Identification of a New Antigen Epitope in the Nuclear Localization Signal Region of Porcine Circovirus Type 2 Capsid Protein

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Key Words

Capsid protein · Epitope mapping · Nuclear localization signal · Porcine circovirus type 2

Abstract

Objective: Porcine circovirus type 2 (PCV2) is the causative agent of postweaning multisystemic syndrome in pigs. The capsid (Cap) protein encoded by ORF2 is the main structural protein involved in the host immune protective response to PCV2. It is therefore important to map the antigenic epitopes of the PCV2 Cap protein. **Methods:** In this study, 5 monoclonal antibodies (mAbs) against the recombinant PCV2 Cap protein, expressed by the baculovirus system in Sf21 insect cells, were generated. The antigenic epitope recognized by these mAbs was located in the Cap A protein by Western blot analysis using 4 overlapping minifragments covering the Cap protein expressed in *Escherichia coli*. To locate the epitope more accurately, 3 sets of overlapping peptides were synthesized. **Results:** The results demonstrated that 4 of the 5 mAbs recognized the same core epitope (²⁶RPWL³⁶VHPRHY³⁶) located in the nuclear localization signal (NLS) region at the N terminus of the Cap protein. The other mAb (1D2) reacted with the recombinant Cap protein only, indicating that it recognizes a potential conformational epitope. This mAb demonstrated a neutralizing effect on PCV2. **Conclusion:** This is the first study to identify an antigenic epitope in the NLS region of the PCV2 Cap protein us-

ing mAbs. The results of this study will facilitate future investigations into the mechanism and function of nuclear localization of this protein.

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Introduction

Porcine circovirus type 2 (PCV2) is recognized as the infectious agent of postweaning multisystemic wasting syndrome. Following the first confirmed case in Canada in 1997 [1–5], PCV2 has become a financial burden on the swine industry in North America, Europe and Asia. PCV2 is a member of the genus *Circovirus*, which belongs to the family *Circoviridae*, the smallest nonenveloped, single-stranded, circular DNA viruses that replicate autonomously in mammalian cells. The viral DNA of PCV2, which is encapsulated by a single viral protein, is a single-stranded negative-sense circularized molecule of 1,767–1,768 nucleotides [6]. Its genome contains 3 major open reading frames (ORFs): ORF1, which encodes 2 replication-associated proteins (Rep and Rep') [7–9], ORF2, which encodes a viral capsid (Cap) protein involved in the host immune response [10, 11], and ORF3, which encodes a protein contributing to virus-induced apoptosis [12, 13]. Forty-one amino acids (aa) in the N terminus of ORF2, which encodes the Cap protein, were associated with localization of the Cap protein in the nucleus, of

which aa 12–18 and 34–41 were critical for localization of Cap in the nucleus [14]. Many studies investigating the potential functions of the PCV2 Cap protein have been reported [15–18]. Monoclonal antibodies (mAbs) against the Cap protein are important tools for investigating the antigen epitopes expressed on the protein. Two epitopes spanning aa residues 69–83 and 117–131 have been identified as specific to the PCV2 Cap protein [19] and at least 5 different, but overlapping, conformational epitopes within aa residues 47–63 and 165–200 and the last 4 aa in the C terminus of the PCV2 Cap protein were located using chimeric PCV1 and PCV2 constructs [20]. Different antigenic epitopes expressed by different PCV2 isolates have been reported too [21, 22].

To study the PCV2 Cap protein and its potential prophylactic use, it is important to map the antigenic epitopes expressed on this protein, especially those located in the nuclear localization signal (NLS) region and those recognized by neutralizing antibodies. In this study, the epitopes were identified using mAbs and one new antigenic epitope located in the NLS region of the PCV2 Cap protein was detected.

