Preparation of polyclonal antibody against human cytomegalovirus UL124 gene product

Jianxiong Zeng [a], Ruilin Li [b], Qiudi Deng [c], Xin Li [d], Xiwen Xing [e], Jie Lu [f], Rong Hai [g], Hua Zhu [h]

Department of Biological Engineering, College of Life Science and Technology, Jinan University, Guangzhou 510632, Guangdong Province, China

[a] 502819859@qq.com, [b] liruilin01@outlook.com, [c] dengqiudi1989@163.com, [d] 757113017@qq.com, [e] xingxiwen@jnu.edu.cn, [f] jie_lu@jnu.edu.cn, [g] guangdong2018@hotmail.com, [h] huazhu2000@gmail.com

Abstract

Human cytomegalovirus (HCMV) is an important and ubiquitous herpesvirus in modern society. HCMV UL124 is a gene encoded by the HCMV open reading frame ORF152, whose specific function is unknown. Given that it is one of proteins encoded by HCMV latency-associated transcripts (CLT), it is presumed to play an important role in latent infection of the virus. In this study, UL124 polyclonal antibody is prepared in order to further investigate the function of UL124 in latent infection of HCMV. The result of western blot showed that the polyclonal antibody specifically recognizes UL124. This work provides an effective tool for further study of UL124.

Keywords

HCMV, Human Cytomegalovirus, UL124, Viral Latency, Polyclonal Antibody.

1. Introduction

Human cytomegalovirus (HCMV) is a ubiquitous beta-herpesvirus that consists of unique long (UL) and unique short (US) segments [1]. HCMV contains a more than 200kb of double-stranded DNA genome, one of the largest human viruses [2], and encodes more than 200 predicted open reading frames (ORFs) [1, 3]. HCMV has a 50-90% seroprevalence rate in the adult population [4]. HCMV infection is usually asymptomatic, but primary infection or viral reactivation in an immunocompromised host can cause serious disease [5]. HCMV poses a serious threat to immunodeficient and immunocompromised Population [7]. Congenital infection among newborns are mainly caused by HCMV [6], which can lead to serious sequelae, including sensorineural hearing loss, microcephaly and mental retardation [8].

Like other herpesviruses, HCMV can cause productive and latent infections [9]. Following a primary productive infection, the virus does not get cleared, but instead, establishes a lifelong latent infection in the host [10]. In order to gain a greater understanding of the mechanism of latent infection, some viral genes expressed during latent infection have been identified, and some studies and discoveries have been made [11]. For example, UL111A expresses LAcmvIL-10 during viral latency, which makes the latently infected cells difficult to be recognized by CD4+T cells [12]. US28 is one of a few viral genes expressed during latency, encoding chemokine receptor homologs [13, 14, 15], and its expression during latency can attenuate cellular signaling pathways to facilitate latent infections [16]. Although many experimental data indicate that some viral genes are expressed during latency, to date, many latency-associated transcripts have also been shown to be expressed during productive infection [10]. Therefore, it is necessary to develop more sensitive and accurate detection tools and techniques to study HCMV latency and reaction.

UL124, originally detected in latently infected granulocyte-macrophage progenitor cells, is mapped to the IE1/IE2 region of the HCMV genome, which is one of the transcripts [ORF59, ORF154 and ORF152 (UL124)] encoded by antisense HCMV latency-associated transcripts (CLT) [17]. At the same time, specific antibodies against the corresponding proteins are detected in HCMV infected individuals, indicating that they are expressed during natural infection (latent infection) [17, 18].

In many studies, it shows that UL124 is conserved in CMVs, encoding a potential membrane protein consisted of 150 amino acids, in which the presence of a signal peptide sequence and a transmembrane domain [19, 20]. In addition, HCMV experienced severe growth defect in human foreskin fibroblast (HFF) after knocking out UL124 [3]. In view of the existing experimental data, it is speculated that UL124 may play an important role in the establishment and maintenance of virus latency, and even in productive infection. However, due to the lack of effective tools, especially specific antibodies, it is difficult to conduct further research.

