UL88 Mediates the Incorporation of a Subset of Proteins into the Virion Tegument

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ABSTRACT Little is known about the human cytomegalovirus (HCMV) tegument protein UL88. Large-scale genomic studies have reported disparate results for UL88-null viruses, reporting both no phenotype and a >1-log decrease in virus titers. UL88 has also been reported to interact with UL69 and UL48, but the functional relevance of this interaction is unknown. Here, we report that UL88, which is conserved among different viral strains, is dispensable for production of infectious HCMV virions in multiple HCMV strains and cell types. However, the specific infectivity of HCMV virions suffers in the absence of UL88, as more genomes are required per PFU. This may be a result of altered virion tegument protein composition, as Western blot analysis shows a significant reduction in the tegument levels of pp71, UL47, and UL48 in viruses lacking UL88. While an interaction between UL88 and UL48 has previously been reported, we show that UL88 can interact with UL47; however, UL88 does not appear to be part of a stable complex consisting of UL47 and UL48. These findings identify an important role for UL88 in incorporating the viral proteins UL47 and UL48 into the virion tegument layer.

IMPORTANCE A better understanding of the role and functions of tegument proteins in HCMV, many of which remain uncharacterized, will contribute to our understanding of the biology of HCMV. The virus has a large genome, greater than 230 kb, and functional annotation of these genes is important for identifying novel targets for improving therapeutic intervention. This study identifies a role for a viral tegument protein with unknown function, UL88, in maintaining the proper tegument composition of HCMV virions. Virions produced in the absence of UL88 exhibit decreased fitness and require more genomes per infectious unit.

KEYWORDS HCMV, UL47, UL48, UL88, pp71, tegument, virus assembly

Human cytomegalovirus (HCMV) is the largest of the human herpesviruses, with a 230-kb genome composed of linear double-stranded DNA. The predicted protein coding capacity varies depending on the report, but the genome likely contains around 200 open reading frames (ORFs) with as many as 751 translated ORFs reported (1, 2). HCMV particles have a complex structure composed of a DNA-containing nucleocapsid surrounded by a tegument layer and lipid envelope. As many as 71 viral proteins are predicted to be incorporated into HCMV particles, of which as many as 35 reside in the tegument (3, 4). The roles of many of these proteins incorporated into the viral tegument layer are unknown, as fewer than half have been ascribed a function (reviewed in reference 5). The tegument proteins of HCMV have been proposed as potential therapeutic targets due to their key functions in the initiation of infection, virion assembly, and particle stability. Understanding the role of these proteins is essential for advancing our understanding of this complex virus and enhancing our treatment capabilities.

The host cell is exposed to tegument proteins upon viral entry; therefore, these proteins are functional immediately upon infection, prior to the onset of viral gene

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expression. The significance of these proteins is evident in their functions in various capacities throughout the course of infection, including viral genome delivery, gene expression regulation, modulation of host immune responses, nuclear egress, and virion envelopment. While the mechanisms of tegumentation and viral assembly are not well understood, these processes are believed to involve the stepwise addition of proteins through protein-protein interactions. Some of these interactions may also play important roles during infection outside tegumentation and virion assembly.

UL88 is a tegument protein of herpesvirus origin that is structurally conserved among different viral strains. The function of this protein in viral replication and pathogenesis is unknown. Viruses lacking UL88 expression have been reported to have either no growth defect (6) or a >1 -log defect in infectious-virion production (7). Since virions are produced in the absence of UL88, its gene is categorized as a dispensable or nonessential gene. Although very little has been reported on the function of UL88, it has been suggested to be a potential egress protein (8, 9). Several proteins have been reported to interact with UL88, including UL69, UL48, and pp71; however, the functional significance of these interactions remains elusive (3, 10). A genetic interaction with *UL138* has also been reported for *UL88*, as deletion of *UL138* led to an upregulation of *UL88* transcripts (11). With limited references to UL88 in the literature, a comprehensive study is needed to determine its role in infection.

In this study, we investigated the role of UL88 during HCMV infection using different cell types and different viral strains to understand its potential role in infection. We identify UL88 as a protein expressed later in infection that localizes to the viral cytoplasmic assembly compartment (cVAC), consistent with its incorporation in the virion tegument layer. Using both the AD169 and TB40/E strains of HCMV, we found that UL88 is completely dispensable for infection in fibroblasts and epithelial cells. Infectious virions are both produced and released in the absence of UL88; thus, our data do not support a role for UL88 as a virion egress protein. We did find, however, a reduction in the virion protein levels of several tegument proteins. This reduction resulted in altered specific infectivity, as more genomes were required per infectious unit. Thus, UL88 plays a role in incorporating a subset of tegument proteins into the virion.

RESULTS

UL88 is expressed during the latter part of HCMV infection and localizes to the cVAC. To understand the role that UL88 plays during infection, we first characterized its expression pattern. We found that *UL88* mRNA started accumulating to appreciable levels between 24 and 48 h postinfection (hpi), and the levels continued to rise throughout infection (Fig. 1A). To correlate this increase in mRNA with protein levels, we next generated a polyclonal antibody against the N terminus of UL88. Using this antibody, we performed a Western blot of lysates from a time course of infected cells. Consistent with the mRNA expression pattern, UL88 was first detected at 48 hpi, albeit just above the detection limit, and its levels increased at subsequent time points late in infection (Fig. 1B). The timing of the increase in protein was similar to that observed for the true late protein pp28 (UL99). To test whether UL88 was expressed with late kinetics, we added acyclovir to prevent DNA replication. As previously described, we observed a moderate decrease in IE1 protein and a significant decrease in IE2 (12), suggesting that the acyclovir treatment was successful. Addition of acyclovir prevented the expression of UL88 (Fig. 1C), suggesting that UL88 is in fact expressed with late kinetics. This is similar to what was reported for UL88 using a quantitative temporal viromics approach and the Merlin strain of HCMV (13). Thus, UL88 is expressed during the late times of infection, which is consistent with some of the proposed roles of UL88.

