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Requirement of the N-terminal residues of human cytomegalovirus UL112-113 proteins for viral growth and *ori*Lyt-dependent DNA replication

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The UL112-113 region of the human cytomegalovirus (HCMV) genome encodes four phosphoproteins of 34, 43, 50, and 84 kDa that promote viral DNA replication. Co-transfection assays have demonstrated that self-interaction of these proteins via the shared N-termini is necessary for their intranuclear distribution as foci and for the efficient relocation of a viral DNA polymerase processivity factor (UL44) to the viral replication sites. However, the requirement of UL112-113 N-terminal residues for viral growth and DNA replication has not been fully elucidated. Here, we investigated the effect of deletion of the N-terminal regions of UL112-113 proteins on viral growth and *ori*Lyt-dependent DNA replication. A deletion of the entire UL112 region or the region encoding the 25 N-terminal amino-acid residues from the HCMV (Towne strain) bacmid impaired viral growth in bacmid-transfected human fibroblast cells, indicating their requirement for viral growth. In co-immunoprecipitation assays using the genomic gene expressing the four UL112-113 proteins together, the 25 N-terminal amino-acid residues were found to be necessary for stable expression of UL112-113 proteins and their self-interaction. These residues were also required for efficient binding to and relocation of UL44, but not for interaction with IE2, an origin-binding transcription factor. In co-transfection/replication assays, replication of the *ori*Lyt-containing plasmid was promoted by expression of intact UL112-113 proteins, but not by the expression of 25-amino-acid residue-deleted proteins. Our results demonstrate that the 25 N-terminal amino-acid residues of UL112-113 proteins that mediate self-interaction contribute to viral growth by promoting their binding to UL44 and the initiation of *ori*Lyt-dependent DNA replication.

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Introduction

Human cytomegalovirus (HCMV), which contains a 235-kb DNA genome, is a ubiquitous pathogen that causes congenital disease and disease in immunocompromised individuals (Mocarski *et al.*, 2013). HCMV DNA replication in lytic infection initiates from the replication origin (*ori*Lyt) within the unique-long (U_L) region of the genome (Anders and Punturieri, 1991). In transient transfection replication assays to determine the replication of *ori*Lyt-containing plasmid in co-transfected cells, 11 genetic loci were identified to be required for efficient *ori*Lyt-dependent DNA replication (Pari and Anders, 1993). These include six replication core proteins: DNA polymerase (encoded by UL54) and its associated polymerase processivity factor (UL44); single-stranded DNA-binding protein (UL57); and a heterotrimer consisting of DNA helicase (UL105), primase (UL70), and primase-associated factor (UL102) subunits. The viral genes encoding these replication core proteins have been found to be highly conserved in the genomes of other herpesviruses, including herpes simplex virus (HSV-1) (Chee *et al.*, 1990; Davison *et al.*, 2003), and are essential for viral replication (Dunn *et al.*, 2003; Yu *et al.*, 2003).

In addition to the six core replication genes listed above, HCMV requires five additional genetic loci (UL36-38, UL84, UL112-113, IE2, and TRS1/IRS1) for efficient *ori*Lyt-dependent DNA replication (Pari and Anders, 1993; Pari *et al.*, 1993; Smith and Pari, 1995; Sarisky and Hayward, 1996). UL84 self-interacts and interacts with IE2 (Colletti *et al.*, 2004), exhibits UTPase activity (Colletti *et al.*, 2005), and is thought to act as a replication initiator protein that recognizes an RNA stem-loop sequence found within the RNA/DNA hybrid region of *ori*Lyt (Colletti *et al.*, 2007). IE2, a strong transactivator encoded by the UL122-123 region, activates expression of the viral replication genes (Iskenderian *et al.*, 1996) and associates with the *ori*Lyt region together with pUL84 (Pari, 2008). The UL36-38 locus expresses viral proteins that inhibit cell death (Goldmacher *et al.*, 1999; Skaletskaya *et al.*, 2001; Goldmacher, 2005). IRS1/TRS1-encoded proteins interact with protein kinase RNA-dependent (PKR) and inhibit its activity in the type I interferon response (Child *et al.*, 2004; Marshall *et al.*, 2009). Therefore, whereas UL84 and IE2 directly act on *ori*Lyt, the proteins encoded from the UL36-38 and IRS1/IRS1 regions appear

to affect DNA replication indirectly by converting the cells to an environment suitable for viral DNA replication.

The UL112-113 region encodes four nuclear phosphoproteins (p34, p43, p50, and p84) with common amino (N)-termini of 252 amino acids (Staprans *et al.*, 1988; Wright *et al.*, 1988; Wright and Spector, 1989). The UL112-113 proteins have been shown to bind to DNA (Iwayama *et al.*, 1994) and enhance the IE2-mediated transactivation of the viral polymerase promoter (Iskenderian *et al.*, 1996; Kerry *et al.*, 1996; Li *et al.*, 1999). UL112-113 proteins have been found to play a role in viral DNA replication through co-transfection replication assays using *ori*Lyt-containing plasmids (Pari and Anders, 1993; Sarisky and Hayward, 1996; Kim and Ahn, 2010). This finding is consistent with genetic studies demonstrating that the expression of UL112-113 proteins is necessary for viral growth (Yamamoto *et al.*, 1998; Dunn *et al.*, 2003; Yu *et al.*, 2003). The UL112-113 proteins have been shown to accumulate in viral pre-replication foci and replication compartments (Penfold and Mocarski, 1997; Yamamoto *et al.*, 1998; Ahn *et al.*, 1999). The UL112-113 gene has also been shown to activate the lytic cycle of Kaposi's sarcoma-associated herpesvirus (Wells *et al.*, 2009).

We previously showed that UL112-113 proteins interact with each other and have cooperative effects with regard to their intracellular localization and their ability to relocate UL44 to pre-replication foci (Park *et al.*, 2006). Furthermore, we demonstrated that UL112-113 p84 is associated with UL44, UL84, and IE2 in virus-infected cells. Although p34, p43, p50, and p84 UL112-113 proteins all physically interact with UL44 *in vitro*, the specific interaction between p84 and UL44 is necessary for efficient viral growth in permissive cells transfected with HCMV bacmid DNAs, and for efficient *ori*Lyt-dependent DNA replication in co-transfection replication assays (Kim and Ahn, 2010).

