Expression, identification, purification of HCMV UL148

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Abstract

Human Cytomegalovirus (HCMV), a herpesvirus, is a common opportunistic infection that is a major cause of morbidity and mortality worldwide especially in organ transplant recipients and neonates. Previous studies have shown that some HCMV low-passage clinical viral strains contain a unique region (UL/B '), and laboratory virus strains lack this region. Without this region, HCMV virulence was also attenuated, indicating that this unique region is critical for the pathogenicity of HCMV. This region contains the UL133-UL151 genes, which potentially encode proteins important for HCMV propagation and pathogenicity. Currently, there are few reports studying UL148 gene in the UL/B 'region, and its role in HCMV pathogenesis. In this report, a prokaryotic expression vector, pET32a-UL148 was constructed to express the UL148 protein. In order to obtain the highest expression level of UL148 protein, the expression conditions including IPTG concentration, temperature and time were optimized. The recombinant UL148 protein was purified using a Ni-NTA column, and the protein was identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (WB). Highly purified recombinant protein was obtained, which will be used for future study of UL148 function.

Keywords

HCMV, UL148, recombinant expression, Purification.

1. Introduction

Collectively, the cytomegaloviruses are ancient viruses that appear to infect the majority of vertebrates. Human cytomegalovirus is a b-herpesvirus with a large dsDNA genome that produces lifelong infections in humans. In most individuals, HCMV infections result in a mild febrile illness, but can lead to severe symptoms in immune-compromised patients or neonates. HCMV infections result in widespread viral dissemination throughout the body, with diverse cell types such as epithelial, endothelial, fibroblast, and smooth muscle cells supporting productive viral infection. To evade the host immune system, the virus induces a myriad of immunomodulatory path-ways to subvert the host innate and adaptive response 1. HCMV is also recognized as the leading cause of in utero viral infection, estimated to occur in approximately 0.64% of pregnancies in the United States 2.

Nucleotide sequence comparisons were performed on a highly heterogeneous region of three human cytomegalovirus strains, Toledo, Towne, and AD169. The low-passage, virulent Toledo genome contained a DNA segment of approximately 13 kbp that was not found in the Towne genome and a segment of approximately 15 kbp that was not found in the AD169 genome. These additional sequences were located at the unique long (UL)/B* (IRL) boundary within the L component of the viral genome. The additional DNA segment within the Toledo genome contained 19 open reading frames not present in the AD169 genome. These findings reveal a dramatic level of genome sequence complexity that may explain the differences that these strains exhibit in virulence and tissue tropism 3. These unique regions include the UL133-UL151 genes, which have been shown to potentially encode for proteins important for HCMV propagation and pathogenicity.

Two of the glycoproteins (gH/gL) expressed in HCMV virions, complexes with glycoprotein O (gO), forming gH/gL/gO to promote viral entry into fibroblasts, a cell type in which fusion events at the

plasma membrane initiate infection 4. Infection of several other types of cells, including monocytes, dendritic cells, endothelial cells, and epithelial cells, requires the pentameric complex of gH/gL and three small glycoproteins—UL128, UL130, and UL131 (UL128-131)—and seems to involve fusion at endosomal membranes 5–13.

In one study the authors identified the ER-resident HCMV UL148 protein as a regulator of gH/gL/gO complexes incorporation into HCMV virions. When UL148 is deleted from the viral genome, incorporation of the trimeric gH/gL/gO complex into virions is strongly impaired, resulting in a reduced capacity of virus particles to establish infection in fibroblast cultures with a corresponding increase in infection in epithelial cell cultures14. Data from Li et al. 14 also show that UL148 only binds to gH/gL when gO or UL128 are not bound, which suggests that binding of UL148 may interfere with binding of gO or UL128 to gL 15. Interestingly, UL130 and UL131 were also found in gH/gL/UL148 coprecipition, a finding which made Li et al.14 propose a model of reversible gH/gL/UL130/148 and gH/gL/UL131/148 complexes competing with formation of gH/gL/UL128-131 and directing gH/gL to formation of gH/gL/gO.