We next examined the localization of UL88 during infection. We generated a virus that expresses an enhanced green fluorescent protein (EGFP)-UL88 fusion protein, with EGFP fused to the N terminus of UL88. We found that EGFP-UL88 accumulated in a juxtanuclear location (Fig. 1D), reminiscent of the viral cytoplasmic assembly compartment. Since UL88 is found in the tegument layer, it is not surprising that it would locate to the assembly region. To confirm that UL88 is found in the cVAC, we stained for both

FIG 1 UL88 is expressed late in infection and localizes to viral assembly compartment. (A) Quantitative PCR of *UL88* transcripts harvested from uninfected (UI) samples and from HCMV-infected (MOI, 3) samples at 24, 48, 72, 96, and 120 hpi. Data are averages for three biological replicates. (B) Western blot analysis of uninfected fibroblasts (Mock) and HCMV-infected (MOI, 3) fibroblasts at 24, 48, 72 and 96 hpi. Blots were probed for UL88, IE1, pp28, and tubulin. (C) Western blot analysis of HCMV-infected (MOI, 3) fibroblasts at 96 hpi treated with DMSO or acyclovir (Acy; 100 μ g/ml). Blots were probed for IE1/2, UL88, and actin. (D) Fluorescence micrograph of fibroblasts infected with HCMV (MOI, 3) expressing EGFP-UL88 from the viral genome at 96 hpi. (E and F) Immunofluorescence analysis of fibroblasts infected with HCMV (MOI, 3) expressing EGFP-UL88 (green) from the genome and stained for the viral proteins (red) pp28 and gB (E) or cellular proteins (red) GM130 and EEA1 (F). Nuclei stained with DAPI (blue). Bars, 10 μ m.

viral and cellular markers of the cVAC in cells infected with the EGFP-UL88 virus. We found that UL88 was in the same region as both pp28 and gB but did not share complete localization with either viral proteins (Fig. 1E). We also found that UL88 localized inside the GM130-containing Golgi ring, in the same area as EEA1-containing endosomes, although very little colocalization was detected with EEA1 (Fig. 1F). Thus, UL88 localizes to the juxtanuclear cVAC during infection.

UL88 is not required for production of infectious virions. We next wanted to investigate a role for UL88 in infection. We first generated two different viruses deficient for UL88 expression (Fig. 2A). In the first virus, we replaced the start codon of *UL88* with *galk* (*UL88-galk*). For the second UL88-deficient virus, the *galk* was removed in a way that replaced the two methionine codons at the beginning of *UL88* with stop codons (*UL88-STOP*). Since the stop codon of *UL87* overlaps the *UL88* start codon, we engineered the mutated region so that the *UL87* stop codon was separated from the mutated *UL88* start codon to preserve the amino acid sequence of UL87, as depicted in Fig. 1A. Western blot analysis of infected human dermal fibroblast (HDF) lysates confirmed that these viruses did not express UL88 (Fig. 2B), while pp28 expression confirmed the presence of virus infection.

Utilizing our UL88-deficient viruses, we infected fibroblasts to determine if UL88 was required for a productive infection. A previous genome-wide study of open reading frames suggested that infection may be moderately affected in the absence of UL88 (7). However, we did not detect any reduction in infectious virions with our two UL88-deficient viruses compared to wild-type (WT) AD169 (Fig. 2C). Since we mutated only the first two methionines, and our antibody was generated against the N terminus of UL88, we could not rule out the possibility that translation initiation occurred from an internal methionine, resulting in a truncated form of UL88 that was functionally sufficient. To rule out this possibility, we generated a third virus which had an added a stop mutation in place of the codon for methionine at amino acid position 216 (*UL88-2xSTOP*) (Fig. 2A). Similar to what was observed for our other UL88-deficient

FIG 2 UL88 is not required for production of infectious virions or spread in fibroblasts. (A) Schematic depicting the approach for generating UL88-null viruses. The green line depicts the start codon of *UL88*. Red lines depict the stop codon of *UL87*. Orange lines indicate the sequence positions of premature stop codons added to *UL88*. The positions of these stop codons in the ORF are depicted by a red X. Arrows indicated the orientation of the ORFs. (B) Western blot analysis of fibroblasts infected (MOI, 3) with wild-type (WT) HCMV or UL88-null viruses (UL88-*galK*, UL88-STOP, and UL88-2xSTOP) at 96 hpi. Antibodies against UL88, pp28, and tubulin were used. (C) Growth curve analysis of fibroblasts infected (MOI, 3) with wild-type HCMV strain AD169 (WT AD169) or UL88-deficient viruses generated on the AD169 background (UL88-*galK*, UL88-STOP, and UL88-2xSTOP). Data are averages for three biological replicates. (D) Light micrographs of fibroblasts infected (MOI, 0.05) with wild-type HCMV, UL88-STOP or UL88-*galK* viruses at 3, 6, and 9 dpi.

viruses, we detected no defect in the production of virus titers. Thus, UL88 is not required for generating infectious AD169 virions in fibroblasts.

Although UL88 was not required for producing a single round of infectious virions, we wondered if it would be required for virus spread. We infected fibroblasts at a low multiplicity of infection (0.05) and monitored the spread throughout the monolayer. The cell monolayer was imaged every 3 days for 15 days. There was no apparent reduction in the virus-induced cytopathic effects of the UL88-deficient viruses compared to the wild type (Fig. 2D). Thus, the absence of UL88 is not required for the spread of AD169 throughout a fibroblast monolayer.

