

Preparation and identification of an antiserum against recombinant UL31 protein of pseudorabies virus

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Summary. – Pseudorabies virus (PRV) late protein UL31 is a homologue of herpes simplex virus 1 (HSV-1) UL31, which is a multifunctional protein important for HSV-1 infection. However, the precise roles of PRV UL31 in virus life cycle are still poorly understood. A relatively crucial tool for uncovering the function of UL31 is an antiserum that specifically detects UL31 in the PRV-infected cells. For this purpose, a recombinant UL31 protein consisting of N-terminal 27 aa of UL31 fused to EYFP and His-tag was expressed, purified and used for the preparation of antiserum in BALB/c mice. Our results show that Western blot analysis and immunofluorescence assay showed that this antiserum could specifically detect the purified recombinant UL31 as well as full-length UL31 in the PRV infected cells. These results demonstrate that the prepared antiserum could serve as a valuable tool for further studies of UL31 functions in PRV infection.

Keywords: pseudorabies virus; UL31 protein; recombinant protein; antiserum

Introduction

Pseudorabies virus (PRV, the genus *Varicellovirus*, the subfamily *Alphaherpesvirinae*, the family *Herpesviridae*) is a pathogen of swine that can cause devastating diseases and economic loss worldwide. PRV is reported to be a useful model for the study of herpesvirus pathogenesis. Furthermore, the neurotropic nature of PRV also makes it a useful tracer of neuronal connections (Pomeranz *et al.*, 2005). Similar to other herpesviruses, PRV genes are believed to be coordinately regulated and expressed at the transcriptional level in a sequential cascade manner and are categorized into

three kinetic classes: immediate-early, early, and late genes (depending upon the requirements for their transcription and the timing of their synthesis) (Pomeranz *et al.*, 2005).

PRV UL31 protein (encoded by *UL31* gene), the homologue of herpes simplex virus 1 (HSV-1) UL31, is the component of the primary tegument with late expression during PRV infection (Granzow *et al.*, 2004). HSV-1 UL31 has been characterized as one of the most important structural proteins of HSV-1. Moreover, HSV-1 UL31 has received notable attention thanks to its interaction with HSV-1 UL34 which is involved in several viral replication processes (Ye and Roizman, 2000; Park and Baines, 2006). For example, UL31 and UL34 (i) co-localize at the nuclear rim (Reynolds *et al.*, 2001); (ii) change localization, interaction and phosphorylation of nuclear lamina components and disrupt the nuclear lamina (Reynolds *et al.*, 2004; Simpson-Holley *et al.*, 2004; Klupp *et al.*, 2007); and (iii) facilitate primary encapsidation and nuclear egress of their respective nucleocapsids, which are regulated by the phosphorylation of UL31 with pUS3 (Reynolds *et al.*, 2002; Mou *et al.*, 2009; Roller *et al.*, 2010). Thus, UL31

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Abbreviations: HSV-1 = herpes simplex virus 1; IFA = immunofluorescence assay; PRV = pseudorabies virus; p.i. = post infection

is a multifunctional protein. However, the exact function of UL31 during PRV infection is still poorly understood.

In this study, a recombinant prokaryotic plasmid that expresses truncated UL31 protein was constructed; protein was expressed and purified by nickel-nitrilotriacetic (Ni^{2+} -NTA) affinity chromatography. Purified protein was used to raise the polyclonal antiserum in BALB/c mice. Finally, the reactivity and specificity of prepared polyclonal antiserum were characterized by Western blot analysis and immunofluorescence assay (IFA). Prepared antiserum against UL31 has a good reactivity with recombinant and also native UL31.

Materials and Methods

Medium, enzymes and other materials. DMEM and FBS were purchased from Gibco-BRL. *Escherichia coli* strains DH5a and BL21 (DE3) were purchased from Invitrogen. Yeast extract and tryptone for bacterial medium preparation were obtained from Promega. Antibiotics and IPTG were bought from Novagen (USA). DNA polymerase KOD-Plus-Neo was purchased from TOYOBO (Japan). Restriction enzymes, DNA ligase, DNA molecular weight markers and protein molecular weight markers were obtained from TaKaRa. Purification of His-tagged protein was performed on a Ni^{2+} -NTA resin (Qiagen).

Cells. PK-15, HEK293T and Vero cells grown in DMEM were supplemented with 10% (vol/vol) heat-inactivated FBS, L-glutamine (2 mmol/l), penicillin (100 $\mu\text{g}/\text{ml}$), streptomycin (100 U/ml), essential sodium pyruvate (1%) and HEPES (10 mmol/l) and cells were maintained at 37°C in 5% CO_2 humidified incubator.

Virus production. PRV-BAC plasmid pBecker2 (Smith and Enquist, 2000) was purified and used for transfection of Vero cells to rescue the virus named vBecker2 as previously described (Li *et al.*, 2011).

