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Deletion of the single putative *N*-glycosylation site of the porcine circovirus type 2 Cap protein enhances specific immune responses by DNA immunisation in mice

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ABSTRACT

The immunogenicity of a putative *N*-linked glycosylation site located at amino acids 143–145 (N143YS) of the porcine circovirus 2 (PCV2) Cap protein was investigated. Eukaryotic vectors expressing wild-type PCV2 Cap (pCap) and *N*-linked glycosylation site mutant Cap (pCap-m) were constructed and the immunogenicity of these proteins was determined following DNA vaccination in BALB/c mice. pCap-m elicited significantly higher Cap-specific T lymphocyte proliferative activity, percentage of $CD8^+$ T cells, ratio of immunoglobulin (Ig) G2a:IgG1 and levels of interferon-γ compared to pCap ($P < 0.05$). These results indicate that deletion of the *N*-glycosylation site in the PCV2 Cap protein enhances specific immune responses and may have a role in Cap-based DNA vaccines with enhanced immunogenicity.

Introduction

Porcine circovirus type 2 (PCV2) is aetiologically associated with a complex of porcine circovirus diseases (PCVD), including post-weaning multisystemic wasting syndrome (PMWS) (Segalés et al., 2005). PMWS primarily affects pigs from 5 to 12 weeks of age and has been associated with morbidities of 5–30%, resulting in substantial economic losses to the pig industry. Pigs affected with PMWS may develop severe immunosuppression (Segalés et al., 2004). PCV2 is also associated with respiratory disease, reproductive failure, hepatitis and porcine dermatitis and nephropathy syndrome (PDNS) (Harding, 2004; Segalés et al., 2005).

PCV2 is a non-enveloped, single-stranded, circular DNA virus with a diameter of 17 nm (Tischer et al., 1982). The PCV2 genome consists of 1767 or 1768 nucleotides and is assumed to have 11 potential open reading frames (ORFs) (Hamel et al., 1998; Zhou et al., 2006). ORF2 encodes the Cap protein, which contains one well-conserved putative *N*-glycosylation site at amino acids 143–145 (N143YS). The consensus sequence for an *N*-glycosylation site is Asn-X-Ser/Thr-X, where X is any amino acid residue except proline, because the Asn residue cannot be glycosylated if a proline occupies the position of the second X.

Blanchard et al. (2003) demonstrated that the ORF2-encoded Cap protein was the major immunogen of PCV2. Glycans can play an important role in modulating the immunogenicity of a viral protein by shielding potential neutralisation epitopes, maintaining an appropriate antigenic conformation and altering the proteolytic susceptibility of proteins (Botarelli et al., 1991; Beyene et al., 2004). However, the effects of *N*-linked glycosylation of PCV2 Cap on specific immune responses mediated by this protein remain elusive. In this study, we investigated the role of putative *N*-linked oligosaccharide chains of the PCV2 Cap envelope protein in the induction of Cap-specific immune responses. The immunogenicity of a Cap *N*-glycosylation mutant was analysed in mice and compared to that of the wild-type Cap protein using a DNA-based vaccination approach.

Materials and methods

Animals

Twenty-four 7- to 8-week-old female PCV2-free BALB/c mice were provided by the Animal Centre of The Nanjing Army Hospital, Jiangsu, China. The mice were maintained under specific pathogen-free conditions and all animal procedures were in accordance with the Guidelines for the Care and Use of Animals at Nanjing Agricultural University (license number SYXK 苏 2010-0005).

PCV2 wild-type Cap and N-glycosylation site mutant Cap eukaryotic expression vectors

The full-length PCV2 *Cap* gene was amplified from genomic DNA of PCV2 strain SH (accession number HM038027.1) using oligonucleotide primers P1 (5′-GTTCTCGAGATGACGTATCCAAGGAGGC-3′) and P2 (5′-CTAGGATCCTTAAGGGTTAAGTGGGGGGT-3′). The amplified gene was subcloned into the *Xho*I and the *Bam*HI sites of the eukaryotic expression vector pcDNA3 (Invitrogen) to construct the wild-type PCV2 Cap recombinant expression plasmid pCap. To generate mutant pCap (Cap-m), the Asn (*N*)-encoding codon located at amino acids 143 in the *N*-glycosylation consensus sequence Asn-X-Thr/Ser was replaced with an Asp (D)-encoding codon. Oligonucleotide primers used for the site-directed mutagenesis reaction were P3 (5′-GGGAGGAGTAGTCTACATAGG-3′) and P4 (5′-AACCTATGACCCCTATGTAGACT-3′). The mutated fragment was amplified by PCR and cloned into the *Xho*I and

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*Bam*HI sites of the eukaryotic expression vector pcDNA3 (Invitrogen) to construct the PCV2 Cap *N*-glycosylation site mutant expression plasmid (pCap-m). The *N*-glycosylation mutant sequence was confirmed by DNA sequence analysis.