There is currently no detail study of the UL148 gene in the UL / B 'region, and so far there have been few reports on the role of UL148 in the pathogenesis of HCMV. Meanwhile, the recombinant cloning expression of UL148 gene has not been reported. Therefore, the recombinant expression of UL148 gene, to facilitate further study of UL148 gene's function is of great value.

2. Materials and Methods

2.1 Plasmids, strains and virus strains

The E. coli strains DH5α was used for the cloning and BL21 (DE3), C41 (DE3), Rosetta (DE3), Rosetta were used for the expression of the UL148 gene. The vectors pET32a was used for the confirmation and transformation of the UL148 gene. E. coli DH-5α and BL21 were cultured on Luria–Bertani (LB) liquid medium or on LB agar at 37 °C, and 100 μg/ml ampicillin was added to maintain the plasmids. The target gene was amplified with HCMV BAC Towne as template.

2.2 Cloning of full-length UL148 gene sequence

UL148 sequence was amplified by Prime STAR® HS DNA Polymerase (Takara, China) using the primers P1 and P2 (Table 1) that were designed using the software Primer 5.0 and Snap gene. Amplification included 34 cycles of a denaturation at 98 ℃ for 10 sec, annealing at 60 ℃ for 5 sec, and elongation at 72 ℃ for 1 min, and a final elongation step at 72 ℃ for 10 min; The UL148 gene was purified by using a DNA Purification Kit (Omega, China). The UL148 gene and the pET32a vector were digested using the restriction enzymes NcoI and XhoI (Angke, China). The digested products were ligated using T4 DNA Ligase (Takara, China). The pET32a-UL148 plasmid, which was constructed by using the pET32a Vector System, was transformed into DH5α competent cells, and the positive clones were isolated by the ammonia resistance method and identified via PCR. Luria-Bertani (LB) medium supplemented with ampicillin (50 mg/mL) was used for culture of the recombinant E. coli strain DH5α. The recombinant pET32a-UL148 plasmid was isolated according to the instructions of the Plasmid Mini Kit (Omega) and was sequenced. The sequence data was analyzed using the online software Nucleotide BLAST from the NCBI (https: //www. ncbi.nlm.nih.gov/).

Table 1. Gene primers

Primers	Primer sequence
UL148-sense-NcoI（P1）	5-CATGCCATGGGCTTGCTGTTCACGCTTGT-3
UL148-anti-sense-xhoI（P2）	5-CCGCTCGAGCCGACGCCGCGACA-3

2.3 Screening optimal expression conditions

The pET32a-UL148 plasmid was transformed into E. coli competent cells of BL21 (DE3), C41 (DE3), Rosetta (DE3), Rosetta. The positive clones were identified by PCR using the above-mentioned primers and digested with NcoI and XhoI. The confirmed colony was selected and inoculated into LB medium supplemented with 50mg/ml ampicillin at 37℃. To improve the efficiency of the expression for further study, the expression conditions, such as the IPTG concentration, temperature and time value, were optimized. The cell pellet samples from the different conditions were harvested, and the UL148 protein of each sample was released from the lysed cells by sonication (power 200, work 5S, intermittent 10S) at 4℃ for 20 min. The extraction of the crude UL148 enzyme solution was subjected to 10% denaturing SDS-PAGE and stained with Coomassie brilliant blue R-250.

2.4 Prokaryotic expression of UL148 gene

The selected transformants were subsequently cultured in LB broth. Once OD_{600} values of 0.5–0.6 were reached, 0.1mM IPTG (Sigma, USA) was added to induce UL148 expression. The cells were cultured for another 5 h at 37℃ under 200 rpm. The cells were subsequently lysed and centrifuged to separhe recombinant UL148 protein was purified using Ni-NTA agarose (Novagen, German) and examinate the soluble and insoluble proteins, and the supernatant was stored -20 ℃ until use.

