Research Article

Human Cytomegalovirus Tegument Protein pUL23 Interacts with Capsid Protein pUL85

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Abstract | The tegument protein pUL23 of human cytomegalovirus (HCMV) plays an important in the virus pathobiology, however, its role in viral assembly and replication is poorly defined. In this study we demonstrated that HCMV pUL23 interacts with an essential component of capsid, the minor capsid protein (mCP, pUL85). Interaction was determined and confirmed with yeast two-hybrid, GST pull-down and co-immunoprecipitation analyses. This interaction may serve as a link in the processes of HCMV capsidation and tegumentation. This study provides further evidences in the functions of tegument proteins in the assembly of HCMV virons.

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Introduction

Human cytomegalovirus (HCMV), one of eight human herpesviruses (Roizmann et al., 1992; Edelman, 2005), is a major cause of morbidity and mortality among newborns and immnocompromised patients, such as AIDS patients and organ transplant recipients (Jacobson and Mills, 1988; Rubin, 1990). The virion of HCMV has a tripartite structure composed of the capsid containing a large, double-stranded DNA genome (240 kb) (Chee et al., 1990), the amorphous tegument, and the outermost envelope (Spaete et al., 1994).

HCMV UL23 ORF could be transcribed into a 284-amino-acid (aa) protein products (33kD) (Chee et al., 1990; Murphy et al., 2003), which was found as a virion tegument components and locates in the site of HCMV tegumentation (Adair et al. 2002). Predication of the conserved domain in pUL23 protein indicates that it contains two copies of conserved sequence motif of CMV US22 superfamily. Therefore, HCMV UL23 gene was identifies to belong to US22 gene family (Kouzarides et al., 1988; Efstathiou et al., 1992). Previous research shows US22 family gene M140 of murine cytomegalovirus (MCMV) is indispensable for viral capsid assembly.

To better understand the role of pUL23 in HCMV replication, we searched for the viral encoded interacting partner of HCMV pUL23 using yeast two-hybrid assay. Using this strategy, we were able to identify the minor capsid protein, pUL85, as an interacting partner of pUL23. The pUL85-pUL23 interaction

was also detected in GST pull-down assay and co-immunoprecipitation assay to validate the yeast two-hybrid findings. In addition, we observed a competitive binding of pUL23-pUL85 and pUL46-pUL85, the later interaction is a key step in HCMV capsid assembly. These results may provide more evidence suggesting pUL23 involving in HCMV capsid assembly and effective viral replication.

Materials and Methods

Cells

Cos-7 cells were maintained in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, 0.1 mg/ml streptomycin (pen/strp, Invitrogen). Transfections of Cos-7 cells were performed using Polyfect reagent (Qiagen) according to the manufacturer's instruction.

Plasmids Constructions

For construction of yeast two-hybrid bait plasmid pGBKT7-UL23, PCR-amplified UL23 ORF was cloned into *Eco*R I and *Sal* I site of pGBKT7 (Clontech). Similarly, PCR-amplified UL85 and its in-frame truncations, UL85-N (containing N-terminal region, residues from 1 to 111aa), UL85-M (containing central region, residues from 104 to 202aa) and UL85-C (containing C-terminal region, residues from 196 to 307aa) ORFs, were inserted into *Eco*R I and *Xho* I sites of pGADT7 vector, respectively.

To express glutathione S-transferase (GST) fusion proteins in prokaryotic cells, UL85 and its truncations region ORFs (UL85-N, UL85-M, UL85-C) were amplified by PCR and cloned into *Eco*R I and *Xho* I sites of pGEX-4T-1 vector, respectively.

To express pUL23, pUL85 and pUL46 in mammalian cells, PCR-amplified Flag-tagged-UL23, HA-tagged-UL85 and cMyc-tagged-UL46 ORFs were inserted into *Eco*R I and *Xho* I sites of pcDNA3.1(+) vector (Invitrogen), respectively.

Library Construction

Karina Schierling's methods (Schierling et al., 2004) were slightly modified to construct the genomic library of the HCMV strain Towne. Briefly, Towne $_{BAC}$, derived from the HCMV strain Towne (Marchini et al., 2001), was extracted and digested with DNase I to a medium size of 0.5-1.5 kb and blunt ended with DNA-Blunt Kit (Takara). Thereafter, a single A nucleotide was added by using A-Tailing Kit (Takara). Then the fragments was ligated into vector pGADT7 that had been digested with *Sma* I and treated with Taq DNA polymerase in the presence of dTTP. The ligation reaction mixture was electroporated into *E.coli.* DH5α. Colonies were harvested, and library DNA was isolated by Alkaline lysis method.