Transient transfection and protein expression

Porcine kidney-15 (PK-15) cells free of PCV were grown in Dulbecco's modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (HyClone). The cells (5×10^5) were plated in 6-well tissue culture plates 24 h before transfection. Each 6-well tissue culture plate of cells was transfected with 4 μg pCap or pCap-m plasmid using Lipofectamine 2000 (Invitrogen). The cells were cultured at 37 °C in a CO_2 incubator and harvested 48 h post-transfection in lysis buffer consisting of 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.02% NaN_3, 0.5% NP-40 and 1% Triton X-100 in phosphate-buffered saline (PBS), along with 1 μg/mL aprotinin and 100 μg/mL phenylmethylsulphonyl fluoride.

Western blot analysis

PK-15 cells were harvested 48 h post-transfection and the cell lysates were centrifuged at 10,000 *g* for 10 min to clear cellular debris. The supernatants were then inactivated at 56 °C for 1 h and aliquots of each protein were digested with 500 U peptide *N*-glycosidase F (PNGase F, New England Biolabs) for 1 h at 37 °C or mock digested as a negative control. The samples were then separated by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a nitrocellulose membrane. The nitrocellulose membrane was blocked overnight with 5% skim milk at 4 °C and then incubated overnight at 4 °C with a 1:100 dilution of a mouse anti-Cap PCV2-specific monoclonal antibody (isotype immunoglobulin G2a, IgG2a; Jeno Biotech) in PBS containing 0.1% Tween-20 (PBST). After three washes with PBST, the membrane was incubated with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H + L) antibody (Kirkegaard and Perry Laboratories) for 1 h. The membrane was then washed again with TBST and developed with an electrochemiluminescence reagent (Biouniquer).

Immunisation

Female BALB/C mice (7- to 8-weeks-old) were split into four groups of mice (six mice per group) and injected intramuscularly with 100 μg of each plasmid DNA in 100 μL PBS on days 0, 14 and 28. The four groups of mice were as follows: a control group (injected with only PBS), a vector group (injected with the pcDNA3 plasmid), a pCap group (injected with the pCap plasmid) and the mutant group (injected with pCap-m plasmid). After the first immunisation, serum samples were collected from the retroorbital sinus every 2 weeks until the end of the experiment. Ten weeks after the first immunisation (post-immunisation, p.i.), all mice from each group were euthanased and sera were collected for flow cytometry (FCM), cytokine measurements and lymphocyte proliferation assays.

Detection of anti-Cap antibodies and antibody isotypes

An indirect ELISA (Shang et al., 2008) was performed to detect the titres of total IgG, IgG1 and IgG2a against the PCV2 Cap protein. The Cap protein was expressed in *Escherichia coli* (Zhou et al., 2005a) and stored in 0.2 M carbonate buffer (pH 9.6). Ninety-six-well plates (Nunc) were coated with 0.1 μg PCV2 Cap protein and incubated at 4 °C overnight. After blocking with 1% bovine serum albumin (BSA) in PBS, 100 μL mouse sera (serially diluted two-fold; lowest dilution 1:64) were added and the plates were incubated at 37 °C for 2 h. The bound antibodies were detected by HRP-conjugated goat anti-mouse IgG, IgG1 or IgG2a antibodies. Tetramethylbenzidine (TMB, Sigma) was used as a chromogen for colour development and the absorbance was measured at 450 nm. Antibody titres were defined as the reciprocal of the highest dilution of the sample for which the absorbance was at least twice that of the control serum sample tested on the same plate. The data were presented as the $\log_2$ value of the titre.