Materials and Methods

Preparation of mAbs and Viruses

Six-week-old, female, BALB/c mice were purchased from the Experimental Animal Center of Harbin Veterinary Research Institute (Chinese Academy of Agricultural Sciences, China). Mice were immunized intraperitoneally with 250 µg of purified PCV2 recombinant Cap protein (PCV2-rCap), prepared using the baculovirus expression system in 0.5 ml of emulsion with complete Freund's adjuvant. Two booster injections with the same dose of emulsion were then given at 2-week intervals. One day after the last injection, the spleens were surgically removed from the mice. Five mAbs, designated 1D2, 2E8, 3A10, 5F2 and 6F10, were prepared using the lymphocyte hybridoma technique. The mAb ascites titers reached 1:2,048,000, 1:512,000, 1:1,024,000, 1:512,000 and 1:1,024,000 for 1D2, 2E8, 3A10, 5F2 and 6F10, respectively. The mAb isotypes were IgG2a for 1D2, 2E8, 5F2 and 6F10; and IgG1 for 3A10. The mAb light chains were λ-chain for 2E8, 3A10, 5F2 and 6F10, and κ-chain for 1D2. Western blot analysis showed that mAbs 2E8, 3A10, 5F2 and 6F10, but not 1D2, reacted with native PCV2-Cap protein, indicating that the epitopes recognized by these mAbs are present within the mature virus particles (fig. 1). mAb 1D2 reacted to PCV2 protein in an immunoperoxidase monolayer assay and an antigen capture ELISA, and could be used to differentiate between PCV1 and PCV2 under the same conditions. mAb 1D2 was also able to neutralize PCV2 in a neutralization test (data not shown). Strain PCV2-JF (GenBank No. HM038022) was isolated and maintained in the pig kidney cell line (PK-15), which was PCV1 free. The truncated Cap peptides were designed according to the ORF2 sequence of strain PCV2-JF. Anti-PCV2-positive polyclonal swine sera and PCV2-negative swine sera were prepared in our laboratory.

Fig. 1. Western blot analysis of the reactivity of PCV2 Cap protein to a range of mAbs. Native virions from strain PCV2/JF were separated by SDS-PAGE, transferred to nitrocellulose membrane, and then incubated with mAbs. The mAbs and the SP20 control are indicated in the figure. Western blot analysis showed that mAbs 2E8, 3A10, 5F2 and 6F10 reacted with native PCV2 Cap protein (indicated by the arrow at approximately 30 kDa), but mAb 1D2 did not.

Primer Design, PCR Amplification and Construction of Recombinant Expression Vectors Containing the Truncated ORF2 Genes

Four pairs of primers were designed from the DNA sequence of strain PCV2-JF which spanned the entire ORF2 gene and contained *Bam*HI and *Xho*I restriction sites (restriction enzyme sites are shown in italics in table 1). The 4 overlapping PCR products amplified using these primer pairs were termed Cap A, B, C and D. PCR (using reaction components from Takara Biotechnology, China) was performed on a thermocycler under the following conditions: 5 min at 94°, followed by 35 cycles of 30 s at 94°, 30 s at 55° and 30 s at 72°, and a final step of 10 min at 72°. After agarose gel purification and restriction enzyme digestion (with *Bam*HI and *Xho*I), the PCR products were inserted into the cloning sites of the expression vector pGEX-6p-1 (Invitrogen, USA). The resulting recombinant plasmid was verified by enzyme digestion and DNA sequencing.

Expression and Identification of Truncated ORF2 Genes

Four confirmed recombinant plasmids (pGEX-Cap A, B, C and D) were transformed in *Escherichia coli* BL21 cells for expression. After shaking of the cell cultures for 1 h, 1 mM of isopropyl-D-thiogalactopyranoside (Pharmacia Biosciences) was added to

Table 1. Primers for amplification of truncated forms of PCV2 ORF2 gene by PCR

Fragment names	Primer sequence	Position of the amplified fragments in ORF2
Cap A	5'-CTGGGATCCATGACGTATCCAAGGAGGCGTTA-3' 5'-CCGCTCGAGTCACGTTTTGACTGTGGTTCGCTT-3'	nt 1–192
Cap B	5'-CTGGGATCCACCTTCGGATATACTATCAAGCG-3' 5'-CCGCTCGAGTCATGTGGCCTTTGTTACAAAGTTA-3'	nt 154–402
Cap C	5'-CTGGGATCCGTTAAGGTTGAATTCTGGCCCTG-3' 5'-CCGCTCGAGTCACAGCTGATTTCTTTTGTGTTTG-3'	nt 301–549
Cap D	5'-CTGGGATCCGTCCTAGATTCCACTATTGATT-3' 5'-CCGCTCGAGTCAAGGGTTAAGTGGGGGTCTTTA-3'	nt 496–699

