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Generation and immunogenicity of porcine circovirus type 2 chimeric virus-like particles displaying porcine reproductive and respiratory syndrome virus GP5 epitope B

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ABSTRACT

Virus-like particles (VLPs) can be used as transfer vehicles carrying foreign proteins or antigen epitopes to produce chimeric VLPs for bivalent or multivalent vaccines. Based on the crystal structure of porcine circovirus type 2 (PCV2) capsid protein (Cap), in addition to alignment of the Cap sequences collected from various isolates of PCV2 and PCV1, we predicted that Loop CD of the PCV2 Cap should tolerate insertion of foreign epitopes, and furthermore that such an insertion could be presented on the surface of PCV2 VLPs. To validate this, the GP5 epitope B of porcine reproductive and respiratory syndrome virus (PRRSV) was inserted into Loop CD of the PCV2 Cap. The 3D structure of the recombinant PCV2 Cap (rCap) was simulated by homology modeling; it appeared that the GP5 epitope B was folded as a relatively independent unit, separated from the PCV2 Cap backbone. Furthermore, based on transmission electron microscopy, the purified PCV2 rCap self-assembled into chimeric VLPs which entered PK-15 cells. In addition, PCV2 chimeric VLPs induced strong humoral (neutralizing antibodies against PCV2 and PRRSV) and cellular immune responses in mice. We concluded that the identified insertion site in the PCV2 Cap had great potential to develop PCV2 VLPs-based bivalent or multivalent vaccines; furthermore, it would also facilitate development of a nano-device to present a functional peptide on the surface of the VLPs that could be used for therapeutic purposes.

1. Introduction

Virus-like particles (VLPs), composed of one to several viral structural protein(s) and absent in viral genomes, not only mimic viruses in overall structure and size, they also retain capacity to entry into cells [1]. Furthermore, that VLPs are readily recognized and processed by antigen presenting cells (APCs) [2,3] make them capable of inducing robust immune responses *in vivo* [4,5]. Collectively, these features make VLPs-based vaccines a viable research focus [6]. In addition, VLPs are also exploited to carry and display foreign antigenic epitopes on the surface, thereby producing so called chimeric VLPs, which are able to elicit strong immune responses against both the VLPs *per se* plus inserted foreign epitope(s) [7–9]. Moreover, VLPs have being used as gene-delivery vectors, providing new approaches for gene and immune therapies [10–12].

Porcine circovirus type 2 (PCV2) is the primary causative agent of porcine circovirus-associated diseases (PCVADs), causing severe economic losses in the swine industry worldwide [13]. The PCV2 virus, which belongs to a member of the genus *Circovirus* in the family *Circoviridae*, contains a circular single-stranded DNA genome approximately 1.7 kb long. The genome contains three major open reading frames (ORFs): ORF1, 2 and 3. Among them, ORF2 encodes the sole structural capsid protein (Cap) and is involved in capsid assembly, viral genome packaging, and cell infection [14–16]. The PCV2 Cap is capable of self-assembling into VLPs *in vitro* [17–21]. Furthermore, assembled VLPs have been successfully used as the main component of commercial vaccines against PCV2 infection [22,23]. In addition, PCV2 VLPs have been engineered as a vector to generate chimeric VLPs displaying foreign epitopes of other

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antigens. For example, *somatostatin* (SS) gene was fused at the C-terminus of the Cap to generate PCV2 chimeric VLPs. Antibodies against both the PCV2 Cap and SS were elicited in pig [24]. In another study, PCV2 chimeric VLPs were produced by replacing the nuclear localization signal region (NLS) amino acids (aa) 1–39 at the N-terminus of the PCV2 Cap respectively with a T-cell epitope (aa 1446–1460), a B-cell epitope (aa 693–716) and a T-cell epitope conjugating with a B-cell epitope of classical swine fever virus (CSFV) [25]. However, no neutralizing antibodies against CSFV were detected. Therefore, the N-terminus of the Cap without a NLS sequence was unsuitable for inserting foreign B-cell epitopes [25]. In addition, a foreign epitope fused at the N-terminus of the PCV2 Cap may hide inside the PCV2 VLPs after assembly [26]. Importantly, if an epitope or antigen gene is exposed on the exterior surface of VLPs, it could be presented to B lymphocytes *via* direct interaction with B cells receptor (BCR) [7]. Therefore, in order to generate an effective chimeric VLPs, it is crucial to identify one or more optimal sites on the exterior surface of the PCV2 VLPs for insertion of foreign epitope (s), which in turn relies on elucidating PCV2 capsid structure.

Crystal structure of the PCV2 Cap was resolved in 2011 [26]. Seven loops connecting eight β-strands in the Cap subunit constitutes a typical jelly-roll structure, previously identified in many other icosahedral viruses [27]. These loops might be responsible for VLPs assembly and determine the surface patterns of the PCV2 capsid, however the precise function of each loop remains to be elucidated.

In this study, PCV2 VLPs were used as a foreign antigen carrier for efficiently presenting the GP5 epitope B (^{37}SHIQLIYNL^{45}) [28–30] of PRRSV on the exterior surface of PCV2 VLPs. This epitope was successfully inserted into one of loops (Loop CD) of the PCV2 Cap; a recombinant Cap (rCap) was subsequently expressed in bacteria and purified. The PCV2 chimeric VLPs, assembled from the purified rCap, were readily visualized with transmission electron microscopy, retained their ability to enter cells, and when injected into mice, induced a immune response to both PCV2 and PRRSV that was comparable a commercial inactivated bivalent vaccine. Hence, these chimeric VLPs had great potential as a novel vaccine for protection against both PRRSV and PCV2.

