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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

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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.

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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 (Grefte *et al.*, 1992a, b).

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Supplementary methods are available with the online version of this paper.

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.*, 1999) 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

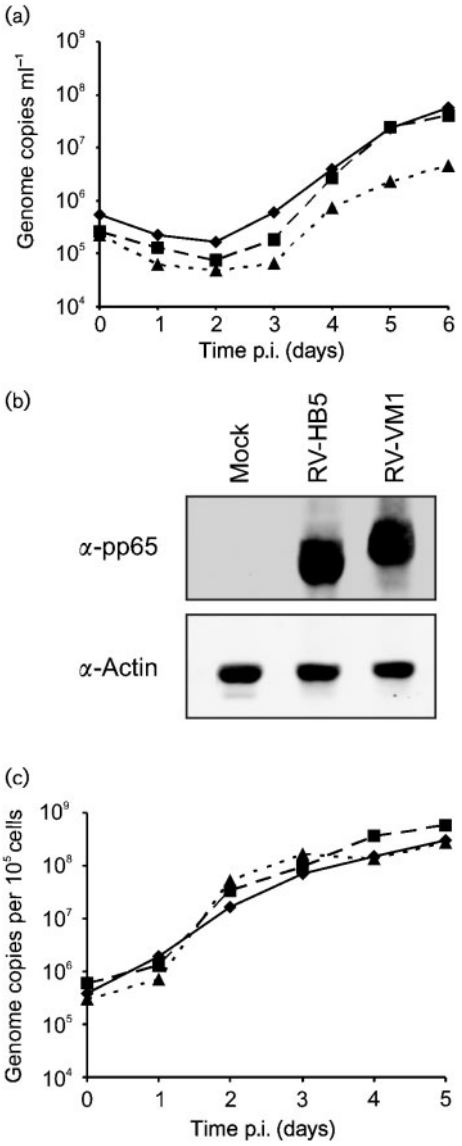

Fig. 1. Release of viral DNA from infected cells, pp65 expression and DNA replication kinetics of mutant RV-VM1. (a) Release of viral DNA, as measured by TaqMan DNA quantification. HFF were infected at an m.o.i. of 0.1. At the indicated time points p.i., culture supernatants were collected and viral DNA (genome copies) was quantified by TaqMan PCR. (b) Immunoblot analysis of pp65 and pp65–VM1 expression in HFF, infected for 4 days with RV-VM1 or the parental strain RV-HB5. Antibodies were directed against pp65 (α-pp65) and against actin (loading control). (c) Viral DNA replication kinetics as measured by TaqMan quantification. Infection conditions of HFF were set up in a way to ensure comparable copies of viral DNA per cell for each virus (four copies per cell at 6 h p.i.). Cells were collected at the indicated time points, DNA was extracted and subsequently quantified by PCR. TaqMan PCR analyses were performed on triplicate samples in each experiment and these experiments were performed three times on independent samples. ◆, RV-HB5; ▲, RV-VM1; ■, RV-VM4.

(pUL99) or pp150 (ppUL32; data not shown). In contrast, the distribution of pUL69 and the MCP was markedly different in RV-VM1-infected cells compared with cells infected with the parental strain. Phase-contrast images of cells infected with parental RV-HB5 or with the pp65-null mutant RVHd65 showed the typical reticulated NIBs (Gilloteaux & Nassiri, 2000; Maeda *et al.*, 1979), which are considered to be sites of virus replication, transcription and capsid assembly (Fig. 3). Surprisingly, these NIBs were undetectable in the nuclei of most RV-VM1-infected HFF, but were replaced by large globular structures (LGS). Staining of RV-VM1-infected cells with a pp65-specific mAb confirmed the nuclear retention of the pp65–VM1 fusion protein in these cells. The protein appeared to be located at the rims of the LGS, but failed to stain the body of these structures (Fig. 3). However, preliminary immunogold electron microscopic (EM) analyses on thin sections of RV-VM1-infected cells showed that pp65 is also contained in the core of these LGS, indicating that the internal parts of LGS are inaccessible to antibody staining in immunofluorescence analyses. The pp65 antibody also stained the nuclear membrane; the latter localization complies with that of wt-pp65 (Sanchez *et al.*, 1998). The pUL69-specific antibody also stained the LGS in RV-VM1-infected cells, showing a rim location. In contrast, pUL69 appeared to be located in NIBs in both wt- and RVHd65-infected cells (Fig. 3). The MCP-specific antibody stained the NIBs of RV-HB5- and RVHd65-infected cells, as expected. In contrast, MCP also localized clearly to the newly formed LGS in RV-VM1-infected cells, also showing a ring-like topology. Taken together, fluorescence analyses and confocal laser-scanning microscopy showed that RV-VM1 infection resulted in disruption of NIBs, formation of LGS and an accumulation of pp65, pUL69 and MCP in these structures.