Table 2. Design of synthesized aa sequence of the truncated Cap A fragment

Peptide names	Peptide sequence
Cap A-a	TYPRRRYRRRRHRPRGHLGQILRRR
Cap A-b	ILRRRPWLHPRHRYRWRRKNGTFN
Cap A-c	KNGTFBPRLSRTFGYTIKRTTVKT
Cap A-b-1	ILRRRPWLHPRHRY
Cap A-b-2	PRHRYRWRRKNGTFN

Table 3. Design of synthesized aa sequence of truncated Cap A-b-1 fragment

Peptide names	Peptide sequence	Peptide position
Cap A-b-1-1	RRRPWLHPRHRYRW	aa 24–38
Cap A-b-1-2	RPWLHPRHRYRWRR	aa 26–40
Cap A-b-1-3	WLHPRHRYRWRRKN	aa 28–42
Cap A-b-1-4	VHPRHRYRWRRKNGT	aa 30–44
Cap A-b-1-5	GQILRRRPWLHPRH	aa 20–34
Cap A-b-1-6	HLGQILRRRPWLHVP	aa 18–32
Cap A-b-1-7	RGHLGQILRRRPWL	aa 16–30
Cap A-b-1-8	RPRGHLGQILRRRPW	aa 14–28

Luria-Bertani broth and the cultures were then agitated for a further 4 h. The bacterial cells were pelleted at 5,000 g for 15 min and lysed by sonication in PBS (pH 7.4). The expression of fusion proteins was confirmed by Western blot analysis with the porcine polyclonal antibody against PCV2-rCap.

Western Blot Analysis

Cell lysates were mixed with an equal volume of sample loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2%

SDS, 0.1% bromophenol blue and 10% glycerol) and separated by SDS-PAGE on 12% polyacrylamide gels. For immunoblotting, the proteins were transferred from the polyacrylamide gel to nitrocellulose membrane. Nonspecific antibody binding sites were blocked with 1% nonfat milk in PBS-T (PBS plus 0.5% Tween-20) overnight at 4°. The membranes were then incubated with the primary antibody at 37° for 1 h. After incubation with the primary antibody, the membrane was washed 3 times with PBS-T (5 min each time). The blot was then probed with a 1:5,000 dilution of HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich) secondary antibody for 1 h at 37°. Finally, the membrane was washed 3 times with PBS-T and immunoreactivity was visualized using the substrate diaminobenzidine (Sigma-Aldrich).

Preliminary Determination of the Epitopes Expressed on the Truncated Cap A Fragment

After preliminary location of the epitopes using the truncated Cap proteins, 3 peptides spanning the entire Cap A fragment deduced from PCV2-JF strain were designed and synthesized (GL Biochem, Shanghai, China). Adjacent peptides had 5 or 6 aa residues in common and were termed Cap A-a, -b and -c (table 2). The peptides were screened by indirect ELISA. Briefly, an ELISA plate was coated with the synthesized peptides (1 µg per well) and incubated overnight. The plate was then blocked with 1% nonfat milk for 1 h at 37°. After 3 washes with PBS-T, 100 µl of hybridoma supernatant containing mAbs was added to each well and incubated at 37° for 1 h. Following 3 further washes with PBS-T, the bound mAbs were detected with a 1:5,000 dilution of HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich). After further washes with PBS-T, 100 µl of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) was added. The reaction was stopped after 30 min with 2 M H₂SO₄ and the absorbance at 405 nm was measured using an ELISA plate reader (Bio-Rad, Hercules, Calif., USA). All samples were analyzed in duplicate and the results are presented as the average of these readings. Based on the results, 2 other peptides spanning the Cap A-b region (Cap A-b-1 and -2) were subsequently synthesized (table 2) and shared 5 aa residues. These peptides were used for further mapping of the epitopes and were screened using the ELISA method described above.