2.5 Purification of the UL148 protein

BL21 broth was obtained with the optimal conditions above. The cell pellet was harvested and resuspended in lysis buffer (50 mmoL/L NaH2PO4, 300 mmoL/L NaCl, 1 mg/mL, lysozyme, pH 8.0). Lysozyme (Dingguo Changsheng Biotech, Beijing, China) was added at a final concentration of 1 mg/mL and incubated at 4 ℃ for 10 min. Triton X-100 was added at a final concentration of 1%, and the UL148 protein was released from the lysed cells by sonication. The sediment containing UL148 was separated from the cellular fraction after centrifugation (10,000×g, 10 min, 4 ℃). At room temperature, the protein was dissolved in 8 mol/L urea solution for 1 h. After that, the supernatant was centrifuged again and filtered through a 0.45 μm filter to perform protein purification. A Ni-NTA column (Bio-Rad , Beijing, China) was used to purify UL148 protein, and different concentrations of imidazole buffer (50 mmoL/L NaH2PO4, 300 mmoL/L NaCl, 50, 100, 150, 200, 250, and 500 mmoL/L imidazole pH 8.0) were used for a graded elution of the proteins and examined by SDS-PAGE and western blotting.

2.6 Western blotting analysis

The purified protein was subjected to 15% (w/v) SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the proteins on both gels were transferred onto a nitrocellulose membrane (Bio-Rad, California, USA). Anti-His monoclonal antibody was used as the primary antibody and Horseradish peroxidase labeled goat anti-mouse was employed as the secondary antibody.

3. Result

3.1 Cloning of full-length UL148 gene sequence

The genomic DNA of HCMV BAC Towne was used as a template to amplify the complete UL148 gene (Fig. 1A). The UL148 gene was ligated to pET32a and was transformed into E. coli DH5α cells. The recombinant plasmid pET32a-UL148 was verified by PCR after digestion with the restriction enzymes NcoI and XhoI. The recombinant plasmid pET32a-UL148 was verified by digestion (Fig. 1B) and PCR (Fig. 1C). The result shows that the recombinant plasmid was successfully transformed into DH5α. The coding sequence of the UL148 gene was 951 bp (GenBank accession number GQ121041.1), and it contained one open reading frame (ORF) that started with an ATG start codon and terminated with a TAG stop codon. The recombinant pET32a-UL148 plasmid was isolated according to the instructions of the Plasmid Mini Kit (Omega) and was sequenced. The sequencing results were compared with the UL148 gene sequence on NCBI. The sequencing results were consistent with the target gene.
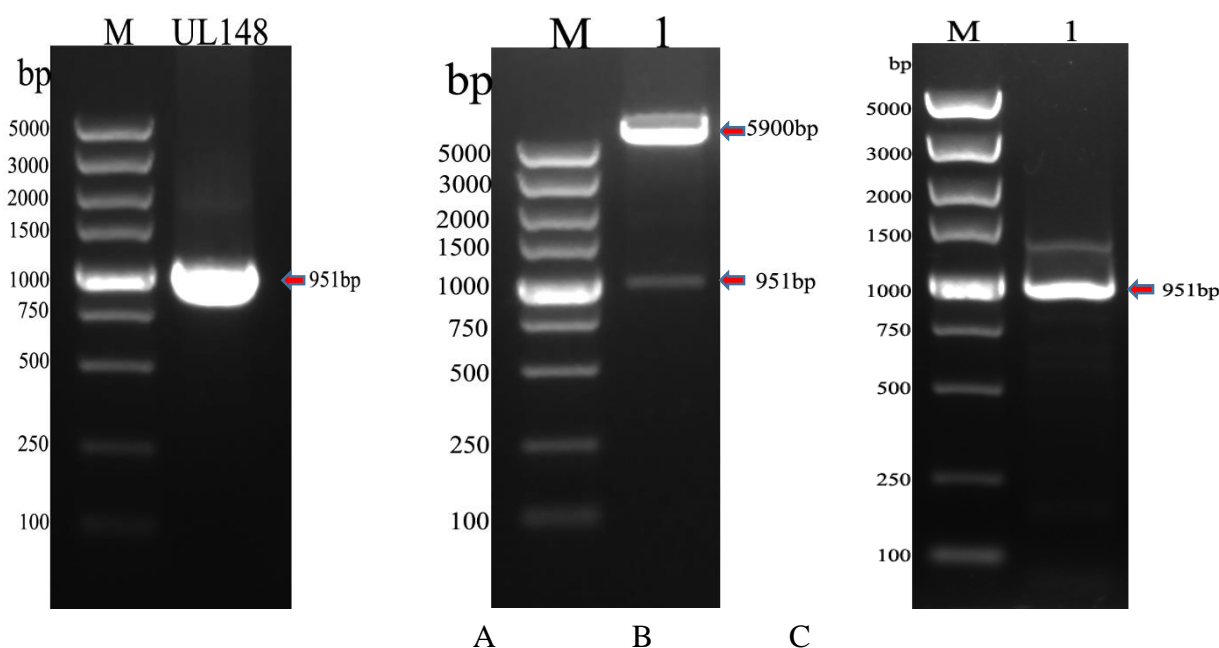
Fig. 1 Cloning and construction of the UL148 expression vector. A To amplify the complete UL148 gene (lane UL148). B Confirmation of the recombinant plasmid pET32a-UL148 by digestion with the restriction enzymes NcoI and XhoI, which produced an UL148 fragment (lane 1: 951bp) and a pET32a fragment (lane 1: 5900bp); C Screening the UL148 gene by PCR with DH5α as a template (lane 1). The result shows that the recombinant plasmid was successfully transformed into DH5α.

