Human cytomegalovirus (HCMV) encodes several tegument and envelope proteins that are essential for the cytoplasmic assembly of infectious virus (Britt et al., 2004; Mach et al., 2005; Silva et al., 2003; Smith & DeHarven, 1973). One such tegument protein is pp28, a true late and abundant outer tegument protein that is encoded by HCMV UL99 ORF (Chee et al., 1990; Meyer et al., 1988). Deletion of the UL99 ORF impairs the production of enveloped and infectious virus particles indicating that pp28 is essential for the cytoplasmic assembly and production of infectious virus (Britt et al., 2004; Silva et al., 2003). When transiently expressed in the absence of viral infection, pp28 is localized to the endoplasmic reticulum–Golgi intermediate compartment (ERGIC), an intracellular trafficking itinerary that requires the N-terminal 30 aa of pp28 (Sanchez et al., 2000b; Seo & Britt, 2006). The pp28 protein is localized to the assembly compartment (AC) in HCMV–infected cells, and viral functions are required for its localization to the AC late in infection (Sanchez et al., 2000a, b). Mutant forms of pp28 that fail to localize to the AC result in the production of non-enveloped and non-infectious cytoplasmic viral particles, findings consistent with the hypothesis that localization of pp28 to the AC is prerequisite for the viral assembly (Seo & Britt, 2007, 2006). In addition, the pp28 protein multimerizes and importantly, its multimerization appears to be a post-localization function within the AC required for the assembly and envelopment of infectious virus (Seo & Britt, 2008).

Using a deleitional mutagenesis, we demonstrated that only the N-terminal 61 aa of pp28 was required for the production of infectious virus and that a mutant virus that expressed only the first 61 aa of pp28 exhibited a viral assembly and replication phenotype indistinguishable from wild-type virus (Jones & Lee, 2004; Seo & Britt, 2006). Within this 61 aa sequence, the pp28 protein contains a stretch of 16 acidic aa (aa 44–59) that is required for its localization to the AC and assembly of infectious virus (Jones & Lee, 2004; Seo & Britt, 2006). Interestingly, a viral mutant containing only the first 50 aa (one-half of the acidic cluster of pp28) exhibited an intermediate phenotype that had normal intracellular trafficking to the AC, but exhibited a phenotype of decreased infectious virus production secondary to a delayed accumulation of this truncated form of pp28 in the AC (Seo & Britt, 2006, 2007). These findings suggested the possibility that phenotypic variations that were observed with different pp28 mutants could be secondary to differences in the functional activities specified within this stretch of 16 acidic aa in the N terminus of pp28.

In this study, we have investigated the possibility that the acidic cluster of pp28 has distinct functional subdomains that are required for its intracellular localization and, for its role in virus assembly. We have approached this question using a distinct functional domains within the acidic cluster of tegument protein pp28 required for trafficking and cytoplasmic envelopment of human cytomegalovirus