UL88 is not required for the production and spread of infectious virions, at least for the AD169 strain of HCMV. Since this strain has been widely propagated in culture and may have developed compensatory mechanisms for growth in the absence of UL88, we sought to investigate a potential role for UL88 using an alternative strain, TB40/E. Using a strategy similar to the one described for Fig. 2A for the UL88-mutant viruses in the AD169 background, we generated a UL88-*galK* virus and a UL88-STOP virus in the TB40/E background. We also generated a revertant strain in which UL88 expression was restored. Western blot analysis confirmed that the UL88-null viruses do not express UL88 and that expression is restored in the revertant strain (Fig. 3A). Of note, we found the ratio of UL88 to pp28 protein to be higher in TB40/E, which we found was due to higher levels of pp28 protein during infection with AD169 (data not shown). We

FIG 3 UL88 is not required for infection when deleted from alternate strains. (A) Western blot analysis of uninfected fibroblasts or fibroblasts infected with HCMV (MOI, 3) strain TB40/E (WT), UL88-deficient viruses generated on the TB40/E background (UL88-*galK* and UL88-STOP), and a revertant virus with restored UL88 expression (UL88-REV) at 96 hpi. Antibodies against UL88, pp28, and tubulin were used. (B) Growth curve analysis of infected-fibroblasts (MOI, 3) using the viruses described for panel A. (C) Spread of HCMV after low-MOI (0.05) infection of fibroblasts, presented as the percentage of nuclei infected at 3, 6, 9, 12, and 15 days postinfection. Data in panels B and C are averages for three biological replicates. (D) Immunofluorescence staining for immediate early protein at 15 dpi (pink) from one representative replicate of the samples used for panel C. Bar, 500 μ m.

performed growth curve analyses using these viruses and found that, similar to AD169, the absence of UL88 did not have any effect on the production of infectious HCMV virions after a single round of infection (Fig. 3B). We also investigated whether the absence of UL88 affected the spread of TB40/E. We found no difference in the spread of the viruses that expressed UL88 (WT TB40/E and UL88-REV) and those deficient for UL88 expression (UL88-*galK* and UL88-STOP), as measured by the percentage of infected cells at 3, 6, 9, 12, and 15 days postinfection (dpi) (Fig. 3C). Immunofluorescent staining of the immediate early proteins showed nearly complete infection of the monolayer for all four viruses (Fig. 3D). Thus, UL88 is not required to produce infectious virions in either the AD169 or TB40/E strain. Since we did not observe a difference between the two strains, the ensuing studies were done using only one strain, TB40/E, and the corresponding UL88 derivatives.

UL88 is not involved in virion egress. UL88 has been mentioned as potentially being involved in cytoplasmic egress (8, 9). Our growth curve titers were calculated from total virus, both cell associated and cell free, and would not be able to distinguish an egress phenotype. To test whether UL88 is involved in cytoplasmic egress, we infected fibroblasts and collected only the supernatant for titer analysis. We found no difference in the cell-free virus from viruses expressing UL88 and those null for UL88 (Fig. 4A). Electron microscopy analysis of the wild-type TB40/E and UL88-STOP viruses found enveloped virions in both the cytoplasm and extracellular space (Fig. 4B). This is consistent with our previous finding that UL88 is dispensable for infection and does not support a role for UL88 in envelopment.

FIG 4 UL88 does not affect spread or egress of HCMV. (A) Extracellular-virus titers of fibroblasts infected (MOI, 3) for 96 hpi with WT TB40/E, UL88-*galK*, UL88-STOP, or UL88-REV. Data are averages for three biological replicates. (B) Electron micrographs of cytoplasmic and extracellular virions at 96 hpi following infection (MOI, 3) of fibroblasts with wild-type TB40/E and UL88-STOP.

Although in the host, HCMV infection can occur in a number of different cell types and tissues, our analysis thus far had been restricted to fibroblasts. Thus, we next wanted to test whether UL88 is required for infection of alternate cell types. We first tested epithelial cells by infecting ARPE-19 cells and monitoring infection over 2 weeks. Using a virus that expresses mCherry from the viral genome, we monitored infection by imaging the infected cells at 3, 7, 10, and 14 days postinfection. We observed no difference in infection between the wild-type and UL88-deficient viruses (Fig. 5A). Thus, UL88 is not required for infection of the epithelial ARPE-19 cells.

We next investigated whether UL88 is required for infection in cell lines of monocytic lineage. Differentiated THP-1 cells have been shown to support a productive HCMV infection (14). Accordingly, we were able to recover infectious virus following infection of differentiated THP-1 cells. However, we observed no difference in virus production between our UL88-expressing and UL88-deficient viruses (Fig. 5B). Thus, UL88 is not required for infection of differentiated THP-1 cells. Our data therefore show that UL88 is not required for a productive HCMV infection in multiple strains and cell types.

Viruses lacking UL88 have altered specific infectivity. While we did not detect a defect in the production of infectious virions in the absence of UL88, one interesting difference we consistently observed between UL88-expressing and UL88-deficient

FIG 5 UL88 does not have a cell type-specific role during infection. (A) Spread of mCherry-expressing WT TB40/E, UL88-*galK*, and UL88-STOP viruses (MOI, 3) on ARPE-19 cells visualized at 3, 7, 10, and 14 dpi. Representative images from one of three biological replicates are shown. Bars, 500 μ m. (B) Virus titers at 120 hpi from differentiated THP-1 cells infected with WT TB40/E, UL88-*galK*, UL88-STOP, and UL88-REV. Data are averages for three biological replicates.

FIG 6 Virions produced in the absence of UL88 have altered specific infectivity. (A) qPCR of DNA isolated from equal infectious units of 5 distinct preparations each of wild-type TB40/E, UL88-STOP, and UL88-REV. (B) Protein from equal infectious units of the WT TB40/E, UL88-*galK*, UL88-STOP, and UL88-REV preparations were subjected to SDS-PAGE separation followed by silver staining. Numbers indicate fold increase ($\pm$ standard deviation) of lane intensity compared to the WT sample used for panel B plus two other independent virus preparations. (C) qPCR of viral DNA isolated from fibroblasts infected (MOI, 3) with wild-type TB40/E, UL88-STOP, UL88-*galK*, and UL88-REV viruses at 6 hpi. Data are averages for three biological replicates. (D) Growth curve analysis of fibroblasts infected with equal genomes (equivalent to an MOI of 3 for the WT) of wild-type TB40/E, UL88-*galK*, UL88-STOP, and UL88-REV. (E) Spread of HCMV in fibroblasts infected with equal genomes (equivalent to an MOI of 0.05 for WT) of HCMV strain TB40/E or the UL88-deficient viruses UL88-*galK*, UL88-STOP, and UL88-REV monitored at 3, 6, 9, 12, and 15 days postinfection by expression of EGFP from viral genomes. Data in panels D and E represent one experiment from three biological replicates.