Yeast Two-Hybrid Screening

To screen the viral encoded interacting partner of pUL23, *Saccharomyces cerevisiae* strain AH109 was simultaneous transformed with the bait plasmid and prey plasmid by the lithium acetate method (Gietz and Woods, 2002). The transformants were selected for growth on SD/-Trp/-Leu/-His/-Ade plates at 30°C. The positive colonies were thereafter analyzed for β-galactosidase activity by filter lift experiments. Interactor plasmids from clones positive in both assays were rescued via transformation of *E.coli.* DH5α and sequenced.

In Vitro Gst Pull-Down Assay

GST fusion proteins were expressed in *E.coli.* BL21 (DE3) and purified with glutathione-Sepharose 4B beads according to the manufacturer (Novagen). Purified GST fusion protein was incubated with lysate of transfected Cos-7 cells in binding buffer (50mM Tris (pH 7.5), 150mM NaCl, 10% glycerol, 0.1mM EDTA, 2mM DTT). After washing with binding buffer, proteins pulled down by the beads were separated by SDS-PAGE and analyzed by immunoblotting.

Co-immunoprecipitation

Transiently transfected Cos-7 cells were lysed in RIPA lysis buffer (20 mM Tris(pH 7.5),150 mM NaCl, 1% Triton, 1mM EDTA). For anti-Flag IPs, 2μg Anti-FLAG (MBL) antibody was incubated with 0.2 ml lysates at 4°C with rotation for 1h before 25 μl Protein A/G bead (GE Healthy) was added and incubated for 3h. Proteins bind to the beads were eluted with 2×SDS-PAGE sample buffer at 95°C for 10min and analyzed by immunoblotting with anti-HA or anti-FLAG antibodies.

Results

pUL23 Physically Interacts with pUL85 in Yeast and in Mammalian Cells

To identify viral encoded interaction partners of pUL23 by yeast two-hybrid assay, we constructed a random