To obtain specific antibody to UL124, we expressed a fusion protein of UL124 *in vitro* and immunized the rabbit with the purified fusion protein. Western blot experiments verified its activity. These efforts provide an effective tool to study the role of UL124 in viral latency and even lytic infections.

2. Methods

2.1 Materials and reagents

E. *coli* DH5α, E. *coli* BL21(DE3), HCMV TB40/E strain and TB40/E BAC, pET32a(+), human foreskin fibroblasts (HFF) cells and human acute monocytic leukemia (THP-1) cells were stored in our lab. HFF cells were cultured in DMEM supplemented with 1% P-S and 10% FBS. THP-1 cells were cultured in RPMI-1640 medium supplemented with 10% FBS. Restriction enzymes HindIII and BamHI were purchased from Thermo Fisher (MA, USA), Ni-NTA were from BIO-RAD (CA, USA). DL1,000 DNA Marker, DL5,000 DNA Marker, TaKaRa Taq™, PrimeSTAR® HS DNA Polymerase and T4 DNA Ligase were from Takara Biotechnology Company (Dalian, China).

2.2 The construction of a recombinant plasmid with His tag

Primers were designed based on the UL124 gene (Gene ID: 3077503) as follows: the forward primer (with BamHI restriction site): 5'-CGGCGCGGATCCATGGAAAGGAACAGTCTGTTAGT CTGT-3'; the reverse primer (with HindIII recognition site): 5'-CGCGCGAAGCTTAACATAGCG TGGGATCTCCAC-3'. Primers were synthesized by Tsingke (Guangzhou, China). HCMV TB40/E BCA was used as template for PCR amplification. The PCR reaction system (50μl) was: 10μl 5×PrimeSTAR Buffer (Mg2+ Plus), 4μl dNTP Mixture (2.5mM each), 2μl (0.2μM) each primer, 1μl template, 0.5μl Prime STAR HS DNA Polymerase (2.5U/μl) and 30.5μl ddH2O. The PCR reaction conditions were: pre-incubation at 98℃ for 5 min, and then denaturation at 98℃ for 10s, annealing at 55℃ for 30s, extension at 72℃ for 28s, cycling 35 times, and then 72℃ for 7min. The PCR products were analyzed on a 1% agarose gel and recovered using a Gel Extraction kit (OMEGA Bio-Tek, USA).

The PCR product was cloned into the pET-32a(+). PCR products and vectors, simultaneously digested with BamHI and HindIII, were ligated with T4 ligase, and then transformed into E. *coli* DH5α, and plated onto ampicillin plates. Transformed bacteria were grown in LB solid medium containing 100μg/ml ampicillin for 12-16h, and plasmids were extracted by E.Z.N.A Plasmid Kit (OMEGA Bio-Tek, USA). Positive clones were screened by double digestion and DNA sequencing was performed by Tsingke (Guangzhou, China).

2.3 Prokaryotic expression and purification of fusion proteins

To express the protein encoded by UL124 gene, the recombinant plasmid pET32a(+)-UL124 was transformed into E. *coli* BL21(DE3) as described above. Positive clones were inoculated in 600mL LB containing 100μg/ml ampicillin and incubated at 37℃ for 2-3h until the OD600 reached 0.4-0.6,

and then Isopropyl β-D-thiogalactopyranoside (IPTG) (Takara) was added with a final concentration of 0.5 mM. The bacteria were incubated at 30℃ with shaking for another 6h.

The bacteria were centrifuged at 10,000 g for 5 minutes at 4℃ and washed three times with PBS. Subsequently the bacteria were resuspended in Buffer1 (50mM sodium phosphate, 300mM sodium chloride, 5mM imidazole and 8M urea, pH8.0) and sonicated at 60W in ice. The lysate was centrifuged at 13,000 g for 30 minutes at 4℃, and the supernatant was passed through a 0.22μm filter and purified by Ni-NTA resin (BIO-RAD). According to the protocol, the method was as follows: The column was equilibrated with equilibration buffer (50mM sodium phosphate, 300mM sodium chloride, pH8.0) at 2ml/min, and then the sample was loaded at 1ml/min. Next the column was washed with 6 column volumes (CVs) of elution Buffer 1-6 (50mM sodium phosphate, 300mM sodium chloride, 8M urea, pH8.0, imidazole concentrations containing 5, 25, 100, 200, 300, 500 mM, respectively) at 1ml/min. Finally, the column was washed with 10CVs elution buffer (50mM sodium phosphate, 300mM sodium chloride, 500mM imidazole, 8M urea, pH8.0) at 2ml/min. Samples were collected in 1ml volume fraction and analyzed by SDS-PAGE.