viruses was a discrepancy in fluorophore expression per cell. Equal numbers of cells were infected, as the multiplicity of infection (MOI) was controlled for, but the fluorescence per cell was much brighter in the absence of UL88. Since this was observed for both mCherry and EGFP, which were driven from two distinct promoters (EGFP from the major immediate early promoter of HCMV and mCherry from the simian virus 40 [SV40] promoter), we hypothesized that the increased fluorophore expression was not merely a factor of altered promoter regulation. Among the possible explanations for the increased fluorophore expression is that there was a higher number of copies present due to an increase in the number of genomes delivered to the cell. To investigate the relationship between genome copies and infectivity, we isolated DNA from equal titers of five independent preparations of WT TB40/E, UL88-STOP, and UL88-REV. Quantitative PCR analysis of the DNA revealed that the UL88-STOP virus had more genomes per PFU than both the wild-type and revertant viruses (Fig. 6A). Thus, more genomes of a UL88-deficient virus are required to obtain an equivalent infectious titer.

We next wanted to investigate if the observed increase in DNA copies per titer correlated with an increase in virion protein. Silver stain analysis of protein harvested from equal titers of WT, UL88-STOP, UL88-REV, and UL88-*galK* virus preparations revealed increased protein content in viruses lacking UL88 (Fig. 6B). Thus, UL88-deficient viruses have altered specific infectivity, requiring more virions per PFU. We next tested whether the altered specific infectivity was a result of decreased entry into cells. We harvested viral DNA from infected cells following trypsinization to remove virions that had attached, but not yet entered, at 6 hpi before the initiation of genome replication. We found an increased number of genomes in cells infected with virions produced in the absence of UL88 (Fig. 6C), suggesting that these virions were able to

enter cells. The increased delivery of genomes from UL88-deficient viruses is also consistent with and could explain the observed increase in fluorophore expression.

To further show that virions produced in the absence of UL88 were less efficient in completing a productive infection, we infected cells with equal genomes from both UL88-expressing and UL88-deficient viruses at amounts corresponding to both high and low MOI, as calculated for the wild-type virus. As expected, we found that the UL88-STOP and UL88-*galK* viruses were able to produce infectious virions; however, this production was delayed and titers were reduced compared to wild-type and UL88-REV viruses at 120 hpi (Fig. 6D). The spread of the UL88-deficient viruses was similarly reduced after a low-MOI infection (Fig. 6E). Thus, taken together, the above data suggest that viruses produced in the absence of UL88 have an altered specific infectivity and require more genomes to initiate an infection equivalent to that caused by wild-type virus.

The virion tegument composition is altered in UL88-deficient viruses. Based on the above results indicating that UL88-deficient virions have an altered specific infectivity, we hypothesized that the virion composition may be altered in the absence of UL88. To test this hypothesis, we separated virions from dense bodies and other contaminants using a glycerol-tartrate gradient and probed for common tegument proteins. We found a significant reduction in the amount of UL47, UL48, and pp71 tegument proteins, while the levels of other tegument proteins were only slightly reduced or unaltered (Fig. 7A). Analysis of cell lysates at 96 hpi revealed a significant reduction in UL48 protein but only a modest reduction in UL47 (Fig. 7B). The reduction in UL48 protein does not appear to be a result of reduced transcripts (Fig. 7C). Similar to what was observed in virions, the cellular levels of other tegument proteins (pp150, pp28, and pp65) were unaffected by the absence of UL88. Thus, the reduction of UL48 protein in the tegument layer may partially be a result of lower cellular levels. Importantly, UL47 cellular levels are not affected by the absence of UL88. In sum, UL88 is required to maintain proper cellular and virion levels of UL48.

Previous studies reported a relationship between UL48 and UL88. UL48 and UL88 have been shown to interact in a yeast two-hybrid system, after cotransfection of cells and during infection (3, 15). UL48 also has a well-documented interaction with UL47 (16–18). We therefore hypothesized that UL88 could be part of a complex with UL47 and UL48. To test for an interaction between UL47 and UL88, we cotransfected plasmids expressing a C-terminally FLAG-tagged UL47 and UL88. A small amount of UL88 coprecipitated with UL47-FLAG, indicating that the two proteins can interact; however, the interaction was not robust, as only a small percentage of UL88 coprecipitated (Fig. 7D). We hypothesized that the interaction may be stabilized in the presence of other viral proteins, such as UL48. To test this, we subjected lysates from infected fibroblasts transduced with a lentivirus expressing UL47-FLAG to immunoprecipitation with FLAG beads. We found that UL48 coprecipitated with UL47, serving as a positive control for the immunoprecipitation (Fig. 7E). However, we did not detect any UL88 in the UL47 pulldown sample. Since we could not detect an interaction between UL47 and UL88 during infection and only a small amount of the transfected proteins interacted, UL88 is not likely part of the stable complex reported for UL47 and UL48. Thus, the UL88-dependent effects on cellular levels of UL48 and virion levels of both UL47 and UL48 are a result of a transient interaction or a mechanism independent of a direct interaction.

DISCUSSION

The tegument layer of HCMV contains a number of proteins, of both viral and cellular origin. Tegument proteins can play a role both early in infection upon viral entry and late in infection during assembly, envelopment, and egress. Thus, describing the role of these proteins is important for establishing a mechanistic understanding of the HCMV replication cycle. While phenotypes have been described for viruses deficient for expressing a number of these tegument proteins, very little is known about UL88. Primary sequence analysis of *UL88* from various HCMV strains, whether those that have been grown in laboratories for decades or recent clinical isolates, reveals a high degree