3.2 Expression and distribution of UL148 recombinant protein in four express strains

The pET32a-UL148 plasmid was transformed into E. coli competent cells of BL21 (DE3), C41 (DE3), Rosetta (DE3), Rosetta. The positive clones were identified by PCR using the above-mentioned primers and digested with NcoI and XhoI, the confirmed colony was selected and inoculated into LB medium supplemented with 50mg/ml ampicillin at 37 ℃.

The pET32a-UL148 plasmid was transformed into four E. coli competent cells, and IPTG induced expression was carried out under the same expression conditions (temperature: 20 ℃ ; IPTG concentration: 1 Mmol / ml; time: 6 hours). SDS-PAGE gel electrophoresis was used to analyze the cells. Compared with the cells not induced by IPTG, it was found that the recombinant plasmids were expressed in four kinds of cells expressing IPTG, and the expression of BL21 (DE3) is slightly higher than other strains (Fig. 2A). After induction of the cells, the cells were ultrasonically lysed and centrifuged to separate the supernatant and the precipitation. Precipitation and supernatant were analyzed by SDS-PAGE gel electrophoresis. The results show that the target protein bands were mostly distributed in the precipitate, and the expression level in BL21 (DE3) was slightly higher than that in other strains (Fig. 2B). The recombinant plasmid pET32a (+) - UL148 was expressed in BL21 (DE3), and the target bands were mainly distributed in precipitation.
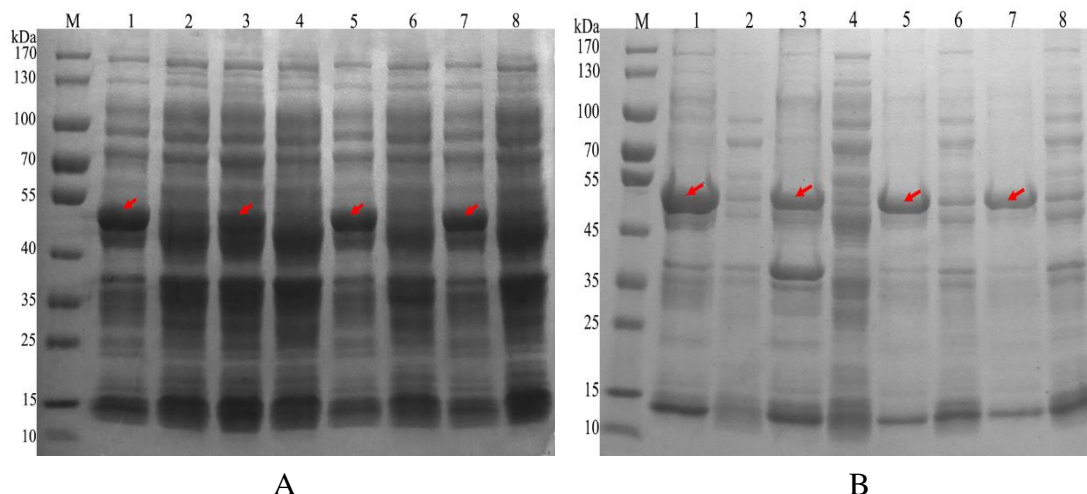
Fig. 2 A: M- Protein Marker：pET32a-UL148 recombinant plasmid was transformed into BL21 (DE3) expressing strain（lane 1: IPTG induction, lane 2: without IPTG）：pET32a-UL148 recombinant plasmid was transformed into C41 (DE3) expressing strain（lane 3: IPTG induction, lane 4: without IPTG）：pET32a-UL148 recombinant plasmid was transformed into Rosetta(DE3) expressing strain（lane 5: IPTG induction, lane 6: without IPTG）：pET32a-UL148 recombinant plasmid was transformed into Rosetta expressing strain（lane 7: IPTG induction, lane 8: without IPTG）. B: M-protein maker; 1 and 2: pET32a-UL148 recombinant plasmid was transformed into BL21 (DE3) expressing strain and induced by IPTG, the cells were ultrasonically sterilized and centrifuged into precipitated (lane 1) and supernatant (lane 2). 