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Human cytomegalovirus UL99-encoded tegument protein pp28 contains a 16 aa acidic cluster that is required for pp28 trafficking to the assembly compartment (AC) and the virus assembly. However, functional signals within the acidic cluster of pp28 remain undefined. Here, we demonstrated that an acidic cluster rather than specific sorting signals was required for trafficking to the AC. Recombinant viruses with chimeric pp28 proteins expressing non-native acidic clusters exhibited delayed viral growth kinetics and decreased production of infectious virus, indicating that the native acidic cluster of pp28 was essential for wild-type virus assembly. These results suggested that the acidic cluster of pp28 has distinct functional domains required for trafficking and for efficient virus assembly. The first half (aa 44–50) of the acidic cluster was sufficient for pp28 trafficking, whereas the native acidic cluster consisting of aa 51–59 was required for the assembly of wild-type levels of infectious virus.
panel of pp28 chimeric proteins in which the acidic cluster of pp28 was replaced with well-defined heterologous intracellular sorting signals: (i) acidic dileucine motif of the cation-dependent mannose-6-phosphate receptor (CD-MPR) [ac-LL (MPR)], (ii) acidic amino acids of CD-MPR [ac(MPR)], (iii) DXXLL motif (DDHLL), (iv) acidic amino acids of furin [ac (Furin)], (v) NPXY motif (NPVY) or finally, (vi) YXXΦ motif (YQAL) to generate the chimeric protein and then, the chimera was fused with EGFP (Fig. 1). All constructs were confirmed by nucleotide sequencing, and expression of the chimeric protein of expected mass was demonstrated by immunoblot analysis following transient expression in 293T cells (data not shown). Each sorting signal is recognized by different domains/proteins and results in directing proteins that express these signals to different intracellular compartments (Bonifacino & Traub, 2003; Crump et al., 2001; Misra et al., 2002; Molloy et al., 1999; Robinson, 2004; Shiba et al., 2002). Previously, we have shown that pp28 is localized to the ERGIC and does not traffic to more distal compartments of the TGN in transiently expressed cells in the absence of other viral proteins (Sanchez et al., 2000b). Moreover, the first 30 aa of pp28 have been shown to be sufficient for its localization to the ERGIC (Seo & Britt, 2006).

We investigated the intracellular localizations of these chimeric proteins using transient expression in COS-7 cells followed by detection of pp28 using fluorescence microscopy (Fig. 2a). Consistent with previous studies (Seo & Britt, 2006), we found that the pp28 mutants, pp28Mut43 and pp28Mut61 in which the acidic cluster of pp28 is deleted or retained, respectively, were both localized to the ERGIC. As predicted, the trafficking of the pp28 chimeric proteins was indistinguishable from that of the pp28 mutants, and overlapped with an ERGIC marker, p115 (Fig. 2a). These results indicated that the intracellular localization of pp28 chimeric proteins only required the first 43 aa of pp28 and that the inserted sorting signals did not redirect trafficking of these proteins.

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**Fig. 1.** Generation of pp28 chimeric proteins. (a) Amino acid sequence of pp28 (190 aa) of HCMV strain AD169. The stretch of acidic amino acids (aa 44–59) of pp28 is identified by bold and underlined letters, and positions are listed above the sequence. (b) Generation of the pp28 chimeric proteins fused with EGFP. The acidic cluster of pp28 truncation mutant (aa 1–43) was replaced by a variety of sorting signals, ac-LL(MPR), ac(MPR), DDHLL, ac(Furin), NPVY and YQAL and then fused with EGFP by cloning into the pEGFP-N2 vector CMV-IE promoter, Cytomegalovirus immediate-early promoter.
Fig. 2. Intracellular localization of pp28 chimeric proteins. (a) The indicated pp28 chimeric proteins were transiently expressed in COS-7 cells. At 2 days post-transfection, the cells were fixed and stained with antibody specific to p115, an ERGIC marker. The pp28 chimeric proteins fused with EGFP are in green, p115 in red and nuclei stained with DAPI in blue. Bar, 10 µm. (b) Human fibroblast cells were electroporated with plasmid DNAs encoding pp28 chimeric proteins fused with EGFP and infected 2 days later with HCMV at an m.o.i. of 0.2. The cells were harvested at 7 days post-infection, fixed and stained with antibody specific to HCMV protein immediate early (IE)-1 to identify nuclei of infected cells and antibody to GM130, a Golgi (a secretory compartment) marker. The Golgi compartment is surrounding the AC in infected cells. Both IE-1 and GM130 are in red. The pp28 chimeric proteins fused with EGFP are in green. Note that only pp28 chimeric proteins containing an acidic cluster (pp28WT-EGFP, pp28Mut61-EGFP, pp28Mut50-EGFP, pp28Mut43-ac-LL(MPR)-EGFP, pp28Mut43-ac(MPR)-EGFP and pp28Mut43-ac(Furin)-EGFP) were localized to the AC in the juxtanuclear position of infected cells. Bar, 10 µm. (c) The mean fluorescent intensity of pp28Muts-EGFP localized in the AC was measured by ZEN microscope and imaging software (Zeiss). The graphs indicate the mean intensity of EGFP per the area of AC in each frame (from 8 to 10 cells of each sample) ±SEM (P<0.01). Note that the mean intensity of pp28Mut50-EGFP and pp28 chimeric proteins containing a foreign acidic cluster [pp28Mut43-ac-LL(MPR)-EGFP, pp28Mut43-ac(MPR)-EGFP and pp28Mut43-ac(Furin)-EGFP] was about 50% of that of pp28WT-EGFP and pp28Mut61-EGFP localized in the AC.
molecules when they were transiently expressed in the absence of virus infection.