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

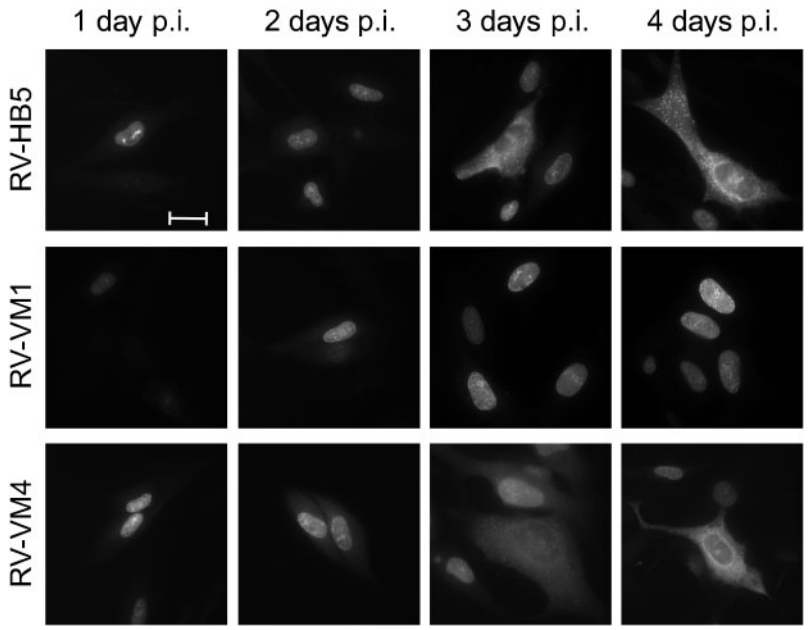

Fig. 2. Subcellular localization of pp65–VM1 in infected HFF. Cells were infected for the indicated times with the different viruses. Cells were subsequently fixed and labelled with a pp65-specific antibody for fluorescence analyses. wt-pp65 was nuclear in RV-HB5- and RV-VM4-infected cells at 1 and 2 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.

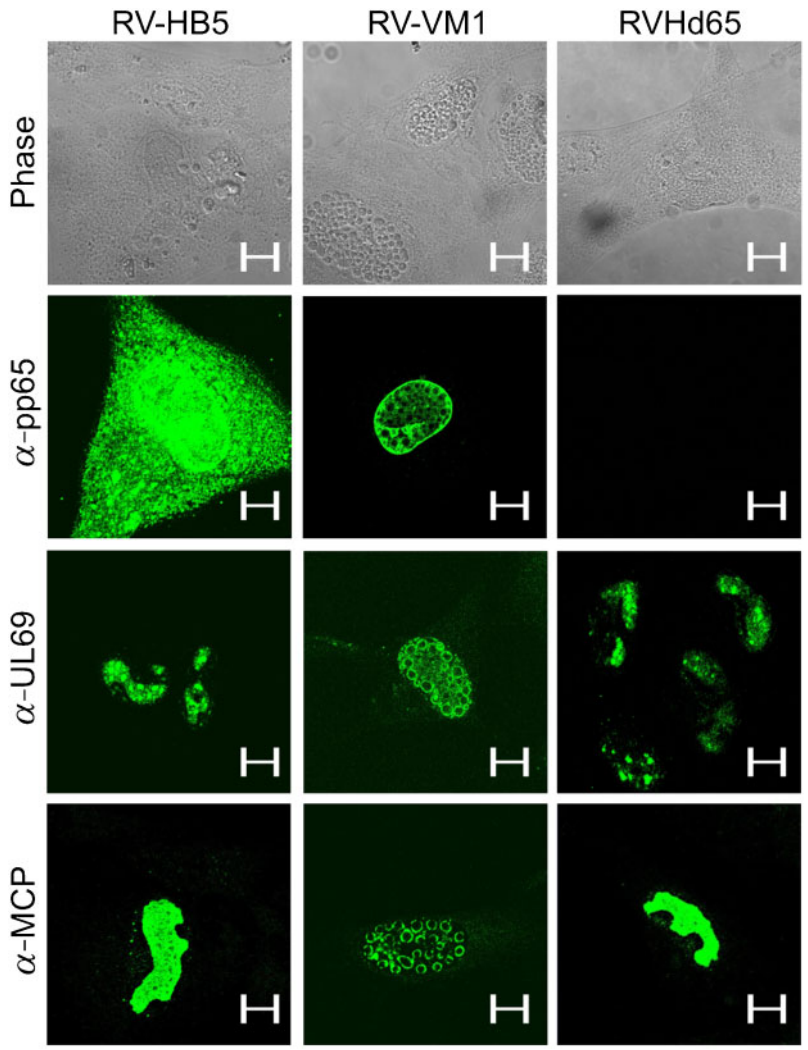

Fig. 3. Phase-contrast and confocal laser-scanning microscopy of infected HFF. Cells were infected for 4 days with the viruses indicated at the top and were subsequently fixed and stained with mAbs against pp65 (α-pp65), pUL69 (α-UL69) or MCP (α-MCP). Cells were analysed by a Zeiss LSM-510 laser-scanning 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.

