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Preparation and characterization of an antiserum against truncated UL54 protein of pseudorabies virus

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Summary. – Pseudorabies virus (PRV) early protein UL54 is a homolog of herpes simplex virus 1 immediate-early protein ICP27, which is a multifunctional protein essential for the virus replication. However, the precise role of the PRV UL54 protein in the virus life cycle is still poorly understood. To shed more light on this problem, we considered it essential to have available an antiserum specifically detecting this protein. Since it was known that a full-length UL54 protein is a too big molecule for efficient expression in prokaryotic systems, it was truncated from 1 to 66 N-terminal amino acids, fused to EYFP-His tag and expressed in *Escherichia coli* through an appropriate expression vector. The truncated protein was purified by Ni-NTA affinity chromatography and used for raising an antiserum in rabbits. Western blot analysis showed that this antiserum specifically recognized the purified truncated as well as full-length UL54 protein in PRV-infected cells. Immunofluorescence assay confirmed the latter finding and also demonstrated localization of this protein first in nucleoli and later in whole nuclei of PRV-infected cells. These results indicate that the prepared antiserum could serve as a valuable tool in further studies of PRV UL54 protein function.

Keywords: pseudorabies virus; UL54 protein; truncated protein; antiserum

Introduction

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, the PRV genes are believed to be coordinately regulated and expressed 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).

UL54, a PRV-encoded early protein consisting of 361 aa, is a potential RNA binding protein (Huang *et al.*, 2005). The homologue of PRV UL54, the herpes simplex virus 1 (HSV-1) immediate-early protein ICP27, is a multifunctional protein with roles in the shutoff of host protein synthesis, transactivation of viral and cellular genes, inhibition mRNA splicing and translation regulation, it stimulates pre-mRNA 3′ processing, shuttling between the nucleus and the cytoplasm, promotes viral RNA nuclear export, transcriptional regulation and formation of virus-induced-chaperone enriched domains (Sandri-Goldin, 2008; Dobrikova *et al.*, 2010; Hernandez and Sandri-Goldin, 2010). Furthermore, ICP27 is proven to be implicated in apoptosis, determining the composition of HSV-1 virions and inhibition of type I interferon signaling (Zachos *et al.*, 2001; Melchjorsen *et al.*, 2006; Johnson *et al.*, 2008; Sedlackova and Rice, 2008; Gillis *et al.*, 2009). To date, ICP27 and its other homologues have been extensively stud-

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Abbreviations: HSV-1 = herpes simplex virus 1; IFA = immunofluorescent assay; Ni^{2+}-NTA = Ni^{2+}-nitrilotriacetic; NLS = nuclear localization signal; NoLS = nucleolar localization signal; p.i. = post infection; PRV = pseudorabies virus

ied, however, the exact functional properties of PRV UL54 are less understood, despite the fact that the aa sequence of UL54 shows 41% identity to ICP27 (Hamel and Simard, 2003; Boyne and Whitehouse, 2006; Melchjorsen *et al.*, 2006; Boyne *et al.*, 2008; Johnson *et al.*, 2008; Sandri-Goldin, 2008; Toth and Stamminger, 2008; Boyne and Whitehouse, 2009; Guo *et al.*, 2009; Juillard *et al.*, 2009; Ding *et al.*, 2010; Ote *et al.*, 2010).

In the present study, the UL54 protein, truncated to 66 N-terminal amino acids and fused to EYFP-His tag was expressed in *E. coli*. The truncated protein was purified by Ni-NTA affinity chromatography and used for raising an antiserum in rabbits. This antiserum (i) detected the truncated as well as the full-length UL54 protein in Western blot analysis and (ii) revealed the localization of the UL54 protein in PRV-infected cells.

Materials and Methods

Medium, enzymes and other materials. DMEM and FBS were purchased from Gibco-BRL. *Escherichia coli* strains DH5α 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. Restriction enzymes, DNA ligase, Ex *Taq* DNA polymerase, 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 μg/ml), streptomycin (100 U/ml), essential sodium pyruvate (1%) and HEPES (10 mmol/l) and cells were maintained at 37°C in a 95% air-5% CO_2 humidified incubator.

Transfection with PRV-BAC. PRV-BAC plasmid pBecker2 (Smith and Enquist, 2000) was extracted to transfect into Vero cells and then used to rescue the virus named vBecker2 as previously described (Marchini *et al.*, 2001; Li *et al.*, 2011a,b).