Although co-transfection assays have demonstrated that the self-interaction of UL112-113 proteins via the shared N-termini is necessary for their intranuclear distribution as foci and for the efficient relocation of UL44 to viral replication sites, the requirement of UL112-113 N-terminal residues for viral growth and their involvement in DNA replication have not been fully elucidated. Here, we investigated the effect of deletion of the N-terminal residues of UL112-113 proteins on viral growth and *ori*Lyt-dependent DNA replication.

Materials and Methods

Cell culture

Human foreskin diploid fibroblast (HF) and 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a 5% CO_2 humidified incubator at 37°C.

Transient DNA transfection and electroporation

293T cells were transiently transfected via the *N, N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid-buffered saline (BBS) (Calbiochem) version of the calcium phosphate method, as previously described (Park *et al.*, 2006). HF cells were transfected via electroporation. The cells (2×10^6) in 200 μl of resuspension buffer were mixed with 3 μg of bacmid DNA, 4 μg of plasmid expressing the transactivator pp71, and 1 μg of plasmid expressing enhanced green fluorescent protein (EGFP, for monitoring the electroporation efficiency), and were then electroporated at 1,300 V and 40 ms using a Microporator MP-100 system (Digital Bio Technology). The cells were plated in T-25 flasks. When the cells became confluent, they were split into new flasks at a ratio of 1:2.

Plasmids

The expression plasmids for p34, p40, p50, and p84 proteins encoded from the UL112-113 region, and N- or C-terminal truncated versions of p84 (p84ΔN25, p84ΔN252, and p84 ΔC347) have been previously described (Park *et al.*, 2006; Kim and Ahn, 2010). The entire UL112-113 genomic gene was cloned into the pENTR vector (Invitrogen) (pMY8). The UL112-113(ΔN25) gene expressing the 25 N-terminal amino acid-deleted versions of the p34, p40, p50, and p84 proteins, and the UL112-113(ΔC347) gene expressing intact p34, p40, and p50 proteins and the C-terminal-truncated form (the 347 N-terminal amino acids) of p84 protein were produced on the pMY8 background by polymerase chain reaction (PCR). Expression plasmids for myc-tagged proteins were produced by moving the cDNAs or the genomic genes from pENTR vectors to a pCS3-MT (with a six-myc tag) (Roth *et al.*, 1991)-based destination vector using LR Clonase (Invitrogen). Plasmids expressing HA-p43 and HA-UL44 (Kim and Ahn, 2010), and plasmids expressing HA-IE2 (wild-type and mutant) (Park *et al.*, 2007) were previously described. A plasmid (pSP38) harboring HCMV replication-origin DNA (*ori*Lyt), and plasmids expressing six core replication proteins (UL54, UL44, UL57, UL105, UL70, and UL102) and auxiliary proteins (IE2 and UL84) have been previously described (Sarisky and Hayward, 1996).

Bacmid mutagenesis

The reagents for the conjugal transfer of sequences to the bacterial artificial chromosome (BAC) DNA (bacmid) in *Escherichia coli* and the HCMV (Towne strain) bacmid (Marchini *et al.*, 2001) were provided by Dr. H. Zhu (University of Medicine and Dentistry of New Jersey, USA). This HCMV bacmid contained the F plasmid sequences, a marker for chloramphenicol resistance (cm^r), and the GFP expression cassette, which was substituted in place of the US1 to US12 gene region. The mutagenesis procedure through conjugal transfer of mutated sequences (cloned in the transfer vector GS284) to HCMV bacmid-containing bacteria was previously described (Lee and Ahn, 2004; Lee *et al.*, 2004). The HCMV genomic DNA fragment encompassing the entire UL112 region and a part of the UL111a and UL113 regions was PCR-amplified using the primers LMV65 and LMV66. A 2.3-kb *Sph*I-*Eco*RI DNA fragment was prepared from this fragment and inserted into the *Sph*I/*Eco*RI sites of pUC18 (pMY1). pMY1 was used as a template for UL112 mutagenesis. To produce ΔUL112, the upstream region of UL112 in pMY1 was PCR-amplified as a *Sph*I-*Bam*HI fragment using

the primers LMV65 and LMV84 and ligated with the large *Sph*I-*Bam*HI fragment of pMY1, resulting in pMY2. UL112 (ΔN25), which has a deletion of the 25 N-terminal amino-acid region, was generated by PCR using the primers LMV 271 and LMV272 (pMY51). To create transfer vectors, the ΔUL112-containing fragment (as a *Nhe*I-*Sac*I fragment) and the UL112(ΔN25)-containing fragment (as a *Sph*I-*Sac*I fragment) were prepared after PCR amplification using the primers LMV85 and LMV86 and cloned into pGS284, a derivative of the positive suicide selection vector pCV442 (Marchini *et al.*, 2001), resulting in pMY4 and pMY52, respectively. The *Nhe*I-*Sac*I fragment containing the wild-type UL112 DNA was also cloned into pGS284 (pMY3) and used to rescue the UL112-mutated bacmids. The primer sequences used were LMV65, 5′-GACGGCATGCTGCGGCGATGC TGTC-3′; LMV66, 5′-GAGACAGCTGCGGCGGTCCTC GCGAC-3′; LMV84, 5′-GATCGGATCCATGACGCGGC-3′; LMV85, 5′-GATCGCTAGCGCATGCTGCGGCGATG CT-3′; LMV86, 5′-GATCGAGCTCGAATTCGGAGGAA CACGG-3′; LMV271, 5′-GACGCTAGCATGAATCAGAC TTTCGAC-3′; LMV272, 5′-GAAGCTAGCGACGCGGCG GCGCAAAGC-3′.

The procedure for conjugative transfer has been described elsewhere (de Lorenzo and Timmis, 1994; Smith and Enquist, 1999). Briefly, to transfer DNA sequences in pGS284 to the HCMV bacmid, *E. coli* S17-λpir containing the GS284 donor plasmid was conjugated with a RecA$^+$ derivative of *E. coli* DH10B (Smith and Enquist, 1999) harboring the bacmid DNA. Exoconjugates were selected sequentially with antibiotics and sucrose. The resultant mutant HCMV bacmid DNAs were examined by DNA sequencing and restriction enzyme digestion.