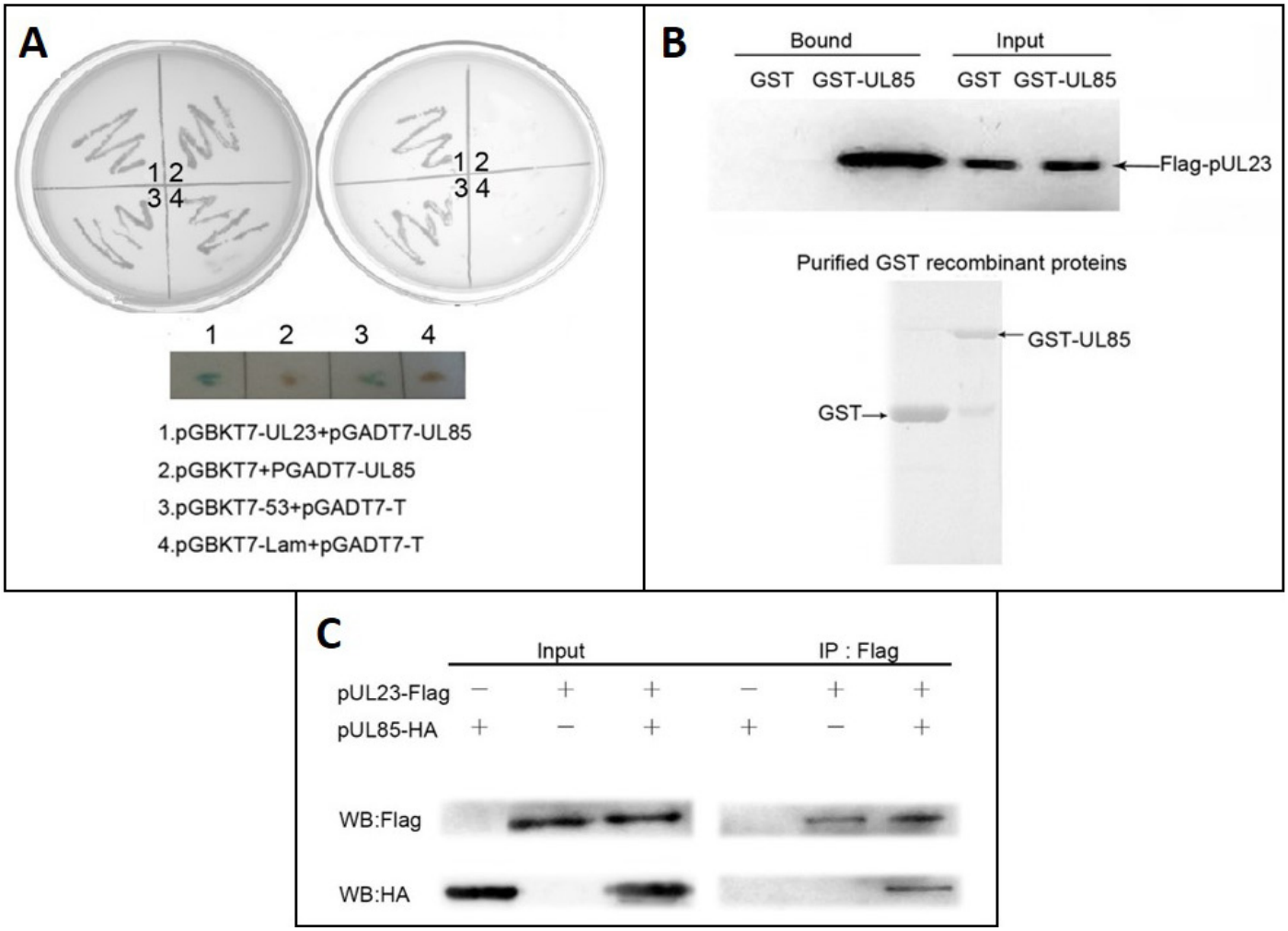

Figure 1: *Identification of the interaction between pUL23 and pUL85.* **A)** *pUL23 interacts with pUL85 in a yeast two-hybrid assay. Yeast clones grown on selective plates and formation of blue colour in filter paper containing X-gal indicate protein-protein interaction between full-length pUL23 and pUL85;* **B)** *GST protein or GST-fusion proteins of pUL85 bound to glutathion-Sepharose bead were incubated with lysates prepared from cos7 cells transfected pcDNA3.1(+)-UL23-Flag. Bound proteins were analyzed by western blot (WB) with anti-Flag Antibody;* **C)** *Identificaiton of the interaction between pUL23 with pUL85 by co-immunoprecipitation (Co-IP) analysis. Cos-7 cell were transiently transfected with vectors expressing both Flag-tagged-pUL23 and HA-tagged-pUL85 fusion proteins (co-transfected) or with either vector alone, lysed, and subjected to immunoprecipitation with anti-Flag antibody. The immunoprecipitated material was electrophoresed and detected with anti-Flag and anti-HA antibody, respectively*

genomic library of HCMV strain Towne fused to the GAL4 activation domain with an average insert size of 0.3 to 1.5 kb (Supplementary Figure S1). This size should ensure good coverage of protein domains. Our library consisted of ≥1×10^9 independent clones, and had titer value ≥5×10^8 cfu/mL, which means the probability that any fragment in the genome will occur at least once in the library is more than 99.99%.

Thereafter, we searched for viral encoded interacting proteins by performing yeast two-hybrid screening using our constructed genomic library with the bait plasmid pGBKT7-UL23 encoding a fusion of GAL4 DNA binding domain (GAL4-BD) with the UL23 open reading frame. We obtained two independent interacting clones, one of them corresponding to amino acid 165 to 286 of pUL85. We re-tested the interaction between full-length pUL23 and pUL85 in yeast with pGBKT7-UL23 and pGADT7-UL85, the result indicated that pUL23 protein could bind to pUL85 protein in yeast two-hybrid system. As positive and negative control, we test the interaction between SV40 large T antigen and p53 protein or human lamin C protein, which has been reported previously (Figure 1A).

To characterize the interaction between pUL23 and pUL85, *in vitro* GST pull-down assay was performed with GST or GST-pUL85 bound to glutathione-Sepharose beads. The beads were incubated with the lyastes prepared form Cos-7 cells transfected with pcDNA3.1 (+) -UL23-Flag, which expressed FLAG-tagged pUL23. Immunoblotting of the proteins bound to the glutathione- Sepharose beads revealed

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that Flag-tagged-pUL23 in the cell lysate was pulled down by GST-pUL85, but not GST alone (Figure 1B), indicating that pUL23 interacts with pUL85 *in vitro*.