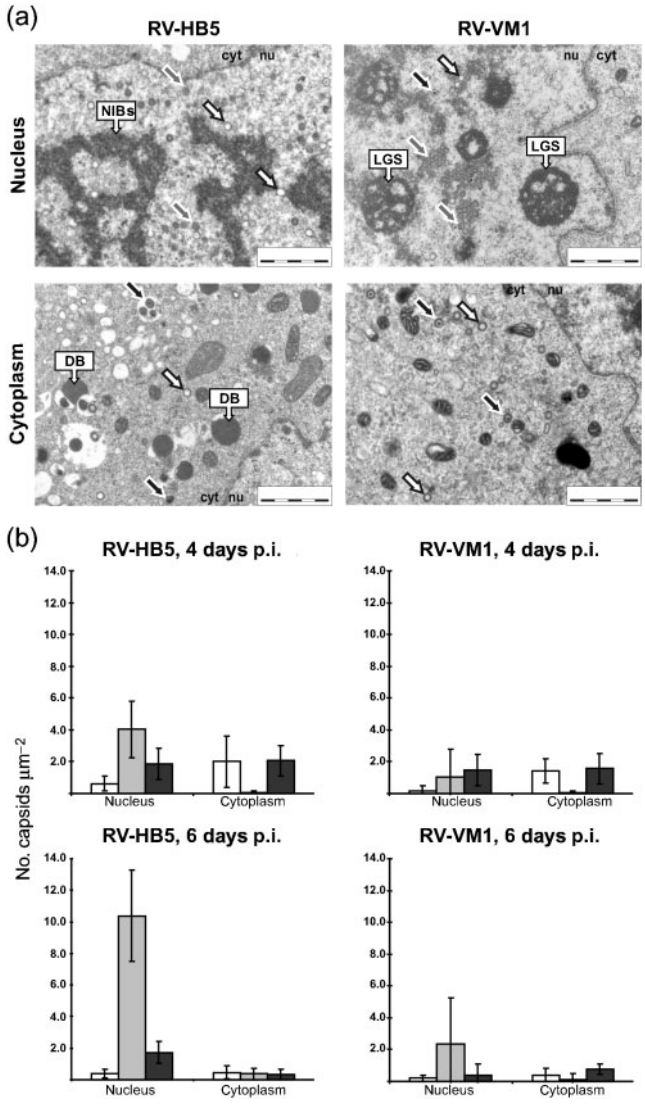

Fig. 4. Transmission electron microscopy analysis of RV-HB5- and RV-VM1-infected HFF. (a) HFF were infected with RV-VM1 or with the parental strain RV-HB5 before being processed for conventional electron microscopy at 6 days p.i. Electron optic analysis revealed the localization of all three types of capsid, A (white arrows), B (grey arrows) and C (black arrows), in the nucleus (nu) of RV-HB5-infected cells. In addition, typical NIBs were found in these cells. In the cytoplasm (cyt) of RV-HB5 cells, only A and C capsids were found in addition to DB. In the nucleus of RV-VM1-infected cells, capsids were found in clusters, located close to newly formed LGS. A and C capsids, but no DB, were found in the cytoplasm of RV-VM1 cells. Bars, 1 μm. (b) Quantification of EM analyses of RV-HB5 and RV-VM1 infected HFF. Cells were infected for 4 or 6 days with RV-VM1 or RV-HB5. A (white bars), B (grey bars) and C (black bars) capsids were counted in representative regions of the nucleus and the cytoplasm of the samples. In each cell, three different squares of 1.5 μm^2 were counted. Data are expressed as means ± SD.

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

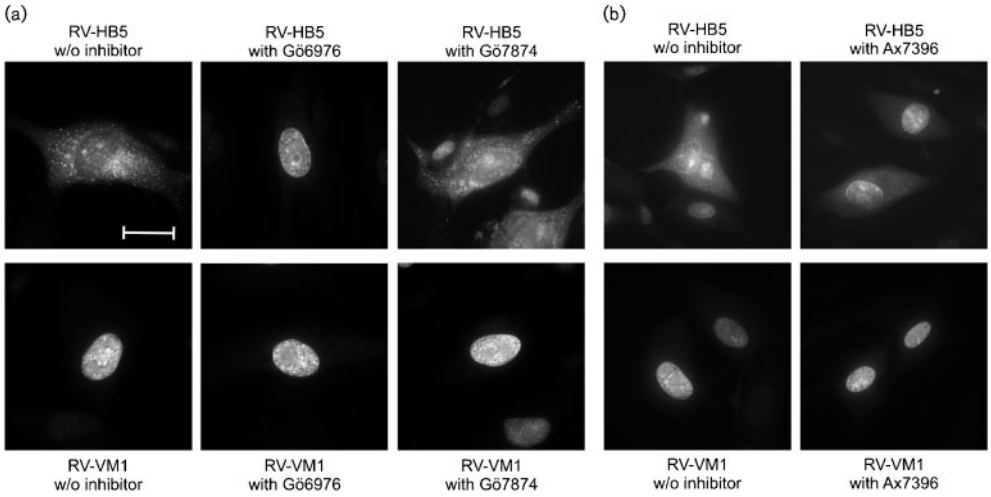

Fig. 5. Impact of pUL97 kinase on nuclear export of pp65–VM1. HFF were infected with wt RV-HB5 or with RV-VM1. Inhibitors Gö6976, Gö7874 (a) or Ax7396 (b) were immediately added to some of the cultures [others were left without (w/o) inhibitor] and cells were incubated for 4 days. After that, HFF were 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.

