl protein loading buffer (2×) and boiled at 95 °C for 10 min. Samples were analysed by Western blotting using antibodies for the detection of immunoprecipitated proteins and the determination of protein expression levels.

Western blot analysis

Cell lysates or protein samples for Western blot analysis were obtained from CoIP or Phos-tag experiments, as well as from cell fractionations. The samples were mixed with protein loading buffer, boiled for 10 min at 95 °C and either frozen at −20 °C or used immediately. The protein samples were separated electrophoretically by SDS-PAGE and transferred to a nitrocellulose membrane at 200 mA for 70 min under standard conditions [20]. Non-specific binding of the antibodies was prevented by incubating the membrane with 5 % skim milk powder solution for 90 min. Proteins of interest were detected by incubating the saturated membrane with the corresponding primary antibody, diluted in 2.5 % skim milk powder solution overnight at 4 °C. After washing the membrane, 3×10 min with PBS0/0.1 % Tween, the HRP-coupled secondary antibody diluted in 2.5 % skim milk powder solution was added to the membrane for 1 h at room temperature (RT). Thereafter, the membrane was washed 3×10 min with PBS0/0.1 % Tween again and briefly incubated with a freshly prepared ECL solution (10 ml ECL solution A, 100 μl ECL solution B and 3.1 μl H2O2). The resulting luminescence was detected using a FUJIFILM luminescent image analyser LAS-1000 (Fujifilm Europe GmbH, Düsseldorf, Germany). The molecular weight marker PageRuler prestained protein ladder was used as a reference for the detected protein bands. Detection of other proteins on the same membrane was performed by incubating the membrane with Roti-Free stripping buffer for 20 min at 56 °C. After extensive washing with PBS0/0.1 % Tween, the membrane was saturated and treated with antibodies again as described above.

Phos-tag labelling

In order to analyse protein phosphorylation, Phos-tag analysis (Wako Chemicals, Neuss, Germany) was performed. This technology is based on a conventional SDS-PAGE, except that Phos-tag and MnCl2 (molar ratio 1 : 2) are added to the running gel. The Phos-tag molecule binds selectively to phosphate ions and thereby decreases the electrophoretic mobility of phosphorylated proteins proportionally to the number of phosphate ions. This results in a separation of phosphorylated and non-phosphorylated forms of a protein after Western blotting and immunodetection. In this study, PEI-transfected 293 T cells were collected and lysed as described above, except that CoIP buffer without EDTA was used and phosphatase inhibitor (1 %) was added to the buffer. After lysis, a SDS-PAGE with a Phos-tag gel (6 % acrylamide, 10 μM Phos-tag and 20 μM MnCl2) was performed at a constant current of 25 mA per gel. As MnCl2 can influence blotting efficiency, it was removed by rinsing the running gel 2×20 min with Western blotting buffer (including 1 mM EDTA) and 1×20 min with conventional Western blotting buffer. The proteins were transferred to a nitrocellulose membrane at 200 mA for 90 min. The membranes were then subjected to Western blot analysis.

Proteomic and bioinformatic analyses

The quantitative proteomics analyses of purified viral particles were performed using ion mobility-enhanced data-independent acquisition on a SYNAPT G2-S mass spectrometer, as recently described [33]. Statistical analysis of the data sets was performed using the ANOVA analysis tool provided by MS-Excel 2010. For detailed information on the methodology used, please refer to [33, 56]. Cyclin docking motifs were identified by a pattern search using the [KR]-x₁-x₀,[FYLIVMP] motif that was derived from known cyclin–ligand complexes [76–78]. The schematic model of a ternary pUL97-cyclin–pp65 complex was generated based on the following several structural and experimental considerations. The pUL97–cyclinB1 interaction has been shown previously and led to the construction of a homology model of this binary complex [54]. For pp65, structural information is currently restricted so that the presence of a functional cyclin docking motif was modelled to the canonical motif binding site on the cyclin domain.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

References