2.4 Preparation of anti-UL124 rabbit polyclonal antibody

To generate an anti-UL124 rabbit polyclonal antibody, the purified protein was concentrated with ultrafiltration tubes as an antigen to immunize New Zealand white rabbits (4 months old, 3kg). The antigen was mixed with Freund's complete adjuvant (Sigma) in a 1:1 (v/v) ratio. Before immunization, 5mL pre-immune serum from rabbit ear veins was collected. The immunizations were conducted as follow: injecting 1mL concentrated protein emulsified with Freund's complete adjuvant into rabbits at a dose of 0.5mg/ml, then boosting the immunization by injecting the purified protein every 2 weeks for a total of three times. Serum was collected 10 days after the last immunization and antibody titer are determined by enzyme-linked immunosorbent assay (ELISA).

2.5 ELISA

The ELISA kit (Xinbosheng Bio) was used according to the manufacturer's protocol. The antigen was diluted to 5μg/ml with a coating solution, added to the Enzyme plate in 100μl per well, and incubated overnight at 4℃. Coating solution was discarded and the plate was washed 3 times by detergent. After washing, the plate was blocked with blocking solution in 200μl per well and incubated at 37℃ for 2h. The plate was washed again three times with detergent, and added with rabbit antiserum (rabbit antiserum is diluted 10, 100, 1k, 10k and 100k times with PBS, respectively) in 100μl per well, incubated at 37℃ for 1h. Serum was discarded, and washed three times with detergent and incubated with goat anti-rabbit IgG H&L (HRP) (1:4000) at 37℃ for 1h. The plate was washed with detergent three times, and added with the coloring solution in 100μl per well. After the color was obvious, adding stop solution in 50μl per well, and measuring the absorbance value OD450 on the microplate reader.

2.6 Verifying the specificity of anti-UL124 rabbit polyclonal antibody

THP-1 cells were infected with HCMV TB40/E strain (MOI=5) and harvested for RNA and protein extraction. Total RNA was extracted using RNAiso Plus (Takara), and used as templates for RT-PCR to verify UL124 expressed in the TB40/E strain. The protein was analyzed by western blot and the specific method was as follows: 15% SDS-PAGE was used to separate cellular proteins and transferred to PVDF membrane, blocked in 5% skimmed milk powder for 1h, incubated with anti-UL124 rabbit polyclonal antibody (1:400) overnight at 4℃, and washed by TBST (containing 0.5% Tween20) three times for 15 min each time. After washing, the membrane was incubated with goat anti-rabbit IgG H&L (HRP) (1:10000, Abcom) at room temperature for 1h, then washed as above. Finally, the immune response signal was detected by LI-COR ODYSSEY FC imaging system (U.S.A.).

3. Results

3.1 The construction of a recombinant plasmid with His tag

To construct a recombinant plasmid, the UL124 gene was amplified from the HCMV TB40/E BAC genome by PCR (Fig. 1A). The full length sequence of UL124 was inserted into the prokaryotic expression plasmid pET32a(+) between BamHI and HindIII restriction sites. The positive recombinant plasmid was confirmed by restriction enzyme double digestion (Fig. 1B) and sequencing analysis (data not shown).

