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Identification of linear B cell epitope on gB, gC, and gE proteins of porcine pseudorabies virus using monoclonal antibodies

Panpan Zhang[a], Lin Lv[a], Haifeng Sun[a], Shihai Li[a], Hui Fan[a], Xianwei Wang[a,b], Juan Bai[a,b,*], Ping Jiang[a,b]

[a] Key Laboratory of Animal Diseases Diagnostic and Immunology, Ministry of Agriculture, MOE International Joint Collaborative Research Laboratory for Animal Health & Food Safety, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China
[b] Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, China

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ABSTRACT

Since 2011, there have been outbreaks of pseudorabies (PR) in several pig farms despite vaccination coverage, which causes substantial economic loss to the swine industry in China. The emergence of a pseudorabies virusvariant strain with high virulence and antigenic variation (e.g., PRV ZJ01), is considered to be the primary cause. In this study, truncated gB, gC, and gE of PRV ZJ01 was expressed and used to generate seven monoclonal antibodies (mAbs) against gB, gC, or gE. An indirect immunofluorescence assay (IFA) revealed that these mAbs were specific against PRV. Subsequently we identified the B cell epitopes recognized by these mAbs by Western blot. The mAbs 5A2 and 6G5 against gB recognized the same B cell linear epitope at ^{576}SAVATAA^{582}, the mAb 5D10 against gC recognized the B cell linear epitope at ^{134}GETFE^{138}, mAb 7C5 against gC recognized the B cell linear epitope at ^{143}RRGRFRSPDAD^{153}, and mAbs 3E1, 3H8, and 4D2 against gE recognized the same B cell linear epitope at ^{151}IGDYL^{155} of gE. Biological information analysis showed that these B cell linear epitopes are highly conserved among different PRV isolates and the epitope ^{143}RRGRFRSPDAD^{153} with a high antigenic index and high hydrophilicity, fully exposed on the surface of the gC, is likely to be an important B cell epitope. These mAbs and their defined epitopes may provide useful tools for the study of the structure and function of the PRV protein, analysis of antigenic epitope characteristics, and establishment of antibody detection methods.

1. Introduction

Pseudorabies (PR) is caused by the pseudorabies virus (PRV) and represents one of the most significant infectious disease affecting the pig industry (Pomeranz et al., 2005; Szpara et al., 2010). Pigs are the only natural host and reservoir of PRV. In general, PRV infects pigs at various phases of development. PRV infections in newborn piglets can lead to nervous system disorders and even death (Muller et al., 2010). In pregnant pigs, PRV infection typically results in abortion (Zuckermann, 2000). In adult pigs, PRV infection often leads to respiratory disease, and the survival of an acute infection results in a chronic lifelong latent infection with the virus.

PRV has 11 types of envelope glycoproteins, of which glycoprotein B (gB) is a major viral antigen and participates in the processes of virus entry and cell-to-cell spread (Peeters et al., 1992; Rauh and Mettenleiter, 1991). The gB antigen epitopes are primarily located at residues 59–126, 216–279, and 540–734 (Zaripov et al., 1999). Most antibodies target epitopes within residues 540–734. The antibodies against immunodominant regions can mediate viral clearance in infected cells and contribute to protective immunity against PRV (Zaripov et al., 1998). Glycoprotein C (gC) is the main component involved in viral adhesion to host cell receptors and is considered to be a potent inducer of the immune response (Rue and Ryan, 2008). Moreover, gC initiates viral invasion by interacting with heparan sulfate glycoprotein (HS) expressed on the surface of host cells (Rue and Ryan, 2002, 2003). Monoclonal antibodies (mAbs) against gC can neutralize the virus in vitro and protect mice and pigs from viral infection (Riviere et al., 1992). Therefore, gC is one of the preferred proteins in PRV subunit vaccine research. Studies have identified three gC B cell epitopes, which are located at amino acids 65–79, 80–94, and 85–99 (Ober et al., 2000). Glycoprotein E (gE) promotes viral fusion with host cells and the spread of viruses between nerves. Moreover, gE is the primary virulence factor of the virus. Although a deletion of gE does not affect PRV replication, the gE-deleted virus plaque becomes smaller and virulence is decreased

* Corresponding author at: Key Laboratory of Animal Diseases Diagnostic and Immunology, Ministry of Agriculture, MOE International Joint Collaborative Research Laboratory for Animal Health &Food Safety, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China.
E-mail address: baijuan116@163.com (J. Bai).

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(Mettenleiter et al., 1994).

The most common strategy employed for the prevention and control of PRV is vaccination. In some developed countries (e.g., the USA, New Zealand, and many members of the European Union) PR was eradicated using gE-deleted vaccines and the accompanying diagnostic test, which could distinguish infected from vaccinated animals (Bouma, 2005; Pannett et al., 1999). However, PR continues to circulate sporadically in many regions throughout the globe (Obaldia, 2005; Wu et al., 2013). Currently, gE-deleted vaccines are widely used to protect animals against PRV in most countries (Elbers et al., 2000). Thus, we can distinguish vaccinated from infected pigs by the detection of gE-specific antibodies. In addition, we can also detect antibody titers against PRV gB or gC induced by vaccination to assess the degree of vaccine efficacy. Therefore, in the present study, mAbs against PRV gB, gC, and gE were prepared and the B cell epitopes recognized by these mAbs were identified. These findings may provide novel insight into the study of the structure and function of PRV proteins, the analysis of antigenic epitope characteristics, and the establishment of antibody detection methods.

2. Materials and methods

2.1. Cells, virus, experimental animals, and main reagents

BHK-21 and SP2/0 myeloma cells were stored in our laboratory. The PRV ZJ01 strain (Accession number: KM061380) was isolated and stored in our laboratory. Female, 8-week-old BALB/c mice were purchased from Yangzhou University Experimental Animal Center. BHK-21 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), at 37 °C in a 5% CO_2 humidified atmosphere. The myeloma SP2/0 cell line was maintained in RPMI-1640 supplemented with 20% FCS. Horseradish peroxidase (HRP)-conjugated rabbit anti-bovine IgG (H + L) was purchased from KPL. Freund's complete and incomplete adjuvants, HAT and HT supplements, and 50% polyethylene glycol 4000 (PEG4000) were purchased from Sigma-Aldrich. RPMI-1640 and Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco. FCS was purchased from Science Cell.