Precise Epitope Mapping

According to the results of ELISAs, the overlapping fragment of Cap A-b-1 (²²ILRRRPWLHVPRHRY³⁶) was truncated by sequentially removing 2 aa from each end. The resulting truncated peptides (designated Cap A-b-1-1 to Cap A-b-1-8) were screened by ELISA as described above (table 3). The immunoreactivity of the artificial peptides was confirmed using PCV2-positive pig sera, and we tested whether the epitope could be recognized by the immune system using an ELISA method similar to that mentioned above.

Results

Amplification of Truncated ORF2 Genes and Identification of Recombinant Expression Vectors

Four truncated fragments of the ORF2 gene were amplified from the DNA of strain PCV2-JF (data not shown). These PCR products were then inserted into the cloning sites (*Bam*HI and *Xho*I) of the expression vector pGEX-6p-1. The desired recombinant plasmid was verified by enzyme digestion and DNA sequencing.

Expression of Truncated ORF2 Proteins and Localization of Antigenic Epitopes

Four truncated proteins were expressed as GST fusions, separated by SDS-PAGE and identified by Western blot analysis with a porcine polyclonal antibody to PCV2-rCap. The truncated proteins (~34 kDa) all showed strong reactivity to the porcine polyclonal antibody (fig. 2). The protein expressed by the pGEX-6p-1 vector (~28 kDa) showed no reactivity and served as the negative control. These 4 proteins displaying antigenic epitopes were identified by 5 mAbs (6F10, 5F2, 3A10, 2E8 and 1D2). Western blot results showed that the truncated Cap A protein was recognized by 4 mAbs (6F10, 5F2, 3A10 and 2E8) and mAb 1D2 did not react with any of the truncated proteins, except the whole Cap protein expressed by recombinant insect baculovirus (fig. 3).

Localization of the mAb Epitopes in the Truncated Cap A Protein

To locate the linear antigenic epitopes within the Cap A protein, 3 overlapping peptides were synthesized and probed by 4 mAbs (6F10, 5F2, 3A10 and 2E8). ELISA results showed that the Cap A-b peptide was recognized by all 4 mAbs. Each peptide was 24 or 25 aa in length and overlapped by 5 or 6 aa with the neighboring peptides, indicating that the linear epitope was located in the Cap A-b peptide and the adjacent peptides showed no antigenicity to these mAbs (fig. 4a).

Fig. 2. Identification of the expression of recombinant fusion proteins by SDS-PAGE and Western blot analysis. SDS-PAGE of the recombinant fusion proteins, together with the vector only and the *E. coli* BL21 cells as controls, was performed and the gel was silver stained to visualize the band corresponding to the recombinant protein (indicated by arrows in **a**). The 4 truncated proteins (~34 kDa) were all strongly reactive with the porcine polyclonal antibody (**b**). The pGEX-6p-1 vector (~28 kDa) was included as a negative control.

To further elucidate the position of the linear antigenic epitope within the Cap A-b peptide, 2 overlapping peptides were synthesized and probed by 4 mAbs (6F10, 5F2, 3A10 and 2E8). ELISA results showed that the Cap A-b-1 peptide was recognized by all 4 of these mAbs, indicating that the linear epitope was located in the Cap A-b-1 peptide (fig. 4a).

Fig. 3. Detection of the reactivity of 5 mAbs (6F10, 5F2, 3A10, 2E8 and 1D2) to the overlapping peptides covering the length of the PCV2 Cap protein by Western blot analysis. Truncated recombinant proteins were analyzed along with the purified Cap protein expressed by Sf21 cells and the expression vector only as positive and negative controls, respectively. Of the 5 mAbs analyzed, 4 (6F10, 5F2, 3A10 and 2E8) only showed reactivity with the pGEX-

Cap A fragment and the Cap-positive control whereas mAb 1D2 showed no reactivity with any truncated fragments and only reacted with the Cap-positive control. The results indicate that the epitopes against the 4 mAbs may be located in the truncated fragment pGEX-Cap A and the remaining 1D2 may be a conformational epitope based on its neutralizing effect against PCV2 (data not shown).

