Preparation of polyclonal antibody against human cytomegalovirus UL146 gene product – α chemokine vCXC-1

Lingling He [a], Mengqiong Xu [b], Xiwen Xing [c], Jie Lu [d], Hua Zhu [e]

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

[a] linglinghehll@hotmail.com, [b] xumengqiong@stu2015.jnu.edu.cn, [c] xingxiwen0025@sina.com, [d]jielujnu@outlook.com, [e]hua.zhu@rutgers.edu

Abstract

The life-long infection of human cytomegalovirus (HCMV) is one of the most common public health threats. HCMV UL146, a HCMV open reading frame (ORF), encodes the α-chemokine vCXC-1 which has been shown to facilitate viral latency and dissemination. However, a detailed molecular mechanism is still largely unclear due to the lack of reagents, such as UL146 specific antibodies. To further investigate the mechanism of vCXC-1 involvement in evading the host immune system, the UL146 specific polyclonal antibodies were generated. The results of Western blots indicated that the polyclonal antibodies are UL146 specific. This study provides the field an effective tool to unveil the role of UL146 in HCMV latency.

Keywords

HCMV, cytomegalovirus, UL146, chemokine, vCXC-1, polyclonal antibody, immune escape.

1. Introduction

Human cytomegalovirus (HCMV) is a double-stranded DNA virus that belongs to the β-herpes virus family. HCMV contains the largest genome in human herpesviruses. It encodes more than 200 open reading frames (ORFs) [1]. HCMV infected between 50%-95% of the general population [2]. In most cases, HCMV infection is asymptomatic, however, in immuno-compromised adults and congenially infected newborns, HCMV infection can cause serious diseases, such as pneumonia, retinitis, encephalitis and hepatitis [3].

Life-long latent infection by gene silencing is a hallmark of HCMV [2]. Several HCMV viral genes contribute to immune evasion [4, 5]. For example, viral encoded US2, US3, US6 and US11 proteins act cooperatively to suppress the host immune response by decreasing the expression of major histocompatibility (MHC) class I proteins [6-9]. UL18 excludes the MHC-1 by binding to MHC-I, which interferes the T cell's ability of recognizing the infected cells [10]. Chemokines are group of immuno modulator proteins which attract white blood cells to the site of infection. They are divided into four types according to structure: C, CC, CXC, and CX3C. HCMV suppresses their function by binding to the chemokines [10] or expressing chemokines-like proteins [11]. For examples, US28 protein inhibits host immune response by binding to C-C chemokines [10]; UL128 protein is a CC chemokine that is a member of pentamer complex (gH/gL/UL128-UL130-UL131A), influencing the tissue tropism[3]; UL146 and UL147 genes encode two viral chemokines vCXC-1 and vCXC-2, respectively [12].

vCXC-1 is the first discovered HCMV-encoded α(CXC) chemokine [12], which has been shown to be a potent neutrophil chemoattractant in vitro[8, 13]. Mounting evidences indicate that neutrophils serve as carriers to aid the virus dissemination into endothelial cells [14]. Thus, vCXC-1 facilitates the systematic spread of HCMV via neutrophils. However, the myeloid lymphocyte NK cells play an important role in clearing initial HCMV infected cells. To avoid the attraction of NK cells to the infected site, viral-encoded vCXC has been shown to preferentially attract neutrophils through binding to CXCR2, which is mainly expressed on the surface of neutrophils [11, 15]. However, a

detailed molecular mechanism of this process is still unclear primarily due to the lack of reagents, particularly vCXC-1 specific antibodies.

To obtain high efficient antibody of UL146, we expressed the fusion proteins in vitro and the purified recombinant proteins were used as antigen to immune rabbits to generate polyclonal antibodies against UL146. The specificity was further verified by Western blots. These works provided a valuable reagent to investigate the mechanism of HCMV UL146 in immune invasion.

2. Methods

2.1 Materials and reagents

E. coli DH5α, E. coli BL21 (DE3), HCMV Towne strain（ATCC VR-977 long version） and Towne BAC, pET32a (+), and human foreskin fibroblast (HFF) cells were stored in our lab. HFF cells were cultured in DMEM supplemented with 10% FBS. Restriction enzymes SalI and BamHI were purchased from Thermo Fisher (MA, USA). Ni–NTA Resin was from BIO-RAD (CA, USA). DNA Marker1,000, DNA Marker 5,000, PrimeSTAR® HS DNA Polymerase and T4 DNA ligase were from Takara Biotechnology Company (Dalian, China).