2.2. Expression and purification of recombinant His-tagged proteins

To construct a plasmid expressing the main antigen coding region of gB (codons 440–740), gC (codons 42–249), and gE (codons 24–269) of the PRV ZJ01 strain, we designed PCR primers according to the gB, gC, and gE gene sequences available in GenBank and amplified the respective gene fragments. The PCR products were purified using agarose gel electrophoresis. The purified PCR products were sequentially cloned into pET-32a or pET-28a.

Escherichia coli DH5a was transformed with plasmids pET-32a-gB, pET-28a-gC, and pET-32a-gE. The recombinant positive plasmid was confirmed by bacterial PCR and enzymatic digestion. The recombinant protein was expressed in an *E. coli* BL21 (DE3) strain by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated for 5 h in Luria-Bertani (LB) medium containing 100 μg/mL kanamycin or 50 μg/mL ampicillin. After the incubation, bacterial cells were centrifuged for 10 min at 8,000 × *g*, washed in phosphate buffered saline (PBS, pH 7.4), and resuspended in a final volume of 500 μL PBS. Bacterial cells were lysed by sonication. Recombinant gB, gC, and gE were identified with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. Recombinant gB and gE were purified by nickel affinity chromatography. Recombinant gC was purified using urea dialysis (Eggenreich et al., 2016; Upadhyay et al., 2014). The concentration of the purified recombinant proteins was measured with a bicinchoninic acid assay (Thermo) according to the manufacturer's instructions. The protein purity was analyzed by SDS-PAGE. Finally, the proteins were stored at −80 °C.

2.3. Immunization of animals

All procedures involving animals for this study were approved by and performed in accordance with the Animal Ethics Committee and Nanjing Agricultural University animal experiment central guidelines, respectively. Female BALB/c mice (aged 6–8 weeks old) were immunized with 50 μg of purified recombinant gB, gC, and gE protein in 0.2 mL, emulsified in the same amount of Freund's complete adjuvant. Two booster injections were administered with equal immunogen with Freund's incomplete adjuvant at three week intervals. Ten days after the last injection, blood samples were collected, and the serum antibody titer against the recombinant proteins was measured using an indirect ELISA. The mouse with the highest antibody titer was intraperitoneally administered with 50 mg of immunogen without adjuvant three days prior to cell fusion.

2.4. Preparation of monoclonal antibodies

The preparation of mAbs against gB, gC, and gE was performed as described previously (Bai et al., 2014). Briefly, three days after the final booster, the mice were euthanized and the spleen cells were fused with SP2/0 myeloma cells at a ratio of 5:1 using 50% polyethylene glycol 4000. The fused cells were plated into 96-well plates maintained in RPMI-1640 supplemented with HAT and 20% FCS. Ten days later, the HAT medium was replaced by HT medium. The hybridoma culture supernatants were assessed with an indirect ELISA with purified PRV used as a coating antigen. Positive hybrids were cloned three times via limiting dilution. After identification, the positive hybridoma cells were grown in RPMI1640 supplemented with 20% FCS and the ascetic fluid of the mAbs was prepared using 10-week-old female BALB/c mice. After preparing the ascites fluid, the mAb titers were measured by an indirect ELISA. The class and subclass of the mAbs were determined using a commercial Mouse Monoclonal Antibody Isotype Elisa Kit (Proteintech) according to the manufacturer's protocol. A Western blot and immunofluorescence assay (IFA) were used to determine the degree of mAb specificity.

2.5. Indirect ELISA

For the indirect ELISA, plates were coated overnight at 4 °C with 200 ng of purified PRV (100 μL/well in 0.1 M NaHCO3, pH 9.6). The plates were washed three times with PBS containing 0.05% Tween-20 (PBST) and blocked with 200 μL of 5% non-fat milk in PBST for 2 h at 37 °C. Aliquots of 100 μL supernatant from the hybridoma cultures were added to the wells. In addition, the supernatant from the SP2/0 culture and sera from mice immunized three times with recombinant protein were used as the negative and positive controls, respectively. The plates were incubated at 37 °C for 1 h. After the incubation, the plates were washed three times with PBST. Then, 100 μL horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was added at a dilution of 1:5,000, and the sample was incubated at 37 °C for 0.5 h. Antibody binding was analyzed by adding 50 μL TMB for 10 min. The color development reaction was stopped with 2 M H_2SO_4, and the absorbance was measured at 450 nm.

2.6. SDS-PAGE and Western blot

Proteins mixed with loading buffer were boiled for 10 min and separated by 10% SDS-PAGE. The gel was either stained with Coomassie brilliant blue or electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 2 h with 10% non-fat milk in TBST at room temperature and washed three times with PBST. The membrane was then incubated at 4 °C overnight with PRV antiserum (1:100; prepared in our laboratory) or a mAb as the primary antibody. After rinsing with PBST, the membrane was treated for 1 h at room temperature with goat anti-mouse IgG-HRP as the

secondary antibody. Following four washes with TBST, the bound proteins were visualized using ECL (electrochemiluminescence) reagents with a Tanon 5200 chemiluminescence imaging system.

2.7. Immunofluorescence assay (IFA)

BHK-21 cells were seeded into a 24-well cell culture plate at a density of 2×10^5 cells/well and the cells were infected with PRV ZJ01 at an MOI of 0.1. When the cells began to develop lesions due to the infection, the medium was discarded and washed three times with PBS. The cells were then fixed in precooled methanol for 30 min at 4 °C and washed three times with PBS. After blocking with 10% FBS, the cells were inoculated with 1:1000 dilution of mAb for 2 h, followed by three washes with PBST. The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse antibody at a dilution of 1:100 for 1 h at 37 °C and washed three times with PBST. The images were captured using a fluorescence microscope.

2.8. Preliminary analysis of antigenic epitopes

To localize the antigen epitope, a series of overlapping truncated gB, gC, and gE genes (Fig. 1) were cloned into pET-32a and expressed in *E. coli* BL21 (DE3) as fusion proteins. Next, these truncated recombinant gB, gC, and gE proteins were detected with a Western blot analysis using the specific mAbs.

