Immunogenicity of porcine circovirus type 2 capsid protein targeting to different subcellular compartments

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Abstract

Porcine circovirus type 2 (PCV2) is known to be associated with post-weaning multisystemic wasting syndrome (PMWS), an emerging disease in swine. The development of effective vaccines against PCV2 infection has been accepted as an important strategy in the prophylaxis of PMWS, and a DNA vaccine expressing the major immunogenic capsid (Cap) protein of PCV2 is considered to be a promising candidate. However, recent studies have revealed that interferons (IFNs), especially IFN-\(\gamma\), can enhance the replication of PCV2, indicating that the high levels of IFN-\(\gamma\) induced by DNA vaccination seem to have potential deleterious effect on protective immunity. Strategies to improve the neutralizing antibody response and simultaneously decrease the IFN-\(\gamma\) response will facilitate the clinical application of DNA vaccines against PCV2. In the present study, four different DNA vaccine constructs encoding cytoplasmic (Cy-ORF2), secreted (Sc-ORF2), membrane-anchored (M-ORF2) or authentic nuclear-targeted (pc-ORF2) Cap protein were generated to evaluate the neutralizing antibody and IFN-\(\gamma\) responses in a mouse model. Although all four DNA constructs could elicit PCV2-specific humoral immune responses, mice inoculated with Sc-ORF2 developed a significantly higher level of neutralizing antibodies than those that received M-ORF2, pc-ORF2 or Cy-ORF2. Furthermore, mice immunized with Sc-ORF2 or M-ORF2 showed a significantly decreased or enhanced IFN-\(\gamma\) level, respectively, compared with those inoculated with pc-ORF2. With respect to neutralizing antibody and IFN-\(\gamma\) levels, Sc-ORF2 is a good candidate for DNA vaccination, and the secreted Cap protein appears to be an ideal antigen for use in development of vaccines against PCV2.

Keywords: Porcine circovirus type 2 (PCV2); Capsid protein; Cellular localization; DNA immunization; Neutralizing antibody; IFN-\(\gamma\)

1. Introduction

Porcine circovirus (PCV) is classified in the genus Circovirus of the Circoviridae family (Todd et al., 2000). Two genotypes of PCV, PCV type 1 (PCV1) and PCV type 2 (PCV2), have been identified. PCV1 was originally identified as a contaminant of porcine kidney cell line PK-15 and is considered non-pathogenic for swine (Tischer et al., 1982), whereas PCV2 has been isolated from pigs affected by post-weaning multisystemic wasting syndrome (PMWS), an emerging disease of swine (Ellis et al., 1998). Characterized clinically by fever, progressive weight loss, dyspnea, tachypnea, anemia, diarrhea, and jaundice in pigs 5–12 weeks old (Harding, 1997), PMWS is currently endemic in many swine-producing countries, and is associated with a serious economic impact on the global swine industry. Accumulated evidence indicates that PCV2 is the primary, but not the sole, causative agent of PMWS (Harms et al., 2001; Fenaux et al., 2000, 2002; Opriessnig et al., 2003, 2004; Roca et al., 2004).

PCV2 is a small non-enveloped single-stranded circular DNA virus with a 1.76-kb ambisense genome (Mankertz et al., 2000). The genome contains at least two major open reading frames (ORFs). ORF1 encodes the replicase proteins (Rep and Rep’), involved in rolling circle PCV2 DNA replication, and ORF2 encodes the capsid protein (Cap protein) (Nawagitgul et al., 2000; Cheung and Bolin, 2002). Because neutralizing monoclonal antibodies to PCV2 react with the Cap protein (McNeilly et al., 1998).
et al., 2001) and neutralizing sera from pigs have also been shown to recognize this protein (Pogranichnyy et al., 2000). Cap protein is a leading target for design of new vaccines against PCV2 infection.