2.2 The construction of recombinant plasmid His-tagged expression vectors

Primers were designed based on the UL146 gene (GenBank: FJ616285.1) as follows; the forward primer (with BamHI restriction site): 5'-CGCGGATCCATGCGATTAATTTTTGGTTCGCT-3'; the reverse primer (with SalI recognition site): 5'-ACGCGTCGACTTCCAACGCGGACGTTTG-3'. Primers were synthesized by Sangon Biotech (Shanghai, China). HCMV BAC Towne library was used as template for PCR amplification. PCR was performed in a 50μL volume containing 10μL 5×Primer STAR Buffer, 4μL (200μM) dNTP Mixture, 2μL (0.2μM) each primer, 1μL template, 0.5μL (1.25units) Primer STAR HS DNA polymerase and 30.5μL ddH2O and under the following conditions: pre-incubation at 98°C for 5 min, and then denaturation at 98°C for 10 sec, annealing at 60°C for 5 sec, extension at 72°C for 30 sec, and 72°C for 10 min, cycling 31 times. The PCR products were analyzed on 2% agarose gel and recovered by using the Gel Extraction kit (OMEGA Bio-Tek, USA.).

The PCR product was cloned into pET-32a (+). PCR products and vectors were double digested with SalI and BamHI, ligated by T4 ligase, transformed into DH5a E. coli, and plated on ampicillin plates. Colonies were grown in LB liquid cultures containing 100ug/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 carried out by Sangon Bioech. Co. Ltd. (Shanghai, China).

2.3 Prokaryotic expression and purification of fusion protein

To express the protein of vCXC-1 encoded by UL146, recombinant plasmid pET32a (+)-UL146 was transformed into E. coli BL21(DE3), as described above. The positive colons were inoculated in 400 mL LB containing 100μg/ml ampicillin and cultured at 37°C for 2-3h until OD600 reached 0.4–0.6. Isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma) was added to a final concentration of 0.5 mM. Cells were incubated at 20°C with shaking for another 10 h.

The cells were then centrifuged at 8000g for 10min, 4℃ and washed with PBS three times. Subsequently, the cells were resuspended in buffer 1 (50 mM Sodium phosphate, 300 mM Sodium chloride, 5 mM Imidazole, 8 M Urea, pH 8.0) and sonicated at 55W in Ice. Lysates were centrifuged at max speed for 30min at 4℃, and a portion of the supernatants were analyzed by 12% SDS-PAGE gels. The remaining supernatants were passed through a 0.22 μm filter and purified by Ni–NTA Resin (BIO-RAD) according to the manufacture's protocol. Briefly, cartridges were equilibrated with equilibration buffer (50 mM Sodium phosphate, 300 mM Sodium chloride, pH 8.0) at 2 ml/min, then loaded with sample at 1ml/min. Cartridges were washed with 6 column volumes (CVs) of wash buffer 1 at 1ml/min followed by washing with 6 CVs of wash buffer 2 (50 mM Sodium phosphate, 300 mM Sodium chloride, 25 mM Imidazole, 8 M Urea, pH 8.0). Purified Protein was eluted from the cartridge

by 10 CVs of elution buffer (50 mM Sodium phosphate, 300 mM Sodium chloride, 500 mM Imidazole, 8 M Urea, pH 8.0) at 2 ml/min, collected in 1ml fractions, and analyzed by SDS-PAGE.

2.4 Preparation of rabbit anti-UL146 (vCXC-1) polyclonal antibody

To produce rabbit anti-UL146 (vCXC-1) polyclonal antibody, the purified protein was concentrated with ultrafiltration and used as antigen for immunization of New Zealand White Rabbit (3-months old, 2 kg). The antigen was mixed and emulsified with Freund's complete adjuvant (Sigma) in a 1: 1(v/v) ratio. Before immunization, we collected 5 mL preimmune serum from rabbit ear veins. Subsequently, the immunizations were conducted as follows: 1 mL purified protein emulsified with Freund's complete adjuvant was injected into the rabbit, then, boosted by injection of the purified protein every 2 weeks for a total of three times. Antiserum was collected 10 days after last immunization, and antibody titer was determined by enzyme-linked immunosorbent assay (ELISA).

2.5 Verifying the specificity of the rabbit anti-UL146 (vCXC-1) polyclonal antibody

HFF cells were infected with HCMV Towne strain (MOI=1), and cells were harvested for protein and RNA extraction. Total RNA was extracted by MiniBEST Universal RNA Extraction Kit (Takara) reversed transcribed into cDNA, and used as template for PCR to verify HCMV UL146 expressed in Towne strain. The protein was analyzed by Western blots. In brief, the cellular protein was separated by 12% SDS-PAGE and transferred to PVDF membranes, blocked in 5% skimmed milk for 1h, incubated with anti-UL146 rabbit polyclonal antibody (1:500) overnight at 4°C , and washed by TBST (containing 1/2000 Tween) three times for 20 minutes each. After washing, membranes were incubated with fluorescent labeled goat anti-rabbit (1:5000; LI-COR) at 4°C for 2-3 h, then washed, and the immune response signal was detected by LI-COR ODYSSEY FC imaging system (U.S.A.).