FIG 7 The composition of the tegument layer is altered in virions produced in the absence of UL88. (A) Western blot analysis of WT TB40/E, UL88-STOP, and UL88-REV purified on a sodium tartrate gradient. The blots were probed for the envelope protein gB and the tegument proteins UL88, UL47, UL48, pp71, pp150, UL71, pp28, and pp65. Gradient purifications were done for three distinct preparations of each virus, and the blots shown represent one of these preparations. (B) Western blot analysis of fibroblasts infected (MOI, 3) with WT TB40/E, UL88-STOP, UL88-*galK*, and UL88-REV at 96 hpi. The blots were probed with antibodies against the tegument proteins UL88, UL47, UL48, pp150, pp28, and pp65 and tubulin as a loading control. Results of quantitative analysis of the blots in panels A and B plus two additional repeats are shown to the right of the blots. Error bars represent standard deviations. (C) Quantitative PCR analysis of *UL48* transcript levels at 96 hpi in fibroblasts infected with WT TB40/E, UL88-STOP, and UL88-REV. Values are relative to the wild-type sample after normalization and are averages for three biological replicates. (D) Western blot analysis of immunoprecipitations using FLAG beads of HEK293TN cell lysates prepared 24 h after cotransfection with UL88 and either UL47-FLAG or vector control. Blots were probed for FLAG and UL88. (E) Fibroblasts expressing a vector control or UL47-FLAG were infected with WT TB40/E (MOI, 3), and lysates were subjected to immunoprecipitation analysis with FLAG beads at 96 hpi. Blots were probed for antibodies against FLAG, UL88, and tubulin. *, IgG heavy chain; ‡, nonspecific band.

of conservation, with only one or two, often very conserved, amino acid changes detected. While much of this primary sequence conservation is lost in *M88*, its murine CMV (MCMV) counterpart, secondary structure predictions of the two proteins reveal the potential for a high degree of structural homology, although this remains to be proven with structural data. To better understand the role of this conserved tegument protein, we determined the properties of UL88 during infection and investigated the impact of its absence on virus propagation.

Based upon the kinetics of transcript and protein accumulation, 48 to 72 h postinfection, UL88 appears to be expressed with delayed early or late kinetics. Furthermore, using a virus that expresses EGFP fused to the N terminus of UL88, we found that it localizes to the cytoplasmic viral assembly complex. This is consistent with its incorporation into the virion tegument layer. It is also consistent with the proposed role for UL88 as an

egress factor (8, 9). Thus, the expression and localization of UL88 to the assembly compartment would be consistent with a role in virion assembly and egress, a potential role suggested by the literature. However, our data show that UL88 does not play a role in these processes.

Our studies investigating the function of UL88 found that infectious virus could be produced in the absence of UL88 expression. Titers of UL88-deficient viruses were similar to those of wild-type virus, and this observation was independent of both the strain and cell type utilized for infection. Our data are consistent with a global mutational analysis study that used transposon mutagenesis to classify UL88 as a nonessential protein (6). These observations differ slightly from a second study that analyzed each ORF of HCMV and found that deletion of UL88 results in a moderate growth defect (10- to 10,000-fold reduction), as determined by titers of virus produced (7). This difference could be due to the strain, as neither our study nor the transposon study utilized the Towne strain of HCMV, or it could be due to the mutagenesis strategy affecting expression of the immediately proximal genes. The open reading frame of *UL88* has a slight overlap with the neighboring ORFs at both its 5' and 3' sites. Our mutagenesis strategy separated the 3' end of *UL87*, an essential gene, from *UL88* so that the sequence of the expressed UL87 protein remained unaltered.

Further analysis of virions from UL88-deficient viruses found that despite being able to produce infectious virions, more genomes were required to obtain a similar level of infection. This indicates that UL88-deficient viruses have altered specific infectivity. We found that genome levels were higher in cells infected with virions produced in the absence of UL88, indicating that these defective virions were able to enter cells. We also found that the viral tegument composition was altered in the UL88-deficient virions, with lower levels of UL47, UL48, and pp71. Interestingly, UL47 and UL48 have been implicated in a postentry, pre-immediate early (IE) gene expression role (18). Furthermore, UL48 has been shown to antagonize innate immunity (19). The tegument protein pp71 also has a well-documented role in establishing infection and antagonizing innate immune signaling (20–24). Thus, virions with reduced levels of these proteins would likely be less efficient at initiating infection, resulting in altered specific infectivity.

The reduced levels of tegument proteins in the absence of UL88 are interesting, as very little is known about how tegument proteins are packaged. The tegument levels of UL25, UL43, UL45, and UL71 are reduced in virions produced in the absence of the most abundant tegument protein, pp65 (25). Interestingly, neither pp65 nor UL71 was affected by the absence of UL88. However, virion levels of UL47 and UL48, which were both unaffected by the lack of pp65, were reduced when UL88 was not present. This indicates that different tegument proteins are responsible for the packaging of selected subsets of other tegument proteins.

How UL88 mediates incorporation of these proteins into the tegument remains to be elucidated. While both UL48 and UL47 protein levels were reduced in lysates from cells infected with UL88-deficient viruses, which could contribute to reduced tegument levels, the reduction in UL47 was not as drastic as for UL48. Therefore, UL88 may play an active role in incorporating these proteins into the tegument layer. Interestingly, UL88 has a reported interaction with both UL48 and UL69 (3, 15), and UL69 is part of the UL47-UL48 postentry, pre-MIEP (major immediate early promoter) expression complex (18) mentioned above. Therefore, we investigated whether UL88 was part of this complex. While we did detect an interaction between UL88 and UL47 when they were cotransfected, only a small percentage of UL88 interacted with UL47, and we did not detect a UL47-UL88 interaction during infection. Thus, UL88 is not likely to be a stable member of this complex, but it could interact with complex members in a transient fashion as part of tegumentation. With respect to pp71, it is important to note that UL88 is listed as protein that copurifies with pp71 from HCMV-infected cells (10). This suggests an interaction between UL88 and pp71 that could be important for tegument incorporation.