Infectious virus assay. To assay infectious virus, the virus was multiplied and titered on PK-15 cells as described previously (Marchini *et al.*, 2001; Li *et al.*, 2011a,b).

Plasmid constructs. UL54 ORF was amplified by PCR from PRV-BAC pBecker2 with the following primers: UL54F (5′-CG GAATTCATGGAGGACAGCGGCAACAG-3′) and UL54R (5′-CGGGATCCTCAAACAGGTGGTTGCAGTAAAAG-3′). The purified PCR product was digested with *Eco*RI and *Bam*HI and ligated into the correspondingly digested pEYFP-N1 (Clontech) to generate pUL54-EYFP as in our previous studies (Li *et al.*, 2011a,b). UL54 ORF was also subcloned into pCMV-N-HA (Beyotime) and pcDNA3.1(+) (Invitrogen) by the same protocol as described above to yield pCMV-HA-UL54 and pcDNA3.1-UL54, respectively. In addition, the DNA fragment encoding N-terminal 66 aa of UL54 amplified from pUL54-EYFP by primers UL54F and N66R (5′-TTGGATCCATCTGCCGCCGCCGCTGCCGCAG-3′) was inserted into pEYFP-N1 to generate pN66-EYFP. The DNA fragment corresponding to the N-terminal 66 aa of UL54 fused to EYFP, digested with *Eco*RI and *Not*I, was subsequently subcloned into the correspondingly digested pET-28a(+) (Novagen) to yield a recombinant prokaryotic expression plasmid pET28a(+)-N66-EYFP. The presence of the appropriate insert in the obtained plasmids pET28a(+)-N66-EYFP and pN66-EYFP was confirmed by PCR using the primers UL54F and EYFP-R (5'-GAAGATCTCGCTT GTACAGCTCGTCCATGCCG-3').

Expression and purification of truncated protein. *Escherichia coli* BL21 (DE3) were transformed with the recombinant plasmid pET28a(+)-N66-EYFP. The expression and purification of the truncated protein were performed as described previously (Zhao *et al.*, 2010; Li *et al.*, 2011c).

Antiserum preparation. Preimmune serum, which served as a negative control, was collected prior to immunization. The preparation of the antiserum was performed as described previously, with the exception that the recombinant truncated UL54 protein was used in this study (Zhao *et al.*, 2010; Li *et al.*, 2011c).

Western blot analysis. Western blot analysis was performed as previously described, except that the antiserum against the recombinant truncated UL54 or the monoclonal antibody against β-actin (Proteintech Group) was used in this assay (Zhao *et al.*, 2010; Li *et al.*, 2011c).

Immunofluorescent assay (IFA). To detect the subcellular localization of UL54 in PRV-infected cells, non-infected PK-15 cells and cells infected with PRV (vBecker2) at a MOI of 0.1 or 1 at 4, 7, and 10 hrs post infection (p.i.) were subjected to IFA by formaldehyde-based fixation method as described in our previous studies, using the preimmune serum or the antiserum against recombinant truncated UL54 (Li *et al.*, 2011a,b).

Results

Construction of a plasmid expressing the truncated UL54 protein

In an effort to obtain the specific antibody against PRV UL54, which may be used to further investigate its biological function, a recombinant plasmid pN66-EYFP was constructed and verified by colony PCR (Fig. 1a, lanes 1 and 2) and restriction analysis (Fig. 1a, lane 3). After that the DNA fragment corresponding to truncated UL54-EYFP gene was subcloned from pN66-EYFP into pET-28a(+) to yield a recombinant plasmid pET28a(+)-N66-EYFP expressing truncated UL54-EYFP-His protein. Subsequently, colony PCR (Fig. 1b, lanes 1 and 2) and restriction analysis (Fig. 1b, lane 3) of pET28a(+)-N66-EYFP showed that it was successfully constructed. Furthermore, DNA sequencing results demonstrated that there was no nucleotide mutation of truncated UL54 protein compared to that of the PRV Becker strain (data not shown).