3 and 4: pET32a-UL148 recombinant plasmid was transformed into C41 (DE3) expressing strain and induced by IPTG, the cells were ultrasonically sterilized and centrifuged into precipitated (lane 3) and supernatant (lane 4). 5 and 6: pET32a-UL148 recombinant plasmid was transformed into Rosetta (DE3) expressing strain and induced by IPTG, the cells were ultrasonically sterilized and centrifuged into precipitated (lane 5) and supernatant (lane 6). 7 and 8: pET32a-UL148 recombinant plasmid was transformed into Rosetta expressing strain and induced by IPTG, the cells were ultrasonically sterilized and centrifuged into precipitated (lane 7) and supernatant (lane 8). The red arrow indicates the expressed recombinant protein. The expression conditions were as follows: temperature: 20℃, IPTG concentration: 1 mmol / ml, sampling time: 6 h.

3.3 Screening optimal expression conditions

Several factors, including the IPTG concentration, temperature and time, were measured to screen for optimal expression conditions. To screen for the optimal IPTG concentration, all other conditions were fixed at sampling time 6 h and 20℃. Once OD600 values of 0.5–0.6 were reached, and IPTG was added into LB media at various concentrations (ranging from 0 to 1.5 mmoL/L) to observe the effect on the expression of UL148.UL148 protein expression levels were varied with different IPTG concentrations (Fig. 3A). The IPTG concentration of 1.0 mmoL/L resulted in the highest level of protein expression. Therefore, an IPTG concentration of 1.0 mmoL/L was chosen for the optimal induction condition.

Screening for the optimal temperature used a sampling time 6 h and the optimal IPTG concentration identified above. The samples were cultured at the fixed induced condition, and the temperature was varied (20℃, 25℃, 30℃, 37℃). The expression of the UL148 showed significantly higher levels at 37℃ and lower levels at 20℃, 25℃ and 30℃, respectively (Fig. 3B). Therefore, 37℃ was chosen as the optimal temperature.