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 Gö6976. 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 Gö6976, release of virus progeny from RV-VM1-infected cells was indeed less sensitive to pUL97 inhibition (Fig. 6). In parallel, control inhibitor Gö7874 showed only a limited impact on the release of progeny from either RV-VM1- or RV-HB5-infected cells. To confirm this, IC_{50} values of Gö6976 for RV-HB5 and RV-VM1 growth in HFF were determined. Serial concentrations of Gö6976 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 Gö6976. Furthermore the IC_{50} value for wt HCMV was in the same range as recently published (Marschall *et al.*, 2002), using a different method. These results showed that RV-VM1 was resistant to inhibition of pUL97.

wt-pp65 and pp65–VM1 form a complex with pUL69 and pUL97 and are *in vitro* substrates of pUL97

The LGS formed in RV-VM1-infected cells accumulated both the pp65 variant and pUL69. Both tegument proteins are known to associate with pUL97, and pUL69 is phosphorylated by the viral kinase (Chevillotte *et al.*, 2009; Thomas *et al.*, 2009). To investigate whether the three proteins formed a complex and whether the two tegument proteins were phosphorylated by the viral kinase in that complex, coimmunoprecipitation (CoIP) experiments in conjunction with *in vitro* kinase assays (IVKA) were performed on lysates of infected cells. HFF were infected with wt HCMV and different mutants for 5 days at an m.o.i. of 0.25 (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 pUL69-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

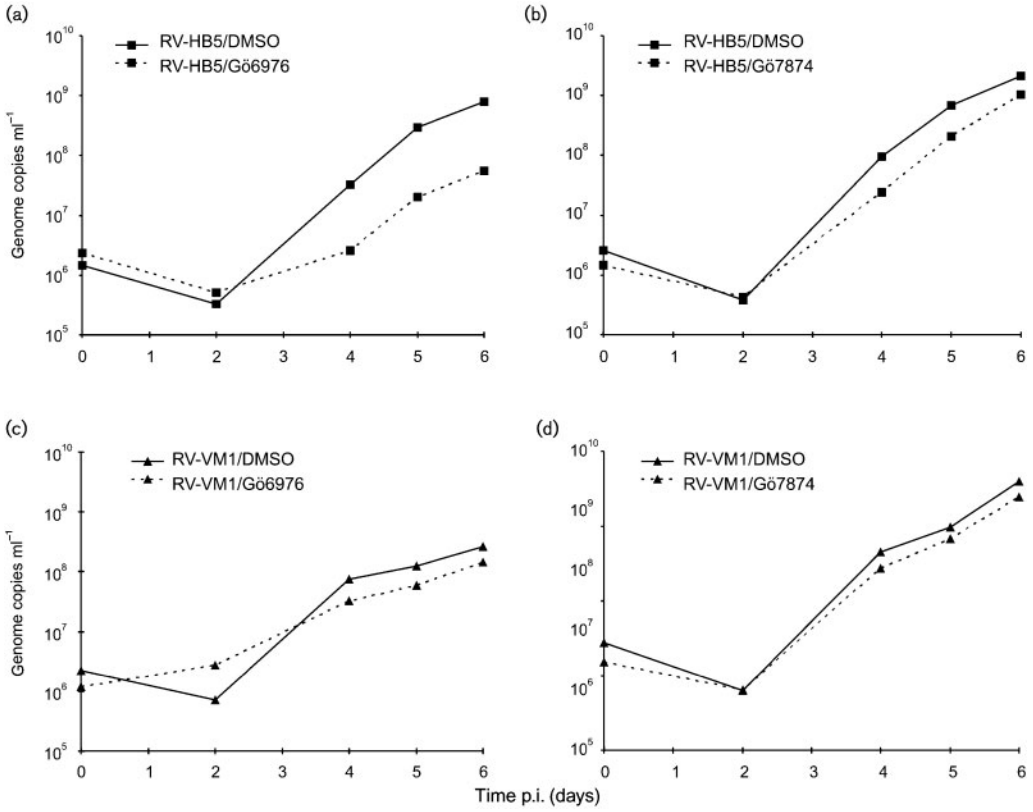

Fig. 6. Resistance of RV-VM1 replication to pUL97 inhibition. HFF were infected with the parental RV-HB5 (a and b; ■) or RV-VM1 (c and d; ▲). Gö6976 (a and c) or Gö7874 (b and d), both kinase inhibitors (dotted lines), or DMSO (solid lines) for control, were added immediately after infection. Infected 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 Gö7874 (b and d).

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 Gö6976 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 HCMVΔUL97-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)

