Production of Monoclonal Antibody Against EP0 Protein of Pseudorabies Virus and Determination of Its Recognized Epitope

Keyu Xiang,^{1*} Yi Cheng,^{1*} Meizhen Zhou,² Leilei Sun,¹ Yikuan Ji,¹ Yu Wang,¹
Baoshi Zhang,¹ Yongwen Luo,¹ and Chunmei Ju¹

Early protein 0 (EP0) is especially important for modulating PRV gene expression and reactivation from the latent state, but the mechanisms have not been elucidated. In this study, six monoclonal antibodies (MAbs) against EP0 protein of PRV were generated and their characterizations were investigated. Western blot analysis showed all six MAbs could react with immunizing antigen, but only 2B12 and 2C6 could react with native EP0 protein from PRV-infected cells. ELISA additivity tests revealed that at least three epitopes in EP0 were defined by six MAbs. The epitope recognized by MAbs 2B12 was further identified in 287–292 aa of EP0 protein using a series of expressed overlapping peptides. These MAbs may provide valuable tools for further research of the functions of EP0 in PRV infection.

Introduction

PSEUDORABIES VIRUS (PRV) is a member of the family Herpesviridae, subfamily Alphaherpesvirinae, and the causative agent of Aujeszky's disease, which causes serious economic losses worldwide among pigs.⁽¹⁾ Like many herpesviruses, PRV has the capacity to establish two types of infections: lytic (or productive) and latent.⁽²⁾ In the lytic pathway, the entire transcription machinery of the herpesvirus is activated, and the progress of infection eventually leads to the production of new virions and the lysis of infected cells. By contrast, in latency, only a limited segment of the herpesvirus genome is transcriptionally active, no new virus particles are produced, and the cells can survive the infection.

The PRV life cycle is primarily controlled at the level of transcription. Gene expression of PRV can be divided into three classes: immediate-early (IE), early (E), and late (L) phases during lytic infection and be regulated in cascade fashion. The IE and E proteins of PRV are especially important for regulating viral gene expression and replication. In the PRV genome, *IE180* is the only true IE gene.^(3,4) and *EP0* gene is one of the E genes.^(5,6) The PRV EP0 shares the characteristics of a transactivator.^(7–9) However, EP0 has the opposite effect on the *vhs*⁽¹⁰⁾ and *gE*⁽¹¹⁾ promoters, reducing gene expression. EP0 has been demonstrated to be present in the virion.⁽⁷⁾ Therefore it is possible that the effects of EP0 might be mediated by the EP0 of the virion in the early phase

of viral infection. Whether EP0 acts directly or indirectly to modulate transcription is not yet established. Additional experiments are needed to prove the mechanisms.

The EP0 gene is located at the end of the unique long region and transcribed in the opposite direction with respect to the large latency transcript (LLT) gene. The lack of EP0 does not impair PRV in reaching and persisting in the trigeminal ganglia of swine after intranasal inoculation, but the amount of viral DNA harbored in trigeminal ganglia tissue is found to be reduced and dexamethasone is not effective in inducing the reactivation of infectious mutant virus.^(12,13) Thus, the *EP0* gene may be important for reactivation from the latent state.

The mechanisms by which the EP0 modulate the PRV gene expression and reactivation from the latent state have not been elucidated. The work presented here describes the production of monoclonal antibody against EP0 protein of PRV and determination of its recognized epitope, which lay the foundation for further research of EP0 functions in PRV infection.

Materials and Methods

Purification of recombinant PRV EP0

Recombinant protein was expressed in *Escherichia coli* BL21(DE3) strain by transforming the pET-EP0 to produce a fusion with six histidine residues.⁽¹⁴⁾ To purify the recombinant protein, cells were harvested, resuspended in binding

¹College of Veterinary Medicine, ²College of Foreign Studies, South China Agricultural University, Guangzhou, P.R. China.

*These authors contributed equally to this work.

buffer (20 mM sodium phosphate, 500 mM NaCl, 50 mM imidazole [pH 7.4]) containing 1 mM phenyl-methylsulfonyl fluoride (PMSF) and lysed by sonication. After centrifugation, the protein from inclusion bodies was solubilized by 8 M urea and purified by nickel affinity chromatography (GE Healthcare Life Sciences, Uppsala, Sweden). The protein in the final column eluate was dialyzed overnight against renaturing buffer R1 (20 mM sodium phosphate, 500 mM NaCl, 0.1 mM PMSF, 4 M urea [pH 7.4]) and dialyzed with renaturing buffer R2 (20 mM sodium phosphate, 0.1 mM PMSF [pH 7.4]) containing 10% glycerol.