Infectious virus assay. To assay infectious virus, the virus was propagated and titered on PK-15 cells as previously described (Li *et al.*, 2011).

Plasmid construction. UL31 ORF was amplified by PCR from PRV-BAC pBecker2 with the following primers: UL31-F (5'-CGAAGCTTCGGAATTCATGTTTGTAGCGACGGCGGCTC-3') and UL31-R (5'-GCAAGCTTAGGATCCGTCGGGCGAGGGGGCGGCGAAA-3'). Purified PCR products were digested with *EcoRI* and *BamHI* and ligated into the correspondingly digested pEYFP-N1 (Clontech) to generate pUL31-EYFP as in our previous studies (Li *et al.*, 2011). In addition, the DNA fragment encoding N-terminal 27 aa of UL31 amplified from pUL31-EYFP by primers UL31-F and UL31 (1-27)-R (5'-TTGGATCCAAGCGATCGCGGGCCGCGCGCG-3') was inserted into pEYFP-N1 to generate pUL31 (1-27)-EYFP. DNA fragment corresponding to the N-terminal 27 aa of UL31 fused to EYFP was digested with *EcoRI* and *NotI* and subcloned into the correspondingly digested pET-28a(+) (Novagen) to yield a recombinant prokaryotic expression plasmid pET28a(+)-UL31(1-27)-EYFP. For confirming the presence of the appropriate insert (pET28a(+)-UL31(1-27)-EYFP) by PCR, the primer EYFP-R (5'-GAAGATCTCGCTTGTA CAGCTCGTCCATGCCG-3') was used.

Expression and purification of recombinant protein. *E. coli* BL21 (DE3) were transformed with the recombinant plasmid. The expression and purification of the recombinant protein were performed as described previously (Li *et al.*, 2010, 2014).

Antiserum preparation. Preimmune serum, which served as a negative control, was collected prior to immunization. Preparation of the antiserum was performed as described previously (Li *et al.*, 2014). Briefly, BALB/c mice were subcutaneously injected with a mixture of 100 μg of purified UL31 recombinant protein mixed with an equal volume of complete Freund's adjuvant (Sigma) on the back and/or proximal limbs. Two weeks later, the mice were boosted twice subcutaneously with the same amount of UL31 recombinant protein mixed with an equal volume of incomplete Freund's adjuvant at a two-week interval. Two weeks after the last immunization, the antiserum was harvested from the sinus orbitalis of BALB/c mice and stored at -80°C for further use.

Western blot analysis. Western blot analysis was performed as previously described (Li *et al.*, 2014). Briefly, protein samples were subjected to 10% SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane by a semi-dry transfer cell (BioRad) at 15V for 0.5 hr. Membrane was blocked at 37°C for 2 hr with 5% non-fat milk in Tris-buffered saline with Tween-20 (TBST, 50 mmol/l Tris-HCl, 200 mmol/l NaCl, 0.1% (v/v) Tween-20, pH 7.5) and probed with the prepared anti-UL31 antiserum (1:500 dilution) at 37°C for 1 hr. After washing with TBST, the membrane was incubated with alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (1:10,000 dilution) (Sigma) at 37°C for 1 hr. Protein bands were developed by 5-bromo-4-chloro-3-indolylphosphate (BCIP)/nitroblue tetrazolium (NBT) and terminated by distilled water.

Immunofluorescence. To detect the subcellular localization of UL31 in PRV-infected cells, Vero cells were infected with PRV (vBecker2) at an MOI of 1 for 0, 8, 12 and 24 hr and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; 137 mmol/l NaCl, 2.7 mmol/l KCl, 10 mmol/l Na_2HPO_4 , and 2 mmol/l KH_2PO_4 , pH 7.4) for 30 min. After fixation, the cells were blocked with PBS containing 5% BSA and 10% FBS for 1 hr at 37°C. Subsequently, the cells were incubated with UL31-specific polyclonal antibody diluted in PBS containing 0.5% BSA for 1 hr at 37°C. Finally, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (Pierce) was added at a dilution of 1:200 and incubated at 37°C for 1 hr. After each incubation step, the cells were washed extensively with PBS. Hoechst staining was performed for cell nucleus visualization. Fixed cells were analyzed using a Zeiss Axio Observer A1 microscope (Germany). Images were processed with Adobe Photoshop (Li *et al.*, 2011, 2014).

