Original Article

Expression and purification of rabies virus protein P: production and characterization of anti-P polyclonal antibodies

Yan Pan[1,3] Xian-Ming Meng[2] Xian-Kai Wei[2] Jun Li[3] Zhuan-Ling Lu[2]

Hai-Bo Tang[2] Jing-Jing Liang[2] Ting Rong Luo[1,2*]

Abstract

Rabies virus (RV) phosphoprotein (P) is a multifunctional protein. However, the functions of this protein are not fully understood. To further investigate this question, the P protein was expressed and used to raise monospecific polyclonal antibodies. The gene coding for P protein was cloned into expression vector pET-32a (+) to yield pET-32a (+)-P. His-tagged P protein was expressed in *E. coli* BL21 cells and analyzed by SDS-PAGE. After purification by nickel affinity chromatography under native conditions, the recombinant P protein was used to raise anti-P polyclonal antibodies in mice. Western blot analysis showed that the P protein was recognized by the polyclonal antibodies. Immunofluorescence assays also showed that the antibody was able to recognize the native P protein in RV-infected cells.

Keywords: rabies virus, P protein, protein expression and purification, polyclonal antibodies

[1]*State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresourses, Guangxi University, Nanning 530004, Guangxi, China*

[2]*Laboratory of Animal Infectious Diseases, College of Animal Sciences and Veterinary Medicine, Guangxi University, Nanning 530004, Guangxi, China*

[3]*Guangxi Key Laboratory of Veterinary Biotechnology, Guangxi Veterinary Research Institute, Nanning 530001, Guangxi , China*

***Correspondence**: tingrongluo@gxu.edu.cn*

Introduction

Rabies virus (RV), a member of the Rhabdoviridae family, causes fatal neurological disease in humans and animals if not treated in a timely manner. Although significant advances have been made in rabies prevention and control, the disease remains a major threat to public health and continues to cause numerous human deaths around the world. Dogs remain the most important reservoir in Asia, Africa, and Latin America, where most human rabies cases occur (Fu et al., 1997).

The RV genome is non-segmented negative-sense single-stranded RNA and comprises five genes encoding nucleoprotein (N), phosphoprotein (P), matrixprotein (M), glycoprotein (G), and large protein (L) in the order 3′-N-P-M-G-L-5′. The P protein is a multifunctional, essential structural protein with 297 amino acid length. P-deficient rabies viruses are unable to replicate (Shoji et al., 2004; Finke et al., 2004), as RV P is a scaffold protein that is associated with the N and L proteins.

Interaction between RV P protein and a component of the microtubular transport complex, the dynein light chain (LC8), has been suggested to have a role in axonal retrograde transport of RV (Raux et al., 2000; Jacob et al., 2000). Antagonizing effects of RV P protein on specific functions of the innate immune system have been recognized. The P protein mediates inhibition of innate immunity by affecting different pathways. First, it inhibits interferon beta (IFN-β) production by impairing IFN regulatory factor-3 (IRF-3) phosphorylation (Brzózka et al., 2005). Second, P protein prevents IFN signaling by blocking nuclear transport of signal transducer and activator of transcription 1 (STAT1) (Brzózka et al., 2006). Third, it alters promyelocytic leukemia (PML) nuclear bodies by retaining PML in the cytoplasm (Blondel et al., 2002).

To further investigate the functions of P protein, a recombinant plasmid was constructed to express a His-tagged P protein. The recombinant protein was expressed in *E. coli* BL21 (DE3) cells and purified using nickel-nitrilotriacetic acid (Ni^{2+}-NTA) affinity resin under native conditions. Subsequently, a polyclonal antibody was raised against the purified P protein in mice. The reactivity and specificity of the polyclonal antibody are discussed.

Materials and Methods

Ethics statement: All animal experiments mentioned in this research were conducted according to the National Guidelines on the Humane Treatment of Laboratory Animal Welfare (MOST of People's Republic of China, 2006) and approved by the Animal Welfare and the Animal Experimental Ethical Committee of Guangxi University (No. Xidakezi2000138). All husbandry procedures were conducted in compliance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals.