Additionally, to screen for the optimal sample time, the optimal IPTG concentration and temperature were used, and the sampling time was varied (from 2 h to 9 h). The effects of the different sampling time on the expression level of the UL148 were analyzed. When the sampling time was 5 h, the

UL148 showed the significantly highest level of expression (Fig. 3C). Therefore, 5 h was chosen as the optimal sample time.
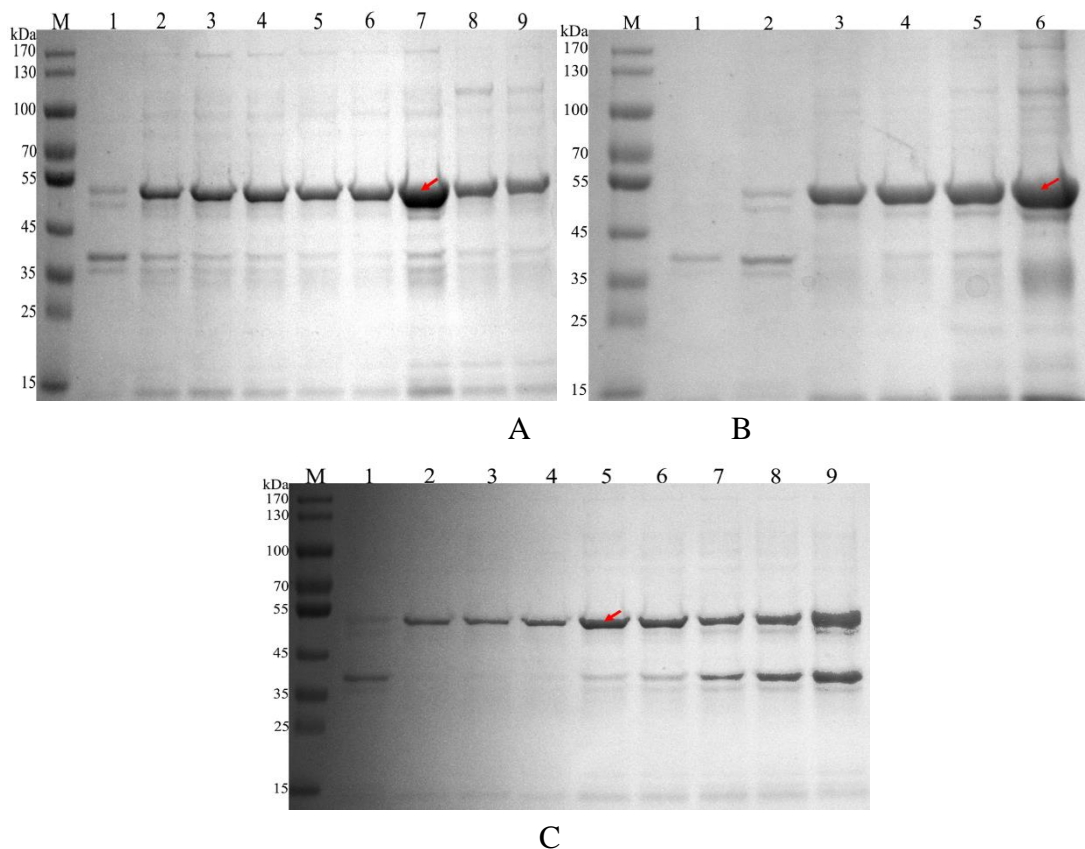

A

B

C

Fig. 3 A: Induction expression of the UL148 protein by different IPTG concentrations. a SDS-PAGE of the proteins expressed with the various IPTG concentrations (2-0.1mmol/L,3-0.2 mmol/L,4-0.4 mmol/L,5-0.6 mmol/L,6-0.8 mmol/L, 7-1 mmol/L, 8-1.3 mmol/L, 9-1.5 mmol/L.) was used to determine the optimal concentration of IPTG. The optimal IPTG concentration was 1.0 mmoL/L. B: Induction of expression of the UL148 protein at different temperatures. a SDS-PAGE with samples produced at different temperatures (3-20℃,4-25℃,5-30℃,6-37℃) was used to determine the best temperature. The optimal condition was 37℃. C: The effects of the different sampling time (2-2h, 3-3h, 4-4h, 5-5h, 6-6h, 7-7h, 8-8h and 9-9 h) on the expression level of the UL148 were analyzed. When the sampling time was 5 h, the UL148 showed the significantly highest level of expression. Therefore, the optimal sample time was 5h.