DNA vaccination is one of significant advances in vaccinology in recent years. Many studies have demonstrated the protective immunity induced by DNA immunization against various viral diseases in animal models. This vaccine strategy has also been tested on PCV2, and is considered an attractive method for vaccination against PCV2 infection (Kamstrup et al., 2004). However, in one study DNA-vaccinated piglets showed intense pyrexia and transiently enhanced PCV2 replication during the early period after challenge, although their growth was delayed to a lesser degree than that of non-vaccinated piglets (Blanchard et al., 2003). Recently, Meerts et al. (2005) found that IFN-γ added to the culture medium before, during, or after inoculation increased the replication of PCV2 in PK-15 cells and porcine monocytes (3D4/31). These observations indicate that high levels of IFN seem to have a deleterious effect on protective immunity. Additionally, lower or even no detectable neutralizing antibody can be induced by some experimental vaccines expressing native Cap protein (Blanchard et al., 2003), although Cap protein is the dominant immunogenic protein of PCV2 and is associated with neutralizing antibody production. The aim of this study was to use a rational molecular design to enhance neutralizing antibody and simultaneously decrease the IFN-γ response to a DNA vaccine encoding Cap protein. We constructed four different expression plasmids encoding Cap protein with different subcellular localizations: cytoplasmic (Cy-ORF2), secreted (Sc-ORF2), membrane-anchored (M-ORF2) and nuclear (pc-ORF2). Using a mouse model, the immunogenicity of the modified Cap proteins on DNA immunization was characterized. Our data clearly showed that DNA vaccine Sc-ORF2 generated higher PCV2-specific neutralizing antibody and a relatively lower IFN-γ response than did DNA viruses M-ORF2 and pc-ORF2, indicating that the Sc-ORF2 is a better candidate for DNA vaccination and the secreted Cap protein should be an ideal antigen for general utility in development of vaccines against PCV2.

2. Materials and methods

2.1. Cells and viruses

A porcine kidney PK-15 cell line free of PCV1 contamination was maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 IU/ml penicillin. The PCV2 strain Yu-A used in this study was originally isolated from a pig with naturally occurring PMWS. Virus was propagated in PK-15 cells as previously described (Nawagitgul et al., 2000).

2.2. Construction and purification of plasmids

pcDNA3.1(+) (Invitrogen) was used as a vector backbone to construct DNA vaccines expressing four different Cap proteins. The genomic DNA of PCV2 strain Yu-A was used as a template in a high-fidelity PCR to amplify the full length or truncated PCV2 ORF2 gene. To generate pc-ORF2, a 705 bp DNA fragment encoding the full length Cap protein was amplified by PCR using the primer pair ORF2s/ORF2r (Table 1). The PCR product was digested with HindIII/SalI and cloned into pcDNA3.1(+). For the construction of Sc-ORF2, the amplified ORF2Δ41 fragment was digested and subcloned into pcDNA3.1(+), encoding the truncated Cap protein (without the 41 N-terminal amino acids) using the primer pair ORF2Δ41s/ORF2Δ41r (Table 1), and the digested PCR product was inserted into the corresponding sites of pcDNA3.1(+). For the construction of M-ORF2, the amplified ORF2Δ41 fragment was digested and subcloned into pcDNA3.1(+)tPA, a modified pcDNA3.1(+) containing the tissue plasminogen activator (tPA) leader sequence (Tong et al., 2006). To construct M-ORF2, the ORF2Δ41 fragment without a stop codon was amplified using primer pair ORF2Δ41s2/ORF2Δ41r2 and subcloned into pcDNA3.1(+)tPA, resulting in pcDNA3.1(+)tPA-ORF2Δ41s. A 216 bp DNA fragment containing the transmembrane anchor domains (TM) of influenza virus hemagglutinin (HA) was amplified by PCR from the plasmid pT-HA with primer pair HA1/HA2 designed from the appropriate GenBank sequence (GenBank accession: AF461521). After digestion, this PCR product was directly cloned into pcDNA3.1(+)tPA-ORF2Δ41s, immediately downstream of the PCV2 ORF2 gene. Schematic diagrams of these recombinant plasmids are shown in Fig. 1. All plasmids were verified by appropriate restriction enzyme digestion and sequencing.