Preparation of monoclonal antibodies against PRV EP0

Female BALB/c mice, 4–6 weeks of age, were immunized subcutaneously with 100 µg purified EP0 protein emulsified

with Freund's complete adjuvant (Sigma-Aldrich, St Louis, MO) in 0.2 mL, respectively, on day 0, and boosted twice on days 14 and 28 with 50 µg antigen emulsified with Freund's incomplete adjuvant (Sigma-Aldrich). The antibody levels were examined by an enzyme-linked immunosorbent assay (ELISA) using the recombinant EP0 protein as coating antigen. The mice with the highest serum antibody titer were injected intraperitoneally using 100 µg of antigen without adjuvant. Three days later, the splenocytes of the immunized mice were isolated and fused with SP2/0 myeloma cells using 50% (w/v) polyethylene glycol (Sigma-Aldrich). Hybridomas were screened for secretion of desired antibodies by ELISA using two antigens (the immunizing antigen and a mock prepared from *E. coli* BL21(DE3) strain by transforming the pET-32a(+)) and cloned twice by limiting dilution method. Monoclonal antibody isotypes were determined by Mouse Mab Isotyping Test Kit (AbD Serotec, Kidlington, United

TABLE 1. REACTIVITY OF EXPRESSED DIFFERENT PEPTIDES OF EP0 PROTEIN WITH MAb 2B12

Peptide	Primer sequence	Peptide position in EP0 protein	Reactivity in WB with MAb 2B12
P1	5'-TAGGAATTCATGGGCTGCACGGTCTCT-3' 5'-TACGAAGCTTGCGGATGGGGTCGCTCTC-3'	1–176	–
P2	5'-TAGGAATTCATCTTCAACTGGCTTTAT-3' 5'-TACGAAGCTTGTCGTCGTCCTGGGTGAG-3'	158–409	+
P3	5'-TAGGAATTCATCTTCAACTGGCTTTAT-3' 5'-CCCAAGCTTCGCCGATATGTCAAACAG-3'	158–221	–
P4	5'-CCGGAATTCGCAAACACCCACGCCCTC-3' 5'-CCCAAGCTTATCCTCCGTGTGCAACCC-3'	207–272	–
P5	5'-CCGGAATTCTCCCCGACGAGGAAGAC-3' 5'-CCCAAGCTTCCCCGAGGCACCATGCTG-3'	255–341	+
P6	5'-CCGGAATTCTCCGGCGAGGGGTCCTCT-3' 5'-TACGAAGCTTGTCGTCGTCCTGGGTGAG-3'	329–409	–
P7	5'-CCGGAATTCTCCCCGACGAGGAAGAC-3' 5'-CCCAAGCTTCAGGCACCGAGTCCGTCT-3'	255–310	+
P8	5'-CCGGAATTCCGGGCGGCCGTGCT A-3' 5'-CCCAAGCTTCCCCGAGGCACCATGCTG-3'	296–341	–
P9	5'-CCGGAATTCGCAAACACCCACGCCCTC-3' 5'-CCCAAGCTTCGCCGGCCTCTGGTCG-3'	207–286	–
P10	5'-CCGGAATTCGCGGACGACCAGAGGC-3' 5'-CCCAAGCTTCCCCGAGGCACCATGCTG-3'	280–341	+
P11	5'-CCGGAATTCGCAAACACCCACGCCCTC-3' 5'-CCCAAGCTTGGCCCTGCGCGGGGA-3'	207–294	+
P12	5'-CCGGAATTCGCAAACACCCACGCCCTC-3' 5'-CCCAAGCTTCCTGCGCGGGGAGCGC-3'	207–293	+
P13	5'-CCGGAATTCGCAAACACCCACGCCCTC-3' 5'-CCCAAGCTTGCGCGGGGAGCGCCTG-3'	207–292	+
P14	5'-CCGGAATTCGCAAACACCCACGCCCTC-3' 5'-CCCAAGCTTCGGGGAGCGCCTGGG-3'	207–291	–
P15	5'-CCGGAATTCGCGCCCAGGCGCTCCCCG-3' 5'-CCCAAGCTTCCCCGAGGCACCATGCTG-3'	286–341	+
P16	5'-CCGGAATTCCCCAGGCGCTCCCCGCG-3' 5'-CCCAAGCTTCCCCGAGGCACCATGCTG-3'	287–341	+
P17	5'-CCGGAATTCAGGCGCTCCCCGCGC-3' 5'-CCCAAGCTTCCCCGAGGCACCATGCTG-3'	288–341	–
P18	5'-CCGGAATTCCGCTCCCCGCGCAGG-3' 5'-CCCAAGCTTCCCCGAGGCACCATGCTG-3'	289–341	–