3.4 Purification of the UL148 protein

The UL148 protein was expressed in 1 L of LB-ampicillin culture. The cell pellet was harvested and sonicated. UL148 protein was separated by centrifugation and adsorbed to a Ni-NTA column. Other proteins were removed using a 150 mmol/L imidazole buffer wash, and UL148 protein was eluted with 200 mmol/L imidazole. The purification conditions of the detected UL148 protein were assessed using 10% SDS-PAGE (Fig. 4A). The purity of the detected UL148 protein was assessed using 10% SDS-PAGE and western blotting
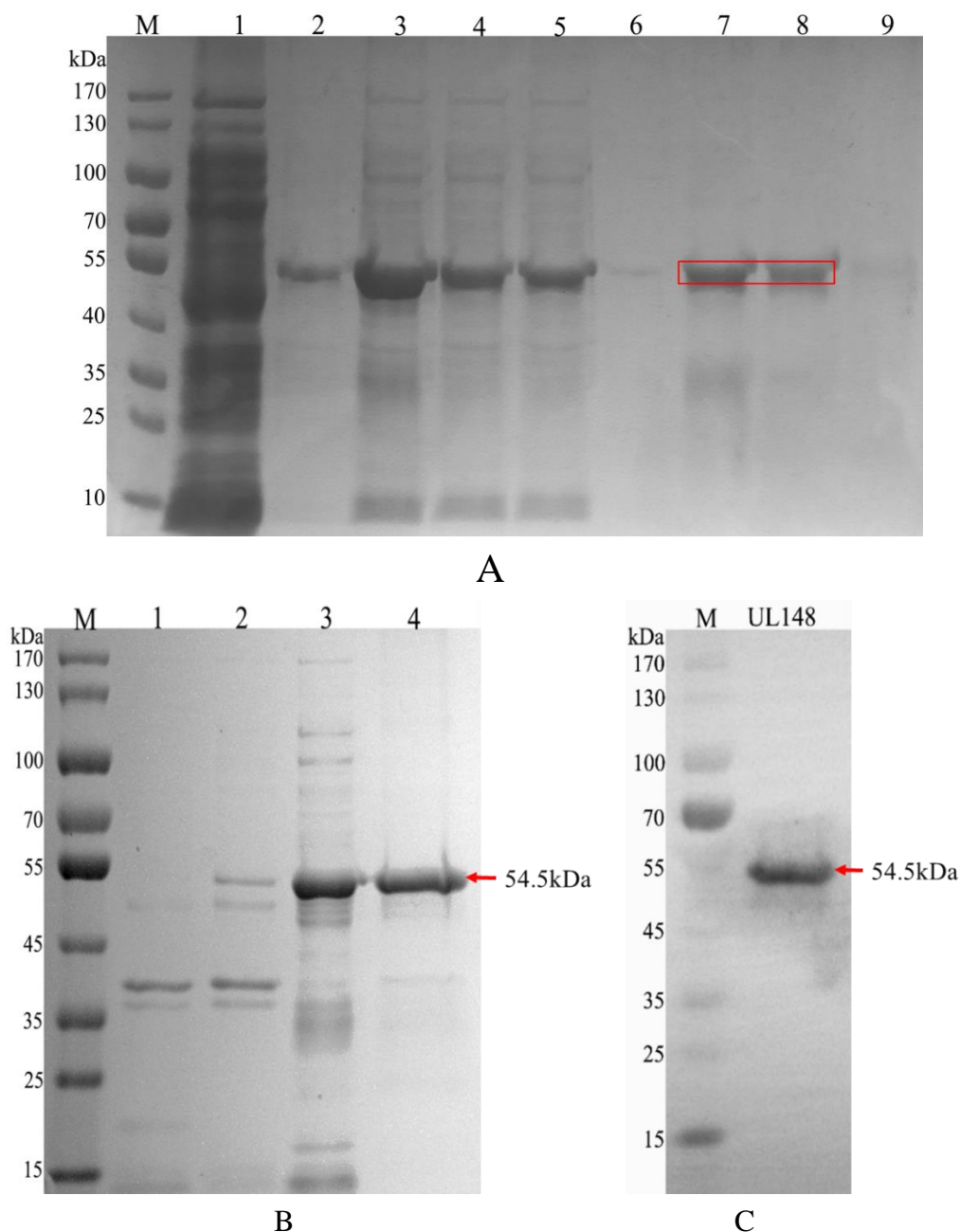
Fig. 4 Purification of the UL148 protein. A: Expression and purification of UL148 protein: lane 1: UL148 protein induced expression, bacterial ultrasonography and supernatant of centrifugation; lane 3:UL148 protein induced expression, bacterial ultrasonography and precipitated of centrifugation; lanes 4-9: Solution of UL148 protein eluted using imidazole buffer with imidazole concentrations of 20, 50, 100, 150, 200 and 500 mmoL/L, suggesting that other proteins were removed by the 150 mmoL/L imidazole buffer, after which the pure UL148 protein was eluted by 200 mmoL/L imidazole. B: The purified UL148 recombinant protein was analyzed by SDS-PAGE: lane 1: expression of the pET32a plasmid transformed into BL21 (DE3) strain at the optimal Conditions; lane 2: UL148 without IPTG induction; lane 3: expression of the UL148 protein at the optimal conditions; lane 4: Purified UL148 fusion proteins via Ni-NTA agarose. C: The purified UL148 recombinant protein was analyzed by western blotting: lane UL148: Purified UL148 fusion proteins.