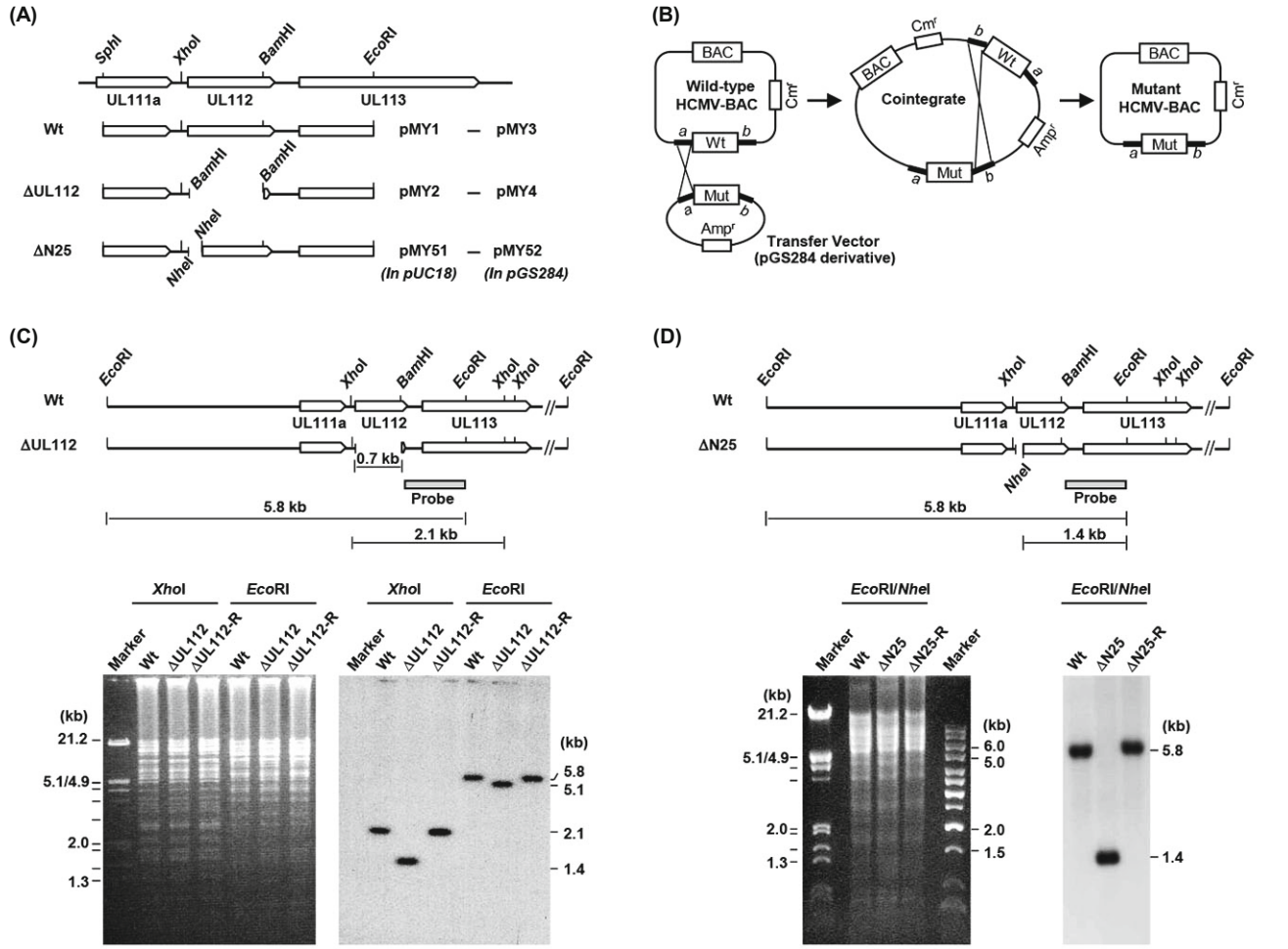

Fig. 1. Construction of HCMV bacmids containing ΔUL112 or UL112(ΔN25). (A) Construction of transfer vectors (in pGS284) for bacmid mutagenesis. The 2.3-kb *Sph*I-*Eco*RI fragment containing the UL112 gene and its derivatives containing the mutant UL122 genes were produced in pUC18 and then moved into pGS284 to produce transfer vectors in pGS284 (see 'Materials and Methods'). (B) Scheme for genetic exchange in *E. coli* (see 'Materials and Methods' for details). (C and D) (Top) Structures of the HCMV (Towne) bacmid genomes (wild-type and ΔUL112). The 7.6-kb *Eco*RI regions encompassing the UL111a and UL112-113 regions and the locations of the restriction enzyme sites used for deletion and mapping by Southern blot analysis are shown. (Bottom) Restriction fragment DNA patterns obtained following *Xho*I or *Eco*RI digestion of three HCMV bacmid DNAs (wild-type, ΔUL122, and revertant) (C) and patterns obtained following *Eco*RI/*Nhe*I digestion of three bacmid DNAs (wild-type, ΔN25, and revertant) (D) are shown on the left, and their Southern blot images obtained with the 769-bp probe (*Bam*HI-*Eco*RI fragment) are shown on right. The sizes (in kb) of λ-*Hin*dIII/*Eco*RI are shown in the marker lane.

Antibodies

Anti-HA rat monoclonal antibody (MAb) (3F10), either conjugated with peroxidase or labeled with fluorescein, and anti-myc mouse MAb 9E10 were purchased from Roche. Anti-myc rabbit polyclonal antibody was raised against bacterially purified his-tagged six-myc fragments. The anti-flag mouse MAb M2 was obtained from Sigma. The anti-UL44 (p52) mouse MAb was purchased from Advanced Biotechnologies, Inc. Secondary antibodies for cell staining were obtained from Jackson ImmunoResearch Laboratories, Inc.