To further study sorting signals required for the localization of pp28 chimeric proteins to the AC in HCMV-infected cells, we performed the transient expression/infection assay. The EGFP plasmids expressing pp28 chimeric proteins were transfected into primary human fibroblast (HF) cells and then infected with wild-type HCMV (Fig. 2b). Previously, we have shown that the acidic cluster of pp28 is required for its localization to the AC in cells infected with HCMV (Seo & Britt, 2006). We co-stained cells with antibodies specific to a HCMV protein immediate early (IE)-1 (P63-27) and a Golgi marker GM130 at 6 days post-infection to allow detection of the nuclei of infected cells and the Golgi compartment to help further define the intracellular localization of pp28 chimeric proteins. The localization of pp28 chimeric proteins and pp28 mutants revealed qualitative and quantitative differences. We measured the mean fluorescent intensity of EGFP accumulated in the AC by confocal microscopy (Fig. 2c). Consistent with previous studies (Seo & Britt, 2006, 2007), the mutant pp28Mut61-EGFP was concentrated in the AC with efficiency comparable to that of pp28WT-EGFP. The intensity of the fluorescence signal from the pp28Mut50-EGFP in the AC was only about 50 % of that from the pp28WT-EGFP (Fig. 2c). The pp28Mut43-EGFP, however, failed to localize to the AC and was distributed peripherally to the AC when compared with pp28WT-EGFP and pp28Mut61-EGFP (Fig. 2b). Interestingly, the intracellular distribution of pp28Mut43-DDHLL-EGFP, pp28Mut43-YPY-EGFP and pp28Mut43-YQAL-EGFP was similar to that of pp28Mut43-EGFP. This result indicated that dileucine, NPXY and YXXΦ motif did not direct the intracellular trafficking of pp28 to the AC in virus-infected cells. In contrast, the pp28Mut43-ac-LL(MPR)-EGFP, pp28Mut43-ac(MPR)-EGFP and pp28Mut43-ac(Furin)-EGFP were localized to the AC, although the concentration of these proteins in the AC appeared decreased compared with that of pp28WT-EGFP and pp28Mut61-EGFP (Fig. 2b, c). These findings indicated that the localization of pp28 to the AC was dependent on a stretch of acidic amino acids rather than other sorting signals such as dileucine, NPXY and YXXΦ motif. The results also indicated that the acidic cluster of pp28 could be interchanged with non-native acidic clusters and the intracellular trafficking of pp28 to the AC maintained.

To examine the effect on the production of infectious virus of altered intracellular localization of pp28 chimeric proteins expressing heterologous sorting signals, we generated a panel of recombinant viruses with stop codons inserted into the pp28Mut43-heterologous sorting signal coding sequence using a linear recombination system and a galactokinase gene (galK) selection system as previously described (Seo & Britt, 2006, 2008; Warming et al., 2005) (Fig. 3a). After electroporation of HF cells with these recombinant bacterial artificial chromosomes (BACs), infectious virus was not recovered from the recombinant BAC in which a stop codon was inserted at codon 44 [BAC-pp28Mut43 (aa 1–43)] or from the recombinant BAC in which the acidic cluster of pp28 was deleted [BAC-pp28Δac (Δ aa 44–59)] (Fig. 3b). Furthermore, we also failed to recover infectious viruses from the recombinant BACs expressing pp28 chimeric proteins containing sorting signal sequences such as dileucine, NPXY and YXXΦ motif ([BAC-pp28Mut43-DDHLL, BAC-pp28Mut43-NPVY and BAC-pp28Mut43-YQAL] (Fig. 3b). However, infectious viruses were recovered from the recombinant BACs expressing pp28 chimeric proteins containing foreign acidic clusters [BAC-pp28Mut43-ac-LL(MPR), BAC-pp28Mut43-ac(MPR) and BAC-pp28Mut43-ac(Furin)] (Fig. 3b). The results indicated that a stretch of acidic amino acids positioned after the first 43 aa of pp28 was sufficient for production of infectious virus and that the acidic cluster of pp28 could be replaced with non-native acidic clusters to yield a chimeric pp28 molecule that could support recovery of infectious virus.