2.3. In vitro transfection, indirect immunofluorescence assay and Western blot

PK-15 cells were seeded at a concentration of 2.5 × 10^5 cells/well into six-well tissue culture plates (Nunc) until the cells reached approximately 70–80% confluence. Transfection was performed with LipofectAMINE 2000 reagent (Invitrogen) as specified by the manufacturer. At 48 h post-transfection, the cells were fixed with absolute methanol and processed for indirect immunofluorescence assay using porcine anti-
PCV2 polyclonal antibodies (1:200)(VMRD, USA), followed by FITC-conjugated rabbit anti-porcine IgG (1:2000) (Sigma). Fluorescent images were examined under an inverted fluorescence microscope (Olympus IX70). For Western blot analysis, the supernatants or cells were collected at 48 h post-transfection and then stained using the immunoperoxidase assay. The neutralization titer was calculated as the reciprocal of the highest serum dilution that was able to completely block PCV2-infection in PK-15 cells.

2.7. ELISPOT detection of IFN-γ producing cells

Ninety-six-well PVDF (polyvinylidene difluoride)-backed microplates pre-coated with a monoclonal antibody specific for mouse IFN-γ (R&D, America) were washed and incubated for approximately 20 min at room temperature with sterile RPMI 1640. The culture medium was removed and replaced with 200 μl of splenocyte suspension (1 × 10⁶/ml), with or without 20 μg/ml recombinant Cap protein, in triplicate to each well for 48 h incubation at 37°C. After culture, the plates were washed and then incubated with 100 μl of 1:120 diluted biotinylated mouse IFN-γ antibody (R&D, America) at 2–8°C overnight. After washing three times, 100 μl of 1:100 diluted Streptavidin-AP was added to each well and incubated for 2 h at room temperature. After washing, spots were developed by adding 100 μl of BCIP/NBT Chromogen to each well and incubating for 1 h at room temperature. After discarding the Chromogen solution and rinsing the microplate with deionized water, plates were allowed to air dry. The spots were evaluated using an automated ELISPOT reader (AID ELR02) coupled with AID ELISPOT 2.6 software. Results were expressed as the number of spots per million splenocytes and represent the average of the triplicate values.

2.8. Real-time PCR analysis of mIFN-γ mRNA expression

Splenocytes (1 × 10⁶/ml) were cultured in 24-well plates for 20 h at 37°C in the presence of 5% CO₂, with or without 20 μg/ml recombinant Cap protein. Total RNA was extracted and one microgram of RNA was reverse transcribed in a 20 μl reaction mixture. The cDNA product (0.5 μl) was amplified in a 25 μl reaction mixture containing SYBR® Green Realtime PCR Master Mix (Toyobo), 0.2 μM each of the forward and reverse gene-specific primers (IFN-γ or β-actin, Table 1). Each cDNA sample was performed in triplicate. PCR amplifications were performed using an Applied Biosystems 7500 Real-Time PCR System (ABI). Thermal cycling conditions were 2 min at 50°C, 10 min at 94°C, and 40 cycles of 15 s at 94°C and 1 min
at 60°C. Gene expression was measured by relative quantity, which compares the threshold cycle (Ct) of the sample of interest to the Ct generated by a reference sample referred to as the calibrator (non-stimulated splenocytes incubated for the same time period as stimulated splenocytes). IFN-γ gene expression was normalized to β-actin expression by subtraction of Ct to provide ΔCt values. The ΔΔCt was calculated as the difference between ΔCt values for Cap-stimulated and non-stimulated splenocytes from the test animal. The relative difference in IFN-γ expression between stimulated and unstimulated cells was determined using the equation 2−ΔΔCt according to the User Bulletin number 2, ABI prism 7500 Sequence Detection System (Applied Biosystems) instructions. This equation is valid only if the amplification efficiencies of β-actin and mIFN-γ are approximately equal.