2.6 Enzyme Linked Immunosorbent Assay (ELISA)

The protein was diluted with carbonic acid-sodium carbonate buffer to 4 μg/mL as coating liquid. Diluted protein was added to the microplate with 100 μL per well and incubated overnight at 4°C. Coating liquid was discarded, and plates were washed three times by PBST. After washing, the microplate was blocked in 5% skimmed milk for 2 h at 37°C, washed by PBST three times, then incubated with rabbit immune serum (rabbit immune serum:1%BSA=1:10, 1:102, 1:103, 1:104, 1:105) be tested at 37°C for 2 h. The serum was discarded, plates were washed with PBST three times and incubated with HRP-labeled goat anti-rabbit at 37°C for 1 h. Plates were subsequently washed with PBST for three times antibody titers from the rabbits were measured by Molecular Devices EMax Plus® (USA)

3. Results

3.1 Construction of recombinant his-tagged expression vectors

To construct the recombinant prokaryotic expression vector, HCMV UL146 gene was amplified from HCMV BAC Towne genome (Figure 1A). The full-length sequence of UL146 was inserted into prokaryotic expression plasmids pET32a (+) between SalI and BamHI restriction sites. The positive clones were confirmed by restriction enzyme digestion (Figure 1B) and sequencing (data not shown).

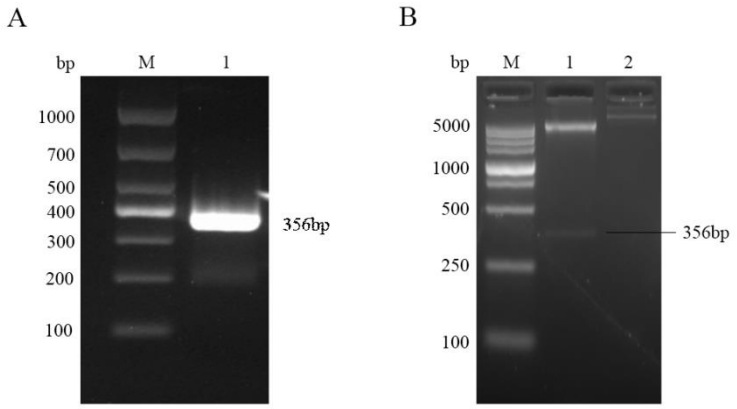

Fig.1 Identification of the products of UL146 amplification and the recombinant plasmids by restriction enzyme digestion.

A. Amplification of UL146 gene, M: DNA marker, lane 1: the PCR product of UL146. B. UL146 expression construct. Lane 1 represents recombinant plasmid digested by BamH I and Sal I, lane 2 represents the plasmid pET32a (+) without digestion.

3.2 Prokaryotic expression and purification of fusion protein

To purify the UL146 protein, pET-UL146 expression vector was transformed into E. coli BL21 (DE3) and inducted by 0.5 mM IPTG at 20°C for 10h. The fusion protein was highly expressed but in an insoluble form (Fig.2A), which was purified by affinity chromatography through Ni–NTA Resin (Fig.2C). The identity of the fusion protein was further confirmed by Western blot using His-tag specific antibodies (Fig.2B).