FIG. 1. SDS-PAGE analysis of polypeptide components of EP0 preparations. Preparations of purified recombinant PRV EP0 (lane 1), IPTG-induced *E. coli* containing pET-EP0 (lane 2), and uninduced *E. coli* containing pET-EP0 (lane 3) were analyzed by SDS-PAGE and stained by Coomassie Brilliant Blue. The 85 kDa recombinant PRV EP0 is indicated by the arrow.

Kingdom). Large quantities of the monoclonal antibodies were produced by intraperitoneal injection of hybridoma cells into liquid paraffin-treated BALB/c mice. After 7–14 days, the ascites containing high concentrations of antibodies were harvested.

Determination of monoclonal antibody specificity

To determine the specificity of the MAbs, Western blots were used to test their reactivity with recombinant EP0, and native EP0 from PRV-infected cell lysates. Mock- and PRV-infected cell lysates were prepared as described previously.⁽⁹⁾ Protein samples were applied onto 12% SDS-polyacrylamide gels and electrophoresed. The separated proteins were then electrophoretically transferred to a nitrocellulose sheet. The sheet was treated sequentially with 5% non-fat milk, a 500-fold dilution of the MAbs, and finally with peroxidase-labeled goat anti-mouse IgG (AbD Serotec). The antigen was detected using 3, 3-Diaminobenzidine tetrahydrochloride (DAB) as the substrate.

ELISA additivity tests

In order to test the MAbs recognizing different epitopes, additivity ELISA tests were performed as described by Etteradossi and colleagues.⁽¹⁵⁾ Ninety-six-well ELISA plates (Corning Inc., Corning, NY) were coated overnight with recombinant EP0 protein diluted in carbonate buffer (pH 9.8). Two MAbs were added either separately or simultaneously,

FIG. 2. Reactivity of MAb 2B12 with recombinant or native EP0 protein by Western blot analysis. The preparations of uninduced *E. coli* containing pET-EP0 (lane 1), IPTG-induced *E. coli* containing pET-EP0 (lane 2), mock-infected (lane 3), and PRV-infected IBRS-2 cells (lane 4) were analyzed by Western blot. Molecular weight of native EP0 protein range from 55 to 70 kDa because of its post-translational modification such as phosphorylation.

and the amount of bound antibody was quantitatively measured. An additivity index (AI), which compares the ODs obtained by two assays (alone and in a mix) under standardized conditions, was calculated for each pair of MAbs according to the formula:

$$AI = \{ [2 \times A_{1+2} / (A_1 + A_2)] - 1 \} \times 100$$

Here A_1 and A_2 are the ODs obtained when the MAbs are assayed separately, and A_{1+2} is the OD when the same amounts of the two MAbs are pooled in the same well. Provided the concentrations of the MAbs are saturating for the antigen, the AI will tend to be zero if both MAbs recognize the same epitope, but close to 100 if the two epitopes are topographically unrelated.

Determination of epitope recognized by MAb 2B12

To assess the precise location and amino acid sequence of the epitope recognized by MAb 2B12, DNA fragments derived from the EP0-coding region were amplified with a series of primers (Table 1) and cloned into pET-32a(+) expression plasmid. The fusion proteins were expressed in *E. coli* BL21(DE3) strain by transforming the plasmids and the reactivity of the expressed proteins with MAb 2B12 was analyzed by immunoblotting. The epitope recognized by MAb 2B12 was identified as the overlapping peptide of expressed fusion proteins reacting with it.