2.9. Statistical analysis

Student’s t-test was used to compare the humoral and cellular immune responses between the different groups. P-values of <0.05 were considered statistically significant.

3. Results

3.1. Plasmid design and localization analysis

Expression of the PCV2 Cap protein in different cellular compartments was achieved by expressing various forms of the Cap protein (Fig. 1). pc-ORF2 contained a full length ORF2 gene, resulting in the authentic nuclear-targeted Cap protein. Because the nuclear localization signal was deleted, Cy-ORF2 was designed to express a cytoplasmic localized form of Cap protein. To achieve secretion of the expressed protein, Sc-ORF2 was constructed by linking a human tPA leader sequence, a strong signal peptide for protein secretion (Tong et al., 2006), immediately upstream of the truncated ORF2 gene as a secretion signal. M-ORF2 contained not only the same secretion signal, but in addition also the fragment encoding the transmembrane domain HA.

To determine whether the different DNA vaccine constructs could express the expected, differently targeted Cap proteins in cell culture, 70–80% confluent PK-15 cells were transfected with pc-ORF2, Cy-ORF2, Sc-ORF2, M-ORF2, or the empty vector pcDNA3.1(+), respectively. Cap protein-specific ELISA antibody was determined using the purified Cap protein as antigen. As shown in Fig. 3a, at 4 weeks after primary immunization, the antibody titer reached a detectable level in all the vaccinated groups except for the group immunized with the empty vector, and a further increase in antibody levels was observed at 6 weeks after primary immunization. Throughout this experiment, mice immunized with Sc-ORF2 produced significantly higher ELISA antibody titers compared with mice vaccinated with pc-ORF2, Cy-ORF2, or M-ORF2. This difference was statistically significant both at 4 weeks (P<0.05) and 6 weeks (P<0.01) after primary immunization. There was no significant difference (P>0.05) between the pc-ORF2 group and M-ORF2 group; however, the ELISA antibody titers in the groups given pc-ORF2 and M-ORF2 were significantly higher than in the group immunized with Cy-ORF2.

Serum samples were further evaluated for the ability to neutralize PCV2 in vitro using serum neutralization assays. As shown in Fig. 3b, mice immunized with Sc-ORF2 or M-ORF2 developed significantly higher PCV2-specific neutralizing antibody titers than mice that received pc-ORF2 or Cy-ORF2 (P<0.01). Although the titer was slightly lower in mice immunized with M-ORF2 compared with mice immunized with Sc-ORF2, the difference was not statistically significant (P>0.05) at either 4 or 6 weeks after primary immunization. In addition, mice immunized with pc-ORF2 showed higher neutralizing antibody titers than those given Cy-ORF2, but the difference was not significant.

3.3. IFN-γ responses induced in mice immunized with different DNA constructs

Antigen-specific IFN-γ secretion from stimulated splenocytes was determined by the frequency of IFN-γ-producing cells in an IFN-γ-specific ELISPOT. As shown in Fig. 4, the highest frequency of IFN-γ-producing cells was detected in M-ORF2-injected mice (geometric mean value, 315 spot-forming cells (SFC)/10^6 splenocytes). The frequency of IFN-γ-producing cells in this group was significantly higher than in the other groups (P<0.01). Relatively lower values could be detected in mice inoculated with Sc-ORF2 (104 SFC/10^6 splenocytes), Cy-ORF2 (69 SFC/10^6 splenocytes) or pc-ORF2 (175 SFC/10^6 splenocytes).