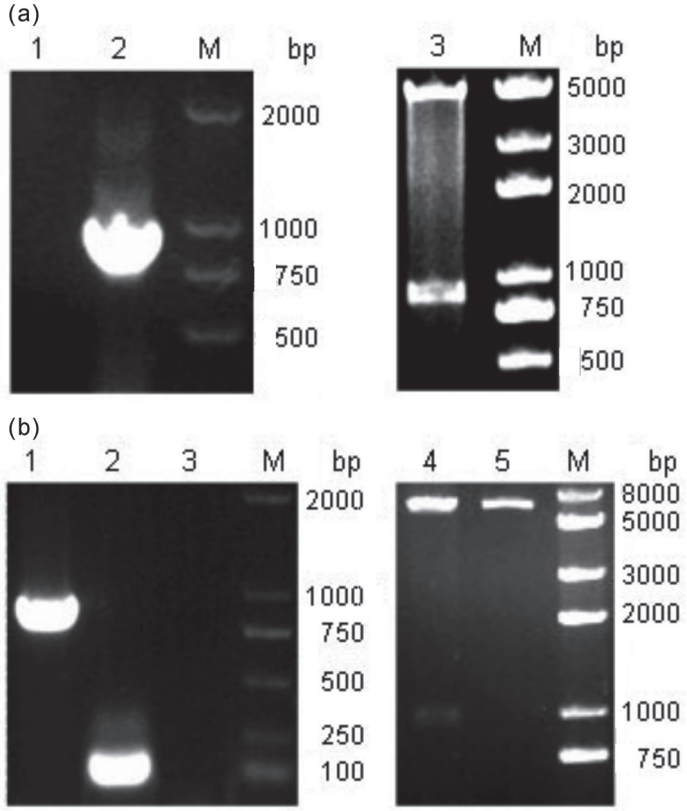

Fig. 1

Verification of plasmid constructs

(a) Cloning of the plasmid pN66-EYFP: PCR and restriction analysis. Negative control (lane 1), product of PCR with primers UL54F and EYFP-R (truncated UL54-EYFP gene) (lane 2), pN66-EYFP digested with *Eco*RI and *Not*I (lane 3), DNA size markers (lanes M). (b) Expression plasmids pET28a(+)-N66-EYFP and pET28a(+): PCR and restriction analysis. pET28a(+)-N66-EYFP, product of PCR with primers UL54F and EYFP-R (truncated UL54-EYFP gene) (lane 1), pET28a(+)-N66-EYFP, product of PCR with primers UL54F and N66R (truncated UL54 gene) (lane 2), negative control (lane 3), pET28a(+)-N66-EYFP digested with *Eco*RI and *Not*I (lane 4), pET28a(+) digested with *Eco*RI and *Not*I (lane 5), DNA size markers (lanes M).

Expression and purification of the truncated UL54 protein

After induction with 1.0 mmol/l IPTG at 37°C for 4 hrs, *E. coli* BL21 (DE3) cells harboring pET28a(+)-N66-EYFP exhibited a high level of protein expression (Fig. 2, lane 2). A distinct band of approximately 36 kDa, corresponding to the expected Mr of truncated UL54-EYFP-His protein, was found only after induction (Fig. 2, lane 2), whereas there was no corresponding protein expression without IPTG induction (Fig. 2, lane 1).

In addition, according to SDS-PAGE analysis of the soluble fraction and cell debris pellet, the induced truncated UL54-EYFP-His protein was found in the soluble fraction as well as in cell debris pellet (Fig. 2, lanes 3 and 4). For purification, soluble fraction of the truncated UL54-EYFP-His protein was used.

Purification of the truncated UL54-EYFP-His protein was performed using a single chromatographic step of im-

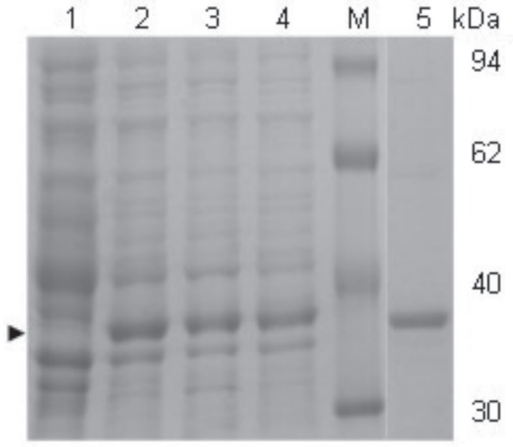

Fig. 2

Expresion and purification of the truncated UL54 protein

SDS-PAGE of total proteins from non-induced *E. coli* BL21 (DE3) cells carrying the plasmid pET28a(+)-N66-EYFP (lane 1), total proteins from induced cells (lane 2) and their soluble (lane 3) and insoluble fractions (lane 4), purified truncated UL54 protein, positive control (lane 5), protein size markers (lane M).