2.9. Biological information analysis

Biological information regarding the presence of the identified epitopes in the different PRV strains was obtained by comparing the identified epitope in gB, gC, and gE of PRV ZJ01 with those from other PRV strains using DNASTAR Megalign software. Simultaneously, structure of the gB was obtained from the RCSB website and the structure of gC and gE was predicted with the SWISS-MODEL website. The spatial characteristics of the identified epitopes in gB, gC, and gE was analyzed by mapping the epitope locations onto a 3D model of gB, gC, and gE using PyMOL software based on the results obtained using the RCSB and SWISS-MODEL online server

3. Results

3.1. Expression and purification of the recombinant proteins

Truncated recombinant His-fused gB, gC, and gE were successfully expressed using the *E. coli* expression system as shown by SDS-PAGE and the Western blot analysis (Fig. 2A, lane 1, 7, and 13). Recombinant His-tagged gB and gE were expressed in the supernatant of IPTG-induced *E. coli* following ultrasonication (Fig. 2A, lanes 4 and 16). Recombinant His-tagged gC was expressed in the inclusion bodies of the IPTG-induced *E. coli* (Fig. 2A, lanes 11). The Western blot results revealed that recombinant His-fused gB, gC, and gE were recognized by PRV-positive serum derived from immunized mice (Fig. 2B, lanes 1, 7, and 13). In contrast, the control was not recognized by the PRV-positive

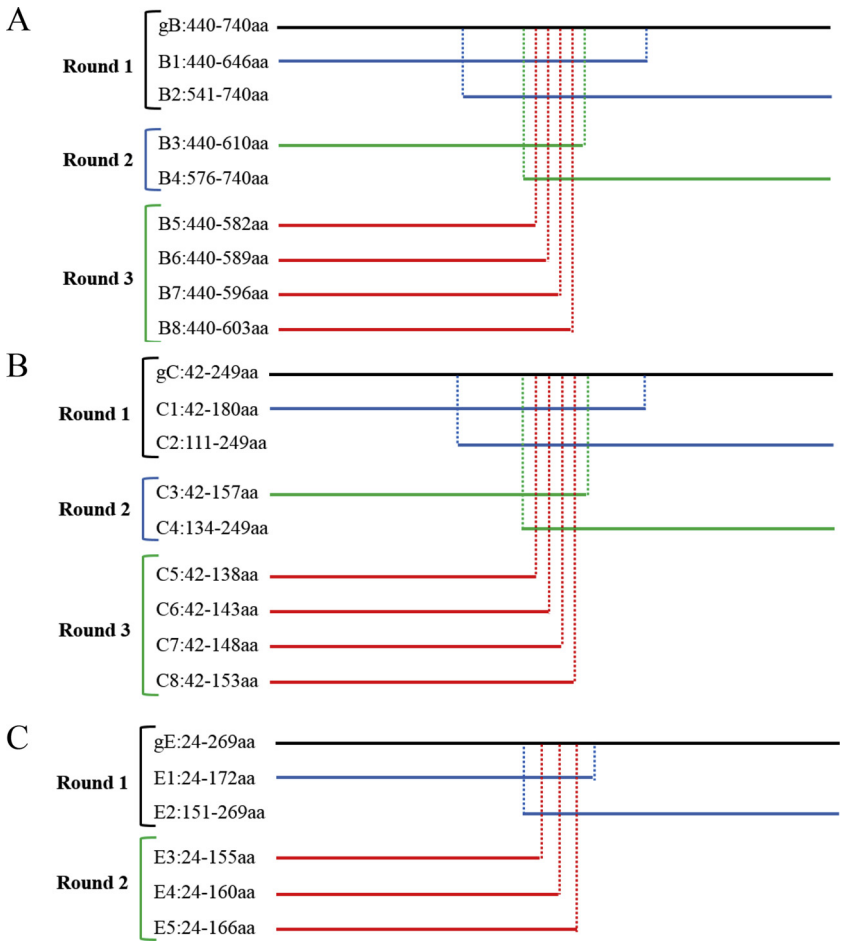

Fig. 1. Schematic representation of the peptides used for B cell epitope mapping. (A) Eight overlapping peptides were expressed to investigate the epitopes of mAbs 5A2 and 6G5. (B) Eight overlapping peptides were expressed to investigate the epitopes of mAbs 5D10 and 7C5. (C) Five overlapping peptides of gE were expressed to investigate the epitopes of mAbs 3E1, 3H8, and 4D2.