To confirm the interaction between pUL23 and pUL85 in human cells, co-immunoprecipitation assays were performed. We transfected Cos-7 cells with expression plasmids for HA-tagged pUL85 and Flag-tagged pUL23 or control vectors. Protein complexes were precipitated with anti-Flag antibody and analyzed by immunoblotting with anti-HA antibody and anti-Flag antibody, respectively. The result demonstrated that pUL85 could be precipitated only when co-expressed with pUL23 in Cos-7 cells (Figure 1C).

N-terminal Region of pUL85 is Dispensable for the Interaction with pUL23

To map the interaction domains between pUL85 and pUL23, series truncations of pUL85 were fused to the GAL4 activation domain (GAL4-AD) or GST (Figure 2A) and then performed analyses by yeast two-hybrid assay or GST pull-down assay. As shown in Figure 2, pUL23 could bind to the central region and C-terminal region of pUL85 both in yeast two-hybrid assay (Figure 2B) and GST pull-down assay (Figure 2C), while the N-terminal 111 amino acids had no interaction with pUL23 in both assays.

pUL23 does not form a pUL23- pUL46-pUL85$_2$ Tetramer

During assembly of the HCMV capsid, pUL85 interacting with pUL46 to form a pUL46-pUL85$_2$ triplex proteins is required before they bind to major capsid-scaffold protein complexes. Therefore, pUL23 might be incorporated into pUL46-pUL85$_2$ triplex through interacting with pUL85. To test this hypothesis, we next investigated whether pUL23 could form a pUL23-pUL46-pUL85$_2$ tetramer. As shown in Figure 3, according to the results obtained in co-immunoprecipitation assays, pUL46 was not co-immunoprecipitated with pUL23 when co-expressed

