Modification of the major tegument protein pp65 of human cytomegalovirus inhibits virus growth and leads to the enhancement of a protein complex with pUL69 and pUL97 in infected cells

Sabine Becke,1 Véronique Fabre-Mersseman,1† Steffi Aue,1 Sabrina Auerochs,2 Tina Sedmak,3 Uwe Wolfrum,3 Dennis Strand,4 Manfred Marschall,2 Bodo Plachter1 and Sabine Reyda1

1Institute for Virology, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany
2Institute for Clinical and Molecular Virology, University of Erlangen-Nürnberg, Erlangen, Germany
3Institute for Zoology, Johannes Gutenberg-University Mainz, Mainz, Germany
41st Department of Internal Medicine, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany

The tegument protein pp65 of human cytomegalovirus (HCMV) is abundant in lytically infected human foreskin fibroblasts (HFF), as well as in virions and subviral dense bodies (DB). Despite this, we showed previously that pp65 is dispensable for growth in HFF. In the process of refining a DB-based vaccine candidate, different HCMV mutants were generated, expressing a dominant HLA-A2-presented peptide of the IE1 protein fused to pp65. One of the mutant viruses (RV-VM1) surprisingly showed marked impairment in virus release from HFF. We hypothesized that analysis of the phenotypic alterations of RV-VM1 would provide insight into the functions of pp65, poorly defined thus far. RV-VM1 infection resulted in nuclear retention of the fusion protein and reorganization of nuclear inclusion bodies. Coimmunoprecipitation experiments suggested that wild-type (wt) pp65 and pp65–VM1 were substrates of the viral pUL97 kinase in vitro and formed a complex with the viral RNA-export protein pUL69 and with pUL97 in lysates of infected cells. No evidence for an impairment of pUL97 within this complex was found. However, RV-VM1 replication in infected cells was resistant to a pUL97 inhibitor, and pUL97 inhibitors mimicked the mutant in terms of pp65 being retained in the nucleus. The results suggest that the life cycle of RV-VM1 was impeded at the stages of early-late transcription, RNA export or capsid maturation. wt-pp65 may play a role at these stages of infection, and complex formation with pUL69 and pUL97 may be important for that function.

INTRODUCTION

Human cytomegalovirus (HCMV) is a ubiquitous pathogen that severely affects individuals with impaired or immature immune-defence functions (Griffiths et al., 2009; Mocarski et al., 2007). The components of the HCMV tegument have recently attracted considerable attention, as these proteins carry functions important for various stages of the virus replication cycle (reviewed by Kalejta, 2008). The phosphoprotein pp65 (ppUL83) has long been known as an abundant tegument constituent (Roby & Gibson, 1986), yet it is dispensable for HCMV growth in human foreskin fibroblast (HFF) cell cultures (Schmolke et al., 1995b). pp65 is an important target antigen of cellular and humoral immune responses (Beninga et al., 1995; McLaughlin-Taylor et al., 1994; Plachter et al., 1990; Wills et al., 1996). It would thus be reasonable to assume that modification or deletion of the protein would be favourable for the virus. However, the sequence of pp65 is highly conserved in laboratory strains and clinical isolates (Dolan et al., 2004; Pande et al., 1991) and lack of pp65 expression in such strains has never been reported. Indeed, detection of pp65 has been used as a reliable diagnostic marker for acute HCMV infection in transplant recipients for over 15 years (Greffe et al., 1992a, b).
Initial studies described an association of pp65 with serine/threonine kinase activity (Britt & Auger, 1986; Somogyi et al., 1990). Subsequent analyses identified the cellular Polo-like kinase 1 (Gallina et al., 1990) and the viral pUL97 kinase (Kamil & Coen, 2007), both serine/threonine kinases, as pp65 interaction partners. pp65 has also been shown to shuttle between the nucleus and the cytoplasm (N. Frankenberg, P. Lischka, T. Stamminger & B. Plachter, unpublished results). One recent report demonstrated that a viral mutant lacking pp65 expression replicated poorly in monocyte-derived macrophage cell cultures (Chevillotte et al., 2009). The same authors showed that pp65 interacts with another tegument protein, pUL69 (Chevillotte et al., 2009), a promiscuous transcriptional transactivator and a nuclear-export factor of unspliced viral RNAs (Chevillotte et al., 2009; Lischka et al., 2006; Toth & Stamminger, 2008). Although all of these studies provided interesting pieces of information, the function of pp65 in HCMV infection is still unknown.