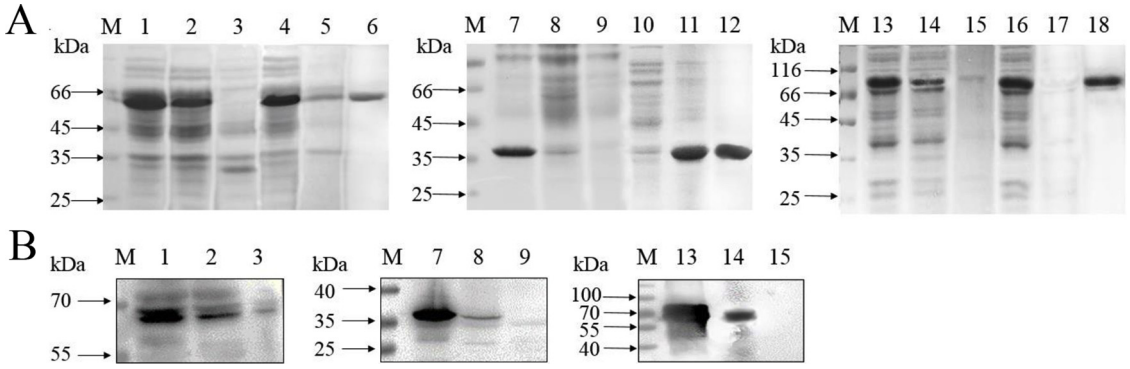

Fig. 2. Expression and purification of recombinant His-tag protein. Bacterial lysates from *E. coli*. BL-21 cells transformed with recombinant plasmids pET-32a-gB, pET-28a-gC, and pET-32a-gE were subjected to SDS-PAGE (A) and Western blot (B) analysis with anti-His mAb. Lane 1, BL21-pET-32a-gB with IPTG induction; lane 2, BL21-pET-32a-gB without IPTG induction; lane 3, BL21-pET-32a with IPTG induction; lane 4, the IPTG-induced BL21-pET-32a-gB supernatant after sonication; lane 5,the precipitation of IPTG-induced BL21-pET-32a-gB after sonication; lane 6, purified gB protein; Lane 7, BL21-pET-28a-gC with IPTG induction; Lane 8, BL21-pET-28a-gC without IPTG induction; lane 9, BL21-pET-28a with IPTG induction; lane 10, the supernatant of IPTG-induced BL21-pET-28a-gC after sonication; lane 11, the precipitation of IPTG-induced BL21-pET-28a-gC after sonication; lane 12, purified gC protein; Lane 13, BL21-pET-32a-gE with IPTG induction; Lane 14, BL21-pET-32a-gE without IPTG induction; lane 15, BL21-pET-32a with IPTG induction; lane 16, the supernatant of IPTG-induced BL21-pET-32a-gE after sonication; lane 17, the precipitation of IPTG-induced BL21-pET-32a-gE after sonication; lane 18, purified gE protein.

serum. The purity of the recombinant protein was analyzed by SDS-PAGE (Fig. 2A, lane 6, 12, and 18).

3.2. Anti-serum analysis of administered mice by indirect ELISA

The anti-serum titer of mice administered gB, gC, or gE was assessed with an indirect ELISA. The results indicate that all of the immunized mice displayed high antibody titers (range: 1:3,200–1:12,800) against the immunogen after three booster immunizations, compared to un-immunized mice (Fig. 3). These results indicate that a successful immunogenic response was induced in response to the recombinant protein.

3.3. Preparation of hybridoma cell lines

Spleen cells (splenocytes) were harvested from the mice exhibiting the highest antibody titer against the immunogen and fused with SP2/0 myeloma cells. The supernatant of the growing hybridoma cells was detected using an indirect ELISA, and positive hybridoma clones were screened. Eventually, seven positive hybridomas were obtained among which, two of the hybridomas secreted mAbs against gB (5A2 and 6G5); two hybridomas secreted mAbs against gC (5D10 and 7C5); and three hybridomas secreted mAbs against gE (3E1, 3H8, and 4D2). Using a commercially available isotype classification kit, the isotyping results indicated that 3E1, 3H8, and 4D2 were of the IgG1 subclass, 5A2, 6G5, and 5D10 were of the IgA subclass, and 7C5 was of the IgG2b subclass (data not shown). The affinity of the mAbs to PRV were determined by an ELISA using serial dilutions of the mAbs. The results indicated that these mAbs had high anti-PRV titers, and the supernatants from the SP2/0 myeloma cell culture were used as the negative control in the ELISA (Fig. 3D).

3.4. mAb specificity analysis

The level of mAb specificity was further investigated by Western blot and IFA. The Western blot showed that the 5A2 and 6G5 mAbs specifically recognized the recombinant gB protein and native gB protein, respectively, from PRV-infected BHK-21 cells. The 5D10 and 7C5 mAbs specifically recognized the recombinant gC protein and native gC protein, respectively, from PRV-infected BHK-21 cells. The 3E1, 3H8,

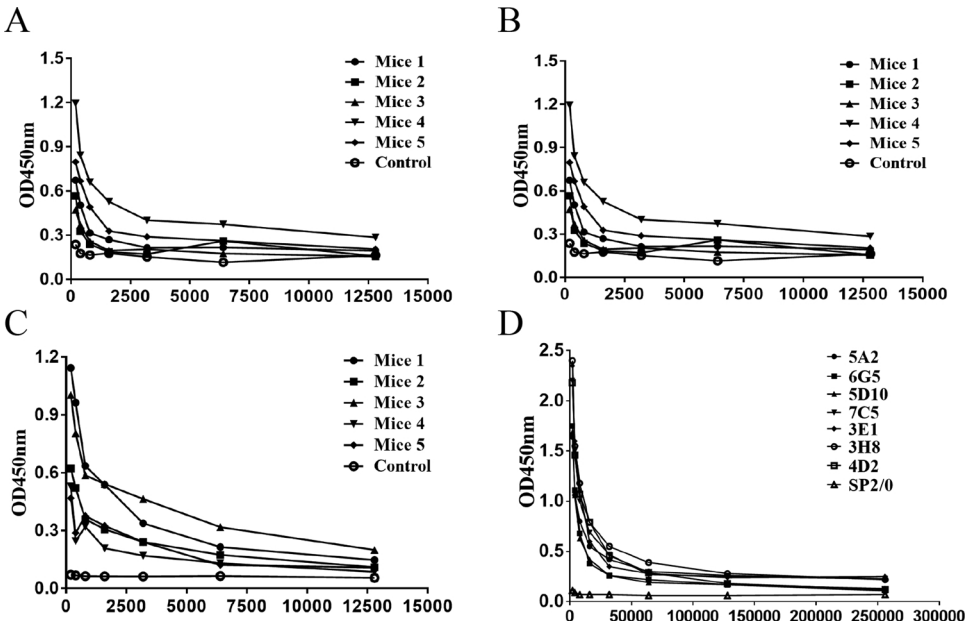

Fig. 3. Serum titer assay and PRV-specific mAb titers. (A, B, and C) Mouse sera titer after booster immunization. After the final booster immunization, serum samples were collected from the mice. Serially diluted serum samples were tested using an indirect ELISA with purified PRV used for coating. Serum samples from unimmunized mice served as the negative control. (D) Serially diluted ascites fluid was measured using an indirect ELISA with purified PRV used for the coating. The supernatant from SP2/0 cells served as a negative control. OD_{450nm}, optical density for ELISA.