Results

Construction of a plasmid expressing recombinant UL31 protein

In an effort to obtain the specific antibody against PRV UL31, which may be used for further investigation of UL31

biological functions, plasmids pUL31-EYFP and pUL31(1-27)-EYFP were constructed. DNA fragment encoding UL31 (1-27)-EYFP digested with *EcoRI* and *NotI* was subcloned from pUL31(1-27)-EYFP into the correspondingly digested pET-28a(+) to produce a recombinant prokaryotic expression plasmid pET28a(+)-UL31(1-27)-EYFP. Subsequently, the recombinant plasmid pET28a(+)-UL31(1-27)-EYFP was verified by colony PCR and restriction analysis. The results showed that it was successfully constructed (data not shown). In addition, DNA sequencing results demonstrated that there was no nucleotide mutation of truncated UL31 protein compared to that of the PRV Becker strain (data not shown).

Expression and purification of recombinant UL31 protein

After induction with 2.0 mmol/l IPTG at 37°C for 4 hr, *E. coli* BL21 (DE3) harboring the recombinant plasmid exhibited a high level of expression. A distinct band of approximately 35 K, corresponding to the expected M_r of recombinant UL31(1-27)-EYFP-His protein, was found only after induction, whereas there was no corresponding protein expression either in BL21 (DE3) harboring pET28a(+) or BL21 (DE3) harboring the recombinant plasmid without IPTG induction (data not shown).

Meanwhile, several expression parameters, including induction by IPTG concentrations and induction times were tested to optimize the expression of the recombinant protein. According to our results, the recombinant protein was found to have high expression under all examined condition (data not shown).

In addition, according to SDS-PAGE analysis of the soluble fraction and cell debris pellet, the induced recombinant protein was found predominantly in the cell debris pellets (data not shown), suggesting that this recombinant protein was mainly insoluble in the form of inclusion bodies.

Purification of the recombinant protein was performed using an immobilized metal affinity chromatography on Ni^{2+} -NTA resin column. SDS-PAGE analysis showed that the recombinant protein was successfully purified and one band corresponding to M_r of about 35 K was detected (data not shown). Approximately 280 mg of recombinant protein per liter of the culture was obtained after purification. Subsequently, the purified protein was injected into BALB/c mice to raise the antiserum.

Characterization of the antiserum against recombinant UL31 protein

After four immunizations, the antiserum was extracted from sinus orbitalis of BALB/c mice. To evaluate the reactivity and specificity of the prepared antiserum we performed Western blot analysis. The antiserum could specifically recognize the purified recombinant protein (Fig. 1, lane 1) and protein in the lysates of BL21 (DE3) harboring the recom-

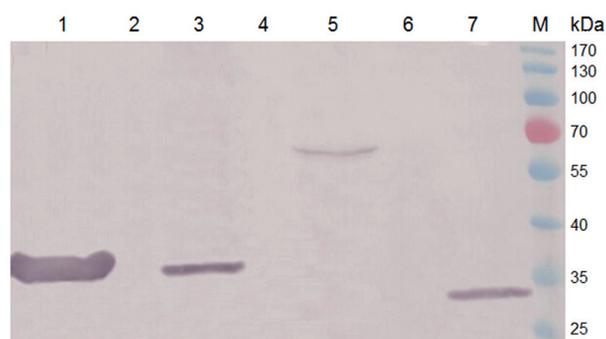


Fig. 1

Characterization of the antiserum against recombinant UL31 protein
Western blot analysis of the antiserum. Purified recombinant protein (lane 1); recombinant plasmid in *E. coli* BL21 uninduced (lane 2) and induced (lane 3) with IPTG; untransfected HEK293T cells (lane 4) or HEK293T cells transfected with pUL31-EYFP (lane 5); mock Vero cells (lane 6) and infected with PRV (lane 7); lane M, protein marker.

binant plasmid after induction with 1.0 mmol/l IPTG at 37°C for 4 hr (Fig. 1, lane 3), but there was no cross-reaction with protein in the lysates of non-transformed BL21 (DE3) (Fig. 1, lane 2) under the same conditions. In addition, Western blot analysis also indicated that the antiserum could clearly detect a band of the EYFP tagged full-length UL31 protein in the lysates of pUL31-EYFP transfected HEK293T cells (Fig. 1, lane 5) whereas no band was observed in the lysates of untransfected HEK293T cells (Fig. 1, lane 4). Moreover, the antiserum was able to recognize UL31 in Vero cells infected with PRV (Fig. 1, lane 7) while no band was detected in mock-infected Vero cells (Fig. 1, lane 6). These results indicate that the prepared antiserum cannot only detect the recombinant UL31 but also the native UL31.