The lack of phenotypic alterations of pp65-deletion mutants in permissive HFF has impeded closer studies to elucidate the function of the tegument protein. In the course of a project aiming at the development of a vaccine (Mersseman et al., 2008a, b), we fortuitously identified an HCMV pp65 mutant (RV-VM1) that was unexpectedly difficult to reconstitute after transfection of the respective HCMV DNA and which showed poor growth in HFF. The severe impairment in virus release of RV-VM1 led us to analyse this mutant further in order to approach the molecular functions of pp65.

**RESULTS**

Insertion of a 30 aa heterologous sequence at Arg387 of pp65 severely impairs release of viral DNA from infected HFF, but does not alter viral DNA replication

The mutant RV-VM1 was generated by using a previously described bacterial artificial chromosome (BAC) mutagenesis strategy (Mersseman et al., 2008a). It encoded a pp65 with a 30 aa insertion at Arg387, encompassing an immunodominant HLA-A2-presented peptide from the non-structural IE1 protein (comprising aa 288–309) and a myc tag. Prior to viral reconstitution, the accuracy of BAC-VM1 and a revertant BAC (BAC-VM4) was verified by restriction-fragment pattern analysis, Southern hybridization and sequencing of the modified region. All experiments showed the expected results (data not shown). Viruses were subsequently reconstituted in HFF.

In a first experiment, viral DNA release from infected cells as a surrogate marker for progeny release from HFF was addressed. This method has been shown previously to provide a reproducible, relative measure of infectious particles (Besold et al., 2009). Release of RV-VM1 DNA from HFF was clearly impaired at all times tested, compared with parental (RV-HB5) and revertant (RV-VM4) viruses (Fig. 1a). To analyse whether the expression of the pp65–VM1 fusion protein was reduced in comparison to wild-type (wt)-pp65, Western blot analysis of infected cells was performed (Fig. 1b). No reduction of the steady-state level of the fusion protein at 4 days post-infection (p.i.) could be detected. This indicated that the mutation did not generally impair pp65 expression.

We next analysed whether the reduced release of RV-VM1 DNA from infected cells was related to an impairment of viral DNA replication. For this, cells were infected with RV-HB5, RV-VM1 or RV-VM4, and DNA was extracted at different time points after infection. The amount of viral DNA was quantified by using TaqMan PCR analysis (Fig. 1c). No differences were seen in intracellular viral DNA levels, showing that DNA replication was unimpaired in cells infected with RV-VM1. These results proved that processes downstream of viral DNA replication were impaired in RV-VM1-infected cells, indicating that pp65 was engaged in events in later stages of the virus life cycle.

pp65–VM1 is retained in the nucleus at late stages of infection

One hallmark of the late stage of HCMV infection in HFF is the shift of pp65 from the nucleus to the cytoplasm (Mersseman et al., 2008a; Schmolke et al., 1995a). This coincides with the formation of cytoplasmic assembly sites (Sanchez et al., 2000a, b). Consequently, we analysed whether pp65–VM1 had retained the cytoplasmic location in late-stage-infected cells. HFF were infected with RV-VM1, wt or revertant viruses and were subsequently analysed by indirect immunofluorescence analysis. As expected, wt-pp65 localized to the nucleus in RV-HB5- and RV-VM4-infected cells at 1 and 2 days p.i. (Fig. 2). At 3 days p.i., and even more pronounced at 4 days p.i., wt-pp65 was mainly located in the cytoplasm. The protein preferentially accumulated in dot-like structures in the cytoplasm of RV-HB5- and RV-VM4-infected cells, probably representing virions and dense bodies (DB). pp65–VM1, in contrast, was retained in the nucleus at all times tested (Fig. 2).