Finally, to determine the kinetics of replication for viruses recovered from the recombinant BACs, we performed virus yield assays. Virus titres were determined by a fluorescent infectivity assay of HF cells infected with BAC-derived wild-type parent or recombinant viruses at the indicated times after infection (Andreoni et al., 1989). The replication kinetics and virus yields of all three recombinant viruses recovered from BAC-pp28Mut43-ac-LL(MPR), BAC-pp28Mut43-ac(MPR) and BAC-pp28Mut43-ac(Furin) were delayed, when compared with that of wild-type virus. The virus yield at 6 days after infection with the recombinant viruses in culture was approximately 10-fold less than that of the wild-type virus (Fig. 3c). The defective replication kinetics of all recombinant viruses were similar to that of pp28STOP50 virus that has been reported previously (Seo & Britt, 2006, 2007). These results were consistent with the results that suggested less efficient localization of pp28 chimeric proteins in the AC in transient expression/infection assay (Fig. 2b, c), arguing that a delayed and decreased viral growth of the recombinant viruses was secondary to delayed trafficking and/or reduced concentration of the pp28 chimeric proteins in the AC. Our findings indicated that although the role of the acidic cluster in pp28 was secondary to delayed trafficking and/or reduced concentration of pp28 chimeric proteins containing foreign acidic clusters, wild-type replication kinetics of HCMV required the expression of the native acidic cluster. Taken together, our results indicated that the native acidic cluster of pp28 has a distinct functional role in the trafficking of pp28 to the AC and in viral assembly within the AC, but that within this stretch of acidic amino acids, subdomains could exist that contributed to the efficiency of pp28 trafficking and/or virion assembly.