TABLE 2. BIOLOGICAL PROPERTIES OF SIX ANTI-EP0 MONOCLONAL ANTIBODIES

MAb reference	Antibody isotype	Reactivity in ELISA with immunizing antigen	Reactivity in WB with recombinant EP0	Reactivity in WB with native EP0
1C4	IgG2a	+	+	—
2B12	IgG2a	++++	+	+
2C6	IgG2a	++++	+	+
4D3	IgG1	++	+	—
5B6	IgG3	+	+	—
6C11	IgG2a	+	+	—

TABLE 3. ADDITIVITY INDEX FOR SIX ANTI-EP0 MONOCLONAL ANTIBODIES

	4D3	1C4	5B6	6C11	2B12	2C6
4D3	7.9 (0.2)	33.5 (3.9)	-3.5 (3.2)	26.7 (7.6)	68.7 (1.2)	73.4 (0.8)
1C4		11.7 (2.1)	6.1 (4.8)	14.5 (7.6)	50.6 (2.1)	55.6 (0.1)
5B6			2.7 (0.8)	11.9 (1.5)	65.7 (4.0)	68.2 (2.2)
6C11				-3.6 (6.1)	66.1 (3.6)	70 (2.1)
2B12					-3.1 (0.3)	-1.7 (2.5)
2C6						1.4 (1.8)

Mean ELISA additivity indexes as calculated for paired MAb in duplicate assays. Figures in parenthesis are standard deviation. Results placed in the same rectangle indicate overlapping epitopes.

Results and Discussion

The recombinant PRV EP0 was purified from *E. coli* BL21 (DE3), transformed with a pET-EP0 plasmid carrying the PRV EP0 gene. After induction with IPTG, an approximate 85 kDa fusion protein was observed by SDS-PAGE analysis (Fig. 1). The purified protein was used as immunogen to inoculate BALB/c mice. After several immunizations, the splenocytes from the BALB/c mice were fused with SP2/0 myeloma cells. Eventually, six hybridomas were selected producing MAbs against the recombinant EP0 protein, identified as 1C4, 2B12, 2C6, 4D3, 5B6, and 6C11 respectively. Their isotypes and reactivity with recombinant EP0, and native EP0 from PRV-infected cell lysates, are shown in Table 2. MAbs 4D3 and 5B6 were of IgG1 and IgG3 isotypes, respectively, and the others were of IgG2a isotype. In ELISA screening assays, MAbs 2B12 and 2C6 were strongly reactive with recombinant EP0 protein, but the others reacted weakly or moderately. Western blot analysis showed all six MAbs could react with immunizing antigen, but only 2B12 and 2C6 could react with native EP0 protein from PRV-infected cells (Fig. 2).

The topographic relationships between epitopes recognized by six MAbs were analyzed by an ELISA additivity test. This assay requires that the antigen be saturated with each antibody tested. The efficient saturation of the antigen was confirmed by low self-AIs obtained with all MAbs (Table 3). AIs calculated for pairs of MAbs showed that four of the recognized epitopes shared close antigenic relationships, and two groups of overlapping epitopes were evidenced—the first one defined by MAbs 5B6 and 4D3 (AI of -3.5%), the second by MAbs 5B6, 1C4, and 6C11 (AIs

ranging from 6.1–14.5%). MAb pairs of 2B12 and 2C6 probably recognize overlapping epitope different from others for their low AI of -1.7% and consistent high AIs with other MAbs.

MAbs 2B12 and 2C6 can react with native EP0 protein from PRV-infected cells. Moreover, they appear very similar in ELISA additivity, and thus might probe the same epitope. Therefore, epitope recognized by 2B12 was determined by its reactivities with a series of fusion peptides of EP0 expressed in *Escherichia coli* (Table 1, Fig. 3). The results showed the recognized epitope located in 287–292 aa of EP0 protein with the corresponding residues of PRRSPR. Analysis by DNASTar software demonstrated that antigenic index of region in 280–300aa of EP0 protein was significantly higher (data not shown). This result is consistent with the location of above-mentioned epitope. Therefore, this region can be chosen in preparation of antibody against EP0 protein in the future. The peptide of PRRSPR is conserved in EP0 protein of different PRV strains. For this reason, the preparation of MAb 2B12 lays the foundation for further research of EP0 functions in PRV infection.