Cells and viruses: Mouse neuroblastoma (NA) and baby hamster kidney (BHK) cell lines were maintained in Dulbecco's modified eagle medium-high glucose (DMEM) supplemented with 10% fetal calf serum (FCS) and incubated at 37°C, 5% CO_2. The rabies virus ERA/Flury strains were used in this study. All viral stocks were prepared in NA cells and stored at -80°C. Viral titers were determined by a focus-forming assay in NA cells.

Construction of prokaryotic expression plasmid for His-tagged P protein: All restriction enzymes used for cloning were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). The open reading frame (ORF) of the RV P gene was amplified from RV (ERA strain) with the forward primer (5′-CCG*AATTC*ATGAGCAAGATCTTTGT-3′, *Eco*R I restriction enzyme site employs ialics), and the reverse primer (5′-CG*AAGCTT*TTAGCAAGATGTA TAGCG-3′, *Hind* III site employs italics). The PCR product was digested with *Eco*R I and *Hind* III and inserted into the prokaryotic expression vector pET-32a (+) digested with the same enzymes to create pET-32a (+)-P.

Expression of recombinant His-tagged P protein: *E. coli* BL21 (DE3) cells (TaKaRa Biotechnology, Dalian, China) were transformed with pET-32a (+)-P and the transformants were inoculated into Luria–Bertani (LB) medium containing 100 μg/ml ampicillin and grown at 37°C, until the cultures reached an OD_{600} of about 0.8. Then, recombinant protein expression was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.4 mM at 28°C for 5 hr. Total bacterial lysate was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Analysis of solubility of recombinant His-tagged P protein: Cell pellets harvested from 100 mL culture medium after induction at 37°C were suspended in 20 mL lysis buffer (20 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole). The sample was frozen in dry ice, thawed in cold water, and then subjected to sonication on ice. Total cellular proteins were then partitioned into soluble and insoluble fractions by centrifugation at 12,000 × *g* at 4°C for 30 min. The supernatant was transferred into a fresh tube and the pellet was resuspended in 2 mL lysis buffer. The soluble and insoluble protein fractions were analyzed by SDS-PAGE.

Purification of recombinant His-tagged P protein: Cells from 1000 ml of culture medium were harvested and resuspended in 20 mL lysis buffer, then subjected to sonication and centrifugation at 12,000 × *g* at 4°C for 30 min. The supernatant was collected and saved on ice and subsequently filtered through a 0.45 μM membrane. The recombinant His-tagged P protein was purified through a gravity flow column prepacked with 2 ml Ni-NTA Agarose (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions.

Immunization of mice and production of polyclonal antibodies: Six-week-old male BALB/c mice were injected intradermally into the back with a mixture of 50 μg purified P protein and an equal volume of complete Freund's adjuvant (Sigma-Aldrich China Inc., Shanghai, China). Normal serum was collected

from the mice before immunization. The mice were boosted twice at 2-week intervals with intradermal injection of the same amount of P protein mixed with an equal volume of incomplete Freund's adjuvant. Two weeks after the final immunization, blood was collected from retro-orbital vessels and the serum was stored at -80°C.

Western blot: BHK-21 cells infected with RV (ERA/Flury strain) were collected. The total proteins in cell lysate were separated by 12% SDS-PAGE and transferred to a polyvinylidene fluoride membrane using a semi dry transfer unit (Bio-Rad Laboratories, Shanghai, China) at 20 V for 2 h. The membrane was blocked with 5% non-fat dry milk made in Tris-buffered saline with Tween 20 (TBST; 50 mM Tris-HCl, 200 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5) at 37°C for 1 h. After washing three times with TBST, the membrane was incubated at 37°C for 1 h with the anti-P polyclonal antibody raised in mice at a dilution of 1:400. Horseradish peroxidase-labeled goat anti-mouse IgG (1:4000 dilution) (Sigma-Aldrich) was used as the secondary antibody. After each incubation step, the membrane was washed four times with TBST. Finally, protein bands recognized by the antibody were developed using the substrates 5-bromo-4-chloro-3-indolylphosphateand nitroblue tetrazolium); the reaction was terminated by adding distilled water.

