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Characterization of specific antigenic epitopes and the nuclear export signal of the *Porcine circovirus* 2 ORF3 protein

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ABSTRACT

Porcine circovirus 2 (PCV2) is the etiological agent of postweaning multisystemic wasting syndrome. PCV2 ORF3 protein is a nonstructural protein known to induce apoptosis, but little is known about the biological function of ORF3 protein. Therefore, we undertook this study to map ORF3 protein epitopes recognized by a panel of monoclonal antibodies (mAbs) and to characterize putative nuclear localization (NLS) and nuclear export (NES) sequences in ORF3. The linear epitopes targeted by two previously published mAbs 3B1 and 1H3 and a novel mouse mAb 3C3 were defined using overlapping pools of peptides. Here, we find that ORF3 in PCV2 infected cells contains a conformational epitope targeted by the antibody 3C3, which is distinct from linear epitopes recognized by the antibodies 3B1 and 1H3 in recombinant ORF3 protein. These results suggest that the linear epitope recognized by 3B1 and 1H3 is masked in PCV2 infected cells, and that the conformational epitope is unique to PCV2 infection. Furthermore, we find that ORF3 protein expressed in cytoplasm in early stages of PCV2 infection and then accumulated in nucleus over time. Moreover, we localize a NES at the N-terminus (residues 1–35aa) of ORF3 which plays critical role in nuclear export activity. These findings provide a novel insight that deepens our understanding of the biological function of PCV2 ORF3.

1. Introduction

Porcine circovirus (PCV) (*Circoviridae, Circovirus*) is a small non-enveloped, single-stranded, circular DNA virus with two subtypes, PCV1 and PCV2 (King et al., 2012). PCV1 is nonpathogenic and was first isolated from a persistently infected porcine kidney cell line PK15 (Tischer et al., 1982, 1974). In contrast, PCV2 is pathogenic and is the causative agent of postweaning multisystemic wasting syndrome (PMWS), and was first isolated in 1996 in western Canada (Clark, 1996, 1997).

Eleven open reading frames (ORFs) have been predicted in the PCV genome that encode viral proteins. ORF1 encodes the Rep and Rep', replication initiation proteins that are essential for virus replication (Cheung, 2004; Steinfeldt et al., 2001). The N-terminus of Rep and Rep' contain three putative nuclear localization signals that contribute to their nuclear import (Finsterbusch et al., 2005). ORF2 encodes the capsid (Cap) protein containing a NLS (Liu et al., 2001), which is a structural protein (Mahe et al., 2000; Nawagitgul et al., 2000) that is directly involved in the intracellular transport of PCV2 through interactions with dynein IC1 (Cao et al., 2014). Antibody binding domains in Cap have been well characterized (Lekcharoensuk et al., 2004; Mahe et al., 2000; Nawagitgul et al., 2000; Shang et al., 2009; Trible et al., 2012). ORF4 was recently identified and has been shown to be nonessential for PCV2 replication. ORF4 plays a role in suppressing caspase activity and prevents activation of $CD4^+$ and $CD8^+$ T lymphocytes during PCV2 infection (He et al., 2013).

The ORF3 protein was first described as a PCV2 nonstructural protein (11.9 kDa) in 2005 and mainly located in the nucleus of PCV2 infected cells (Lin et al., 2011; Liu et al., 2005). The ORF3 of PCV2 induces apoptosis by competing with p53 to binding to Pirh2 and thereby deregulating p53 homeostasis and is associated with PCV2 pathogenicity (Karuppannan et al., 2009; Liu et al., 2005, 2006, 2007). However, dendritic cells harbor infectious PCV2 without any cell modulation including apoptosis (Vincent

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et al., 2003). Furthermore, comparison of pigs with PMWS and healthy pigs showed that the higher the PCV2 viral load and the severity of PMWS lesions, the lower the apoptosis rate in tissues (Resendes et al., 2004), further contradicting the involvement of apoptosis in PCV2 pathogenesis (Krakowka et al., 2004, 2005). Also, it was demonstrated that ORF3 was limited in inducing pathogenicity (Juhan et al., 2010). Therefore it is important to further evaluate the biological role of ORF3 during PCV2 replication.

Nuclear localization signal (NLS) of viral protein may be required for efficient virus replication (Li et al., 2012). A recent study suggests two clusters of basic amino acid residues ^{53}FQKFSQPAEISDKRYR^{68} and ^{85}HSSRQVTPLSLRSRSSTLHQ^{104} of PCV2 ORF3 are potential NLSs (Lin et al., 2011). However, nuclear transport of small proteins (<50 kDa), like ORF3, can generally be achieved by slow, passive diffusion through the nuclear pore complex (NPC) (Macara, 2001) but larger proteins require active diffusion (Boisvert et al., 2014). Molecules can only get into or out of the nucleus through the NPC. Large proteins including most of protein–nucleic acid complexes require a NLS or nuclear export signal (NES) to enter or leave the nucleus through NPCs. The NLS is usually recognized by importins (Bayliss et al., 2000; Cingolani et al., 1999; Conti et al., 1998; Weis et al., 1995). In contrast, nuclear export factors, such as XPO1, bind to the NES sequences of export cargo in the nucleoplasm in a stable trimeric complex containing RanGTP. Upon entry to the cytoplasm, RanGTP is converted to RanGDP and the complex is dissociated (Marfori et al., 2011). NES is generally short stretches of amino acids characterized by multiple hydrophobic residues with typical spacing, a classical leucine—rich export signal consisting of the consensus sequence Φ-XX(X)-Φ-XX (X)-Φ-X-Φ, where Φ represents L, I, F, V or M, and X represents any amino acid (Macara, 2001).