Indirect immunofluorescence assay

Cells were washed in cold methanol at -20°C for 5 min, and then permeabilized with phosphate-buffered saline (PBS) for 5 min. Cells were then incubated with appropriate primary antibodies in PBS at 37°C for 1 h, followed by incubation with affinity-purified secondary antibodies at 37°C for 1 h. For double labeling, antibodies were incubated together. All slides were examined and photographed with a Carl Zeiss Axioplan 2 confocal microscope system running LSM510 software (Carl Zeiss).

Co-immunoprecipitation (co-IP) assays

293T cells (8×10^5) were harvested 2 days after transfection and sonicated in 0.7 ml of co-IP buffer [50 mM Tris-Cl; pH 7.4, 50 mM NaF, 5 mM sodium phosphate, 0.1% Triton X-100, containing protease inhibitors (Sigma)] by a microtip probe (Vibra-Cell; Sonics and Materials, Inc.) for 10 sec (pulse on: l sec, pulse off: 3 sec). Cell lysates were incubated with appropriate antibodies for 16 h at 4°C. Thirty microliters of a 50% slurry of protein A- and G-Sepharose (Amersham) were added and then the mixture was incubated for 2 h at 4°C to allow adsorption. The mixture was then pelleted and washed seven times with co-IP buffer. The beads were resuspended and boiled for 5 min in loading buffer. Each sample was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with appropriate antibodies.

Co-transfection replication assay

HF cells (2×10^6) were co-transfected via electroporation with plasmids (1 μg each) expressing replication proteins (replication core proteins, IE2, UL84, and UL112-113), and a plasmid (1 μg) containing the HCMV replication origin (pSP38). Transfected cells were plated on 100-mm dishes. At 5 days after co-transfection, total cellular DNAs were isolated using QIAamp DNA Mini Kits (QIAGEN). DNAs (10 μg each) were digested with *Xba*I and *Dpn*I and the digested DNA fragments were resolved by electrophoresis on a 1% agarose gel at 4°C, 100 V for 3 h. Replication products were analyzed by Southern blotting with [α-P^{32}]-labeled *Kpn*I-digested pSP38 DNA as a probe.

Results and Discussion

Deletion of the 25 N-terminal amino-acid residues of UL112-113 proteins impairs viral growth

Self-interaction of UL112-113 proteins is mediated via the shared N-terminal region encoded by the UL112 region. We previously showed that the 25 N-terminal amino-acid residues are required for the self-interaction of UL112-113 p84. To investigate the role of the N-terminal residues of UL112-113 proteins in the context of virus infection, we produced a mutant HCMV bacmid (GFP^+) containing a deletion of the 25 N-terminal amino-acid region, designated UL112(ΔN25). We also produced a mutant HCMV bacmid with the entire UL112 region deleted (ΔUL112) (Fig. 1A and B). The revertant bacmids were also produced. Deletions within the UL112 region in the mutant bacmids and their restoration in the revertants were confirmed by Southern blot analysis (Fig. 1C and D) and direct sequencing.

To determine the infectivity of the ΔUL112 and ΔN25 HCMV bacmids, the wild-type, mutant (ΔUL112 or ΔN25), and revertant bacmids were introduced into permissive HF cells and the appearance and spread of the GFP signals was monitored. Similar to the ΔUL112 bacmid, we found that the ΔN25 bacmid did not produce progeny virions, whereas the wild-

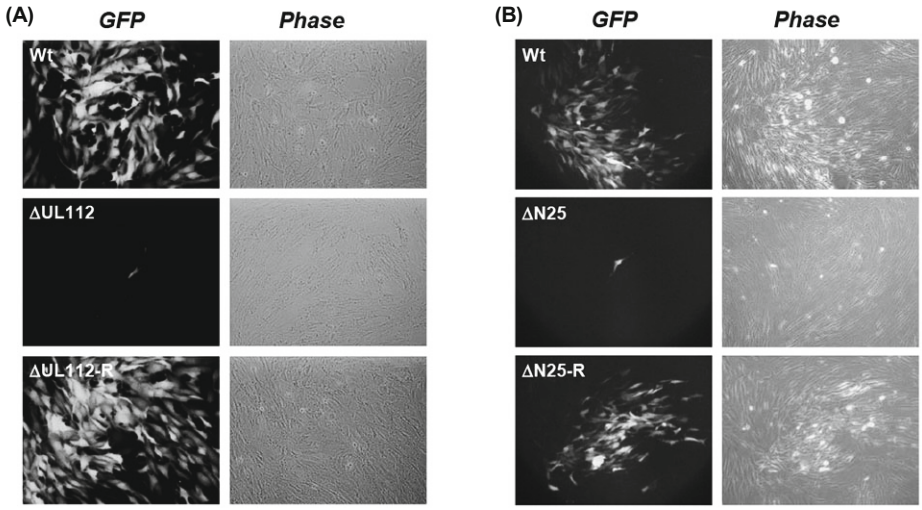

Fig. 2. Infectivity of HCMV ΔUL112 or UL112(ΔN25) bacmids. (A and B) HF cells were electroporated with HCMV bacmids: wild-type, ΔUL112, or its revertant (ΔUL112-R) for (A) and wild-type, ΔN25, or its revertant (ΔN25-R) for (B), and were monitored for the spread of GFP signals (see text). GFP images (left panels) and their phase-contrast images (right panels) were photographed at 16 days for (A) and 12 days for (B) after electroporation. HF cells that received ΔUL112 or ΔN25 HCMV-bacmids usually did not show a GFP-positive signal in most microscopic fields.

type and revertant bacmids efficiently produced progeny virions (Fig. 2). These results provide genetic evidence that the 25 N-terminal amino-acid residues of UL112-113 proteins are necessary for efficient viral growth.

The 25 N-terminal residues are necessary for stable expression of UL112-113 proteins and their self-interaction and interaction with UL44

We investigated the effect of deletion of the N-terminal residues of UL112-113 proteins on their stability and abilities to self-interact and interact with UL44. To express the myc-tagged p34, p43, p50, and p84 proteins together, an expression plasmid containing the UL112-113 genomic gene with a six-myc tag at its 5′ end was produced. A plasmid expressing the 25 N-terminal residue-deleted UL112-113, ΔN25, was also produced using this plasmid. A UL112-113 expression plasmid containing a stop codon within the UL113 region, ΔC347, which expressed myc-tagged p34, p43, p50, and C-terminally truncated p84 (Kim and Ahn, 2010), was also used as a control (Fig. 3A).