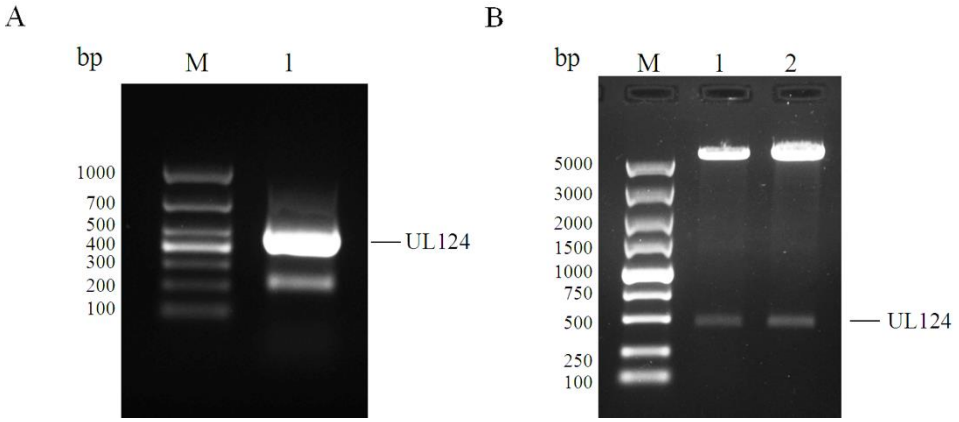

Fig. 1. Identification of UL124 amplification product and the recombinant plasmids by double digestion. A. Amplification of UL124 gene. M: DL1,000 DNA marker, Lane1: PCR product of UL124. B. double digestion of UL124 recombinant plasmid. M: DL5,000 DNA marker, Lanes1, 2 represent double digestion of recombinant plasmids with BamHI and HindIII. B: 459bp UL124 insert was observed.

3.2 Prokaryotic expression and purification of fusion protein.

To obtain pure fusion protein, the pET32a(+)-UL124 recombinant plasmid was transformed into E. *coli* BL21(DE3) and induced by 0.5mM IPTG at 20℃ for 6h. The fusion protein was highly expressed but mainly in an insoluble form (Fig. 2A), which was purified by affinity chromatography through Ni-NTA resin (Fig. 2B). The purified protein was confirmed to be the target protein by western blot using a His-tag-specific antibody (Fig. 2C).

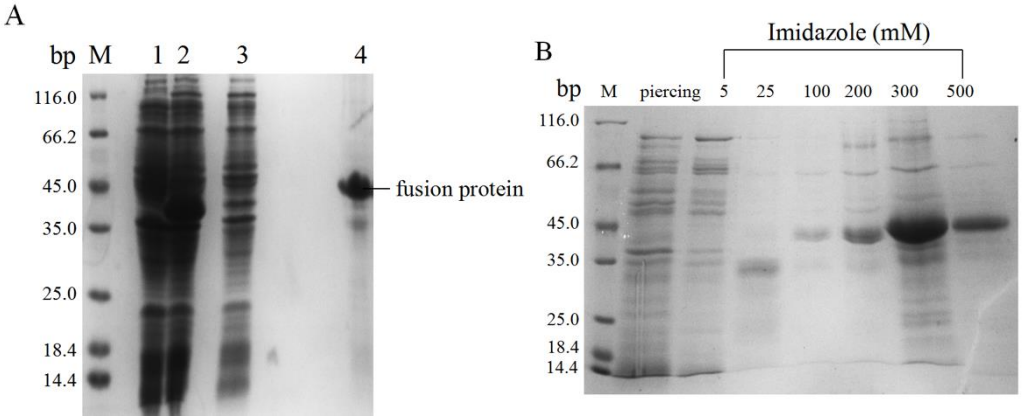

Figure. 2. Prokaryotic expression and purification of UL124 fusion protein. A. Identification of the expression site of the fusion protein by SDS-PAGE. M: 116.0kDa protein Marker, Lane1: without IPTG-induced bacterial lysate; Lane2: IPTG-induced bacterial lysate; Lane3: IPTG-induced bacterial supernatant; Lane4: IPTG induced bacterial inclusion bodies. B. Analysis of the purification of the fusion protein by SDS-PAGE. M: 116.0kDa protein Marker. C. The purified protein was verified by western blot using a His-tag-specific antibody.