RV-VM1-infected cells show reorganization of nuclear inclusion bodies (NIBs) and redistribution of the major capsid protein (MCP) and pUL69

It has been reported that pp65 interacts with the tegument protein pUL69 (Chevillotte et al., 2009) and colocalizes with other tegument proteins in the cytoplasm at late stages of HCMV infection (Sanchez et al., 2000a). To investigate whether the alterations in pp65–VM1 affected the localization of different tegument proteins, RV-VM1-infected HFF were analysed at 4 days p.i. by conventional immunofluorescence microscopy and laser-scanning microscopy. No alterations were seen in the subcellular distribution of the tegument proteins pp71 (pUL82), pp28
Modification of pp65 impedes HCMV infection

IE1 insertion at position Arg387 of pp65 leads to nuclear capsid accumulation and prevents DB formation

To further investigate how the impairment in progeny release of RV-VM1 was related to alterations in virus morphogenesis, cells infected for 6 days were subjected to transmission EM (Fig. 4a). RV-HB5-infected cell nuclei showed the network characteristic for NIBs of late-stage-infected fibroblasts (Gilloteaux & Nassiri, 2000; Maeda et al., 1979). Intranuclear A, B and C capsids were associated with the reticulated NIBs. In contrast, the NIBs appeared to be disrupted in the nuclei of RV-VM1-infected cells, confirming the results obtained with light microscopy. Spherical structures, probably reflecting the LGS seen in light microscopy, became apparent. In some nuclei, viral B capsids of RV-VM1 appeared to be accumulated in the nucleoplasm. C capsids were seen only rarely in the cytoplasm of RV-HB5- or RV-VM1-infected cells. These were mostly associated with cytoplasmic assembly sites (Sanchez et al., 2000a). DB were visible in the cytoplasm of RV-HB5-infected cells, but not in RV-VM1-infected cells. The results obtained with cells...
**Fig. 2.** Subcellular localization of pp65–VM1 in infected HFF. Cells were infected for the indicated times with the different viruses. Cells were subsequently fixed and labelled with a pp65-specific antibody for fluorescence analyses. wt-pp65 was nuclear in RV-HB5- and RV-VM4-infected cells at 1 and 2 days p.i. The signal shifted to the cytoplasm at 3 days p.i. and was even more pronounced at 4 days p.i. Dot-like structures in the cytoplasm are possibly related to cytoplasmic virions and dense bodies (DB). The residual nuclear protein showed a rim staining, concordant with previous reports of a pp65-association with the nuclear lamina (Sanchez et al., 1998). The pp65–VM1 in RV-VM1-infected cells, in contrast, was retained in the nucleus at all times tested. Bar, 10 μm.

**Fig. 3.** Phase-contrast and confocal laser-scanning microscopy of infected HFF. Cells were infected for 4 days with the viruses indicated at the top and were subsequently fixed and stained with mAbs against pp65 (α-pp65), pUL69 (α-UL69) or MCP (α-MCP). Cells were analysed by a Zeiss LSM-510 laser-scanning microscope and Zeiss Software. Phase-contrast microscopy (Phase) revealed nuclear inclusion bodies (NIBs) in RV-HB5- and RVHd65-infected cells, which were replaced by LGS in RV-VM1-infected cells. Confocal images were taken as single slice photographs and showed that pUL69, pp65 and MCP localized to LGS in RV-VM1-infected cells. In addition, the pp65 antibody stained the nuclear membrane in these cells. Bars, 10 μm.
infected for 6 days were also obtained with HFF infected for 4 days (data not shown).