Subcellular localization of UL31 protein in PRV-infected cells

Thus far we had shown that in Western blot analysis the prepared antiserum could specifically detect UL31 in cells infected with PRV. Here, IFA was carried out to identify the subcellular localization pattern of UL31 in PRV-infected Vero cells at different stages. After 0, 8, 12 and 24 hr post infection (p.i.), PRV-infected Vero cells were fixed and permeabilized. The cells were blocked with BSA to eliminate nonspecific binding and incubated with the prepared antiserum. As shown in Fig. 2, UL31 was distributed throughout the nucleus after 8 hr p.i., and after 24 hr p.i. It was localized predominantly at nuclear rim or nuclear membrane. In contrast, no specific staining was observed in PRV-infected cells 0 hr p.i. (Fig. 2). These results suggest that the prepared antiserum against the recombinant protein has a good reactivity and specificity against the native UL31 protein in infected cells, and also reveals that UL31 is a nuclear-targeted protein.

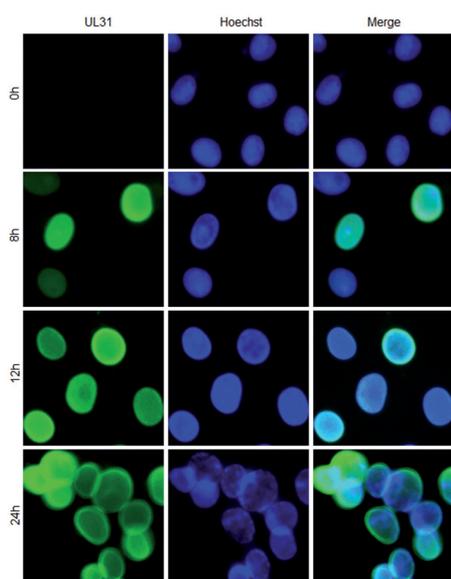


Fig. 2

Subcellular localization of the UL31 protein in PRV-infected Vero cells

Immunofluorescence of cells infected with PRV vBecker2 at an MOI of 1, after 0, 8, 12 and 24 hr p.i. with the antiserum against the recombinant UL31 protein.

Discussion

Here we raised an antiserum against the recombinant UL31 protein which could specifically recognize the full-length UL31 protein. To establish an efficient approach to purify the recombinant UL31, a Ni²⁺-NTA resin column, which exhibits a high binding capacity allowing for a very rapid and single-step purification, was applied to purify the recombinant UL31 protein (Arnau *et al.*, 2006). Additionally, GFP or its mutant has been already used as probe to detect protein expressed in live cells, therefore, truncated UL31 protein was tagged with EYFP in this study (Chalfie *et al.*, 1994). Furthermore, for the expression of recombinant UL31 protein, the *E. coli* strain BL21 (DE3) was used. This strain has the advantage of being deficient in both the lon and ompT proteases and harbors the T7 bacteriophage RNA polymerase gene, which permits the specific expression of heterologous genes driven by the T7 promoter (Studier *et al.*, 1990; Mierendorf *et al.*, 1998).

It is well known that diverse intracellular localizations might reflect different roles of viral proteins, and the intracellular localization of viral proteins may also vary at different times after infection (Feng, 2002). For instance, it has been shown that the localization of HSV-1 UL31 can re-locate from the nucleus to small dense nuclear bodies during the viral replication cycle in Vero cells infected with HSV-1 (Lin *et al.*, 2010), and PRV UL54 relocated from nucleolus to the nucleus as the virus replicates in PK-15 cells at low MOI (Li *et al.*, 2011, 2012). Thus, using the prepared antiserum, the subcellular localization of UL31

in PRV-infected Vero cells was observed at various times post infection. The results showed that after 8 hr p.i. UL31 mainly localized in the nucleus of Vero cells infected with PRV, however, later during the infection, UL31 redistributed from nucleus to the nuclear membrane. It was reported that UL31 and UL34 are required for nuclear egress of herpesviruses from all three subfamilies. UL34 is targeted to the nuclear envelope even in the absence of other viral proteins, whereas UL31 is found diffusely distributed in the nucleus in transfected cells but is relocated to the nuclear rim in the presence of UL34 (Reynolds *et al.*, 2001, 2002, 2004; Fuchs *et al.*, 2002; Simpson-Holley *et al.*, 2004; Klupp *et al.*, 2007; Mou *et al.*, 2009; Roller *et al.*, 2010). Besides, HSV-1 UL31 and UL34 have separate but related functions in recruiting appropriate components to nucleocapsid budding sites at the inner nuclear membrane (Wills *et al.*, 2009). Furthermore, HSV-1 UL31 might form a network to enable the anchorage of viral products for the synthesis and/or packaging of viral DNA into virions (Chang *et al.*, 1997). Moreover, HSV-1 UL31 is also implicated in the optimal activation of NF- κ B and expression of viral gene products (which are frequently related with the nucleus) (Roberts and Baines, 2011), it is therefore not unexpected that UL31 is a primarily nuclear associated protein.

Taken together, the antiserum raised against the recombinant UL31 could recognize not only the purified UL31 but also the native UL31 in PRV-infected cells. Accordingly, this antiserum may serve as a valuable tool for further study of the biological functions of UL31 during PRV infection.

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