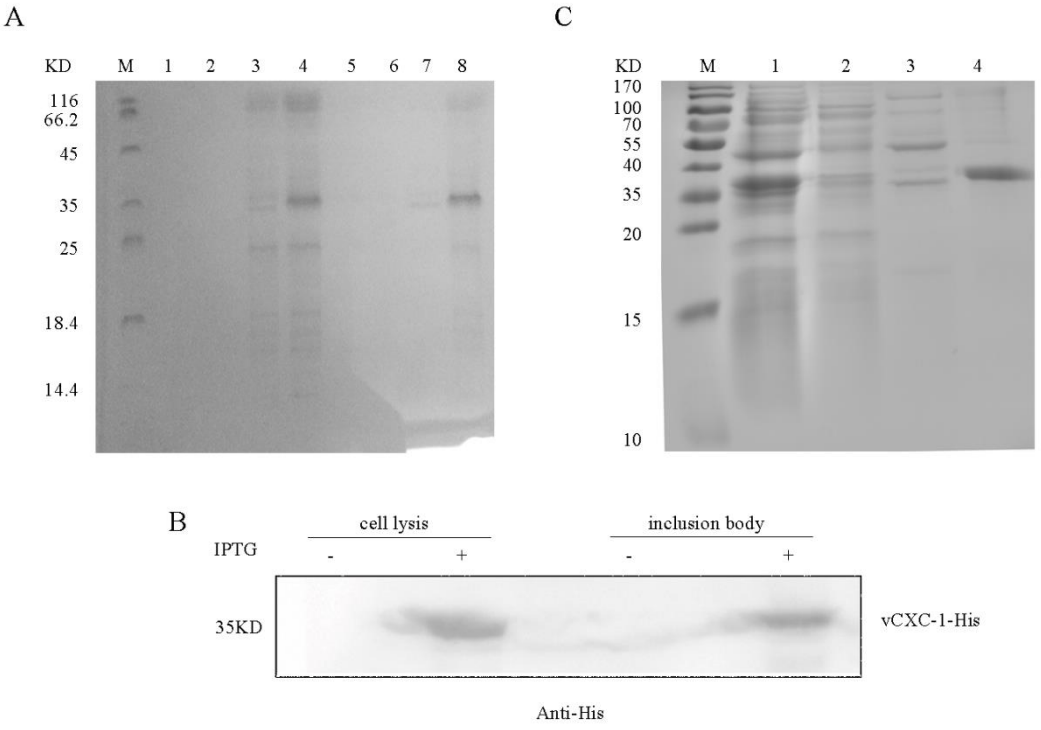

Fig.2 Prokaryotic expression and purification of the HCMV UL146 protein.

A. Identification of the expression sites of vCXC-1-his fusion protein by SDS-PAGE. Lane 1 and 2: culture supernatant; lane 3 and 4: cell lysis; lane 5 and 6: soluble protein; lane 7 and 8: the inclusion body; lane 1,3,5,7: samples without IPTG induction; and lane 2,4,6,8: samples induced by IPTG. B. Verifying the expressed proteins induced by IPTG were vCXC-1-His by Western blot. C. Analyzing

the purification of fusion protein by SDS-PAGE. Lane 1：inclusion body solution; lane 2 and 3: the unbound proteins, lane 4: the purified proteins. M indicates protein ladder.

3.3 Production and tittering of UL146 (vCXC-1) specific polyclonal antibodies

New-Zealand rabbits were injected with purified vCXC-1-His fusion proteins to produce polyclonal UL146 antibodies. The pre-immune and immune serum were collected to test the antibodies activities by ELISA. The immune serum and the pre-immune serum were diluted from 1:10 to 1:100,000. ELISA titers indicated that the post immune sera were specific against UL146 protein. The ratio of immune serum to pre-immunized serum was greater than two is considered positive.

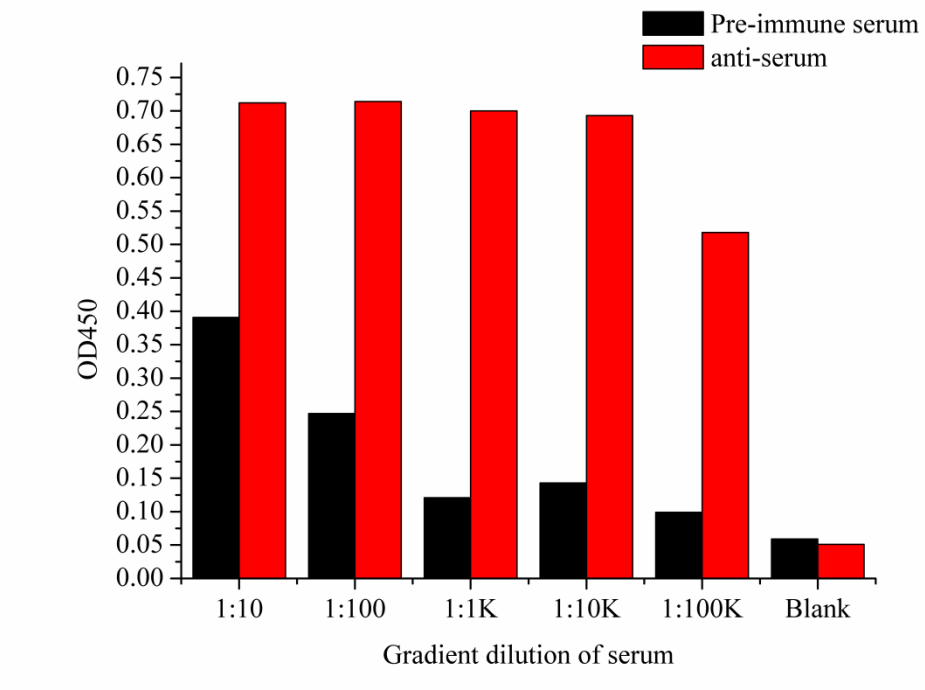

Fig.3 The poly clonal antibodies are specific against UL146. The specificity of antibodies against UL146 were evaluated by ELISA.