To measure the number of particles more accurately, an additional experiment was performed. A, B and C capsids of RV-HB5 and RV-VM1 were counted on representative sections of HFF infected for 4 and 6 days, respectively (Fig. 4b). Despite the accumulation of B capsids close to LGS in the nuclei of RV-VM1-infected cells, the overall count of these particles appeared lower than that of the parental virus. In addition, there appeared to be a tendency towards lower numbers of C capsids at 6 days p.i. in the nuclei of RV-VM1-infected cells. However, these differences may be caused by the considerable variations of counting HCMV particles by EM. Finally, as already seen in the qualitative EM analysis, only limited numbers of capsids were found in the cytoplasm and no clear differences became apparent between the two viruses. Taken together, these results showed that RV-VM1-infected cells are altered in their nuclear architecture compared with wt-infected cells, with a disruption of replication compartments and an attenuation of capsid formation.

Inhibition of pUL97 by Gö6976 and Ax7396 leads to nuclear pp65 retention in wt-HCMV-infected cells

The ability to translocate pp65 from the nucleus to the cytoplasm is one obvious characteristic that distinguished wt-HCMV-infected cells from RV-VM1-infected cells. The regulation of pp65 translocation is unknown. As the viral kinase pUL97 interacts with pp65 (Chevillotte et al., 2009; Cui et al., 2009; Kamil & Coen, 2007), involvement of that enzyme in the transport of the tegument protein was likely. To investigate this, wt-HCMV-infected cells were treated with the pUL97 kinase inhibitor Gö6976 or, for control, with the chemically related inhibitor Gö7874, which is inactive against pUL97 (Marschall et al., 2001, 2002) (Fig. 5a). Inhibition of pUL97 by Gö6976 indeed blocked pp65 translocation to the cytoplasm. To corroborate these findings, the experiment was repeated using quinazoline Ax7396 to inhibit pUL97 (Herget et al., 2004). Again, inhibition of pUL97 impaired the cytoplasmic translocation of wt-pp65, although, in this case, incompletely (Fig. 5b). For control, RV-VM1-infected cells were also treated with the inhibitors. As expected, pp65–VM1 was retained in the nucleus, irrespective of the presence of the kinase inhibitors. This showed that pUL97 is involved in the cytoplasmic translocation of pp65. It also suggested that the interaction of pUL97 with pp65 was functionally impaired with respect to the cytoplasmic translocation of the tegument protein in RV-VM1-infected cells.

RV-VM1 is resistant to inhibition of pUL97 by Gö6976

The pUL97 kinase has been reported to be involved in essential processes of the virus life cycle, including DNA
replication (Wolf et al., 2001) and nuclear capsid egress (Hamirally et al., 2009; Krosky et al., 2003; Marschall et al., 2005; Milbradt et al., 2010). Although DNA replication was unimpaired in RV-VM1-infected cells, a possible explanation of the phenotypic alterations was that pp65–VM1 interfered with essential activities of pUL97 at later stages. We hypothesized that, in this case, RV-VM1 should be resistant to pUL97 inhibitors.