Lymphocyte proliferation assays

Immunogenicity was assayed by testing in vitro proliferation of immunised splenocytes. Inoculated mice were sacrificed 10 weeks p.i., the spleen was placed in a cell strainer and the plunger end of a syringe was used to mash the spleen through the cell strainer into a Petri dish to recover splenocytes. After washing the splenocytes three times with RPMI 1640, the cells were incubated in 96-well flat-bottom microtitre plates (100 μL per well of 4×10^6 cells/mL in RPMI 1640 containing 5% fetal bovine serum) and cultures were stimulated with a final concentration of 1 μg/mL (100 μL per well) Cap protein. At least five replicated wells per sample were prepared. Control cultures without sample were also included. Cultures were incubated at 37 °C with 5% CO_2. The optical density at 570 nm (OD_{570}) was measured after 2 d using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based method (Zhou et al., 2005b). The stimulation index (SI) was calculated using the following formula: $SI = (OD_{vaccine} - OD_{blank})/(OD_{control} - OD_{blank})$.

ELISA-based cytokine measurements

Splenocytes were stimulated with a final concentration of 1 μg/mL (100 μL per well) Cap protein. After incubation for 48 h, splenocyte culture supernatants were collected for cytokine detection. Interleukin (IL)-4 and interferon (IFN)-γ were detected in supernatants from splenocytes cultured from vaccinated mice using commercially available mouse cytokine ELISA kits (Jingmei Corporation). In each assay, a control recombinant mouse cytokine was diluted over the recommended detection range to generate a standard curve. Sample concentrations were interpolated from the standard curve.

Flow cytometry assay

Splenocytes (1×10^6 cells) were cultured and stimulated as described above and then stained with 100 μL blocking buffer (PBS containing 1% BSA and 0.1% NaN_3) containing 1.25 μL phycoerythrin (PE)-conjugated anti-CD3, followed by 0.5 μL fluorescein isothiocyanate (FITC)-labelled anti-CD4 and 1.25 μL PE-Cy5-labelled anti-CD8 monoclonal antibodies and incubated for 30 min at 4 °C. FCM was performed on ⩾10,000 live cells and analysed by the FACScan flow cytometer (BD, LSR) using the Cell Quest software (BD).

Statistical analysis

One-way ANOVA and Student's *t* tests were used to compare results between different groups. All statistical analysis was performed using SPSS version 16.0. $P < 0.05$ was considered to be statistically significant.

Results

Expression of pCap and pCap-m by Western blot analysis

PK-15 cells were transfected with the pCap and pCap-m plasmids. Cells were harvested 48 h post-transfection and cell lysates were digested with *N*-glycosidase, which cleaves *N*-linked glycans. Western blot analysis using an anti-Cap PCV2 monoclonal antibody showed that digestion of the 30 kDa wild-type Cap protein with PNGase F yielded a product of approximate 28 kDa (Fig. 1). Both the mock- and PNGase F-digested Cap-m protein migrated at the same position as the PNGase F-digested wild-type Cap protein (Fig. 1A). The housekeeping gene β-actin had a constant expression level and was used as an internal control (Fig. 1B).

Anti-Cap response

The production of Cap-specific IgG antibodies by mice in each group was monitored by indirect ELISA after immunisation. Seroconversion occurred between weeks 2 and 4 p.i., and IgG was detected at the start of week 4 p.i (Fig. 2). Mice immunised with pCap exhibited slightly higher anti-Cap IgG titres. There were no significant differences in the ELISA antibody responses of mice between the pCap and pCap-m groups.

We next analysed the IgG1/IgG2a isotypes against PCV2 Cap protein to determine the profile of antibody responses and to deduce the primary type of immune response induced by different

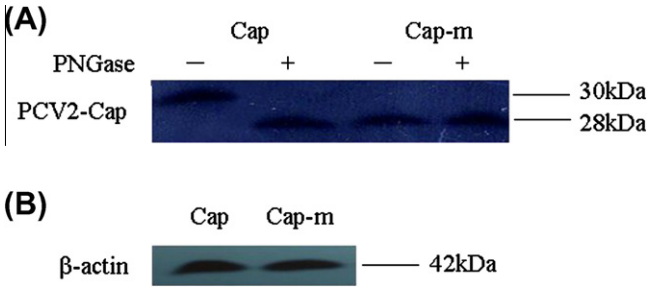

Fig. 1. Western blot analysis of the porcine circovirus type 2 (PCV2) wild-type Cap protein and the *N*-glycosylation mutant protein expressed in PK-15 cells. (A) Protein expressed in PK-15 cells was incubated with PNGase F (+) or buffer only (–). (B) β-actin was used as internal control.