3.3 Production and titration of UL124 specific polyclonal antibodies

New Zealand rabbits were injected with purified fusion protein at a dose of 0.5mg/rat to generate polyclonal antibodies. Pre-immune serum and anti-serum were collected for antibody activity by ELISA. The anti-serum and pre-immune serum were diluted 10, 100, 1k, 10k and 100k times, respectively. ELISA titer show that the serum after immunization was specific for the fusion protein of UL124. The titer represented the highest dilution and the ratio of anti-serum to pre-immune serum was greater than 2 (OD450) (Fig. 3).

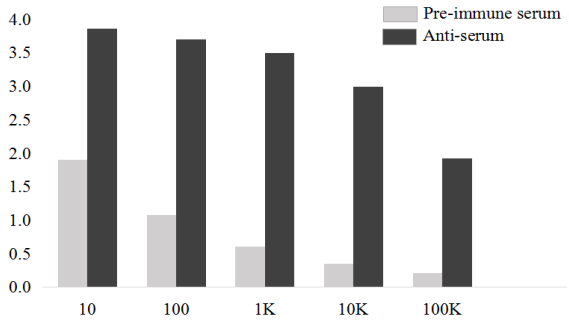

Fig. 3. Polyclonal antibodies were specific for UL124. The specificity of polyclonal sera were assessed by ELISA. The titer of polyclonal antibodies against UL124 in the ELISA exceeded 100k. Pre-immune serum are negative controls.

3.4 Further confirmation of the specificity of the polyclonal antibody against UL124 using the fusion protein from E. coli and the HCMV-infected sample by western blot

To further confirm the specificity of the polyclonal antibody, western blot was performed using the fusion protein from E. *coli* and HCMV infected cells. To ensure transcription of UL124 in infected samples, RT-PCR was performed using UL124-specific primers. The results showed the presence of UL124 mRNA, indicating the transcription of UL124 in infected samples at 3d post infection (Fig. 4A). Western blot revealed that a 35kD fusion protein of UL124 could be detected in IPTG-induced E. *coli* lysates (Fig. 4B). More importantly, an excepted 15kD viral protein (UL124) could be detected in HCMV infected cell lysates, but not in mock infected (4C). These results show that the polyclonal antibody produced by the UL124 gene product in this study not only recognizes the UL124 protein expressed in E. *coli*, but also detects the UL124 protein in HCMV infected cells.

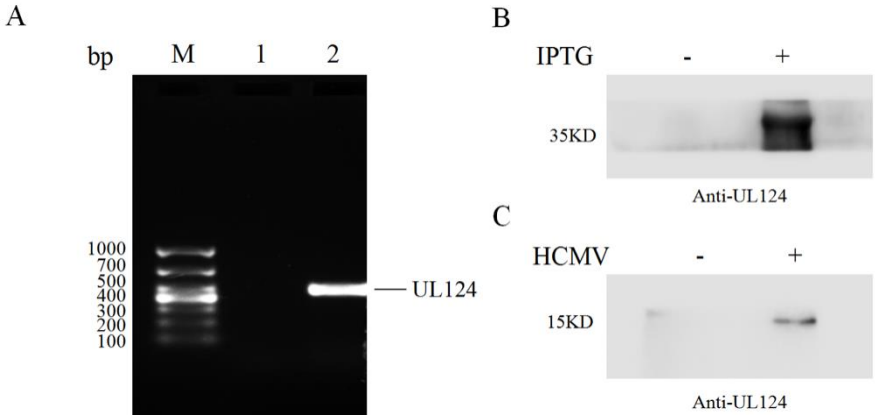

Fig. 4. Specific recognition of the fusion protein and the intracellular protein of UL124. A. RT-PCR analysis of UL124 expressed in 3d infected THP-1 cells. Lane1: cDNA from uninfected cells. Lane2: cDNA from HCMV infected THP-1 cells. B. The fusion protein of UL124 was detected by western blot using the polyclonal antibody against UL124. C. The UL124 protein from HCMV infected cells was detected by western blot using the polyclonal antibody against UL124.

4. Conclusion

In conclusion, we have successfully produced polyclonal antibodies against the UL124 protein. This provides a valuable reagent for further study of the function of UL124 in HCMV infection, latency and reactivation.