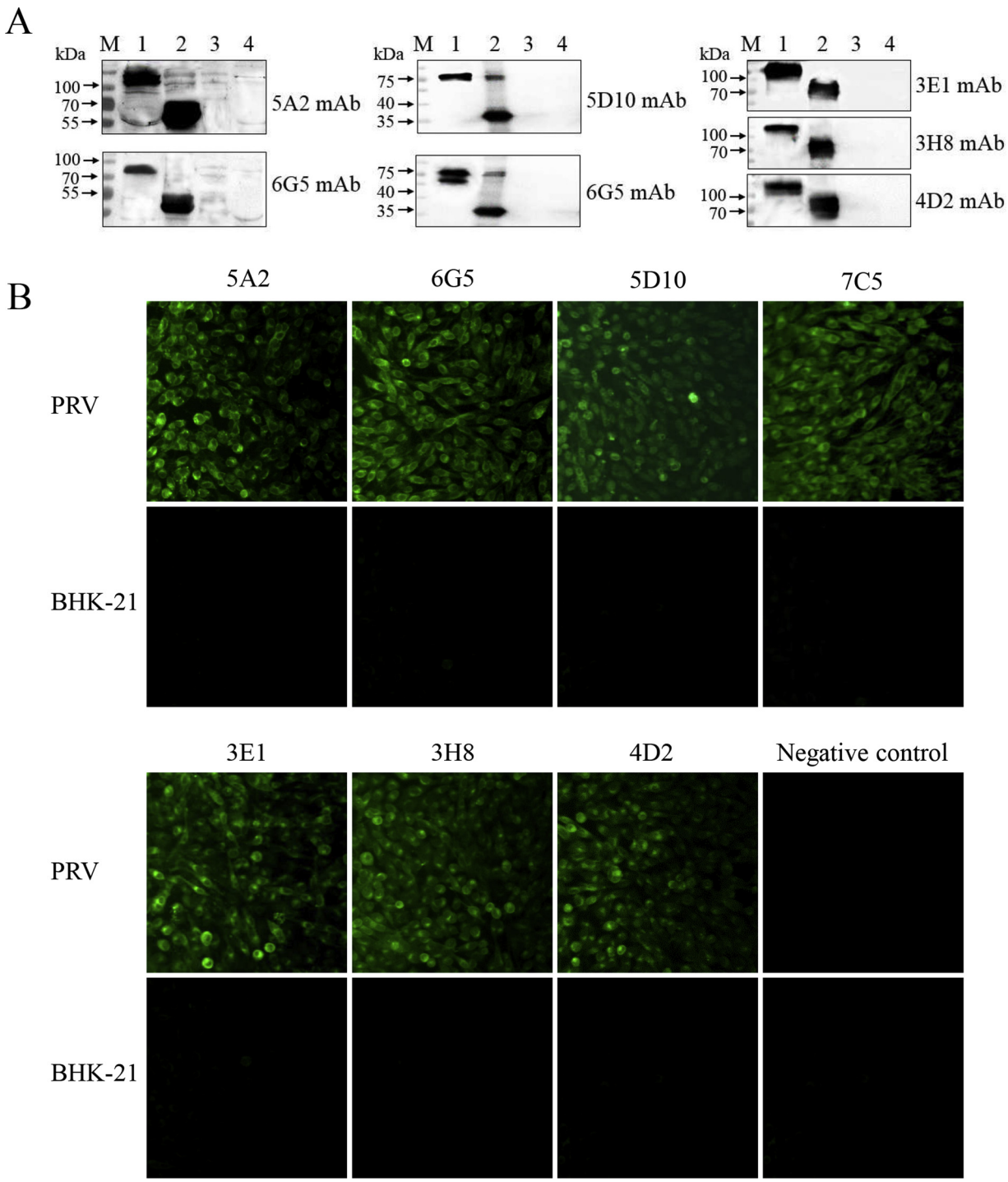

Fig. 4. Characterization and identification of the mAbs. (A) Characterization of mAbs by Western blot. BHK-21 cells were uninfected and infected with PRV ZJ01. *E. coli.* BL21 were transformed with an empty vector or a plasmid encoding His-tagged gB, gC, or gE. The cells were then subjected to Western blot with mAbs. Lane 1, the cells infected with PRV ZJ01; lane 2, expression of His-tagged gB, gC, or gE with IPTG induction; lane 3, expression of the empty vector; lane 4, the cells uninfected with PRV ZJ01.(B) The mAbs recognized in ZJ01-infected BHK-21 cells by IFA. BHK-21 cells were mock infected and infected with PRV ZJ01 at an MOI of 0.01. After approximately 24 h, the cells were fixed and primed with the mAbs and supernatant of the SP2/0 cells for IFA.

and 4D2 mAbs specifically recognized the recombinant gE protein and native gC protein, respectively, from PRV-infected BHK-21 cells (Fig. 4A). Furthermore, IFA revealed that these mAbs reacted with BHK-21 cells infected with PRV, but the mock-infected BHK-21 cells did not (Fig. 4B).

3.5. Identification of the epitope recognized by the mAbs

To identify the epitope recognized by the mAbs, eight overlapping fragments of gB, eight overlapping fragments of gC, and five overlapping fragments of gE were expressed and subjected to a western blot. As shown in Figs. 5A, A2 and 6 G5 mAbs specifically reacted with eight truncated recombinant gB proteins. As shown in Fig. 5B, 5D10 mAb specifically reacted with eight truncated recombinant proteins and 7C5