Fig. 4. ELISAs to determine the antigenic epitopes on the PCV2 Cap protein. **a** Detection of the reactivity of 4 mAbs with 5 peptides from the Cap A region by indirect ELISA. Ninety-six-well microtiter plates were coated with synthesized overlapping peptides. After blocking with nonfat milk, mAbs (6F10, 5F2, 3A10 and 2E8) were added, followed by the addition of secondary antibody, HRP-conjugated goat anti-mouse IgG. Synthesized overlapping peptides were then detected with the 4 mAbs. The results showed that the epitopes to the 4 mAbs were located in the truncated fragment Cap A-b, and further identification indicated that they were more specifically located in the mimic truncated fragment Cap A-b-1. **b** Determination of the minimal unit of the epitope by ELISA. A series of truncated peptides (Cap A-b-1-1 to 8) derived from the

Cap A-b-1 peptide were synthesized and subjected to ELISA to locate the minimal unit of the epitope required for recognition by the 4 mAbs. Removal of more than 4 aa residues from the N terminus of the Cap A-b-1 peptide, and as long as removal of 1 aa residue from C terminus of Cap A-b-1 peptide, resulted in no reactivity with the mAbs. The minimal epitope for the mAbs therefore comprised 11-aa residues: ²⁶RPWL VHP RHRY³⁶. **c** Detection of the 11-aa peptide (²⁶RPWL VHP RHRY³⁶) with the 4 mAbs the polyclonal PCV2-positive swine sera (together with the SP20 supernatant) and the PCV2-negative swine sera as controls by indirect ELISA. The peptide reacted positively with the 4 mAbs (6F10, 5F2, 3A10 and 2E8) and PCV2-positive swine sera only, indicating that this epitope is recognized by the immune system.

Fine Mapping of the mAb Epitopes in the Truncated Cap A-b-1 Peptide

For further epitope determination, a series of truncated peptides (Cap A-b-1-1 to Cap A-b-1-8) derived from the Cap A-b-1 peptide were synthesized and subjected to ELISA. The results showed that truncation of more than 4 aa residues from the N terminus of the Cap A-b-1 peptide, and as long as truncation of 1 aa residue from the C terminus of the Cap A-b-1 peptide, resulted in no reactivity with any of the mAbs. This indicated that the core aa residues comprising the epitope were the same for each mAb, i.e. ²⁶RPWLHVPRHRY³⁶ (fig. 4b). This epitope was also reactive with the polyclonal PCV2 positive swine sera, confirming that the epitope is recognized by the immune system (fig. 4c).

Discussion

mAbs are a powerful tool for identifying linear epitopes or mimic epitopes of proteins and have been used to investigate proteins from a variety of infectious agents [23–25]. Of the 5 mAbs used in this study to investigate the antigenic epitopes of PCV2, 4 recognized the same linear epitope located in the NLS of the N terminus of the Cap protein. The other mAb did not react with any of the truncated Cap proteins but did react with the whole recombinant Cap protein, which was considered a conformational epitope based on its neutralizing effect against PCV2 (data not shown). The methods used in this study were unable to identify conformational epitopes: other approaches are needed for further elucidation.

To avoid the inhibition caused by the NLS on the expression of recombinant proteins, previous studies on the Cap protein have focused on the protein lacking the NLS [22, 26]. To date, no studies have effectively verified the epitopes on the NLS, so the function and mechanism of localization of the PCV2 Cap protein remain to be elucidated. In this study, we identified a new epitope located on the NLS, of which 3 aa residues were critical for nuclear localization of the Cap protein. And we can also conclude that this epitope is one of the predominant domains in the Cap protein because the 4 mAbs are against the same epitope. This is the first study to identify an antigenic epitope in the NLS region of the PCV2 Cap protein using mAbs, and our findings will facilitate further studies on the function and mechanism of nuclear localization of this protein. Moreover, mAb 1D2 was strongly active with native PCV2 particles, and could be used to differentiate between PCV1 and PCV2 under the same conditions. So, for further studies, the mAb would be a useful tool for molecular diagnostic and antigen epitope analysis of the virus. And after accurate epitope location for the neutralizing mAb, it would facilitate the development of new vaccines against PCV2.

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