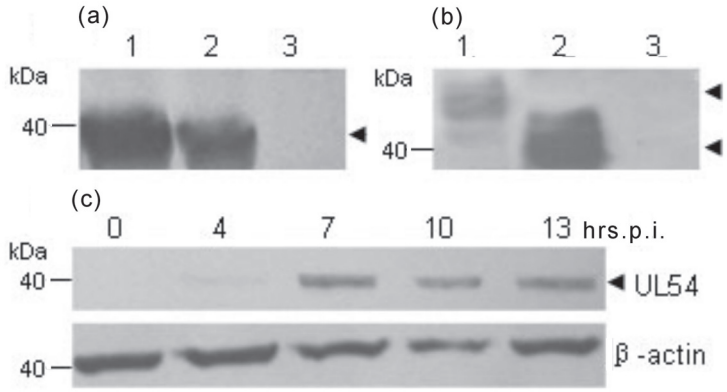

Fig. 3

Characterization of the antiserum against truncated UL54 protein

Western blot analysis. (a) Purified truncated UL54 protein, positive control (lane 1), lysate of *E. coli* expressing truncated UL54 protein (lane 2), negative control (lane 3). (b) Lysates of HEK293T cells expressing full-length UL54 protein from pCMV-HA-UL54 (lane 1) and pcDNA3.1-UL54 (lane 2), negative control (lane 3). (c) Lysates of PK-15 cells infected with vBecker2 prepared at 0–13 hrs p.i. and probed with the antiserum against truncated UL54 and with the monoclonal antibody against β-actin, respectively.

mobilized metal affinity chromatography on Ni^{2+}-NTA resin column. SDS-PAGE analysis showed that the truncated UL54-EYFP-His protein was successfully purified, because only one clear band corresponding to Mr of about 36 kDa was detected (Fig. 2, lane 5). Approximately 400 mg of the recombinant protein per liter of culture was obtained after purification. Subsequently, the purified protein was injected into New Zealand White rabbit to raise the antiserum against the truncated UL54 protein.

Characterization of the antiserum against the truncated UL54 protein

After three immunizations, the antiserum was collected from the carotid artery of New Zealand White rabbit, then Western blot analysis was performed to evaluate the reactivity and specificity of the prepared antiserum. It was found that the antiserum could specifically recognize the purified truncated UL54-EYFP-His protein (Fig. 3a, lane 1) and the protein in the lysates of BL21 (DE3) cells harboring pET28a(+)-N66-EYFP after the induction with 1.0 mmol/l IPTG at 37°C for 4 hrs (Fig. 3a, lane 2), but does not cross-react with the proteins in the lysates of non-transformed BL21 (DE3) cells (Fig. 3a, lane 3) under the same conditions. Furthermore, Western blot analysis also demonstrated that the antiserum was able to recognize the hemagglutinin (HA)-tagged (Fig. 3b, lane 1) and untagged full-length UL54 protein (Fig. 3b, lane 2) in the lysates of pCMV-HA-UL54 and pcDNA3.1-UL54 transfected HEK293T cells, respectively, whereas no band was observed in the lysates of untransfected HEK293T cells (Fig. 3b, lane 3). These results indicate that the prepared antiserum can not only detect truncated recombinant UL54 but also full-length UL54.

Moreover, the antiserum could clearly detect a protein with an apparent Mr of approximately 40 kDa in PK-15 cells infected with PRV (Fig. 3c). In contrast, no band was detected in non-infected PK-15 cells at 0 hr p.i. (Fig. 3c). Additionally, the viral protein UL54 became detectable at 4 hrs p.i. and obvious at 7 hrs p.i. (Fig. 3c), as previously described (Huang and Wu, 2004).

Subcellular localization of UL54 protein in PRV-infected cells

Thus far we have shown that the antiserum could specifically identify the UL54 protein in PRV-infected cells in Western blot analysis. Here, IFA was carried out to characterize the subcellular localization of UL54 in PRV-infected PK-15 cells at different stages. At 4, 7, and 10 hrs p.i., non-infected and PRV-infected PK-15 cells were fixed and permeabilized as described in Materials and Methods. Then, the cells were blocked with BSA to remove nonspecific binding and incubated with the antiserum. As shown in Fig. 4, in cells infected at low MOI (MOI of 0.1), UL54 exhibited mainly a speckle-like distribution in the nucleus that resembles the nucleolus at early virus infection, then it localized throughout the nucleus at 10 hrs p.i. (Fig. 4a). In contrast, UL54 localized throughout the nucleus at almost all time points examined, when cells were infected with PRV at an MOI of 1 (Fig. 4b). In contrast, no specific staining was observed in non-infected cells that were incubated with the prepared antiserum (Fig. 4c) or in PRV-infected cells incubated with