The N-terminal region of UL112-113 proteins is expected to be highly ordered (Fig. 3B). Therefore, we tested the effect of deletion of the 25 N-terminal residues on protein stability. The results of cycloheximide (CXH) chase assays showed that these 25 residues are necessary for stable expression of UL112-113 proteins, particularly p50 and p34.

We previously observed that deletion of the 25 N-terminal amino acids in p84 was sufficient to abrogate its self-interaction (Park *et al.*, 2006). We also investigated the effect of deletion of these 25 residues on self-interaction of UL112-113 proteins under conditions in which the four UL112-113 proteins were expressed together. In co-transfection/co-IP assays, myc-UL112-113 proteins (Wt) interacted with HA-p43, but these interactions were markedly reduced when ΔN25 UL112-113 proteins were used, indicating a role of these N-terminal residues for self-interaction. In a control experiment, expression of myc-tagged p34, p43, and p50, but not full-length p84 from UL112-113(ΔC347) was sufficient for binding HA-p43, suggesting that the reduced p43 binding by ΔN25 UL112-113 proteins was not due to reduced expression of specific UL112-113 proteins (Fig. 3D). These results demonstrated that the 25 N-terminal amino acids are necessary for efficient self-interaction of UL112-113 proteins even under conditions in which the four UL112-113 proteins are expressed together.

We next tested the requirement of the N-terminal residues of UL112-113 proteins on their interaction with UL44 using similar co-IP assays. We previously found that p34,

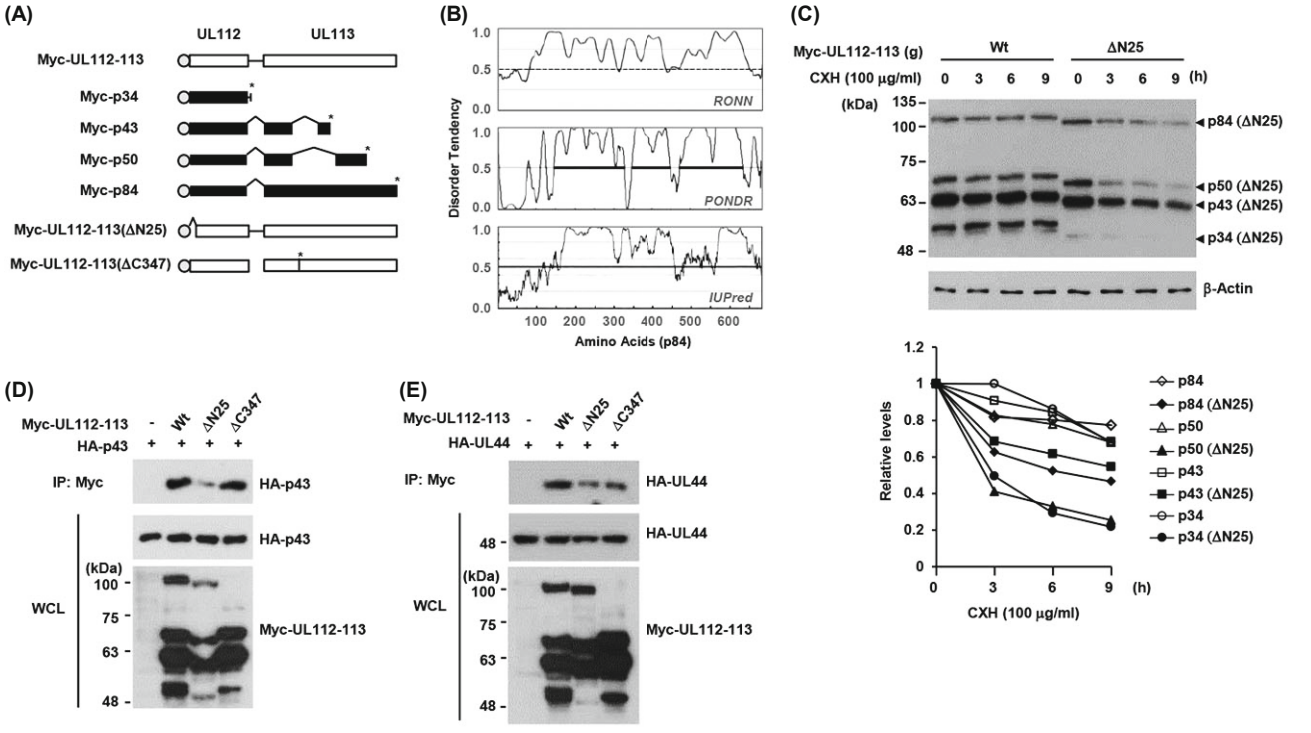

Fig. 3. Effects of deletion of the N-terminal residues of UL112-113 proteins on their stability and ability to self-interact and interact with UL44. (A) A diagram showing the structure of the wild-type and mutant UL112-113 genes used. The open bars indicate the UL112-UL113 genomic gene. The six-myc tags are indicated as filled circles. Asterisks indicate the positions of the stop codons. The mutant UL112-113 genomic genes (ΔN25 or ΔC347) are also indicated. (B) Prediction of the disorder tendency of UL112-113 p84 using the RONN, PONDR, and IUPred programs. The threshold used was 0.5 and higher scores reflect a propensity for disorder. (C) 293T cells were transfected with plasmids expressing myc-tagged UL112-113 proteins (wild-type or ΔN25). Cells were untreated or treated with 200 μg/ml of cycloheximide (CHX) and then further incubated for the indicated times prior to immunoblot assays using anti-myc or anti-β-actin antibodies. Changes of the relative levels of UL112-113 proteins are also indicated as graphs. (D and E) 293T cells were co-transfected with plasmids expressing myc-tagged UL112-113 proteins (wild-type or mutant) and either HA-p43 (D) or HA-UL44 (E) as indicated. At 48 h after transfection, total cell lysates were prepared and immunoprecipitated with anti-myc antibody. The total cell lysates and immunoprecipitated samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with anti-myc or anti-HA antibodies.