2. Materials and methods

2.1. Viruses, cell lines and vector constructs

The PRRSV 08HuN strain (GenBank accession no. GU169411, isolated in our laboratory) was propagated and titrated in MARC-145 cells as described previously [31]. The PCV2 strain (GenBank accession no. KP112484, isolated in our laboratory) [32] was propagated on PK-15 cells as reported previously [25]. PRRSV GP5 epitope B (^{37}SHIQLIYNL^{45}) peptide was synthesized commercially (GenScript, Piscataway, NJ, USA), whereas the PCV2 *cap* gene (GenBank accession no. JF504708) was synthesized and deposited in our laboratory. The PCV2 *cap* gene was cloned into pET100 (Invitrogen, Carlsbad, CA, USA) to generate the recombinant plasmid (namely pET-PCV2-Cap) using for the production of PCV2 Cap. A recombinant DNA fragment containing the PCV2 *cap* and the PRRSV GP5 epitope B in Loop CD of the *cap* was constructed by overlap polymerase chain reaction (PCR). The first pair of primers (F1-*Nde*I: CGC***catatg***CGG GGTTCTCATCATCATC; R1: GCCCGACGTTAAGC-TATAAATCAACTGAATATGAGAG CCACCGCCACCCGGTGG) was used to generate the first fragment, whereas the second pair (F2: GGCTCTCATATTCAGTTGATTTATAGCTTAACGTCGGGCAGCAACC-CGCGTTCTG; R2-*Bam*HI: CGC***ggatcc***TCACGGGTTCAGCGGTGG) was used for the second fragment. These PCR products were mixed equally to generate the fusion gene PCV2-Cap-GP5B by PCR, using F1 and R2 primers. The resulting overlap PCR products were purified (DNA gel extraction kit, Omega Bio-tek, Norcross, GA, USA) and subsequently cloned into a protein expression vector pET100. The recombinant plasmid (namely pET-PCV2-Cap-GP5B), was confirmed by DNA sequencing.

2.2. Amino acid sequence alignment, 3D structure displaying and simulation

Amino acid sequences of the Caps from isolates of PCV1 and PCV2 were collected from GenBank (http://www.ncbi.nlm.nih.gov/). Sequences from 3 PCV1 Caps (GenBank accessions no. AF071879, KC447455 and JN133302) and 14 PCV2 Caps (GenBank accessions no. AB072302, EU148506, AY754017, EU886638, AY180396, AY146991, AB426905, AF201309, AY146993, AY256459, AF201308, AF201310, AY256455 and JF504708) were aligned using web server UniProt (http://www.uniprot.org/). The 3D structure of the PCV2 rCap was simulated by protein homology modeling, with a template consisting of the PCV2 Cap (PDB accession no. 3R0R) [26]. Modeling software (http://www.salilab.org/modeller/) and web server of protein homology modeling (http://swissmodel.expasy.org/) were exploited for protein simulation, whereas PyMoL (version 1.7.4.4; http://www.pymol.org/) was used to display 3D protein structures and surfaces.

2.3. Protein expression and purification

The recombinant plasmid (pET-PCV2-Cap-GP5B) was transformed into BL21 competent cells (Trans Gen, Beijing, China). The PCV2 rCap was expressed as described previously [18]. Cells were harvested by centrifugation (5000 × *g* for 20 min at 4 °C), re-suspended with buffer A (0.1 M $NaH_2PO_4{\cdot}2H_2O$, 0.1 M Na_2HPO_4, 20 mM imidazole, 10 mM Tris-base, 300 mM NaCl, 50 mM KCl, 2 mM $MgCl_2$, 0.1 M ammonium citrate and 5% glycerol, pH 8.0) with 0.5% Triton X-100, 5 mM β-mecapitalethanol and protease inhibitors (0.1 mM PMSF and 1 U/ml Leupeptin), and then immediately ruptured by sonication and cell lysates cleared by centrifugation (18,000 × *g* for 30 min at 4 °C). Supernatants were filtered through a 0.22 μm membrane, then loaded into the packed His-Trap column (GE-Healthcare Life Sciences, New York, USA). Targeted proteins (rCap) were purified by an automated fast protein liquid chromatography (FPLC) system ÄKTA Purifier Plus 10 (GE-Healthcare Life Sciences). Non-specific proteins were purged from the column with 10 column volumes (CV) of buffer B (50 mM $NaH_2PO_4{\cdot}2H_2O$, 20 mM imidazole and 500 mM NaCl, pH 8.0) before elution. Thereafter, 10 CV of buffer C (300 mM imidazole and 300 mM NaCl, pH 6.0) were applied to completely elute the targeted protein. The eluted rCap was detected by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. For blotting, the rCap was detected with a primary antibody anti-6 × His mouse monoclonal antibody (Roche, Basel, Switzerland) and a second antibody alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Promega, Madison, WI, USA), applied sequentially. After incubation with antibody and washing with phosphate-buffered saline (PBS, pH 7.2) blotting signals on the membrane were developed in a buffer of western blue stabilized substrate for AP (Promega).

2.4. Preparation and characterization of chimeric VLPs

The purified rCap was dialyzed against 1 l of buffer A for 48 h at 4 °C (buffer changed three times). Dialysis product was further subjected to size exclusion chromatography using a Sephacryl S-300 16/26 prepacked column (GE-Healthcare). Following negative staining of the protein sample, formation of chimeric VLPs was confirmed with transmission electron microscopy (CM100, Philips