Studies of homologues from other herpesviruses often provide insight into the function of HCMV proteins, as many of the homologues have at least some degree of functional overlap. UL88 has been suggested to be a homologue of the HSV protein

UL21, BTRF1 protein of Epstein-Barr virus (EBV), and ORF23 of Kaposi's sarcoma-associated herpesvirus (KSHV) (9). Of these putative homologues, UL21 has been the most studied. In HSV-2, UL21 has been shown to be essential for viral replication (26). Deletion of UL21 from HSV-1, although not essential, has also been reported to adversely affect propagation of the virus (27, 28). While our results show an increase in defective viral particles in the absence of UL88, UL88-deficient viruses still produce infectious virions at wild-type levels. UL21 has a number of reported protein interactions, including being part of a complex with UL16 and UL11 (29). These interactions are not conserved, as UL88 does not interact with UL94 and UL99, the homologues of UL16 and UL11 (3). Thus, UL88 does not appear to share many attributes with its putative alphaherpesvirus homologues, and more research is necessary to investigate potential shared functions with its gammaherpesvirus counterparts.

While the results show that UL88 is not required to produce infectious virions, it is clear that it has a very important role in the tegumentation process, a process of which very little is known. The altered tegument results in altered specific infectivity and defective virus particles requiring more genomes per PFU. Although the exact mechanism of how this happens is not known yet, these results identify UL88 as a key tegument protein that loads or packages other tegument proteins into the virions.

MATERIALS AND METHODS

Cell culture. Normal human dermal fibroblasts (HDFs) (106-05n; Cell Applications Inc.), normal human lung fibroblast MRC-5 cells (CCL-171; ATCC) and HEK-293TN cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Corning) containing 10% fetal bovine serum (HyClone), 2 mM GlutaMAX (Gibco), 100 U/ml penicillin (Millipore Sigma), and 100 μ g/ml streptomycin (Corning). Retinal pigmented epithelium cells of the ARPE-19 line (CRL-2302; ATCC) were cultured in DMEM-F-12 medium (HyClone) supplemented as described above. The human monocyte cell line THP-1 (TIB-202; ATCC) was maintained in RPMI (Corning) supplemented with 10% fetal bovine serum (HyClone), 2 mM GlutaMAX (Gibco), 100 U/ml penicillin, 100 μ g/ml streptomycin (Corning), and 1.75 μ M β -mercaptoethanol. Prior to infection, THP-1 cells were treated with 8 nM phorbol myristate acetate (PMA) for 48 h. All cells were maintained at 37°C under 5% CO₂.

Cloning and BAC mutagenesis. A wild-type AD169 bacterial artificial chromosome (BAC) and three separate TB40/E BACS were used for recombineering: WT TB40/E, TB40/E expressing mCherry (30), and TB40/E expressing EGFP downstream of the IE2 protein and an inserted T2A sequence (31). Recombinant mutant viruses were generated using BAC mutagenesis in *Escherichia coli* strain SW105 and *galK* selection as described previously (32). Since the beginning of the *UL88* ORF overlaps the end of the *UL87* ORF, the recombineering strategy for the *UL88* mutants included separating these two ORFs to rule out a contribution of *UL87* to any observed phenotype (as depicted in Fig. 2A). The following primers were used for recombineering: *UL88-galK-For*, 5'-GTTGACGGCAGTTCTGAACCCACGTCGCCGCGAGCGCGGTTTGATACACGCTGTGACAAATATCATCGGCA; *UL88-galK-Rev*, 5'-CCCGGCGTCGGACGCTCTCCGGACGAAACGCCGCGGCGGAGCGCGGCTATTTTCACTGCTGCTCTT; *UL88-STOP-sense*, 5'-GGCAGTCTGTAAACCCAGTCGCGCGAGCGCGGTTTGATACGTTAACTTAATAGCGGCGCTGCCGCGCGGCGTTTGTCCGGAGGAGCGTCCGAC; *UL88-STOP-antisense*, 5'-GTCGGACGCTCTCCGGACGAAACGCCGCGGCGGAGCGCGCGCTATTAAGTTAACGTGATGCAAACCGCGCTCGCGCGAGCTGGGTTTCAAGACTGCC; *UL88-REV-sense*, 5'-CGGCAGTTCTGAACCCACGTCGCCGCGAGCGCGGTTTGATACGATGATGGAAGCCGCGCGCGCTGCCGCGCGGCTTCTCCGGAGGAGCGTCCGA; *UL88-REV-antisense*, 5'-TCGGACGCTCTCCGGACGAAACGCCGCGGCGAGCGCGCGGCTTCCATCATCTGATGCAAACCGCGCTCGCGCGAGCTGGGTTTCAAGACTGCC; *EGFP-UL88-For*, 5'-GTTGACGGCAGTTCTGAACCCACGTCGCCGCGAGCGCGGTTTGATACGATGGTGA GCAAGGGCGAG; and *EGFP-UL88-Rev*, 5'-CCCGGCGTCGGACGCTCTCCGGACGAAACGCCGCGGCGGCGAGCGCGCGGCTTCCATCATAGATCTGAGTCCGGACTT. BAC insertion sites were PCR amplified from purified BAC DNA and were verified by Sanger sequencing (Genewiz).

The pEGFPC1-*UL88* plasmid was cloned by amplifying the *UL88* ORF sequence from TB40/E BAC DNA with primers that introduced a 5' *Xho*I site and 3' *Eco*RI site for insertion into the corresponding sites of pEGFPC1. The *UL88* ORF was then amplified using primers that added a 5' *Xba*I and a 3' *Eco*RI sequence for insertion into the corresponding sites in pCDH to make pCDH-*UL88*. To generate pCDH-*UL47-FLAG*, the *UL47* ORF was amplified from TB40/E BAC DNA with primers that added a 5' *Eco*RI site, 3' 2 $\times$ FLAG sequence, and stop codon followed by a *Bam*HI sequence. The PCR product was inserted into the corresponding sites of the pCDH vector. All plasmid sequences were verified by Sanger sequencing (Genewiz).