In conclusion, a prokaryotic expressed recombinant EP0 protein was used as antigen to immunize mice. Hybridoma technique was used to produce PRV EP0-specific monoclonal antibody and six MAbs were generated. By Western blot analysis, all MAbs reacted with immunizing antigen, but only 2B12 and 2C6 reacted with native EP0 protein from PRV-infected cells. At least three epitopes in EP0 are defined by six MAbs in ELISA additivity assay. The epitope of the MAb 2B12 was further mapped in 287–292 aa of EP0 protein using a series of expressed overlapping peptides. We believe that these MAbs would be important tools for

FIG. 3. Reactivity of MAb 2B12 with partial fusion peptides of EP0 expressed in *Escherichia coli* by Western blot analysis. The preparations of peptide located in 207–294 aa (lane P11), 207–293 aa (lane P12), 207–292 aa (lane P13), 207–291 aa (lane P14), 286–341 aa (lane P15), 287–341 aa (lane P16), 288–341 aa (lane P17), 289–341 aa (lane P18) of EP0 protein were analyzed by Western blot with 2B12 as primary antibody.

illustrating the functions and characteristics of EP0 in PRV infection.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (grant no. 31001074).

Author Disclosure Statement

The authors have no financial interests to disclose.

References

1. Tombácz D, Tóth JS, and Boldogkői Z: Effects of deletion of the early protein 0 gene of pseudorabies virus on the overall viral gene expression. *Gene* 2012;493:235–242.
2. Pomeranz LE, Reynolds AE, and Hengartner CJ: Molecular biology of pseudorabies virus: impact on neurovirology and veterinary medicine. *Microbiol Mol Biol* 2005;69:462–500.
3. Feldman LT, Demarchi JM, Ben-Porat T, and Kaplan AS: Control of abundance of immediate-early mRNA in herpesvirus (pseudorabies)-infected cells. *Virology* 1982;116:250–262.
4. Ihara S, Feldman L, Watanabe S, and Ben-Porat T: Characterization of the immediate-early functions of pseudorabies virus. *Virology* 1983;131:437–454.
5. Cheung AK: Cloning of the latency gene and the early protein 0 gene of pseudorabies virus. *J Virol* 1991;65:5260–5271.
6. Tombácz D, Tóth JS, Petrovski P, and Boldogkői Z: Whole-genome analysis of pseudorabies virus gene expression by real-time quantitative RT-PCR assay. *BMC Genomics* 2009;491:1–22.
7. Ono E, Watanabe S, Nikami H, Tasaki T, and Kida H: Pseudorabies virus (PRV) early protein 0 activates PRV gene transcription in combination with the immediate-early protein IE180 and enhances the infectivity of PRV genomic DNA. *Vet Microbiol* 1998;63:99–107.
8. Ho TY, Wu SL, Chang TJ, Hsiang CH, Chang SH, and Hsiang CY: Pseudorabies virus early protein 0 trans-activates the TATA-associated promoter by stimulating the transcription initiation. *Virus Res* 1999;61:77–86.
9. Watanabe S, Ono E, Shimizu Y, and Kida H: Pseudorabies virus early protein 0 transactivates the viral gene promoters. *J Gen Virol* 1995;76:2881–2885.
10. Chang YY, Lin HW, Wong ML, and Chang TJ: Regulation of the vhs gene promoter of pseudorabies virus by IE180 and EP0, and the requirement of a Sp1 site for the promoter function. *Virus Genes* 2004;28:247–258.
11. Chang YY, Wong ML, Lin HW, and Chang TJ: Cloning and regulation of the promoter of pseudorabies virus (TNL Strain) glycoprotein E gene. *Virus Genes* 2002;3:235–241.
12. Cheung AK: Latency characteristics of an EP0 and LLT mutant of pseudorabies virus. *J Vet Diag Invest* 1996;8:112–115.
13. Wesley RD, and Cheung AK: A pseudorabies virus mutant with deletions in the latency and early protein 0 genes: replication, virulence, and immunity in neonatal piglets. *J Vet Diag Invest* 1996;8:21–24.
14. Sun L, Cheng Y, Liang Z, Zhou H, and Ju C: Cloning of EP0 gene of pseudorabies virus and its expression in *E coli*. *China Ani Hus Vet Med* 2012;39:57–60.
15. Etteradossi N, Toquin D, Rivallan G, and Guittet M: Modified activity of a VP2-located neutralizing epitope on various vaccine, pathogenic and hypervirulent strains of infectious bursal disease virus. *Arch Virol* 1997;142:255–270.

Address correspondence to:

Chunmei Ju
College of Veterinary Medicine
South China Agricultural University
Wushan Road 483
Guangzhou 510642
P.R. China

E-mail: juchunmei@scau.edu.cn

Received: May 5, 2014

Accepted: September 2, 2014