p43, p50, and p84 UL112-113 proteins all interact with UL44, and that p84 requires both the shared N-terminal region and the specific near-C-terminal region for UL44 binding (Kim and Ahn, 2010). Therefore, an expression plasmid containing the UL112-113(ΔC347) gene was also used as a control. The result of co-IP assays showed that the UL112-113 proteins expressed from both the ΔN25 and ΔC347 genes bound to UL44 less efficiently than did wild-type UL112-113 proteins (Fig. 3E). These results provide important insight into the interaction between UL112-113 and UL44 under conditions where the four UL112-113 proteins are expressed together. First, the 25 N-terminal residues of UL112-113 proteins were necessary for UL44 binding. Given the important role of these residues in self-interaction, it appears that the self-interaction of UL112-113 proteins facilitates UL44 binding. Second, although the specific C-terminal region of p84 was not necessary for self-interaction, the same region was required for efficient UL44 binding even in the presence of p34, p43, and p50. We previously showed that a mutant HCMV bacmid that expressed p34, p43, p50, and a C-terminally truncated from of p84 did not produce progeny virions (Kim and Ahn, 2010). Therefore, our results also demonstrate that the self-interaction-independent binding of UL112-113 p84 with UL44, which is mediated via the C-terminal region of p84, also plays a critical role in HCMV growth.

The 25 N-terminal residues are required for intranuclear localization of UL112-113 proteins as foci and their ability to relocate UL44

We also investigated the localization patterns of the 25 N-terminal residue-deleted UL112-113 proteins and their ability to relocate UL44. Consistent with a previous report (Park *et al.*, 2006), UL112-113 proteins were distributed as nuclear foci in HF cells; however, ΔN25 UL112-113 proteins were diffusely distributed in the nucleus (Fig. 4A). In cells co-transfected with both Flag- and HA-tagged UL112-113 proteins, wild-type UL112-113 proteins were efficiently co-localized as foci, whereas expression of both the wild-type and ΔN25 proteins together showed an intermediate distribution pattern compared to the patterns of the wild-type or ΔN25 protein alone (Fig. 4B). Furthermore, we investigated whether these 25 residues are required for relocation of UL44 by UL112-113 proteins. As we previously reported (Park *et al.*, 2006), UL44 showed a nuclear diffuse pattern, and co-expression of wild-type UL112-113 proteins effectively relocated UL44 to UL112-113 foci; however, this relocation of UL44 was not observed when ΔN25 proteins were co-expressed (Fig. 4C). These results indicate that the 25 N-terminal residues of UL112-113 proteins are required for UL44 relocation. Notably, ΔN25 UL112-113 proteins were largely co-localized with UL44 in nuclear diffuse forms (Fig. 4C). We attribute this pattern to the weak interaction between ΔN25 UL112-113 proteins and UL44 (as seen in Fig. 3E). Collectively, our data suggest that the absence of UL44 relocation by ΔN25 UL112-113 proteins is largely due to their inability to self-interact.

UL112-13 proteins interact with IE2 via the shared N-terminal regions in a self-interaction-independent manner

UL112-113 proteins have been shown to interact with IE2 in virus-infected cells (Kim and Ahn, 2010). However, it remains unknown whether p34, p43, p50, and p84 all interact with IE2 and whether their self-interaction affects IE2 binding. To address this issue, we carried out co-transfection/co-IP assays using plasmids expressing each one of the four UL112-113 proteins or plasmids expressing all four proteins together (Fig. 5A). We found that p34, p43, p50, and

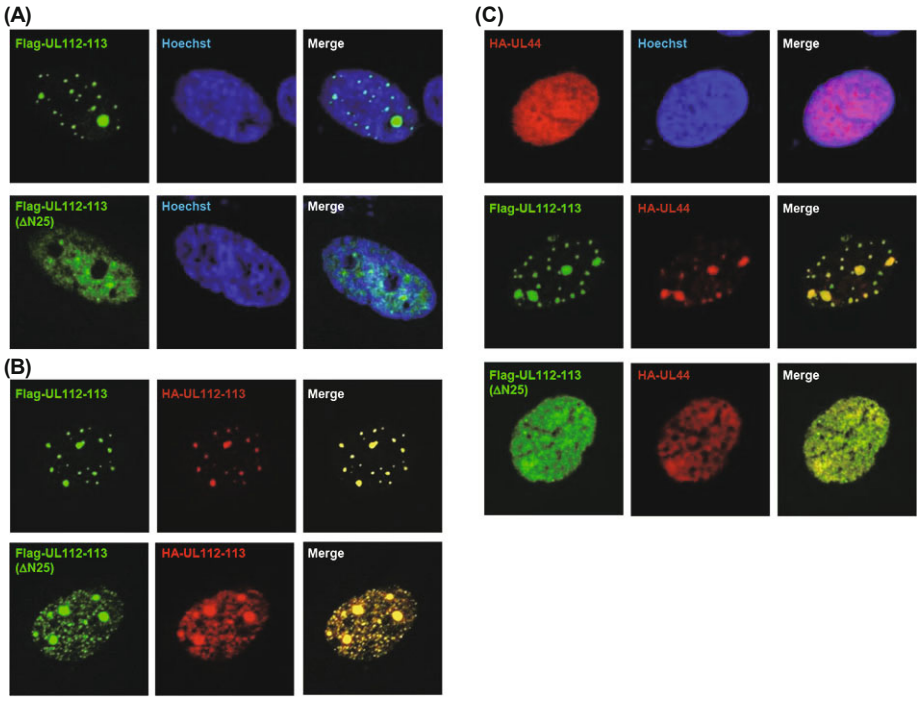

Fig. 4. Relocation of UL44 by UL112-113 proteins. (A and B) HF cells were transfected with Flag-UL112-113 proteins (wild-type or ΔN25 mutant) (A), or co-transfected with plasmids expressing Flag-UL112-113 proteins (wild-type or ΔN25 mutant) and HA-UL112-113 proteins (B). (C) HF cells were transfected with HA-UL44 (top panels), or co-transfected with plasmids expressing Flag-UL112-113 proteins (wild-type or ΔN25 mutant) and HA-UL44 (middle and bottom panels) as indicated. At 48 h after transfection, the cells were fixed with cold methanol and a confocal double-labeled indirect immunofluorescence assay was performed with anti-HA and anti-flag antibodies. To stain the cell nucleus, mounting solutions containing Hoechst were used. Two side-by side panels of single-labeled images and a third panel of the merged image are shown.