Indirect immunofluorescence microscopy: NA cells were infected with RV (ERA/Flury strain) at an MOI of 10 and fixed for 48 hr after infection with 4% paraformaldehyde made in phosphate-buffered saline (PBS) for 20 min. After fixation, the cells were blocked with PBS containing 5% BSA and 10% fetal bovine serum (HyClone, Beijing, China) for 1 hr at 37°C. Subsequently, the cells were incubated with P-specific polyclonal antibody (1:100 dilution). Finally, fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Sigma-Aldrich) was added at a dilution of 1:300 and incubated at 37°C for 1 h. After each incubation step, the cells were washed extensively with PBS. The cells were then visualized using a Nikon Eclipse TE2000-U microscope (Nikon, Tokyo, Japan).

Results

Construction of RV P protein prokaryotic expression plasmid: The P gene was amplified successfully from the RV (ERA/Flury strain) genome (Fig. 1, lane 1). The PCR product was inserted into pET-32a (+) to yield the recombinant plasmid pET-32a (+)-P. The pET-32-P construction was verified by restriction enzyme digestion (Fig. 1, lane 2) and DNA sequencing. Sequencing of the inserted DNA fragment showed that there was no mutation of the P gene compared with RV (ERA/Flury strain) (data not shown).

Expression of recombinant His-tagged P protein: Cell extracts from *E. Coli* BL21 (ED3) transformed with the plasmid pET-32a (+)-P after induction showed a protein band with a molecular weight of about 50 kDa, corresponding to the expected molecular weight of the His-tagged P protein (Fig. 2, lane 2); cells transformed with the control plasmid pET-32a (+) (Fig. 2A, lane 1) or cells without induction by IPTG (Fig. 2B, lane 3) did not show this protein band. Furthermore, according to SDS-PAGE analysis of the soluble and insoluble fractions (Fig. 2, lanes 4-6), a major proportion of the recombinant P protein was found in the soluble fraction, suggesting that the His-tagged P protein was soluble.

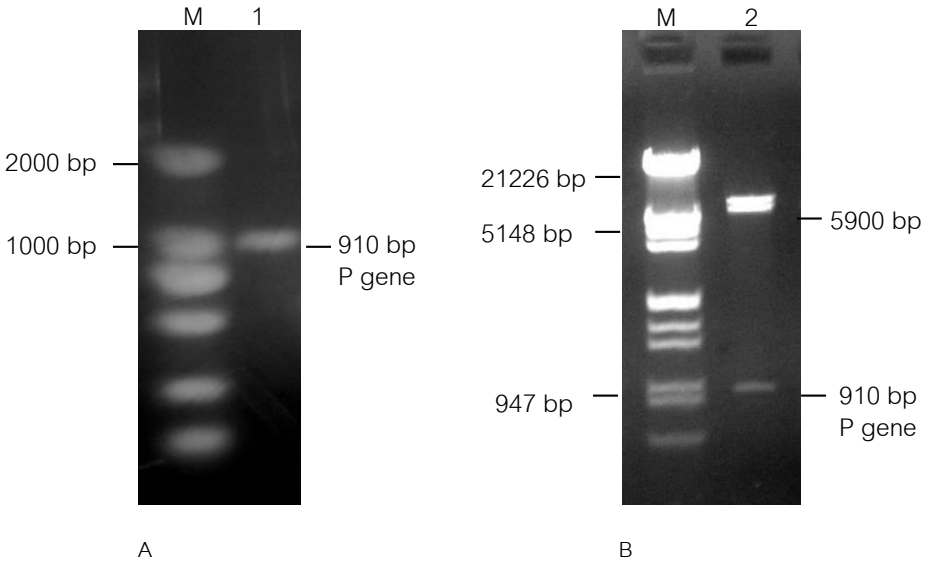

Figure 1 Construction of recombinant plasmid pET-32a (+)-P. (A) Lane 1, PCR product of the P gene; (B) lane 2, recombinant plasmid pET-32a (+)-P digested with *Eco*R I and *Hind* III. Lane M, DNA molecular weight marker.