4. Discussion and conclusion

Despite the clear importance of alternative gH/gL complexes in cell tropism of several beta and gamma herpesviruses, the mechanisms that regulate their relative abundance during infection have

for the most part remained elusive. Previous studies suggest that HCMV makes use of a virally encoded protein, UL148 to modulate the relative abundance on virions of two alternative gH/gL complexes by influencing their assembly and/or maturation. A finding that suggests a novel mechanism for regulation of viral tropism in a herpesvirus16. It is intriguing to note that another research group recently found that the rhesus cytomegalovirus (RhCMV) homolog of UL148, Rh159, binds to natural killer (NK) cell activating ligands within the ER and prevents their movement to the cell surface. Interestingly, Rh159 was previously identified as an epithelial tropism factor of RhCMV 17.

To verify the function of UL148 and to obtain a sufficient amount of purified protein for the future study. His-tagged UL148 fusion protein was produced by establishing a high-level protein expression system in E. coli BL21 (DE3) using the pET32a plasmid. The UL148 protein band (55 kDa) was identified by SDS-PAGE and analyzed by western blotting. It has been reported that factors such as IPTG and temperature affect the expression level and solubility of a target protein 18, 19. Thus, it was necessary to optimize the expression conditions in order to obtain sufficient protein for further study. By calculating the expression levels of the recombinant UL148, we obtained optimal expression based on the IPTG concentration (1.0 mmoL/L), temperature (37 ℃) and sample time (5 h). At the same time, the conditions for purification of the UL148 protein with a Ni-NTA column were also determined. Other proteins were removed using a 150 mmol/L imidazole buffer, and UL148 protein was eluted with 200 mmol/L imidazole. In summary, we successfully cloned and expressed UL148 gene from the HCMV BAC Towne with optimized expression and purification conditions.

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