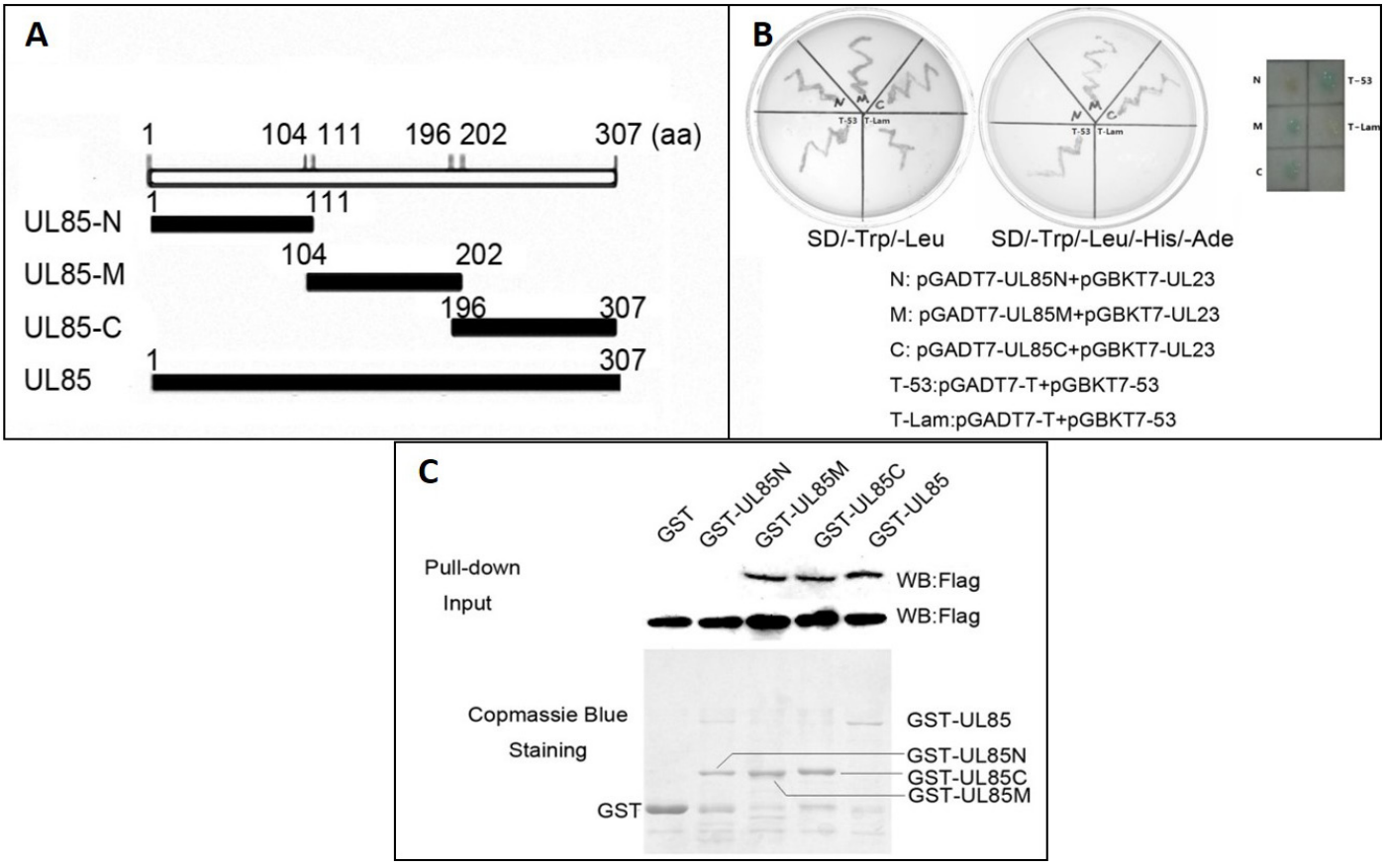

Figure 2: *Map the interaction domains between pUL85 and pUL23.* **A)** *Schematic illustration of pUL85 structures and indicated region truncated. The cDNA fragments encoding region 1-111aa, 104-202aa and 196-307aa of pUL85 were obtained by PCR and cloned into vector pGADT7 and pGEX4T-1, respectively, for yeast two-hybrid assay and GST Pull-down assay;* **B)** *Mapping the pUL85 binding domain to pUL23 by yeast two hybrid. Yeasts transformants were streaked on SD/-Trp/-Leu and SD/-Trp/-Leu/-His/-Ade selective plates and cultured (Left). Yeast colonies were analyzed for expression of the reporter gene β-galactosidase by filter lift assays (Right);* **C)** *Mapping the pUL85 binding domain to pUL23 by GST Pull-down. GST protein or GST-fusion proteins of pUL85-N / M / C bound to glutathion-Sepharose bead were incubated with lysates prepared from cos-7 cells transfected with pcDNA3.1(+)-UL23-Flag.Bound proteins were analyzed by western blot (WB) with anti-Flag Antibody*

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with pUL85, while pUL85 still interfere with pUL23 (Figure 3). It indicates that pUL23 might not simultaneously interact with pUL46 and pUL85 to form an oligomer.

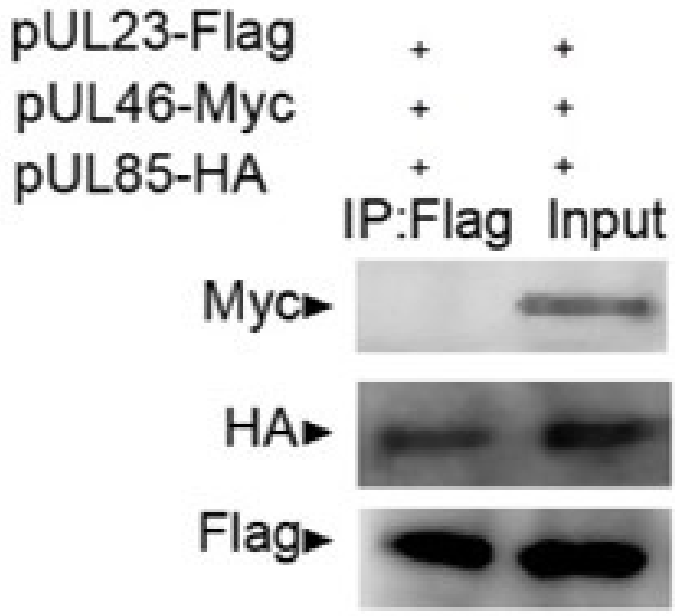

Figure 3: *No interaction between HCMV pUL23 and pUL46 was detected in over-expressed Cos-7 cells. Cos-7 cell were transiently transfected with vectors expressing both Flag-tagged-pUL23, HA-tagged-pUL85 fusion proteins and Myc-tagged-pUL46 (co-transfected), lysed, and subjected to immunoprecipitation with anti-Flag antibody. The immunoprecipitated material was electrophoresed and detected with anti-Flag, anti-HA antibody and anti-Myc antibody, respectively*