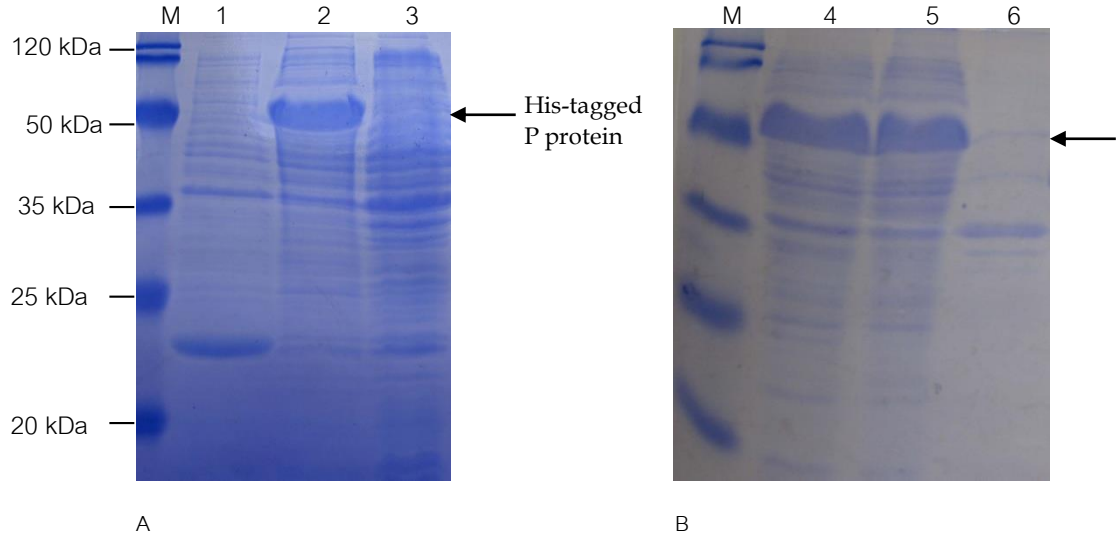

Figure 2 Expression and solubility analysis of His-tagged P protein. (A) Lane 1, total protein from *E. coli* BL21 (DE3) cells transformed with pET-32a (+); lane 2, total protein from *E. coli* BL21 (DE3) transformed with pET-32a (+)-P after IPTG induction; lane 3, total protein from *E. coli* BL21 (DE3) transformed with pET-32a (+)-P without IPTG induction. (B) Lane 4, total protein from *E. coli* BL21 (DE3) transformed with pET-32a (+)-P after IPTG induction; lane 5, lysate supernatant from induced *E. coli* cells; lane 6, lysate pellet from induced *E. coli* cells. Lane M, prestained protein molecular weight marker. Arrow indicates the position of the His-tagged P protein.

Purification of recombinant His-tagged P protein: The His-tagged P protein was purified under native conditions by Ni-NTA chromatography. The SDS-PAGE results verified successful purification, as only a single band with molecular weight of about 50 kDa was observed (Fig. 3A). Lanes 2 to 9 indicated eight fractions of the purified His-tagged P protein from the cell lysate supernatant with 150 mM imidaxole eluation buffer in different washed times over a nickel column. The amount of purified His-tagged P protein was different in the different fractions during purification.

Furthermore, the purified His-tagged P protein could be recognized by both anti-His monoclonal antibodies (mAbs) (Fig. 3B, lane 1) and polyclonal anti-serum against RV (Fig. 3B, lane 2), which demonstrates that the His-tagged recombinant P protein possessed good immunogenicity.