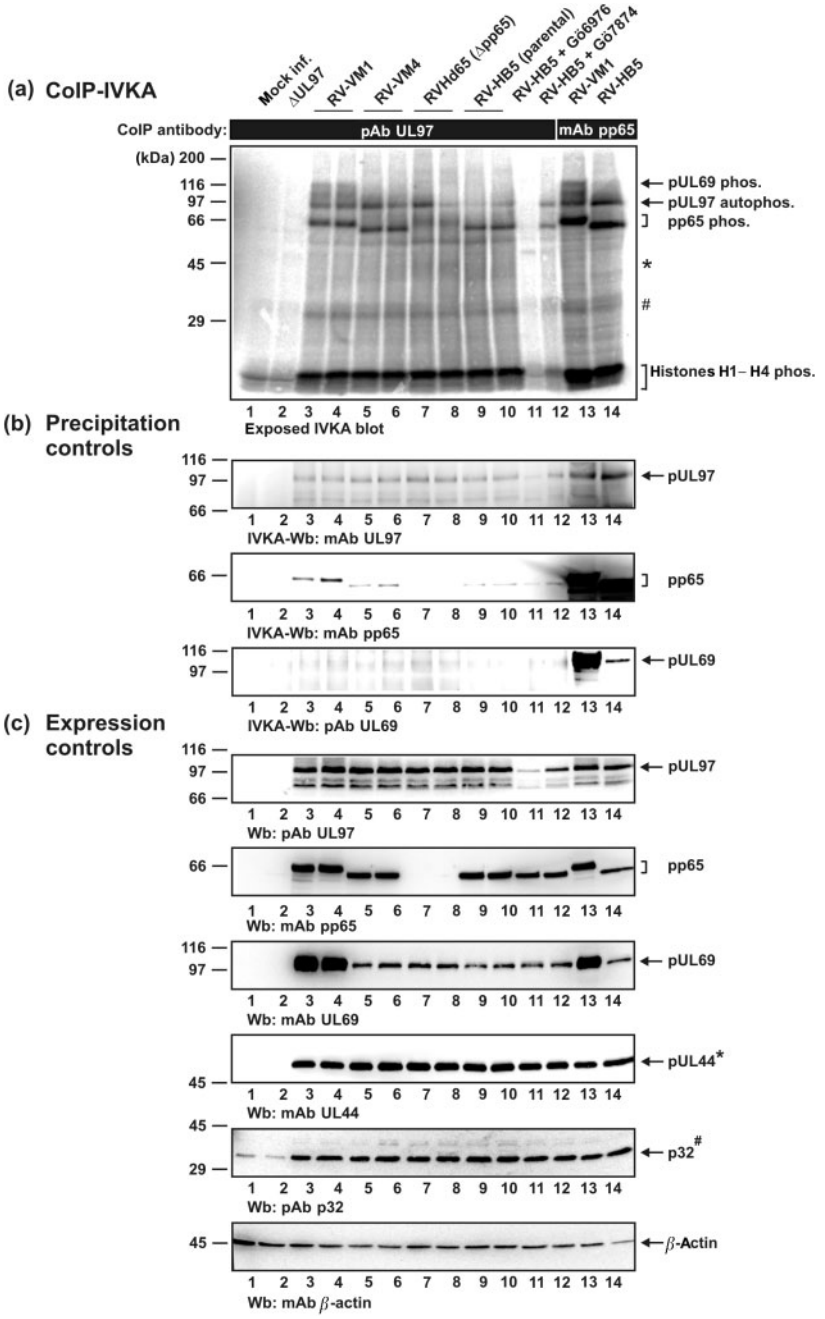

Fig. 7. Phosphorylation of substrate proteins by viral kinase pUL97: comparative analysis with RV-HB5, RV-VM1 and RV-VM4. A combined coimmunoprecipitation and *in vitro* kinase assay (CoIP-IVKA) was performed with coimmunoprecipitates obtained from total lysates of HCMV-infected HFF. HFF were infected with RV-HB5 or HCMV mutants as indicated at an m.o.i. of 0.25 and cultured for 5 days. Protein kinase inhibitors were added to the RV-HB5-samples shown in lanes 11 and 12, 15 h before harvesting the cells. Cells were lysed and subjected to immunoprecipitation using the indicated precipitation antibodies (directed to protein kinase pUL97 or to the putative substrate pp65). (a) The coimmunoprecipitates were used for a pUL97-specific IVKA and labelled phosphorylation products were separated by SDS-PAGE, followed by exposure to autoradiography films (see phosphorylation signals indicated on the right by arrows and brackets; additional faint signals probably represent poorly coimmunoprecipitated pUL44 and p32, indicated by * and #, respectively). A standard substrate of pUL97, purified histones H1–H4, was added exogeneously to all reactions. (b) Control immunostaining of the IVKA-Wb (precipitation controls) was subsequently performed using detection antibodies mAb UL97, mAb 28-77 (against pp65) and pAb UL69, respectively. (c) Reliable expression of pUL97, viral substrates of pUL97 (pp65, pUL69 and pUL44) and a cellular substrate (p32) was monitored on control immunoblots that had been produced with total cell lysates collected prior to the addition of CoIP antibodies. As loading control, the immunoblots were restained with a mAb directed against β-actin (AC15; Sigma-Aldrich).

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. Azzeh *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-glyosidic 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.

METHODS

Cells and viruses. Primary HFF and 293T cells were cultured as described previously (Besold *et al.*, 2009). HCMV strains RV-HB5 (Borst *et al.*, 1999), RVHd65 (Besold *et al.*, 2007) and AD169delUL97 (Marschall *et al.*, 2005) were employed for controls.

BAC recombineering. Modification of HCMV BACs was performed according to the *galK* positive/negative selection of Warming *et al.* (2005) as described previously (Mersseman *et al.*, 2008a); see Supplementary Methods (available in JGV Online) for details.

Growth kinetics and IC_{50} determination. A quantitative real-time PCR using the TaqMan technology was used to quantify intra- and extracellular viral genomes. See Supplementary Methods for details.