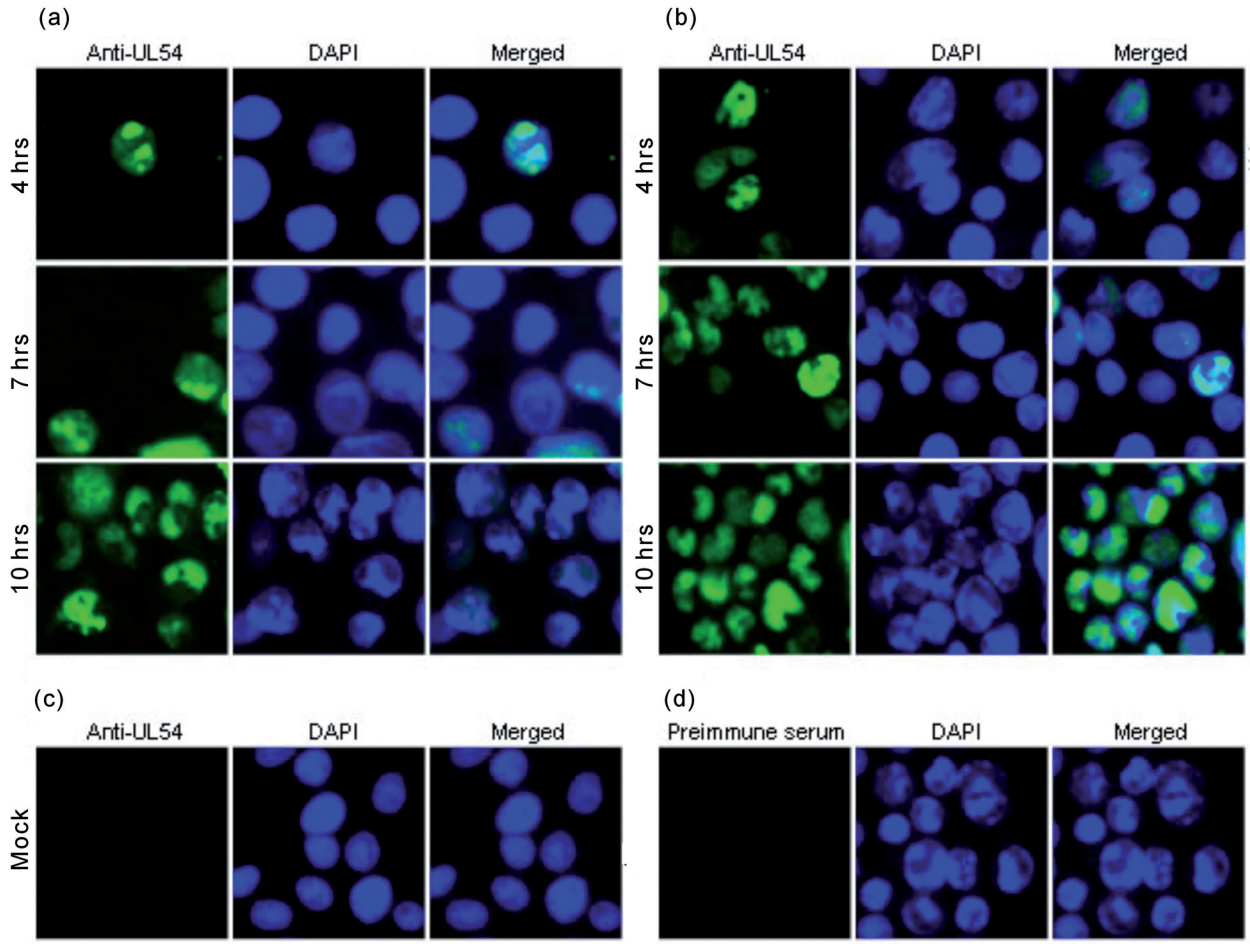

Fig. 4

Subcellular localization of the UL54 protein in PRV-infected PK-15 cells

Cells infected with PRV vBeker2 at MOI of 0.1 (a) and 1 (b), respectively, non-infected control (c). IFA was performed at 4, 7, and 10 hrs p.i. with the antiserum against truncated UL54 protein (anti-UL54) or with the preimmune serum as the negative control (d).

pre-immune serum (Fig. 4d). These results suggest that the antiserum against the truncated UL54 protein has a good reactivity and specificity against the native UL54 protein in infected cells.