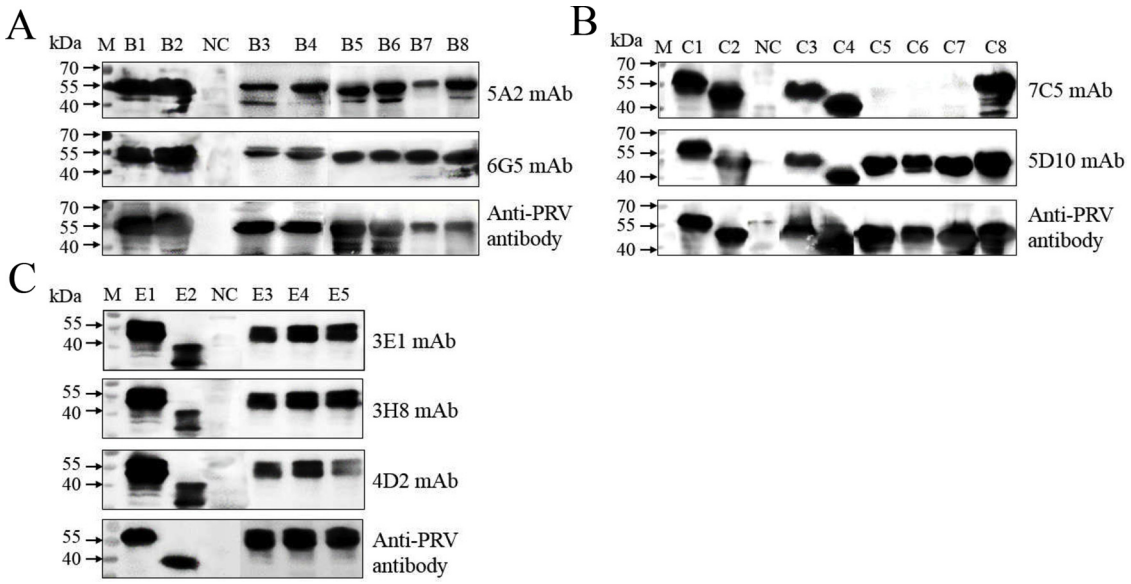

Fig. 5. Identification of the linear epitopes recognized by mAbs. (A) Eight overlapping recombinant peptide fragments, B1-B8, spanning the 440–740 aa of gB were expressed and subjected to a Western blot with mAbs 5A2 and 6G5. (B) Eight overlapping recombinant peptide fragments, C1–C8, spanning the 42–249 aa of gC were expressed and subjected to a Western blot with mAbs 5A2 and 6G5. (C) Five overlapping recombinant peptide fragments, E1–E8 spanning the 24–269 aa of gE were expressed and subjected to a Western blot with mAbs 3E1, 3H8, and 4D2. BL21- pET-32a protein was used as the negative control.

mAb specifically reacted with the C1, C2, C3, C4, and C8 recombinant proteins, but did not react with the C5, C6, and C7 recombinant proteins. As shown in Figs. 5C, 3 E1, 3 H8, and 4 D2 specifically reacted with five truncated recombinant gE proteins. The results indicate that 5A2 and 6G5 recognize the same epitope located at ^{576}SAVATAA^{582}; 5D10 recognizes the epitope located at ^{134}GETFE^{138}; 7C5 recognizes the epitope located at ^{143}RRGRFRSPDAD^{153}; and 3E1, 3H8, and 4D2 recognize the same epitope located at ^{151}IGDYL^{155}.

3.6. Amino acid alignment of the identified epitopes

To evaluate the conservation of the epitope identified by the mAbs among different PRV strains, we aligned the amino acid sequences of the B cell epitopes for gB, gC, and gE with the predicted amino acid sequences from other representative strains of PRV. As shown in Fig. 6, the four linear epitopes are indicated with red boxes. We found that the B cell epitopes, ^{576}SAVATAA^{582}, ^{134}GETFE^{138}, ^{143}RRGRFRSPDAD^{153}, and^{151}IGDYL^{155} are highly conserved among the different PRV isolates.

3.7. Spatial location of epitope binding

Structural analysis of the antigenic epitopes was performed using an online computer software program. The epitope ^{576}SAVATAA^{582} recognized by 5A2 and 6G5 is predicted to be partially buried (Fig. 7A, marked in red), forming a part of α-helix (Fig. 7B, marked in red). The epitope ^{134}GETFE^{138} recognized by 5D10 is predicted to be partially buried (Fig. 7A, marked in green), and the epitope ^{143}RRGRFRSPDAD^{153} recognized by 7C5 is predicted to be exposed on the surface of the gC (Fig. 7A, marked in blue), both exhibiting random coil (Fig. 7B, marked in green and blue, respectively). The epitope ^{151}IGDYL^{155} recognized by 3E1, 3H8, and 4D2 is predicted to be fully exposed on the surface of the gE (Fig. 7A, marked in yellow), forming part of an α-helix (Fig. 7B, marked in yellow). We also analyzed the epitope sequence using PROTEAN. As shown in Fig. 7C, epitope ^{143}RRGRFRSPDAD^{153} showed a high antigenic index and hydrophilicity. Furthermore, this epitope is predicted to be located on the surface of the gC, suggesting that the epitope ^{143}RRGRFRSPDAD^{153} is likely to be an important B-cell epitope on the gC of PRV.

4. Discussion

Porcine PR is prevalent throughout the majority of the world, and several countries have implemented measures to eradicate PR (Thawley and Morrison, 1988). During the 1970s, the Bartha-K61 strain attenuated vaccine was imported from Hungary to China, and PR was effectively controlled in some regions for several decades (Freuling et al., 2017). Since 2011, highly pathogenic PRV variants have emerged in China and spread quickly among pig herds (Chang et al., 2014; Tong et al., 2015); however, the current vaccines do not provide complete protection against new emerging variants of PRV, which have resulted in severe economic losses to the swine industry in China. In 2012, our laboratory isolated a PRV mutant strain (ZJ01 strain). Animal experiments have shown that the virulence of PRV strain ZJ01 was significantly enhanced compared with the classical LA strain (Gu et al., 2015). Therefore, further understanding of the structures and antigenic properties of PRV proteins is urgently required for the development of effective vaccines and immunodiagnostic approaches.

Although the viral proteins expressed by eukaryotic cells exhibit complete biological activity (Popa et al., 2016), the level of expression is too low to purify. In comparison, the prokaryotic system allows for the purification of large quantities of recombinant proteins in a short period of time and involves a simple, inexpensive bacterial cell culture (Porowinska et al., 2013). Thus, the main antigen coding region of the gB, gC, and gE genes was cloned from the vaccine strain PRV ZJ01 and expressed in *E. coli* BL21 (DE3). To improve the level of protein expression, we optimized the IPTG concentration, as well as the induction temperature and time. The gB and gE proteins were expressed in the supernatant, thus allowing for purification via nickel affinity chromatography. To improve the purity of the gB and gE proteins, we optimized the concentration of imidazole in both the binding and wash buffers. Since the gC protein formed insoluble inclusion bodies, to obtain a soluble and highly pure active protein, we washed the inclusion bodies to remove any membrane-bound proteins and dissolved the washed inclusion bodies, which was followed by refolding. The results were assessed by SDS-PAGE.