Acknowledgements

This research was supported by grants from National Natural Science Foundation of China (No. 21708014, No. 81672006), Guangdong Innovative and Entepreneurial Research Team Program (No. 2014ZT05S136).

References

[1] Murphy E: Reevaluation of human cytomegalovirus coding potential. Proceedings of the National Academy of Sciences of the United States of America, Vol. 100 (2003) No.23, p.13585-13590.
[2] Yu D: Functional Map of Human Cytomegalovirus AD169 Defined by Global Mutational Analysis. Proceedings of the National Academy of Sciences of the United States of America, Vol. 100 (2003) No.21, p.12396-12401.
[3] Dunn W: Functional profiling of a human cytomegalovirus genome. Proc Natl Acad Sci U S A, Vol. 100 (2003) No.24, p.14223-14228.
[4] Staras S A: Seroprevalence of cytomegalovirus infection in the United States. Clinical Infectious Diseases, Vol. 46 (2006) No.9, p.1143-1151.
[5] Jackson J W: There Is Always Another Way! Cytomegalovirus' Multifaceted Dissemination Schemes. Viruses, Vol. 10 (2018) No.7, p.383.
[6] Manicklal S: The "Silent" Global Burden of Congenital Cytomegalovirus. Clinical Microbiology Reviews, Vol. 26 (2013) No.1, p.86-102.
[7] Mocarski E S: *Fields Virology(*Knipe, DM, 2007), p.1960-2014.
[8] Schleiss M R: Cytomegalovirus in the neonate: immune correlates of infection and protection. Clinical & Developmental Immunology, Vol. 2013 (2014) No.2013, p.501801.
[9] Strang B L: Viral and Cellular Sub-Nuclear Structures in Human Cytomegalovirus Infected Cells. Journal of General Virology, Vol. 96 (2015) No.2, p.239-53.
[10] Slobedman B: Human cytomegalovirus latent infection and associated viral gene expression. Future Microbiology, Vol. 5 (2010) No.6, p.883-900.
[11] Mocarski ES: *Cytomegaloviruses and Their Replication. In: Fields Virology (4th Edition) (*Lippincott Williams & Wilkins, Philadelphia, PA, USA 2001) p.2629–2673.

[12] Cheung A K: The role of the human cytomegalovirus UL111A gene in down-regulating CD4+ T-cell recognition of latently infected cells: implications for virus elimination during latency. Blood, Vol. 114 (2009) No.19, p.4128-4137.
[13] Chee MS: Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. (1990) No.154, p.125-169.
[14] Welch AR: Cytomegalovirus homologs of cellular G protein-coupled receptor genes are transcribed. J. Virol. Vol. 65 (1991) No.7, p.3915-3918.
[15] Zipeto D: Kinetics of transcription of human cytomegalovirus chemokine receptor US28 in different cell types. J. Gen. Virol. Vol. 80 (1999) No.3, p.543-547.
[16] Krishna B A: Latency-Associated Expression of Human Cytomegalovirus US28 Attenuates Cell Signaling Pathways To Maintain Latent Infection. Mbio, Vol. 8 (2017) No.6, p.01754-17.
[17] Landini M P: Humoral immune response to proteins of human cytomegalovirus latency-associated transcripts. Biology of Blood & Marrow Transplantation Journal of the American Society for Blood & Marrow Transplantation, Vol. 6 (2000) No.2, p.100-108.
[18] Kondo K: Human cytomegalovirus latent gene expression in granulocyte-macrophage progenitors in culture and in seropositive individuals. Proceedings of the National Academy of Sciences of the United States of America, Vol. 93 (1996) No.20, p.11137-11142.
[19] Davison A J: The human cytomegalovirus genome revisited: comparison with the chimpanzee cytomegalovirus genomeFN1. Journal of General Virology, Vol. 84 (2003) No.1, p.17-28.
[20] Rigoutsos I: In Silico Pattern-Based Analysis of the Human Cytomegalovirus Genome. Journal of Virology, Vol. 77 (2003) No.7, p.4326-4344.