Virus preparation, titration, infections, and growth curves. TB40/E, AD169, and derivatives listed above were generated from BAC stocks. BAC DNA was isolated from an overnight bacterial culture. Bacterial pellets were resuspended in resuspension buffer (50 mM Tris [pH 8.0], 10 mM EDTA, containing 200 μ g/ml RNase A) by gentle swirling on ice-water slurry and incubated 5 min at room temperature before addition of lysis buffer (0.1 M NaOH, 0.5% sodium dodecyl sulfate [SDS]). After 5 min, incubation neutralization buffer (sodium acetate, pH 5.5) was added, and samples were placed on ice for 10 min before centrifugation for 15 min at 4°C and 16,000 $\times$ g. DNA was precipitated by addition of an equal

volume of 2-propanol and centrifugation for 10 min at 4°C. The pellet was dissolved in 10 mM Tris (pH 8.0)–1 mM EDTA–150 mM NaCl for 10 min and placed at –80°C for 15 min following addition of 1 ml 100% ethanol. Samples were centrifuged 10 min at 4°C, and the pellet was dried and dissolved in 25 μ l of TE (10 mM Tris [pH 7.4], 0.1 mM EDTA). Purified BAC DNA was electroporated with pCGN-pp71 (a gift from D. Spector) into MRC-5 cells using a Gene Pulser Electroporation System (Bio-Rad) at a setting of 260 V and 960 μ F. Cells were plated and supplemented the next day with MRC5 cells. Upon reaching a 60% or greater cytopathic effect (CPE), cells were collected and stored in freeze medium (10% DMEM with 5% dimethyl sulfoxide [DMSO]) at –80°C as P0 stocks. For virus propagation, P0 stocks were added to MRC-5 cells in roller bottles (Greiner) and harvested 3 to 5 days after 100% CPE was observed. Virus was collected from cells and supernatant, and virions were concentrated by ultracentrifugation on a 20% sorbitol cushion at 20,000 rpm for 1 h at 20°C in a Beckman SW32 rotor. Titers were determined by serial dilutions on MRC-5 cells and were quantified by the immunological detection of immediate early proteins using the monoclonal D4 antibody against IE proteins (33). Images of stained monolayers were acquired on a Nikon Eclipse Ti inverted microscope, and fluorescent nuclei were quantified using NIS Elements software.

HCMV infections were carried out at an MOI of 0.05 for multistep growth curves and at an MOI of 3 for single-step growth curves (HDFs). For equal-genome experiments, the numbers of genomes required for MOI of 3 and 0.05 were calculated for wild-type virus, and the corresponding number of genomes was added for each of the other viruses. Cell counts were determined using a TC20 automated cell counter (Bio-Rad). For high-MOI infection of HDFs, virus was incubated with cells for 3 h at 37°C before the medium was replaced with fresh medium. THP-1 cells were differentiated 48 h prior to infection as described above, and virus was added by centrifugation at $1,000 \times g$ for 30 min at 22°C. The cells were then incubated at 37°C for 24 h, washed twice with $1 \times$ phosphate-buffered saline (PBS), and provided fresh medium. Virus was harvested for determination of titers at the time points postinfection indicated in the text and respective figure legends by scraping the cells into the medium, sonicating 10 times with 1-s pulses, vortexing for 15 s, and centrifuging at $18,000 \times g$ for 10 min. Supernatants were collected and flash-frozen in liquid nitrogen before storage at –80°C. Titers were calculated as described above. For spread assays, HDFs or ARPE-19 cells were infected with WT TB40/E, UL88-STOP, UL88-*galk*, and UL88-REV viruses expressing from the viral genome either mCherry from the SV40 promoter (30) or EGFP from the MIEP (31). The spread of infection was monitored by imaging cells on a Nikon Eclipse Ti inverted microscope for increase in total fluorescence. Alternatively, cells were fixed at the indicated times postinfection and stained with antibody against IE proteins, and the percent infected nuclei (visualized by DAPI [4',6-diamidino-2-phenylindole]) was quantified using NIS Elements software. For spread assays, images were taken every 3 or 4 days, as indicated.

Virion gradient purification. Virus was purified from cells and supernatants from 15-cm dishes as described above. Purified virus particles were resuspended in 2 ml of PBS and then layered onto 30-to-0% glycerol, 15-to-30% tartrate gradients (34) in 0.04 M sodium phosphate, formed by pouring a 7-layer step gradient as described previously (35). Gradients were centrifuged for 120 min at 6°C and 22,000 rpm in an SW-41 rotor. The virion layer was visualized with incandescent light and collected from the gradient. Virions extracted from the gradient were diluted with the sodium phosphate buffer and centrifuged at 22,000 rpm for 1 h using an SW-41 rotor. The virion pellets were resuspended in PBS and processed for Western blot analysis as described below.

Antibodies. An antibody against a UL88 peptide was generated by GenScript. Generation of monoclonal antibodies against IE1/IE2 and UL71 was previously described (31, 33). Commercial antibodies against pp28 (5C3; Virusys), CMV gB (2F12; Virusys), tubulin (DM1A; Millipore Sigma), GM130 (BD Biosciences), beta-actin (BA3R; Thermo Scientific), EEA1 (F.43.1; Thermo Scientific), and FLAG M2 (Millipore Sigma) were purchased. Antibodies against the following proteins were received as kind gifts: IE1 and IE2 from Jim Alwine (36), UL48 from Wade Gibson (37), pp150 (36-14) and pp65 (7B4) from David Spector (38), pp71 (2H10-9) from John Purdy (39), and UL47 from Jens von Einem (16). Anti-FLAG M2 magnetic beads for immunoprecipitations were purchased from Millipore Sigma. Horseradish peroxidase-conjugated secondary antibodies for Western blotting were purchased from Jackson Laboratories (goat anti-rabbit immunoglobulin) and GE Healthcare (sheep anti-mouse immunoglobulin).