Indirect immunofluorescence analysis, confocal laser-scanning microscopy and EM. Indirect immunofluorescence analysis and confocal laser-scanning microscopy were carried out as described previously (Mersseman *et al.*, 2008a; Schmolke *et al.*, 1995a). Transmission EM was performed as described previously (Mersseman *et al.*, 2008a); see Supplementary Methods for details.

pUL97-specific coimmunoprecipitation–*in vitro* kinase assay (CoIP-IVKA). A combined CoIP and IVKA for pUL97 was performed

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 BS510 (against pUL44; 1 : 100; Biotest); 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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REFERENCES

Azzeh, M., Honigman, A., Taraboulos, A., Rouvinski, A. & Wolf, D. G. (2006). Structural changes in human cytomegalovirus cytoplasmic assembly sites in the absence of UL97 kinase activity. *Virology* **354**, 69–79.

Becke, S., Aue, S., Thomas, D., Schader, S., Podlech, J., Bopp, T., Sedmak, T., Wolfrum, U., Plachter, B. & Reyda, S. (2010). Optimized recombinant dense bodies of human cytomegalovirus efficiently prime virus specific lymphocytes and neutralizing antibodies without the addition of adjuvant. *Vaccine* **28**, 6191–6198.

Beninga, J., Kropff, B. & Mach, M. (1995). Comparative analysis of fourteen individual human cytomegalovirus proteins for helper T cell response. *J Gen Virol* **76**, 153–160.

Besold, K., Frankenberg, N., Pepperl-Klindworth, S., Kuball, J., Theobald, M., Hahn, G. & Plachter, B. (2007). Processing and MHC class I presentation of human cytomegalovirus pp65-derived peptides persist despite gpUS2–11-mediated immune evasion. *J Gen Virol* **88**, 1429–1439.

Besold, K., Wills, M. & Plachter, B. (2009). Immune evasion proteins gpUS2 and gpUS11 of human cytomegalovirus incompletely protect infected cells from CD8 T cell recognition. *Virology* **391**, 5–19.

Borst, E. M., Hahn, G., Koszinowski, U. H. & Messerle, M. (1999). Cloning of the human cytomegalovirus (HCMV) genome as an infectious bacterial artificial chromosome in *Escherichia coli*: a new approach for construction of HCMV mutants. *J Virol* **73**, 8320–8329.

Britt, W. J. & Auger, D. (1986). Human cytomegalovirus virion-associated protein with kinase activity. *J Virol* **59**, 185–188.

Chevillotte, M., Landwehr, S., Linta, L., Frascaroli, G., Luske, A., Buser, C., Mertens, T. & von Einem, J. (2009). Major tegument protein pp65 of human cytomegalovirus is required for the incorporation of pUL69 and pUL97 into the virus particle and for viral growth in macrophages. *J Virol* **83**, 2480–2490.

Cui, Z., Zhang, K., Zhang, Z., Liu, Y., Zhou, Y., Wei, H. & Zhang, X. E. (2009). Visualization of the dynamic multimerization of human cytomegalovirus pp65 in punctuate nuclear foci. *Virology* **392**, 169–177.

Dolan, A., Cunningham, C., Hector, R. D., Hassan-Walker, A. F., Lee, L., Addison, C., Dargan, D. J., McGeoch, D. J., Gatherer, D. & other authors (2004). Genetic content of wild-type human cytomegalovirus. *J Gen Virol* **85**, 1301–1312.

Dunn, W., Chou, C., Li, H., Hai, R., Patterson, D., Stolc, V., Zhu, H. & Liu, F. (2003). Functional profiling of a human cytomegalovirus genome. *Proc Natl Acad Sci U S A* **100**, 14223–14228.

Gallina, A., Simoncini, L., Garbelli, S., Percivalle, E., Pedrali-Noy, G., Lee, K. S., Erikson, R. L., Plachter, B., Gerna, G. & Milanesi, G. (1999). Polo-like kinase 1 as a target for human cytomegalovirus pp65 lower matrix protein. *J Virol* **73**, 1468–1478.

Gilloteaux, J. & Nassiri, M. R. (2000). Human bone marrow fibroblasts infected by cytomegalovirus: ultrastructural observations. *J Submicrosc Cytol Pathol* **32**, 17–45.

Grefte, J. M., van-der-Gun, B. T., Schmolke, S., van-der-Giessen, M., van-Son, W. J., Plachter, B., Jahn, G. & The, T. H. (1992a). Cytomegalovirus antigenemia assay: identification of the viral antigen as the lower matrix protein pp65. *J Infect Dis* **166**, 683–684.

Grefte, J. M., van-der-Gun, B. T., Schmolke, S., van-der-Giessen, M., van-Son, W. J., Plachter, B., Jahn, G. & The, T. H. (1992b). The lower matrix protein pp65 is the principal viral antigen present in peripheral blood leukocytes during an active cytomegalovirus infection. *J Gen Virol* **73**, 2923–2932.

Griffiths, P. D., Emery, V. C. & Milne, R. (2009). Cytomegalovirus. In *Clinical Virology*, 3rd edn, pp. 475–506. Edited by D. D. Richman, R. J. Whitley & F. G. Hayden. Washington, DC: American Society for Microbiology.