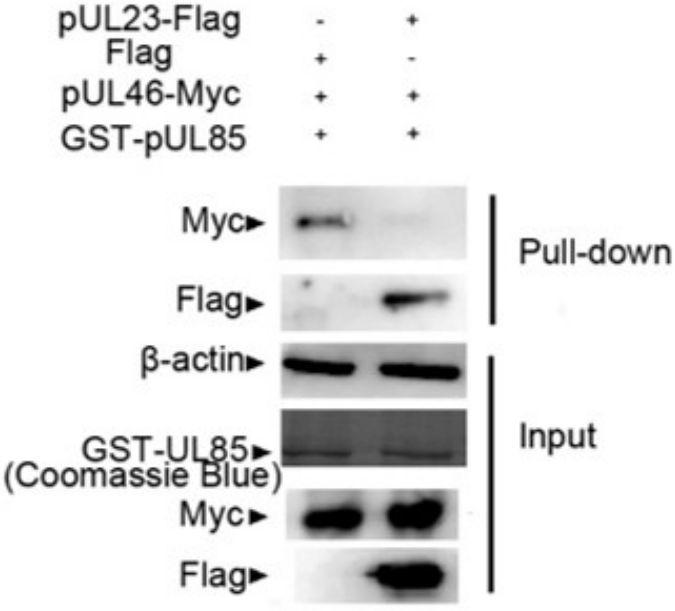

Figure 4: *Existence of pUL23 impairs the interaction between pUL85 and pUL46. GST-Fusion proteins of pUL85 bound to glutathion-Sepharose bead were incubated with Myc-tagged pUL46 together with Flag-tagged pUL23. Bound proteins were analyzed by western blot (WB) with anti-Myc and anti-Flag antibody, respectively*

pUL23 Competitively Binds to pUL85 against pUL46

Previous studies indicated that C-terminal region of pUL85 mediated the interaction between pUL85 and pUL46 in capsid assembly. Therefore, we next investigated whether the interaction between pUL23 and pUL85 jeopardize the formation of pUL46-pUL85$_2$ triplex by performing the *in vitro* GST pull-down assay as described before (Park et al., 2000). The same amount GST-pUL85 was incubate with cMyc-tagged-pUL46 along or with cMyc-tagged-pUL46 and Flag-tagged-pUL23, as shown in Figure 4, there were a huge decrease in the amount of pUL46 pulled down when pUL23 existed in the pull-down system (Figure 4). That indicated that pUL23 could inhibit *in vitro* interaction of pUL85 with pUL46.

Discussion

The results presented here are to our knowledge the first evidence of the interaction between the HCMV tegument protein pUL23 and the capsid protein pUL85. This physical interaction was confirmed by both *in vitro* and *in vivo*. However, a confirmation of this interaction in viral prolific growth still needs further studies.

The capsid assembly for HCMV is a highly-organized process that takes place in the nucleus, which is a quite conserved process shared by nearly all the other herpesviruses including α, β and γ subfamily (Gibson, 2006). The protein-protein interactions play an indispensable role in HCMV capsid assembly, which mainly involve pUL86 (major capsid protein, MCP) interacting with pUL80a (maturational protease precursor) and pUL80.5 (assembly protein precursor) to form the capspmeres; and pUL85 (minor capsid protein, mCP) together with pUL46 (mCP binding protein, mCBP) to form triplexes. Due to the fact that the nuclear localization signal (NLS) only exists in pUL46, but not in pUL85, the nuclear transport of HCMV pUL85 requires its interaction with pUL46. Moreover, it has been proved that mutation of C terminal region of mCP (pUL85) would greatly impair its interaction with mCBP (pUL46) in herpesviruses (Wang et al., 2011), which indicates this region is an essential part for capsid formation.

In our research, the observation that C-terminal region of pUL85, the key region mediating the interaction with pUL46, also involves in the interaction with

pUL23 lights on the possibility that pUL23 involves in the viral capsid assemble by regulation the interacting between pUL85 and pUL46. This hypothesis was partially proved by GST pull-down assay *in vitro*, although there still need more evidence to support this assumption.

In summary, we could show an interaction between the HCMV tegument proteins pUL23 and capisd pUL85, and this interaction thereby impairs the formation of pUL46-pUL85$_2$ triplex *in vitro*. These protein-protein interactions might be a mechanism to preciously control the whole process of HCMV capsid assembly.

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Authors' Contribution

S.L and J.H designed and performed the experiments; S.L analyze the data and drafted the manuscript; J.H revised the manuscript. All authors read and approved the final manuscript.

Supplementary Material

There is supplementary material associated with this article. Access the material online at: http://dx.doi.org/10.17582/journal.bjv/2016.3.5.133.139

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