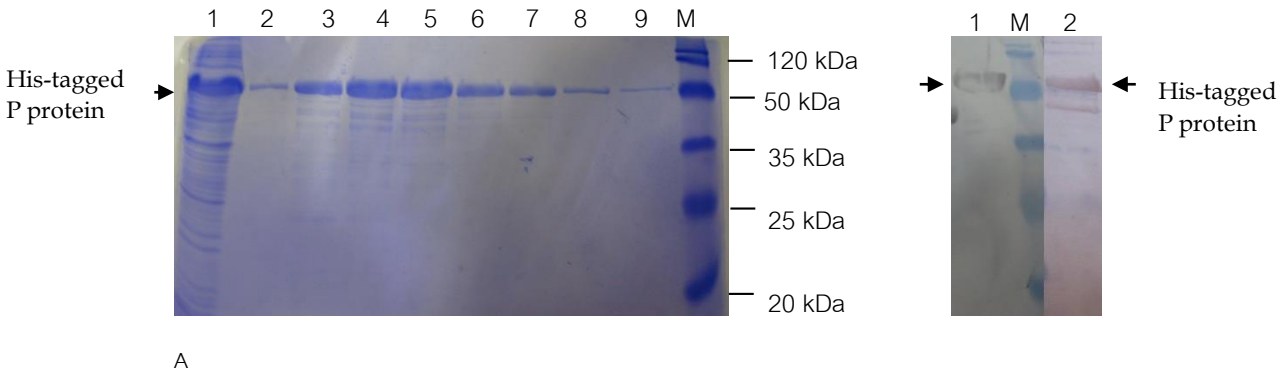

Figure 3 Purification and identification of His-tagged P protein. (A) Purification of the His-tagged P protein. Lane 1, induced total cellular protein; lanes 2-9, 150 mM imidazole elution buffer fraction of His-tagged P protein purified from cell lysate supernatant on a Ni-NTA column. (B) Western blot identification of P protein using anti-His mAbs (lane 1) or anti-RV polyclonal serum (lane 2). Lane M, prestained protein molecular weight marker. Arrows indicate the position of the His-tagged P protein.

Assessment of P-monospecific antibodies by western blot and immunofluorescence assay: After three immunizations, blood was drawn from the mice for serum collection. The western blot results demonstrated that the anti-P polyclonal antibodies reacted specifically with only one protein band with an apparent molecular weight of 37 kDa in the total cell lysate of BHK-21 cells infected with RV (Fig. 4, lane 1); anti-G mAbs, used as a positive control, detected the G protein (Fig. 4, lane 4). In contrast, there were no specific bands in the mock-infected cells (Fig. 4, lane 2) or in the RV-infected cells analyzed using mouse preimmune serum (Fig. 4, lane 3).

The immunofluorescence assays also revealed that the anti-P polyclonal antibodies recognized the native P protein expressed in NA cells infected with RV (Fig. 5A). The Anti-N mAbs, used as a positive control, reacted with the N protein in the RV-infected NA cells (Fig. 5C), the granular-like fluorescence specific to RV N protein could be observed in cytoplasm, but no reaction was detected in the mock-infected NA cells (Fig. 5B) or mouse preimmune serum-incubated cells (Fig. 5D). These results suggest that the polyclonal antibodies had good reactivity and specificity against RV P protein in infected cells.

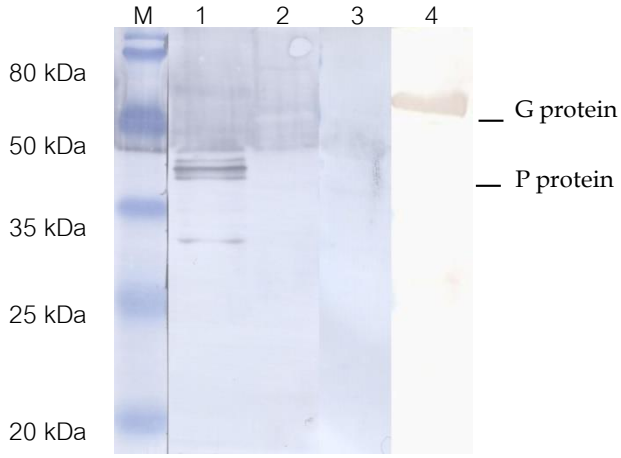

Figure 4 Western blot reactions of P protein with mouse anti-P polyclonal antibodies. Lane 1, lysate of RV-infected BHK-21 cells reacted with anti-P polyclonal antibodies; lane 2, lysate of mock-infected BHK-21 cells analyzed with anti-P polyclonal antibodies; lane 3, lysate of RV-infected BHK-21 cells analyzed with preimmune serum; lane 4, lysate of RV-infected BHK-21 cells reacted with anti-G mAbs. Lane M, prestained protein molecular weight marker.