Hamirally, S., Kamil, J. P., Ndassa-Colday, Y. M., Lin, A. J., Jahng, W. J., Baek, M. C., Noton, S., Silva, L. A., Simpson-Holley, M. & other authors (2009). Viral mimicry of Cdc2/cyclin-dependent kinase 1 mediates disruption of nuclear lamina during human cytomegalovirus nuclear egress. *PLoS Pathog* **5**, e1000275.

Herget, T., Freitag, M., Morbitzer, M., Kupfer, R., Stamminger, T. & Marschall, M. (2004). Novel chemical class of pUL97 protein kinase-specific inhibitors with strong anticytomegaloviral activity. *Antimicrob Agents Chemother* **48**, 4154–4162.

Kalejta, R. F. (2008). Tegument proteins of human cytomegalovirus. *Microbiol Mol Biol Rev* **72**, 249–265.

Kamil, J. P. & Coen, D. M. (2007). Human cytomegalovirus protein kinase UL97 forms a complex with the tegument phosphoprotein pp65. *J Virol* **81**, 10659–10668.

Krosky, P. M., Baek, M. C. & Coen, D. M. (2003). The human cytomegalovirus UL97 protein kinase, an antiviral drug target, is required at the stage of nuclear egress. *J Virol* **77**, 905–914.

Lischka, P., Rosorius, O., Trommer, E. & Stamminger, T. (2001). A novel transferable nuclear export signal mediates CRM1-independent nucleocytoplasmic shuttling of the human cytomegalovirus transactivator protein pUL69. *EMBO J* **20**, 7271–7283.

Lischka, P., Toth, Z., Thomas, M., Mueller, R. & Stamminger, T. (2006). The UL69 transactivator protein of human cytomegalovirus interacts with DEXD/H-box RNA helicase UAP56 to promote cytoplasmic accumulation of unspliced RNA. *Mol Cell Biol* **26**, 1631–1643.

Maeda, F., Ihara, S. & Watanabe, Y. (1979). Morphogenesis of nuclear inclusions and virus capsids in HEL cells infected with temperature-sensitive mutants of human cytomegalovirus. *J Gen Virol* **44**, 419–432.

Marschall, M., Helten, A., Hechtfischer, A., Zach, A., Banaschewski, C., Hell, W. & Meier-Ewert, H. (1999). The ORF, regulated synthesis,

and persistence-specific variation of influenza C viral NS1 protein. *Virology* **253**, 208–218.

Marschall, M., Stein-Gerlach, M., Freitag, M., Kupfer, R., van den Bogaard, M. & Stamminger, T. (2001). Inhibitors of human cytomegalovirus replication drastically reduce the activity of the viral protein kinase pUL97. *J Gen Virol* **82**, 1439–1450.

Marschall, M., Stein-Gerlach, M., Freitag, M., Kupfer, R., van den Bogaard, M. & Stamminger, T. (2002). Direct targeting of human cytomegalovirus protein kinase pUL97 by kinase inhibitors is a novel principle for antiviral therapy. *J Gen Virol* **83**, 1013–1023.

Marschall, M., Freitag, M., Suchy, P., Romaker, D., Kupfer, R., Hanke, M. & Stamminger, T. (2003). The protein kinase pUL97 of human cytomegalovirus interacts with and phosphorylates the DNA polymerase processivity factor pUL44. *Virology* **311**, 60–71.

Marschall, M., Marzi, A., aus dem Siepen, P., Jochmann, R., Kalmer, M., Auerochs, S., Lischka, P., Leis, M. & Stamminger, T. (2005). Cellular p32 recruits cytomegalovirus kinase pUL97 to redistribute the nuclear lamina. *J Biol Chem* **280**, 33357–33367.

McLaughlin-Taylor, E., Pande, H., Forman, S. J., Tanamachi, B., Li, C. R., Zaia, J. A., Greenberg, P. D. & Riddell, S. R. (1994). Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for $CD8^+$ virus-specific cytotoxic T lymphocytes. *J Med Virol* **43**, 103–110.

Mersseman, V., Besold, K., Reddehase, M. J., Wolfrum, U., Strand, D., Plachter, B. & Reyda, S. (2008a). Exogenous introduction of an immunodominant peptide from the non-structural IE1 protein of human cytomegalovirus into the MHC class I presentation pathway by recombinant dense bodies. *J Gen Virol* **89**, 369–379.

Mersseman, V., Bohm, V., Holtappels, R., Deegen, P., Wolfrum, U., Plachter, B. & Reyda, S. (2008b). Refinement of strategies for the development of a human cytomegalovirus dense body vaccine. *Med Microbiol Immunol (Berl)* **197**, 97–107.

Milbradt, J., Webel, R., Auerochs, S., Sticht, H. & Marschall, M. (2010). Novel mode of phosphorylation-triggered reorganization of the nuclear lamina during nuclear egress of human cytomegalovirus. *J Biol Chem* **285**, 13979–13989.

Mocarski, E. S., Shenk, T. & Pass, R. F. (2007). Cytomegaloviruses. In *Fields Virology*, 5th edn, pp. 2701–2772. Edited by D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott Williams & Wilkins.