The titer of polyclonal antibody against UL146 was more than 100,000 in ELISA. Blank is the negative control. The titer represents the highest dilution with the ratio of anti-serum to pre-immunized serum more than2 (OD450).

3.4 Specificity of polyclonal antibody anti-UL146 was further confirmed by Western blot using vCXC-1 protein expressed in E. coli and from HCMV infected samples

To further confirm the specificity of polyclonal antibodies, Western blot analysis was performed using vCXC-l expressed in E. coli or HCMV infected cells. To ensure the expression of vCXC-1 in infection samples, RT-PCR was performed using UL146 specific primers. The results indicated the presence of UL146 mRNA, which suggested the expression of vCXC-1 in infection samples (Fig.4A). Western blot showed that a 35-kD His-tag fusion protein can bewas detected in IPTG-induced UL146 E. coli lysis (Fig.4B). More importantly, a 13-KD viral protein (UL146) can bewas detected in HCMV-infected cell culture media, but not in HCMV-infected cell lysis (4D). These results demonstrated and proved that UL146 gene product, vCXC-1, is secreted out of the infected cells, and the polyclonal antibody generated in this study not only recognizes the protein vCXC-1 expressed in E. coli, but also detects vCXC-1 protein in HCMV infected cells.

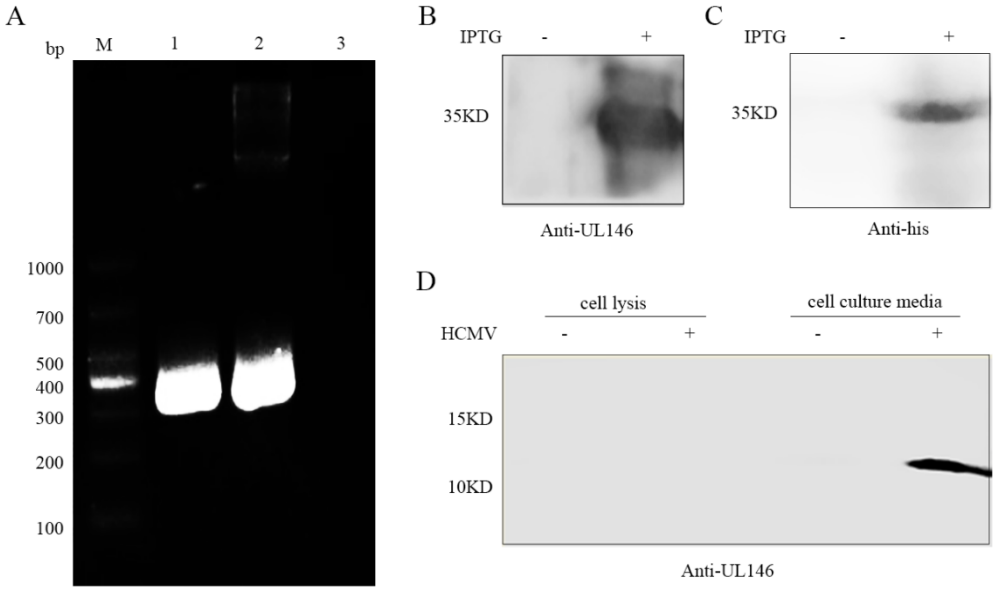

Fig.4 The specific recognition of endogenous protein vCXC-1 and exogenous expressed fusion protein vCXC-1-His.

HFF cells were infected with HCMV Towne strain for 96 h. A. RT-PCR analysis of UL146 expressed in HCMV Towne strain after 96 h infection. Lane 1: cDNA derived from HCMV-infected HFF cells were used as templates; lane 2: BAC Towne library was used as template as a positive control; lane 3: cDNA derived from mock infected HFF cells used as a negative control. B. The specific recognition of exogenous expressed protein vCXC-1-His with anti-UL146. C. The specific recognition of exogenous expressed protein vCXC-1-His with anti-His, used as a positive control. D. The specific recognition of endogenous protein vCXC-1 with anti-UL146.

4. Conclusion

In summary, we have successfully generated a polyclonal antibody against UL146, which provide a valuable tool for further characterizing the HCMV UL146 gene.

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