HFF were infected with RV-HB5 or mutant RV-VM1 in the presence or absence of pUL97 kinase inhibitor Go6976. Viral DNA titres in the culture supernatant of infected cells were determined by quantitative real-time PCR (Besold et al., 2007). Whilst growth of RV-HB5 was severely affected by the addition of Go6976, release of virus progeny from RV-VM1-infected cells was indeed less sensitive to pUL97 inhibition (Fig. 6). In parallel, control inhibitor Go7874 showed only a limited impact on the release of progeny from either RV-VM1- or RV-HB5-infected cells. To confirm this, IC50 values of Go6976 for RV-HB5 and RV-VM1 growth in HFF were determined. Serial concentrations of Go6976 inhibitor were added to the culture medium at the time of infection. Viral DNA loads in the supernatant of the different cultures were determined by quantitative PCR at day 5 p.i. The mean values of two independent experiments were 74.5 ± 44.9 nM for RV-VM1 and 3.1 ± 1.5 nM for RV-HB5, respectively. Consequently, RV-HB5 was 24 times more sensitive than RV-VM1 to Go6976. Furthermore the IC50 value for wt HCMV was in the same range as recently published (Marschall et al., 2001; Fig. 7). Protein kinase inhibitors were added in some cases. Cells were subsequently lysed. pUL97- or pp65-specific antibodies were used to coimmunoprecipitate associated proteins (Fig. 7). A pUL97-specific antiserum coprecipitated pp65, as seen by probing the precipitate with a pp65-specific antibody in immunoblots. Only a faint band was seen, however, using a pp66-specific antibody for immunoblot detection. Surprisingly, pUL69 appeared to be more abundant in the complex from RV-VM1-infected cells compared with the wt-HCMV control.

To test whether pUL97 was capable of phosphorylating the proteins in the precipitated complex, an IVKA was performed. The constant phosphorylation signal obtained for histones H1–H4 (Fig. 7a, lanes 3–10, 13–14), used as a standard substrate, showed a high activity of pUL97 under these conditions (compared with the negative control of HCMVΔUL97, lane 2) and demonstrated the reliability of

**Fig. 5.** Impact of pUL97 kinase on nuclear export of pp65–VM1. HFF were infected with wt RV-HB5 or with RV-VM1. Inhibitors Go6976, Go7874 (a) or Ax7396 (b) were immediately added to some of the cultures [others were left without (w/o) inhibitor] and cells were incubated for 4 days. After that, HFF were fixed and stained with a pp65-specific antibody for indirect immunofluorescence analyses. Inhibition of pUL97 blocked the translocation of pp65 to the cytoplasm of wt-HCMV (RV-HB5)-infected cells. The nuclear localization of pp65–VM1 was unaffected by pUL97 inhibition in RV-VM1-infected cells. Bar, 10 μm.
the assay. Autophosphorylation of pUL97 was detectable in samples from HCMV-infected cells, including RV-VM1, at comparable amounts, indicating that this function of the viral kinase was maintained in the precipitated complexes. The pUL97 inhibitor G6976 almost completely blocked pUL97 activity in terms of autophosphorylation and substrate phosphorylation (e.g. histones H1–H4, lane 11). The results suggest that pp65 is a substrate of pUL97 (Fig. 7a, see labelling of phosphorylated proteins at the right). This is the first description of direct in vitro phosphorylation of wt-pp65 by pUL97, which had been suggested but not shown previously (Kamil & Coen, 2007; Prichard et al., 2005). Notably, wt-pp65, pp65–VM1 and pp65–VM4 were phosphorylated to a similar extent, indicating a normally regulated pp65 phosphorylation by pUL97, and this was also the case for mutant RV-VM1. However, this does not prove that pp65 and pp65–VM1 were phosphorylated at the same sites.

As far as the previously reported pUL97 substrate, pUL69, is concerned (Thomas et al., 2009), an increased pUL69 phosphorylation signal became detectable in the case of RV-VM1, whereas phosphorylation of pUL69 by pUL97 remained largely below the detection limit in the controls (Fig. 7a, lanes 3–4, 13). This is probably related to the large amount of pUL69 precipitated with a pp65-specific antibody from RV-VM1-infected cells (Fig. 7b, pUL69, lane 13). Further substrates of pUL97, such as the polymerase processivity factor pUL44 (Marschall et al., 2003) and cellular p32 (Marschall et al., 2005), were only faintly detectable for all viruses used under these conditions. Interestingly, the mere expression level of cellular p32 was clearly increased in HCMV-infected cells, (Fig. 7c, p32, lanes 3–14) compared with mock-infected (lane 1) or HCMV UL97-infected (lane 2) controls. The increased expression of p32 was also seen in RV-VM1-infected cells (lanes 3–4, 13). Taken together, these experiments showed that: (i) pp65, pUL69 and pUL97 are components of a molecular protein complex (or several complexes) that is enhanced in RV-VM1-infected cells; (ii) pp65–VM1 and pUL69, as well as wt-pp65, are phosphorylated by pUL97 in coimmunoprecipitates from infected cells; and (iii)