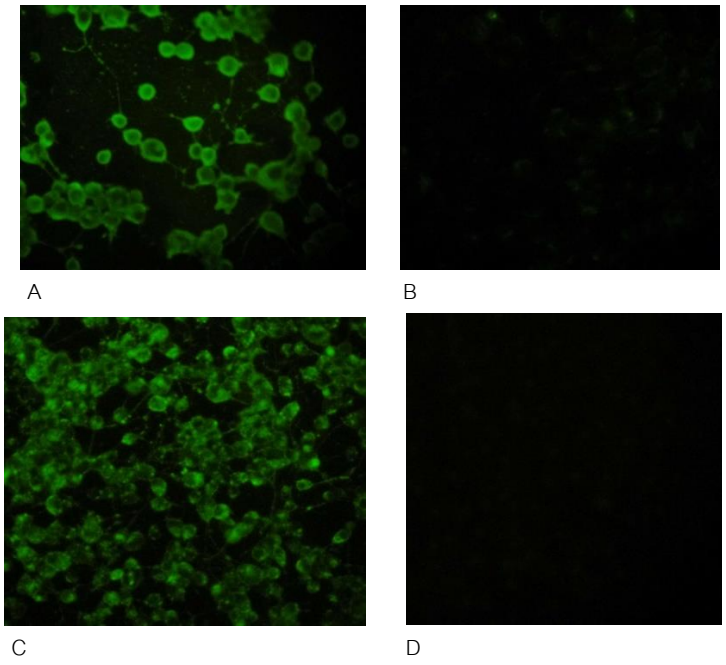

Figure 5 Indirect immunofluorescence tests of P protein in NA cells infected with RV. (A) RV-infected NA cells probed with mouse anti-P polyclonal antibody; (B) mock-infected NA cells probed with mouse anti-P polyclonal antibody; (C) RV-infected NA cells probed with anti-N mAbs; (D) RV-infected NA cells probed with preimmune serum. Magnification: 100×.

Discussion

Prokaryotic expression systems are outstanding for rapid and effective production of recombinant proteins. Nevertheless, these systems often result in the formation of insoluble, inactive inclusion bodies accompanying high-level expression of recombinant proteins in *E. coli* (Oneda and Inouye, 1999). In this study, the pET-32a (+) vector was chosen to produce soluble protein. This vector carries a Trx protein that can increase protein yields in soluble form. Different temperatures, IPTG concentrations, and incubation times can affect the expression level of recombinant P protein. Our results showed that the fusion His-tagged P protein was highly expressed after induction at 28°C with 0.4 mM IPTG for 5 h.

Affinity purification using immobilized metal affinity chromatography on Ni-NTA affinity resin was used in this study to prepare the purified, His-tagged P protein. The 6-His tag is very useful and particularly convenient as a fusion partner for protein purification (Cai et al., 2009). A clear band corresponding to molecular weight of about 50 kDa was observed on the SDS-PAGE gel following Coomassie blue staining, after elution with equilibration buffer containing imidazole. The western blots showed that the fusion His-tagged P protein was recognized by the mouse anti-P IgG, which indicates that the protein had good immunogenicity. The fusion His-tagged P protein was detected with P protein antiserum by western blot, confirming that the recombinant P protein induced an antibody response with high specificity. In addition, this antiserum reacted specifically with RV P protein of about 37 kDa, derived from the RV-infected cells, in the western blot experiments. These results indicate that the antiserum had high sensitivity and specificity. To date, there are no commercial P protein antibodies, which greatly limits the understanding of the functions of RV P protein. This paper appears to represent a method that will contribute to expanding the body of knowledge regarding the functions of RV P protein and associated areas of research.