Discussion

Here we raised an antiserum against the truncated UL54 protein, which could specifically recognize the full-length UL54 protein. Moreover, the truncated UL54 contains both of the nuclear localization signal (NLS) and nucleolar localization signal (NoLS), which have been described before, thus makes the prepared antiserum a good tool for further studying the functions of NLS and NoLS of UL54 or/and full-length UL54 (Li *et al.*, 2011b).

To establish an efficient approach to purify the recombinant truncated UL54, a Ni^{2+}-NTA resin column, which exhibits a high binding capacity, allowing for a very rapid and single-step purification, was used to purify the truncated UL54 protein (Arnau *et al.*, 2006). Additionally, the truncated UL54 protein was also tagged with EYFP in this study, as GFP or its mutants have been used as a probe to detect protein expressed in live cells (Chalfie *et al.*, 1994). For the expression of the truncated UL54 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; Cai *et al.*, 2009b).

It is thought to be a universal phenomenon that almost every protein is marked with a correct location signal in

cell and proteins must be localized at their appropriate subcellular compartment to perform their desired functions. Different intracellular localizations may reflect different functions of viral proteins, and the intracellular localizations of viral proteins may also vary at different times after the infection (Feng, 2002; Cai *et al.*, 2009a). Early studies demonstrated that UL54 is a nuclear protein, which can localize exclusively to the nucleolus in the absence of other viral proteins, whereas the native UL54 in PRV-infected cells is delocalized from the nucleolus to the nucleus in a structure that might resemble the viral replication compartment during infection (Monier *et al.*, 2000; Calle *et al.*, 2008; Li *et al.*, 2011b). In line with previous studies(Li *et al.*, 2011a,b), our results here showed that the localization of UL54 was changed from nucleolus to the entire nucleus as the virus replicated in PK-15 cells at low MOI, suggesting that the subcellular localization pattern of UL54 is associated with viral replication cycle at low MOI (MOI = 0.1) (Li *et al.*, 2011a,b). Recently, a report showed that the localization of HSV-1 UL3 has changed from the nucleus to small dense nuclear bodies as the viral replication cycle in Vero cells infected with HSV-1 progressed (Lin et al., 2010). However, UL54 only located to the nucleus when PK-15 cells were infected with PRV at high MOI (MOI = 1) at all time points examined, implying that the subcellular localization of UL54 also correlates with the virus titer (Lin *et al.*, 2010). Indeed, the correlation between viral gene expression and virus MOI has been described before and multiplicity-dependent growth phenotype has also been observed for several viruses, including PRV, HSV-1, human cytomegalovirus, African swine fever virus and adenovirus (Nevins, 1981; Cai and Schaffer, 1992; Chen and Silverstein, 1992; Moore *et al.*, 1998; Bresnahan and Shenk, 2000; Everett *et al.*, 2004; Fang *et al.*, 2004; Li *et al.*, 2011b). Therefore, the different subcellular localization of UL54 suggests that UL54 perhaps plays an important role in the gene expression, efficient PRV production or some other processes in the course of viral replication cycle.

A previous study shows that while UL54-null PRV is viable, the mutant exhibits aberrant expression of several early and late genes and is highly attenuated in a mouse model. Moreover, UL54 is demonstrated to target to the nucleus through a classic Ran-, importin β1- and α5-dependent nuclear import mechanism. The NLS and NoLS contained in the truncated UL54 is critical for its nuclear and nucleolar import, which have also been well described in ICP27 (Mears *et al.*, 1995; Chen *et al.*, 2002; Huang *et al.*, 2005; Schwartz *et al.*, 2006; Li *et al.*, 2011a,b). Furthermore, the recombinant virus with mutations of the NLS and/or the NoLS in UL54 can have modest or severe defects in DNA synthesis and viral gene expression, as well as in production of infectious progeny, demonstrating that the nuclear targeting of UL54 is required for efficient production of PRV (Li *et al.*, 2011b). These studies indicate that UL54 is a multifunctional protein that might be involved in the transcriptional and/or posttranscriptional regulation of gene expression during infection (Schwartz *et al.*, 2006; Li *et al.*, 2011b). Therefore, the interactions between UL54 and other viral proteins, the exact roles of UL54 in the viral life cycle, as well as the corresponding molecular mechanisms, are all interesting questions to be answered.

Taken together, the antiserum raised against the recombinant truncated UL54 could recognize not only the purified recombinant UL54 but also the native UL54 in PRV-infected cells. Accordingly, this antiserum may serve as a valuable tool for further studies of the biological functions of UL54 during PRV infection.

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