![Fig. 6. Resistance of RV-VM1 replication to pUL97 inhibition. HFF were infected with the parental RV-HB5 (a and b; ■) or RV-VM1 (c and d; ▲). G6976 (a and c) or G7874 (b and d), both kinase inhibitors (dotted lines), or DMSO (solid lines) for control, were added immediately after infection. Infected cell-culture supernatants were collected at the indicated time points and analysed by quantitative TaqMan DNA PCR analysis for the number of viral genomes. TaqMan PCR analyses were performed on triplicate samples in each experiment and these experiments were performed twice on independent samples. RV-VM1 proved to be resistant to inhibition of pUL97 (c), whereas the parental strain RV-HB5 was sensitive (a). As expected, both viruses were resistant to the control G7874 (b and d).](http://vir.sgmjournals.org)
pUL69 steady-state levels are distinctly increased in RV-VM1-infected cells.

**DISCUSSION**

Deletion of the UL83 gene, encoding pp65, has no effect on the efficiency of HCMV release from infected HFF (Besold et al., 2007; Dunn et al., 2003; Schmolke et al., 1995b). This has significantly impeded functional analyses of the tegument protein. Surprisingly, expression of a mutated form of pp65 by RV-VM1 had considerable impact on virus release, thus rendering this virus attractive for further investigation of pp65 function. The only alteration compared with wt-pp65 was the insertion of a 30 aa sequence following position Arg387. This insertion was responsible for the phenotypic alterations of RV-VM1, as they were lost upon reversion. Insertion of the same sequence in other regions of pp65 or fusion of a larger fragment of the IE1 protein to the very C terminus did not lead to the same phenotypic changes of the resulting viruses (Mersseman et al., 2008a, b; Becke et al., 2010).

One hallmark of the infection with RV-VM1 was the dissolution of NIBs and the formation of nuclear LGS. Similar nuclear structures had been reported to be formed...
following inhibition of pUL97 with maribavir or after infection with a pUL97-deletion mutant (Prichard et al., 2005, 2008). These authors also reported the formation of large cytoplasmic aggregates, which seemed to accumulate tegument proteins, including pp65. This is in contrast to RV-VM1, where pp65 was exclusively located in the nucleus and no cytoplasmic aggregates were found. Azzez et al. (2006) showed that infection of HFF with a pUL97-deletion mutant or inhibition of pUL97 in wt-HCMV-infected cells by NGIC-I (non-glycosidic indolocarbazole I) resulted in a reorganization of cytoplasmic assembly sites, including a redistribution of the late protein pp28 and the formation of 2–8 μm wide vacuoles. We observed neither the formation of vacuoles in the cytoplasm nor the redistribution of pp28 after infection with RV-VM1 (not shown).

The differences that were seen in wt-HCMV-infected cells treated with pUL97 inhibitors and RV-VM1-infected cells indicate that different processes were affected in both instances. pUL97 has been reported to be involved in DNA packaging and nuclear capsid egress (Krosky et al., 2003; Marschall et al., 2005; Wolf et al., 2001). There was a tendency towards lower numbers of capsids in the nucleus of RV-VM1-infected cells, with clustering particularly of B capsids. However, as A, B and C capsids were seen, the process of capsid assembly per se appeared functional, although the efficiency of capsid maturation seemed to be compromised. Consequently, processes preceding capsid maturation like viral transcription or RNA export were probably impaired in RV-VM1-infected cells.