Western blotting. Whole-cell lysates were prepared from HCMV-infected cells (MOI, 3) at the indicated times postinfection by harvesting lysates in radioimmunoprecipitation assay (RIPA) buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM aprotinin, 0.2 mM Na_3VO_4 , and 1 μ g/ml leupeptin. Where applicable, cells were treated with DMSO or 100 μ g/ml acyclovir (Millipore Sigma) at the time of infection. Samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes blocked with 5% milk in Tris-buffered saline (0.1% Tween). Blots were developed using SuperSignal West Pico Plus chemiluminescent substrate (Thermo Scientific) and imaged on a ChemiDoc MP imaging system (Bio-Rad).

qPCR. For detection of *UL88* transcripts, total RNA was isolated using an RNeasy minikit (Qiagen) according to the manufacturer's instructions with DNase treatment using a DNA-free DNA removal kit (Qiagen). Equal amounts of RNA were used to generate cDNA using the Invitrogen SuperScript III first-strand synthesis system (Thermo Scientific) with oligo(dT) primers according to the manufacturer's protocol. cDNA samples were cycled as follows on a StepOnePlus real-time PCR system (Thermo Scientific): 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, 60°C for 60 s, and 55°C for 30 s. Primers used for quantitative PCR are as follows: UL88-For, 5'-CGGTGATTCGGACATGTTT; UL88-Rev, 5'-CTCATTGCGAAACGGTGATA; GAPDH-For, 5'-ACCCACTCTCCACCTTTGAC; GAPDH-Rev, 5'-CTGTTGCTGTAGCCAAATTCGT; UL48-For, 5'-CAACGTTTCGTAAACCAAGCGA; and UL48-Rev, 5'-CTGCAGCGCTTCAGAA

TTTC. To calculate absolute transcript numbers, a standard curve was generated using a dilution series of the pEGFPC1-UL88 plasmid (1.62×10^5 to 1.62×10^9).

Virion genomes. For quantification of viral genomes from virion preparations, virions were treated with 250 U of Benzonase (Millipore Sigma) prior to isolation using the DNeasy blood and tissue kit (Qiagen) according to the manufacturer's protocol. For quantification of genomes in cells, DNA from HDFs infected with the wild-type, UL88-STOP, or UL88-REV viruses (MOI, 3) was harvested at 6 hpi using the aforementioned kit and manufacturer's protocol. qPCR was performed on the extracted DNA using the following primers: for HCMV UL99, 5'-GTGTCCTCCATCCCGACTCG-3' (forward) and 5'-TTCACAACGTC CACCCACC-3' (reverse), and for β -globulin, 5'-ACG TGG ATG AAG TTG GTG GT (forward) and 5' GCC CAG TTT CTA TTG GTC TCC (reverse). To generate a standard curve for UL99, 10-fold dilutions (2.77×10^3 to 2.77×10^9 copies) of the HCMV XbaI plasmid C (provided by David Spector) were subjected to qPCR analysis. A standard curve for β -globulin was obtained by diluting the β -globulin sequence from 8.86×10^5 to 8.86×10^{10} . For cellular samples, UL99 genomic equivalents were normalized to β -globulin.

Immunofluorescence microscopy and imaging. Coverslips containing HCMV-infected cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were blocked in PBS containing 10% human serum, 0.5% Tween 20, and 5% glycine. Triton X-100 (0.1%) was added for permeabilization. Primary and secondary antibodies were diluted in blocking buffer. Alexa Fluor 647- and Alexa Fluor 568-conjugated secondary antibodies (Invitrogen) were used as secondary antibodies. Coverslips were mounted with Prolong Diamond antifade mountant with DAPI (Thermo Scientific). Images were taken on a C2+ confocal microscope (Nikon). Images were processed using NIS Elements software.

Electron microscopy. Cells were seeded on 60-mm Permax tissue culture dishes (Nalge Nunc International). Cells were infected at an MOI of 3 for 96 h. At 96 hpi, cells were washed with PBS and were fixed at 4°C in fixation buffer (0.5% [vol/vol] glutaraldehyde, 0.04% [wt/vol] paraformaldehyde, 0.1 M sodium cacodylate). Cells were processed by the Microscopy Imaging Facility (Pennsylvania State University College of Medicine). Briefly, the fixed samples were washed three times with 0.1 M sodium cacodylate, followed by postfixation in 1% osmium–1.5% potassium ferrocyanide overnight at 4°C. Samples were then washed 3 times in 0.1 M sodium cacodylate, dehydrated with ethanol, and embedded in Epon 812 for staining and sectioning. Images were acquired using a JEOL JEM-1400 digital-capture transmission electron microscope.

Lentivirus production and cell selection. Lentivirus was made using the third-generation packaging system. The plasmids pMDLg/pRRE and pRSV-Rev were a gift from Didier Trono (plasmids 12251 and 12253; Addgene), and pCMV-VSV-G was a gift from Bob Weinberg (plasmid 8454; Addgene). Lentiviruses expressing UL47-FLAG or the pCDH vector were generated as follows: 293TN cells were transfected with the respective plasmids using the X-tremeGENE HP DNA transfection reagent (Roche) and harvested 72 h posttransfection. Supernatant was centrifuged at $2,000 \times g$ at 4°C for 10 min, flash-frozen, and stored at -80°C . For lentivirus delivery, subconfluent HDFs were transduced with lentivirus in the presence of 8 $\mu\text{g/ml}$ Polybrene (Sigma-Aldrich). Transduced cells were then passaged under 2 $\mu\text{g/ml}$ puromycin (Thermo Fisher Scientific) selection. For infection experiments, HDFs were transduced with lentivirus expressing the pCDH vector or UL47-FLAG. Following selection in puromycin, HDFs were infected at an MOI of 3 with TB40/E.

Immunoprecipitation. For immunoprecipitations of transfected cells, 293TN cells were transfected with 1 μg each of pCDH-UL47-FLAG and either the pCDH empty vector or the pCDH-UL88 expression construct by using the X-tremeGENE HP DNA transfection reagent (Roche) according to the manufacturer's protocol. Total protein was harvested 24 h posttransfection. For infected cells, lysates were harvested at 96 hpi. All cells were harvested in lysis buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA [pH 8.0], 10 mM tetrasodium pyrophosphate, 100 mM sodium fluoride, 17.5 mM β -glycerophosphate) containing protease inhibitors, sodium orthovanadate, and PMSF. Immunoprecipitations were performed with 500 μg of total protein incubated with FLAG antibody magnetic beads as per the manufacturer's protocol. Briefly, lysates were rotated overnight at 4°C, washed three times with 20 mM Tris–1.15 M NaCl–2.5% Tween 20, and prepared for Western blot analysis as described above.

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