Pande, H., Campo, K., Tanamachi, B. & Zaia, J. A. (1991). Human cytomegalovirus strain Towne pp65 gene: nucleotide sequence and expression in *Escherichia coli*. *Virology* **182**, 220–228.

Plachter, B., Klages, S., Hagelmann, S., Britt, W., Landini, M. P. & Jahn, G. (1990). Procaryotic expression of phosphorylated tegument protein pp65 of human cytomegalovirus and application of recombinant peptides for immunoblot analyses. *J Clin Microbiol* **28**, 1229–1235.

Prichard, M. N., Britt, W. J., Daily, S. L., Hartline, C. B. & Kern, E. R. (2005). Human cytomegalovirus UL97 kinase is required for the normal intranuclear distribution of pp65 and virion morphogenesis. *J Virol* **79**, 15494–15502.

Prichard, M. N., Sztul, E., Daily, S. L., Perry, A. L., Frederick, S. L., Gill, R. B., Hartline, C. B., Streblow, D. N., Varnum, S. M. & other authors (2008). Human cytomegalovirus UL97 kinase activity is required for the hyperphosphorylation of retinoblastoma protein and inhibits the formation of nuclear aggresomes. *J Virol* **82**, 5054–5067.

Rechter, S., Scott, G. M., Eickhoff, J., Zielke, K., Auerochs, S., Muller, R., Stamminger, T., Rawlinson, W. D. & Marschall, M. (2009). Cyclin-dependent kinases phosphorylate the cytomegalovirus RNA export protein pUL69 and modulate its nuclear localization and activity. *J Biol Chem* **284**, 8605–8613.

Roby, C. & Gibson, W. (1986). Characterization of phosphoproteins and protein kinase activity of virions, noninfectious enveloped particles, and dense bodies of human cytomegalovirus. *J Virol* **59**, 714–727.

Sanchez, V., Angeletti, P. C., Engler, J. A. & Britt, W. J. (1998). Localization of human cytomegalovirus structural proteins to the nuclear matrix of infected human fibroblasts. *J Virol* **72**, 3321–3329.

Sanchez, V., Greis, K. D., Sztul, E. & Britt, W. J. (2000a). Accumulation of virion tegument and envelope proteins in a stable cytoplasmic compartment during human cytomegalovirus replication: characterization of a potential site of virus assembly. *J Virol* **74**, 975–986.

Sanchez, V., Sztul, E. & Britt, W. J. (2000b). Human cytomegalovirus pp28 (UL99) localizes to a cytoplasmic compartment which overlaps the endoplasmic reticulum–Golgi–intermediate compartment. *J Virol* **74**, 3842–3851.

Schmolke, S., Drescher, P., Jahn, G. & Plachter, B. (1995a). Nuclear targeting of the tegument protein pp65 (UL83) of human cytomegalovirus: an unusual bipartite nuclear localization signal functions with other portions of the protein to mediate its efficient nuclear transport. *J Virol* **69**, 1071–1078.

Schmolke, S., Kern, H. F., Drescher, P., Jahn, G. & Plachter, B. (1995b). The dominant phosphoprotein pp65 (UL83) of human cytomegalovirus is dispensable for growth in cell culture. *J Virol* **69**, 5959–5968.

Somogyi, T., Michelson, S. & Masse, M. J. (1990). Genomic location of a human cytomegalovirus protein with protein kinase activity (PK68). *Virology* **174**, 276–285.

Thomas, M., Rechter, S., Milbradt, J., Auerochs, S., Muller, R., Stamminger, T. & Marschall, M. (2009). Cytomegaloviral protein kinase pUL97 interacts with the nuclear mRNA export factor pUL69 to modulate its intranuclear localization and activity. *J Gen Virol* **90**, 567–578.

Toth, Z. & Stamminger, T. (2008). The human cytomegalovirus regulatory protein UL69 and its effect on mRNA export. *Front Biosci* **13**, 2939–2949.

Toth, Z., Lischka, P. & Stamminger, T. (2006). RNA-binding of the human cytomegalovirus transactivator protein UL69, mediated by arginine-rich motifs, is not required for nuclear export of unspliced RNA. *Nucleic Acids Res* **34**, 1237–1249.

Warming, S., Costantino, N., Court, D. L., Jenkins, N. A. & Copeland, N. G. (2005). Simple and highly efficient BAC recombineering using galK selection. *Nucleic Acids Res* **33**, e36.

Wills, M. R., Carmichael, A. J., Mynard, K., Jin, X., Weekes, M. P., Plachter, B. & Sissons, J. G. (1996). The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T-cell receptor usage of pp65-specific CTL. *J Virol* **70**, 7569–7579.

Winkler, M., Rice, S. A. & Stamminger, T. (1994). UL69 of human cytomegalovirus, an open reading frame with homology to ICP27 of herpes simplex virus, encodes a transactivator of gene expression. *J Virol* **68**, 3943–3954.

Wolf, D. G., Courcelle, C. T., Prichard, M. N. & Mocarski, E. S. (2001). Distinct and separate roles for herpesvirus-conserved UL97 kinase in cytomegalovirus DNA synthesis and encapsidation. *Proc Natl Acad Sci U S A* **98**, 1895–1900.