Nuclear retention of pp65 was seen both in RV-VM1- and in wt-HCMV-infected cells treated with Gö6976 and Ax7396 inhibitor. This suggests that, under these conditions, either pp65 was unavailable for export or that an interaction of pUL97 with pp65, essential for export of the tegument protein, was blocked in RV-VM1-infected cells. A direct inhibition of pUL97 by pp65–VM1 appeared unlikely, as the kinase was active in CoIP experiments. An alternative explanation was that pUL97 was sequestered aberrantly together with pp65. Complex formation of pp65 with pUL97 would be concordant with the resistance of RV-VM1 against pUL97 inhibition. The results would, however, also be concordant with a physiological rather than an aberrant complex formation of pUL97 with pp65 and pUL69 and an impairment of complex dissociation in RV-VM1-infected cells. This would imply that the formation and dissociation of the complex were functionally relevant for the virus life cycle and were tightened in RV-VM1-infected cells.

Distinct changes were seen in the subcellular localization and steady-state levels of pUL69. This protein is a multifunctional polypeptide that transactivates a number of promoters (Winkler et al., 1994) and is involved in the export of unspliced viral RNAs (Lischka et al., 2001, 2006; Toth et al., 2006; Toth & Stamminger, 2008). It has been reported to be an interaction partner and a substrate of pUL97 (Chevillotte et al., 2009; Thomas et al., 2009). The steady-state levels of pUL69 in RV-VM1-infected cells were strikingly increased compared with those in cells that were infected with either wt-pp65-competent or pp65-deficient viruses. It appears unlikely that high pUL69 levels were a consequence of enhanced gene expression in RV-VM1-infected cells, although this has not been formally addressed at this time. It is more likely that pUL69 displays an increased half-life in the presence of pp65–VM1 as opposed to wt-pp65. In this regard, it is an intriguing finding that the amount of the protein complex pUL69–pp65 coimmunoprecipitated from RV-VM1-infected cells is increased dramatically compared with HCMV-infected control cells. An explanation for a regulatory consequence of the altered pUL69–pp65 interaction could be that pp65 was involved in the regulation of the shuttling function of pUL69, possibly by a direct interaction of the two proteins, as shown by Chevillotte et al. (2009). One possible scenario could be that pp65 attracts pUL97 to pUL69, which is, in turn phosphorylated by the viral kinase. Phosphorylation by pUL97 has been shown to be important for the nuclear RNA-export function mediated by pUL69 (Thomas et al., 2009). In the absence of pp65, pUL97 may have lower avidity for pUL69, but other cellular kinases could cover up for the loss (Rechter et al., 2009). This could serve as an explanation why pp65 deletion does not have a considerable impact on HCMV gene expression and progeny production. In RV-VM1-infected cells, however, pUL69 dissociation and consequently viral mRNA export may be impaired, leading to the phenotypic alterations.

Although this model is hypothetical at this stage, the insertion mutagenesis applied here provides an experimental approach to study this issue further and to elucidate the function of the most abundant tegument protein pp65 of HCMV.
with minor modifications as described previously (Thomas et al., 2009); see Supplementary Methods for details.

**Immunoblot analysis.** Protein samples were denatured under reducing conditions and separated by SDS-PAGE as described previously (Marschall et al., 1999). Blots were incubated with mAb-UL97 (dilution 1:5000), polyclonal antibody (pAb) UL97 (1:5000); both kindly provided by Detlef Michel, Ulm, Germany; mAb 65-33 (1:10) or mAb 69-66 (1:1000), both kindly provided by William Britt, University of Alabama, AL, USA; mAb BSS10 (against pUL44; 1:100; Bioteck); pAb-p32 (1:500; kindly provided by William Russell, University of St Andrews, UK); and an antibody against actin (Rockland). Horseradish peroxidase-labelled secondary antibodies were used for detection (Dianova).

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