An evaluation of immunization efficiency and the identification of vaccine immunization or wild-type infection is primarily dependent on the serological detection. The serological tests most commonly used for

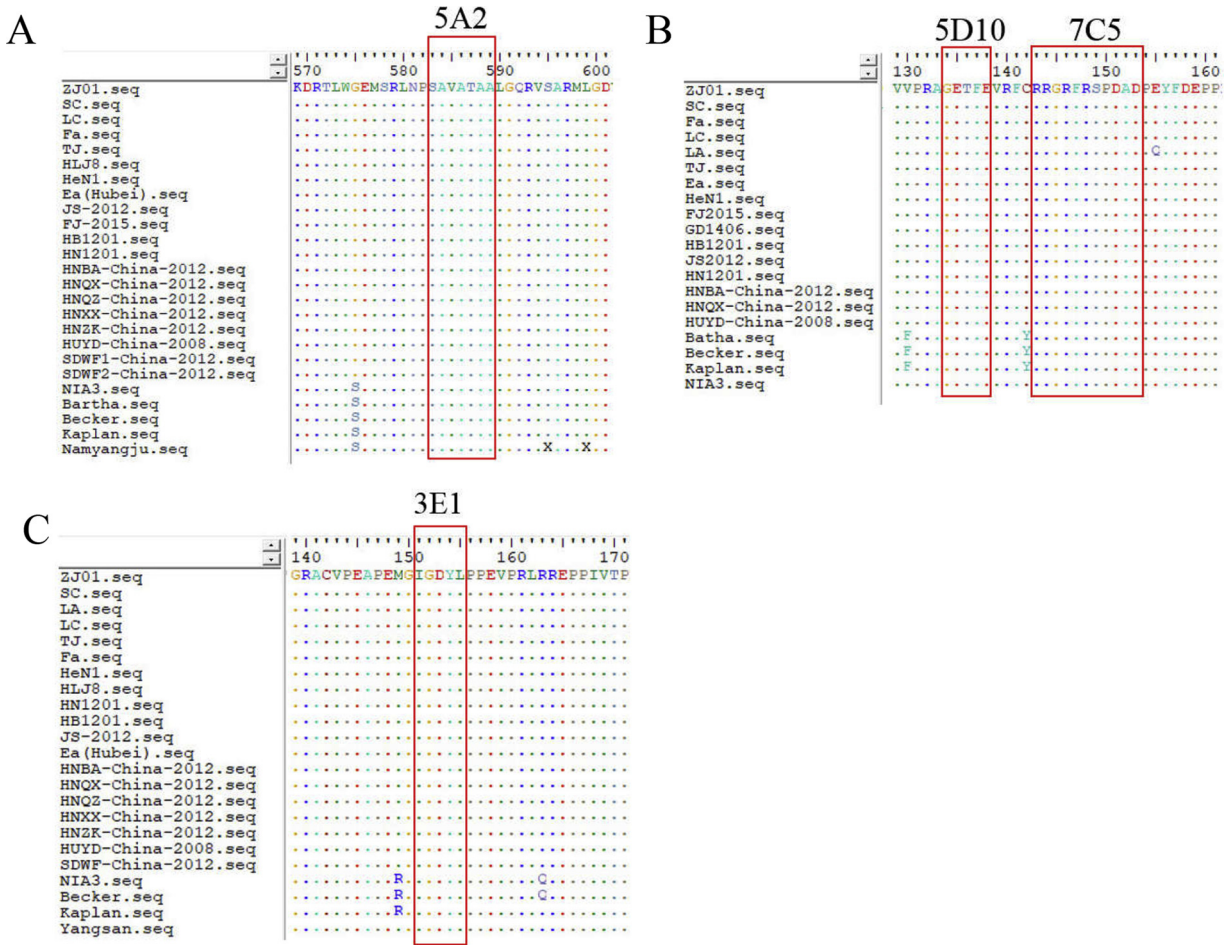

Fig. 6. Sequence alignment analysis of the identified epitope. (A) The amino acid sequence analysis of the gB epitope among the different PRV isolates. (B) The amino acid sequence analysis of the gC epitopes among the different PRV isolates. (C) The amino acid sequence analysis of the gE epitopes among the different PRV isolates. Matching residues are represented by ".".

PRV antibody determination are a virus neutralization test, ELISA, and latex agglutination test. Among these, an ELISA is capable of obtaining a rapid and simple diagnosis for a large quantity of clinical samples. Therefore, the ELISA has been rapidly developed and is currently widely used for clinical diagnosis (Gut et al., 1999; Kit et al., 1990; McGinley et al., 1992). Moreover, the ELISA is also an OIE recommended serological method for diagnosing PR. Moreover, mAbs are highly important for the establishment of ELISA methods.

The associated advantages of mAbs include a uniform composition, as well as both high specificity and sensitivity. Furthermore, mAbs can reduce the serological cross-reactivity between different cells and microbial species or strains, which greatly improves the specificity and sensitivity of the diagnosis (Buss et al., 2012). In the present study, to prepare mAbs against the gB, gC, and gE proteins, antigen-sensitized B lymphocytes were fused with SP2/0 cells to form hybridoma cells. B lymphocytes at different stages substantially influence the ability to obtain positive hybridomas. Some scholars believe that B lymphocytes in the transition period fuse more easily, and despite being the peak period of antibody production, the number of positive hybridoma cells is reduced seven to eight days after immunization (Lemieux and Bazin, 1993). Therefore, in the present study, mouse splenocytes were collected for cell fusion on the third to fourth day after the booster immunization. To prevent SP2/0 cells from reverting to their original state, we used a medium containing 15 μg/mL 8-azaguanine as an adaptation culture prior to fusion. Since the fusion of successful hybridoma cells is rare, we added feeder cells to the fused cells to create a favorable environment for the growth of the fused cells. After the cells had fused, they were placed in a 96-well plate. The number of cells per well should be within optimal levels as too many cells are not conducive to the growth of the fused cells, resulting in a decreased fusion rate. Thus, the cell density was controlled at about 2×10^5 cells per well. To improve the specificity of the screening mAb, we used the purified PRV protein as the coating antigen, which ensured that the mAbs against the prokaryotic expression vector could be effectively eliminated during the screening process. In the ELISA detection of the hybridoma supernatant after fusion, we found that if only one or two liquid exchanges were performed, the rate of positive ELISA detection was particularly high, and there was an increase in the number of false positives. Therefore, we cleaned the fused cell wells three times with RIMl640 three days before the detection of the hybridoma cell supernatant after fusion, and added RIMl640 containing 20% FBS and HAT nutrient solution. Finally, seven hybridoma cell lines that could stably secrete mAbs against gB, gC, or gE were prepared using indirect ELISA screening and subcloning.