The rabies virus P protein is a multifunctional protein that is involved in viral RNA synthesis and in disrupting host innate immune responses. Furthermore, P is a crucial antagonist in the host type I IFN system. RV P prevents the production of IFN upon infection by inhibiting phosphorylation of the IFN transcription factors IRF7 and IRF3. Particularly, P protein disturbs the expression of IFN-stimulated genes (ISGs) by two strategies: first, P binds to activated STAT1 and STAT2 in the cytoplasm; and second, P protein prevents STAT1 and STAT2 from accumulating in the nucleus. In addition, RV P binds to the PML protein, as known as the tripartite motif protein 19 (TRIM19), which is resistant to viral replication, indicating that P is an antagonist of the antiviral PML function (Blondel et al., 2010). The full-length P protein is 297 aa and is encoded by the longest ORF of the P gene. Chenik et al. (1995) found that four truncated P proteins were initiated in virus-infected cells by a ribosomal leaky scanning mechanism from the downstream second, third, fourth, and fifth AUG initiation condons. The truncated P protein can interfere with IFN induction and signaling when expressed from plasmids. In a study by Marschalek et al. (2012,) recombinant RV, in which synthesis of P protein was directed by the poliovirus or foot-and-mouth disease virus internal ribosome entry site (IRES), produced full-length (P1) or truncated P protein (P2), respectively. The P2 overexpressing virus had attenuated growth in IFN-incompetent cells, yet it was superior to P1 overexpressing virus in preventing expression of host ISGs. This indicates that P2 may play an important role in preventing JAK/STAT signaling in RV-infected cells and may thereby determine the resistance of RV to IFN.

Our group is particularly interested in the transcription and replication of RV. Therefore, the recombinant P protein and anti-P polyclonal antibodies obtained will be used as a molecular tool to help clarify its participation in different events of the viral cycle. It will also be used in coimmunoprecipitation experiments with the L protein to evaluate ribonucleoprotein complex in infected NA cells, which could contribute to improve the understanding of RV transcription and replication.

In summary, the present work describes the production of recombinant P protein and specific anti-P antibodies, both of which will be useful for further defining the role of the RV P protein in viral replication and transcription. These reagents will also be useful in studies related to evasion of the innate immune system by rabies virus.

Acknowledgements

The study was supported by the National Natural Science Foundation of China (No. 31570147). The manuscript was kindly edited by Dr. Antonio Garmendia of the University of Connecticut, USA.

Declaration: The authors declare no competing financial interests.

References

Blondel D, Regad T, Poisson N, Pavie B, Harper F, Pandolfi PP, De Thé H and Chelbi-Alix MK 2002. Rabies virus P and small P products interact directly with PML and reorganize PML nuclear bodies. Oncogene. 21(52): 7957-7970.

Blondel D, Kheddache S, Lahaye X, Dianoux L and Chelbi-Alix MK 2010.Resistance to rabies virus infection conferred by the PMLIV isoform. J Virol. 84(20): 10719-10726.

Brzózka K, Finke S and Conzelmann K K 2005. Identification of the rabies virus alpha/beta interferon antagonist: phosphoprotein P interferes with phosphorylation of interferon regulatory factor 3. J Virol. 79(12): 7673-7681.

Brzózka , Finke S and Conzelmann K K 2006. Inhibition of Interferon Signaling by Rabies Virus Phosphoprotein P: Activation-Dependent Binding of STAT1 and STAT2. J Virol. 80(6): 2675-2683.

Cai MS, Cheng AC, Wang MS, Zhao LC, Zhu DK, Luo QH, Liu F and Chen XY 2009. His6-tagged UL35 protein of duck plague virus: expression,

purification, and production of polyclonal antibody. Intervirology. 52(3): 141-151.

Chenik M, Chebli K and Blondel D 1995. Translation initiation at alternate in-frame AUG codons in the rabies virus phosphorotein mRNA is mediated by a ribosomal leaky scanning mechanism. J Virol. 69(2): 707-712.

Finke S, Brzózka K and Conzelman, K K 2004. Tracking fluorescence-labeled rabies virus: enhanced green fluorescent protein-tagged phosphoprotein P supports virus gene expression and formation of infectious particles. J Virol. 78(22): 12333-12343.

Fu ZF 1997. Rabies and rabies research: past, present and future. Vaccine. 15(suppl): 20-24.