Recently, it has been reported that pp28 interacts with HCMV UL94, and that this interaction is important for their localization to the AC and production of infectious virus (Phillips & Bresnahan, 2012; Phillips et al., 2012). Deletion of an interacting domain (aa 37–39) of pp28 lead to mislocalization of both proteins and reduced production of progeny virions (Phillips et al., 2012), suggesting that the first 43 aa of pp28 are required for its localization to the AC through interactions with UL94. In our transient
Recovery infectious HCMV from UL99 recombinant BACs and replication kinetics. (a) Recombinant AD169 BACs expressing pp28 chimeric proteins were generated by the replacement of an acidic cluster (aa 44–59) of pp28 with the indicated heterologous sorting signal sequences and the insertion of a stop codon before aa 60 of pp28 using a homologous recombination system. A wild-type virus derived from the parent AD169 BAC was used as a positive control (Borst et al., 1999). Recombinant AD169 BACs containing a deletion of acidic cluster (aa 44–59) of pp28 or an insertion of a stop codon after aa 43 of pp28 were used as negative controls. Mutations were confirmed by sequence analysis. Recombinant viruses were recovered by electroporation of BAC DNA into HF cells. (b) Successful recovery of infectious viruses is listed by the (+) symbol on the right of the diagram. In all cases, at least three independent attempts were made to recover infectious virus. The infectious viruses were only recovered from a recombinant AD169 BACs containing a UL99 chimeric ORF encoding an acidic domain [BAC-pp28WT, BAC-pp28Mut43-ac-LL(MPR), BAC-pp28Mut43-ac(MPR) and BAC-pp28Mut43-ac(Furin)]. Although small, slowly expanding plaques could be observed from transfections of the other recombinant AD169 BACs that contained a UL99 that lacked an acidic cluster (−), we could neither isolate cell-free infectious virus from these cultures, nor did the plaques expand and infect the monolayer. (c) HF cells were infected with a parent AD169 BAC-derived wild-type virus or BAC-derived recombinant viruses at a m.o.i. of 0.1, and supernatants and cells were harvested at the indicated times. Virus yield was quantified by a fluorescence-based virus infectivity assay. Data are presented as means of duplicate samples and are representative of two individual experiments. Wild-type (■) versus pp28Mut43-ac-LL(MPR) (○), pp28Mut43-ac(MPR) (○) or pp28Mut43-ac(Furin) (▲) virus.
transfection and infection assay, only three pp28 chimeric proteins containing an acidic cluster, ac-LL(MPR), ac(MPR) and ac(Furin) localized to the AC late in infection, indicating that an acidic cluster as well as the first 43 aa of pp28 was required for its authentic intracellular localization. The localization patterns of the pp28 chimeric proteins were similar to those of pp28WT and pp28Mut61, indicating that the acidic cluster of pp28 can be replaced with foreign acidic clusters composed of homologous sequences and that localization of pp28 to the AC was independent of specific sequences in this domain. Consistent with previous studies, recombinant viruses that expressed pp28 chimeric proteins with specific sequences in this domain. Consistent with previous studies, recombinant viruses that expressed pp28 chimeric proteins were localized to the AC resulted in the production of infectious progeny virions. In contrast to these chimeric proteins, other chimeric proteins containing dileucine, NPXY and YXXΦ motif failed to localize to the AC and also failed to produce infectious virus. Thus, as previously claimed, localization of pp28 chimeric proteins to the AC was prerequisite for assembly of infectious virus and provided evidence that previously reported interactions between the product of UL94 and pp28 required for virus assembly could depend on the presence of at least a portion of the native acidic cluster. In addition, similar to findings from our studies of pp28Mut50, the results from pp28 chimeric proteins that contained foreign acidic clusters underscored the importance of efficient trafficking to the AC for optimal production of infectious progeny virions. Lastly, taken together our data indicated that the native acidic cluster of pp28 was required for wild-type levels of virion production rather than in simply acting qualitatively as a trafficking motif that directed this protein to the AC.

There are two possibilities for the requirement of this native acidic cluster of pp28 in its role of virus assembly. One possibility is that the acidic cluster of pp28 has separate domains with distinct functions. The first portion (aa 44–50) of the acidic cluster could be required for intracellular trafficking of pp28 and the second half (aa 51–59) plays a role in protein–protein interactions leading to efficient virion envelopment. The other possibility is that the intact acidic cluster was required for wild-type levels of virion envelopment in that a full-length acidic cluster (16 acidic aa) or specific sequence composition (a duplicated GEDDD sequence) could be required for efficient interactions with viral or cellular proteins that participate in virion envelopment. Even the pp28 chimeric protein containing a long stretch of acidic dileucine motif [ac-LL(MPR), EEESEERDDHLLPM, 14 aa] which is composed of seven acidic aa (EEESEERDD) together with a dileucine motif (DDHLLPM) did not fully restore virus production in the mutant virus to wild-type levels of viral envelopment and assembly. These results suggested that the requirement of the native acidic cluster of pp28 for envelopment of infectious virus was secondary to a unique sequence composition related to a duplicated GEDDD motif, although the requirement for an acidic amino acid domain of at least 16 aa regardless of sequence composition cannot be excluded.

In conclusion, the acidic cluster of pp28 has distinct functional domains, the first half of the acidic cluster of pp28 appears to be sufficient for its trafficking to the AC and can be interchanged with non-native acidic clusters, whereas the second half of the native acidic cluster is required for envelopment of infectious virus and the production of wild-type levels of infectious particles.

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