The subtypes, titers, reactivity, and specificity of these mAbs were evaluated. All mAbs generated in this study specifically reacted with PRV-infected BHK21 cells and represented a high antibody titer against PRV, indicating the potential for the development of an ELISA diagnostic method. Moreover, we used DNASTAR software to analyze the conservation of the identified epitopes among the different PRV strains. We found that all four of the identified epitopes were highly conserved. The epitope locations inputted onto a 3D model showed that the epitope ^{576}SAVATAA^{582} and ^{151}IGDYL^{155} both form a part of an α-helix, and the epitope ^{134}GETFE^{138} and ^{143}RRGRFRSPDAD^{153} both exhibit a

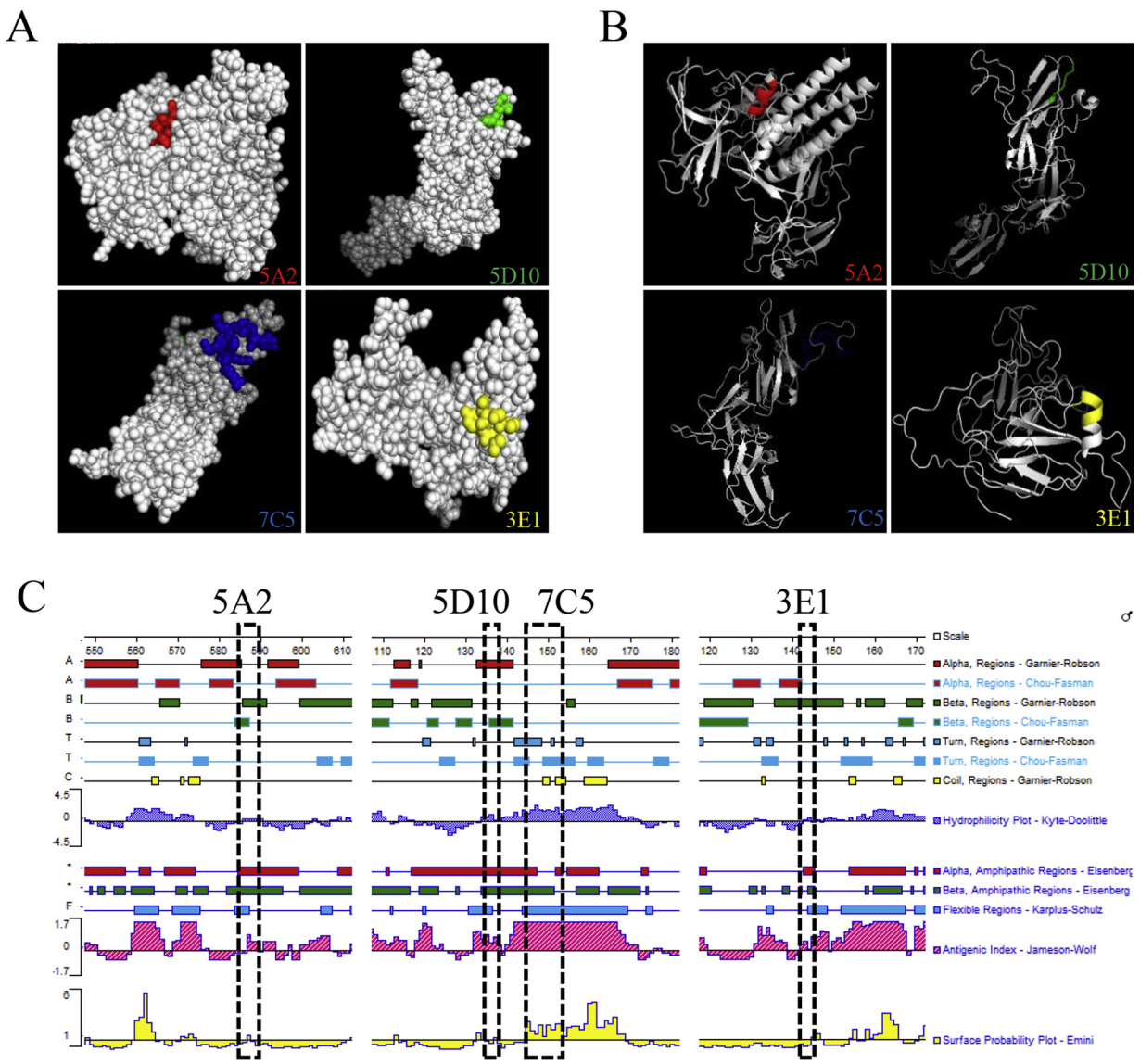

Fig. 7. Localization of the identified epitopes. The relative localization of the identified epitopes of 5A2 marked in red, 5D10 marked in green, 7C5 marked in blue, and 3E1 marked in yellow in a partially predicted 3D structure of gB, gC, and gE is highlighted in spheres (A) and a cartoon representation(B). (C) Structural features of gB, gC, and gE was predicted using PROTEAN software. All four epitopes are shown in boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

random coil. Among those epitopes, ^{143}RRGRFRSPDAD^{153} with a high antigenic index and high hydrophilicity, fully exposed on the surface of the gC, is likely to be an important B cell epitope. Thus, the mAbs and their defined B cell linear epitope may provide valuable tools for the development of immunodiagnostic approaches for PRV. Moreover, the study of the structure and function of gB, gC, and gE, and may also provide important information to further our understanding of their antigenic structures.

Conflict of interest

None.

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