Jacob Y, Badrane H, Ceccaldi PE and Tordo N 2000. Cytoplasmic dynein LC8 interacts with lyssavirus phosphoprotein. J Virol. 74(21): 10217-10222.

Marxchalek A, Drechsel L and Conzelmann K K 2012. The importance of being short: The role of rabies virus phosphoprotein isoforms assessed by differential IRES translation initiation. Eur J Cell Biol. 91(1): 17-23.

Oneda H and Inouye K 1999. Refolding and recovery of recombinant human matrix metalloproteinase 7 (matrilysin) from inclusion bodies expressed by Escherichia coli. J Biochem. 126(5): 905-911.

Raux H, Flamand A and Blondel D 2000. Interaction of the rabies virus P protein with the LC8 dynein light chain. J Virol. 74(21): 10212-10216.

Shoji Y, Inoue S, Nakamichi K, Kurane I, Sakai T and Morimoto K 2004. Generation and characterization of P gene-deficient rabies virus. Virology. 318(1): 295-305.

บทคัดย่อ

การแสดงออกและการทำให้บริสุทธิ์โปรตีนพีของเชื้อไวรัสโรคพิษสุนัขบ้า: การผลิตและการแสดงลักษณะของโปรตีนต่อต้านเชื้อโรคในร่างกาย-พี ชนิดโพลีโคลนอล

ยาน ปัน[1,3] เซียน-หมิง เมง[2] เซียน-ไก เว่ย[2] จูน ลี[3] ซวน-ลิง ลู[2]
ไฮ-โบ ตาง[2] จิง-จิง เหลียง[2] ติง รง เลา[1,2]*

ฟอสโฟโปรตีน (P) ของเชื้อไวรัสพิษสุนัขบ้า (RV) เป็นโปรตีนที่มีหลายหน้าที่ อย่างไรก็ดี หน้าที่ของโปรตีนนี้ยังไม่เป็นที่เข้าใจทั้งหมด เพื่อเป็นตอบคำถามนี้ ฟอสโฟโปรตีนถูกนำมาแสดงออกและใช้ในการสร้างโพลีโคลนอลแอนติบอดีที่จำเพาะ ยีนส์ที่ใช้จับกับฟอสโฟโปรตีนถูกโคลนในตัวนำพาชนิด pET-32a (+) เพื่อสร้าง pET-32a (+)-P ตัวจับกับฟอสโฟโปรตีน (His-tagged P protein) ถูกแสดงออกในเซลล์แบคทีเรีย *E. coli* BL21 ตรวจวิเคราะห์โดยวิธี SDS-PAGE หลังจากผ่านการทำให้บริสุทธิ์โดยกระบวนการนิเกิลโครมาโตกราฟฟีภายใต้ภาวะเป็นลบ ฟอสโฟโปรตีนถูกใช้ในการสร้างแอนติบอดีในหนูทดลอง ผลการศึกษาด้วยวิธี Western blot ฟอสโฟโปรตีนถูกจดจำได้ด้วยโพลีโคลนอลแอนติบอดี ผลการตรวจด้วยวิธีอิมมูโนฟลูโอเรสเซนซ์ก็แสดงให้เห็นว่า แอนติบอดีสามารถจดจำฟอสโฟโปรตีนพื้นฐานได้ในเซลล์ที่ถูกทำให้ติดเชื้อด้วยเชื้อไวรัสพิษสุนัขบ้าได้

คำสำคัญ: เชื้อไวรัสโรคพิษสุนัขบ้า โปรตีนพี การแสดงออกของโปรตีน การทำให้บริสุทธิ์ โพลีโคลนอลแอนติบอดี

[1]*State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresourses, Guangxi University, Nanning 530004, Guangxi, China*

[2]*Laboratory of Animal Infectious Diseases, College of Animal Sciences and Veterinary Medicine, Guangxi University, Nanning 530004, Guangxi, China*

[3]*Guangxi Key Laboratory of Veterinary Biotechnology, Guangxi Veterinary Research Institute, Nanning 530001, Guangxi , China*

***ผู้รับผิดชอบบทความ** E-mail: tingrongluo@gxu.edu.cn*