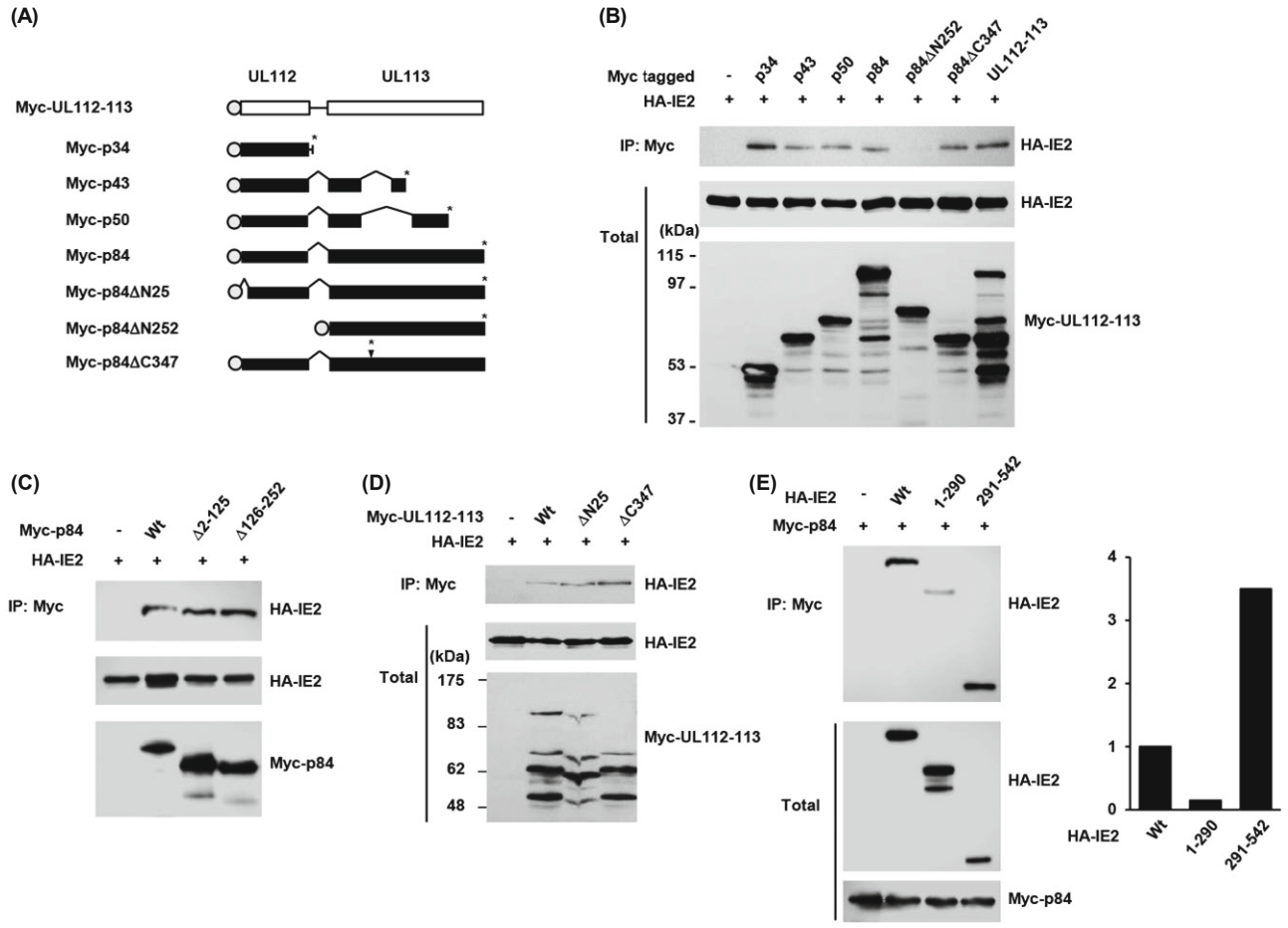

Fig. 5. Co-immunoprecipitation assays showing the self-interaction-independent binding of UL112-113 proteins with IE2. (A) A diagram showing the structures of p34, p43, p50, and p84 (closed bars) expressed from the UL112-113 genomic gene (open bars). The three mutant versions of p84 used are also indicated. The six-myc tags are indicated as filled circles. Asterisks indicate the position of the stop codons. (B) 293T cells were co-transfected with plasmids expressing myc-tagged UL112-113 proteins or HA-IE2 as indicated. At 48 h after transfection, total cell lysates were prepared and immunoprecipitated with anti-myc antibody. The total cell lysates and immunoprecipitated samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with anti-myc or anti-HA antibodies. (C and D) 293T cells were co-transfected with plasmids expressing myc-p84 (wild-type or mutant) and HA-IE2 (C), or plasmids expressing myc-tagged UL112-113 proteins (wild-type or mutant) and HA-IE2 (D) as indicated. At 48 h after transfection, co-IP assays were performed as described for (B). (E) 293T cells were co-transfected with plasmids expressing HA-IE2 (wild-type or mutant) and myc-p84 as indicated, and co-IP assays were performed as described for (B). The relative amounts of co-immunoprecipitated IE2 proteins after normalization with the amount of input myc-p84 are shown as graphs.

p84 interacted with IE2 individually as effectively as when all four proteins were expressed together, indicating that the shared 252 N-terminal amino acids may be involved in this interaction (Fig. 5B). Consistently, an N-terminal-truncated mutant of p84 (ΔN252) that lacked the 252 N-terminal amino-acid region did not interact with IE2, whereas its C-terminal-truncated mutant version (ΔC347) still effectively bound to IE2 (Fig. 5B). We also found that deletion of 125 N-terminal residues or residues from position 126 to 252 from p84 did not affect IE2 binding, suggesting that several regions within the 252 N-terminal amino acids are involved in the ability of p84 to bind to IE2 (Fig. 5C). Consistent with this result, when similar co-IP assays were performed using the UL112-113 genomic genes, deletion of the 25 N-terminal residues from the four UL112-113 proteins did not affect IE2 binding (Fig. 5D). These results indicated that the interaction between UL112-113 proteins and IE2 occurs in a manner independent of the self-interaction of UL112-113 proteins. Furthermore, the expression of p34, p43, p50, and a C-terminally truncated form of p84 from the UL112-113 (ΔC347) gene still allowed for effective IE2 binding, indicating that the interaction of UL112-113 protein with IE2 does not require the C-terminal region of p84, which is unique and not shared by other UL112-113 proteins (Fig. 5D). It appeared that the C-terminal half of IE2 was largely involved in p84 binding (Fig. 5E).