Previously, our laboratory identified monoclonal antibodies (mAbs), 3B1 and 1H3, that recognized the ORF3 protein produced by *Escherichia coli* and transfected cells (He et al., 2012), but they did not react with the ORF3 protein produced in PCV2-infected cells. Here, we have developed a new monoclonal antibody 3C3, which is capable of recognizing ORF3 protein in PCV2-infected cell. We further mapped the mAb-recognition sites for 3B1, 1H3 and 3C3 in the ORF3 protein and characterized the biological functions of the putative NLS and NES sequences in ORF3.

2. Materials and methods

2.1. Viruses, cell lines, and monoclonal antibodies

The PCV2 strain HZ0201 (HZ0201) was isolated from pig farms with naturally occurring PMWS in 2006 (Zhou et al., 2006) and propagated in PK15 cells. A PCV2 ORF3-deficient HZ0201k; strain (mHZ0201) was constructed by our laboratory (He et al., 2012). PCV-free PK15 cells were maintained in minimal essential medium supplemented with 6% fetal bovine serum (Gibco, Carlsbad, CA), at 37 °C in a humidified 5% CO_2 incubator. The anti-ORF3 mAbs 3B1 and 1H3, anti-Cap mAb and rabbit anti-Cap polyclonal serum were developed previously in our laboratory (He et al., 2012; Shang et al., 2009).

2.2. Expression and purification of recombinant His tagged ORF3 protein

The following primers were synthesized using the published DNA sequence for HZ0201 (Genbank no. AY188355): PV2-3-*Eco*RI-F (5′-GCGAATTCATGGTAACCATCCCACCACT-3′) and PV2-3-*Xho*I-R (5′-GCCTCGAGTTACTGATGGAGTGTGGAGC-3′) to amplify the full length sequence of the PCV2 *ORF3* gene. Genomic DNA extracted from PCV2 infected PK15 cells was used as the PCR template. To construct the recombinant expression vector pET-28a-ORF3, the PCR products were purified using the Axygen® AxyPrep™ PCR Clean-Up Kit (Product #AP-PCR-250), digested with the restriction enzymes *Eco*RI and *Xho*I (TaKaRa Biotechnology Co., Ltd., Dalian), and then cloned into the expression vector pET-28a (Novagen, Madison, WI). The resulting plasmids were transformed into *E. coli* Rosetta (DE3) cells. The *E. coli* were induced to express recombinant his-ORF3 with 1 mM isopropyl-β-D-thiogalactopyranoside and allowed to grow 4 h at 37 °C. The cells were collected by centrifugation at 4000 × *g* for 5 min, washed, resuspended in 1/10 the volume of phosphate-buffered saline (PBS), and lysed using sonication treatment. The lysates were pelleted by centrifugation at 10,000 × *g* for 5 min. The supernatant was removed and the pellet was resuspended in an equivalent volume of buffer A (8 M urea, 100 mM NaH_2PO_4, 20 mM imidazole, 10 mM Tris-HCl, pH 8.0) at 4 °C. The supernatant and pellet were analyzed by SDS-PAGE and Western blot. Recombinant His-ORF3 protein was purified using NTA-agarose affinity resin (QIAGEN GmbH, QIAGEN Strasse1, 40724Hilden, Germany). The protein concentration was determined with a bicinchoninic acid (BCA) assay kit (Thermo, Waltham, MA).

2.3. Preparation of the monoclonal antibody against PCV2 ORF3 protein

As previously published (He et al., 2012), four Balb/c mice were immunized subcutaneously with an emulsion mixture containing equal volumes of complete Freund's adjuvant (Sigma–Aldrich, St. Louis, MO) and purified his-ORF3 protein (80 μg his-ORF3 protein per mouse). The mice received two booster immunizations two weeks (subcutaneous, 80 μg his-ORF3/mouse emulsified in incomplete Freund's adjuvant (Sigma–Aldrich)) and four weeks (intraperitoneally, 100 μg his-ORF3 protein/mouse emulsified in incomplete Freund's adjuvant) after the initial immunization. Three days after the final immunization, the anti-ORF3 antibody titer was determined by ELISA and the mouse with the highest titer was bled and sacrificed. The mouse splenocytes were collected and fused with SP2/0 myeloma cells. The hybridoma cells were cultured in RPMI-1640 containing HAT (hypoxanthine–aminopterin–thymidine) and HT (aminopterin–thymidine) for 10–14 days. The hybridomas were screened for antibody production against ORF3 by his-ORF3-ELISA and an immunofluorescence assay (IFA) with PCV2 infected PK15 cells. The hybridomas producing anti-ORF3 antibodies were then subcloned 4–5 times by limiting dilution assay, and the ascites containing the mAb against ORF3 were prepared as previously reported (He et al., 2012; Uchiyama et al., 1981). The isotype of the mAb was determined using the SBA Clonotyping™ System HRP system according to the manufacturer's protocols (Southern Biotechnology Associates, Birmingham, AL).

2.4. Peptide-ELISA and peptide-dot-blot

Ten overlapping peptides (P1–P10) spanning the full length of the PCV2 ORF3 amino acid sequence and seven additional truncated peptides of P9 (P11–P17) (Table 1 and Fig. 3A) were synthesized using solid-phase peptide synthesis on a Symphony Multiplex Peptide Synthesizer (Protein Technologies, Inc., USA). The peptide purity was greater than 90% as assessed by HPLC and mass spectrometry. During synthesis, a cysteine residue was added at the N-terminal of each peptide to link to the carrier protein BSA. The peptides were connected to BSA using a heterobifunctional cross-linker Sulfo-SMCC (Sigma). The BSA-labeled peptides were tested for their reactivity with mAbs 3B1, 1H3 and 3C3 by ELISA. Briefly, the peptides and his-ORF3 were used as the coating antigens for an ELISA as described previously