Requirement of the 25 N-terminal residues of UL112-113 proteins for efficient *ori*Lyt-dependent DNA replication

We further investigated whether the self-interaction of UL112-113 proteins is required for *ori*Lyt-dependent DNA replication in co-transfection replication assays using an HCMV *ori*Lyt-containing plasmid (pSP38) (Fig. 6A). HF cells were co-transfected with pSP38 and plasmids encoding six core

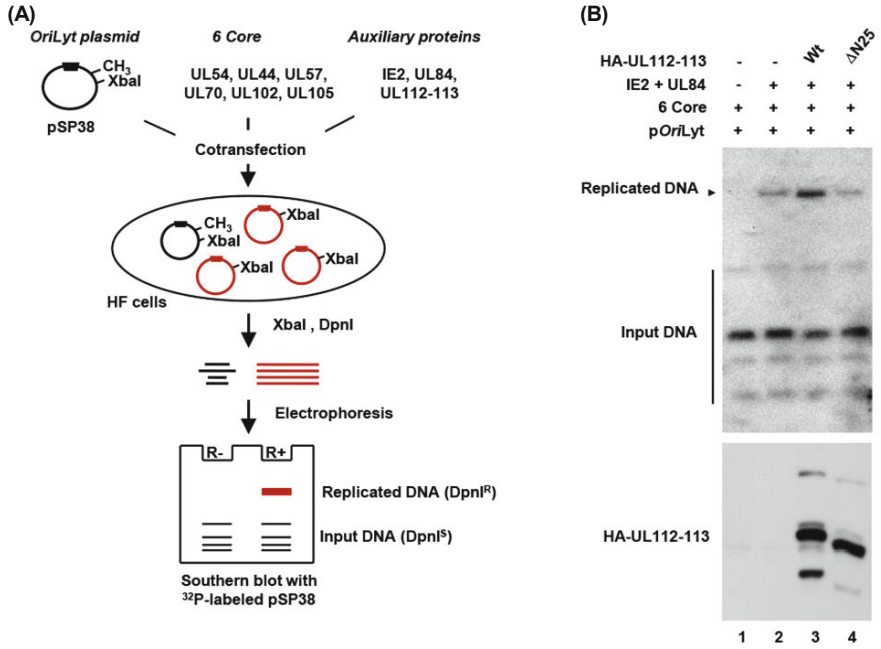

Fig. 6. Co-transfection replication assays using the HCMV *ori*Lyt-containing plasmid. (A) The scheme of co-transfection replication assays. HF cells were co-transfected with plasmids containing the HCMV lytic replication origin (*ori*Lyt) (pSP38), expressing six core replication proteins (UL54, UL44, UL57, UL105, UL70, and UL102), and auxiliary proteins (UL84, IE2, and UL112-113). At 5 days after co-transfection, total cellular DNAs isolated and digested with *Xba*I and *Dpn*I. DNA fragments were separated by electrophoresis and Southern blot analysis was performed with the [^{32}P]-labeled *Kpn*I-digested pSP38 DNA as a probe. The *Dpn*I digestion-resistant replicated DNAs are detectable if the replication is successful (R^+). (B) HF cells were co-transfected with plasmids encoding the HCMV replication origin (pSP38), six core replication proteins, UL84, IE2, and UL112-113 (wild-type or ΔN25 mutant) as indicated. The replicated *ori*Lyt-containing plasmid DNAs were detected by Southern blotting using pSP38 DNA as a probe, as described for (A).

replication proteins, UL84, IE2, and UL112-113 (wild-type and ΔN25 mutant). Consistent with earlier reports (Sarisky and Hayward, 1996; Kim and Ahn, 2010), the expression of the six core replication proteins, UL84, and IE2 together resulted in replication of *ori*Lyt-containing plasmid DNAs (Fig. 6B; lanes 1 and 2), and the addition of the UL112-113 plasmid expressing all of p34, p43, p50, and p84 markedly enhanced the replication levels (Fig. 6B; lanes 2 and 3). When a plasmid containing the UL112-113(ΔN25) gene was used, it did not enhance *ori*Lyt-dependent DNA replication (Fig. 6B; 3 and 4). The results of these co-transfection replication assays demonstrate that efficient replication of the *ori*Lyt-containing plasmids requires the 25 N-terminal residues of UL112-113 proteins.

UL112-113 proteins have been suggested to play a role in the formation of the replication initiation complex (Kim and Ahn, 2012). This stems from the findings that UL112-113 proteins interact with both replication origin-binding proteins (UL84 and IE2) and the viral polymerase processivity factor (UL44) that associates with the viral polymerase (UL54) (Kim and Ahn, 2010). In the present study, we found evidence that the 25 N-terminal amino-acid residues of UL112-113 proteins are necessary for efficient viral growth and that this activity correlates with the requirement of these residues in the self-interaction among four UL112-113 proteins and their interaction with UL44. Furthermore, we demonstrated that the self-interaction facilitated by these 25 residues is necessary for relocation of UL44 and *ori*Lyt-dependent DNA replication. Based on the analysis of a disorder tendency, the 25 N-terminal residues appear to be critical to form the proper structures of UL112-113 proteins. However, precisely how self-interaction among the four UL112-113 proteins promotes UL44 binding is not clear. Nevertheless, it can be inferred from this study that the self-interaction of UL112-113 proteins plays a critical role in the formation of the replication initiation complex via UL44 binding. An interesting question to address in future research is whether the interaction between UL112-113 proteins and UL44 directly regulates processivity of the viral polymerase complex.

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