Quantitative real-time RT-PCR was also performed to analyze the level of IFN-γ mRNA expression in Cap protein-restimulated splenocytes. Similar to the ELISPOT assay, the highest IFN-
γ mRNA expression was found in restimulated splenocytes from mice immunized with M-ORF2. The IFN-γ expression in this group was significantly higher than that of groups immunized with Sc-ORF2, Cy-ORF2 or pc-ORF2 ($P < 0.01$). The mean IFN-γ expression increased 13-fold, 5.1-fold, 3.7-fold and 3.25-fold in the groups immunized with M-ORF2, pc-ORF2, Sc-ORF2 or Cy-ORF2, respectively. The IFN-γ expression in mice immunized with pc-ORF2 encoding native Cap protein was also significantly higher than that in mice injected with Sc-ORF2 and Cy-ORF2 ($P < 0.05$). The differences observed between Sc-ORF2 and Cy-ORF2 injected mice were not statistically significant (Fig. 5).
Fig. 3. Antibody responses in mice immunized with different DNA constructs encoding Cap protein with different subcellular localization. 6–8-week-old BALB/c mice (eight per group) were intramuscularly vaccinated with 100 μg plasmid DNA at weeks 0 and 2. Serum was obtained on weeks 4 and 6 to determine the antibody level. (a) The Cap protein-specific ELISA antibody. *P < 0.01 compared with other four groups. (b) PCV2-specific neutralizing antibody titers. *P < 0.01 compared with pc-ORF2-, Cy-ORF2- or control vector-immunized groups. Data represent the mean ± S.D. for eight mice.

4. Discussion

PMWS has become a major disease currently faced by the global swine industry (Ellis et al., 1998; Morozov et al., 1998). Although other co-factors have been reported to contribute to this disease, there is no doubt that the expression of the clinical disease is dependent on the presence of PCV2 (Blanchard et al., 2003). Despite application of preventive measures in housing and herd management, high PCV2 seroprevalence and PMWS are found in many herds. Thus, development of an effective vaccine against PCV2 infection has been accepted as a strategy for the prophylaxis of PMWS (Fenaux et al., 2004). Because the yield of PCV2 in cell culture is low, and it requires treatment with α-glucosamine (Allan and Ellis, 2000), it is difficult to develop a traditional inactivated vaccine against PCV2. There is currently a trend towards the development of gene-engineered vaccines based on the major immunogenic Cap proteins. Such approaches include DNA vaccine (Blanchard et al., 2003; Kamstrup et al., 2004), recombinant vaccines of PCV1-PCV2 (Fenaux et al., 2004), recombinant adenovirus (Wang et al., 2006), recombinant baculovirus (Blanchard et al., 2003), and recombinant pseudorabies vaccines (Song et al., 2007). Although these experimental vaccines expressing native Cap protein have been demonstrated to induce protective immunity to some degree, their efficacy is unsatisfactory. Alternative strategies should be utilized to improve the protective efficacy of these experimental vaccines.

Fig. 4. Determination of antigen-specific induction of the frequency of IFN-γ producing splenocytes in ELISPOT assays. Mice were immunized as in Fig. 3. Splenocytes were isolated 6 weeks after primary immunization and stimulated with purified Cap protein. Cell suspension (2 × 10⁵ cells/well) was transferred on to ELISPOT plates for 48 h incubation. The number of spots per well was calculated using an automated ELISPOT reader. Results were expressed as the number of spots per million splenocytes and represent the mean ± standard error. *P < 0.01 compared with Cy-ORF2-, Sc-ORF2, or control vector-immunized groups.

Fig. 5. IFN-γ relative gene expression in splenocytes harvested from immunized mice after in vitro stimulation with purified Cap proteins. Splenocytes were isolated 6 weeks after primary immunization and were incubated with or without purified Cap proteins for 20 h. RNA was extracted and subjected to quantitative RT-PCR. Relative quantity of cytokine mRNA was determined by relative quantitative RT-PCR using β-actin gene expression as housekeeping gene. Mean relative quantity of IFN-γ mRNA ± standard error is shown. *P < 0.01 compared with other four groups.
In the present work, two factors were considered to optimize the DNA vaccine expressing the Cap protein. Firstly, previous studies have revealed that IFN-γ seems to enhance the replication of PCV2 (Meerts et al., 2005), suggesting a possible adverse effect on immune protection if a high level of IFN-γ is induced by DNA vaccination. It is therefore necessary to decrease or eliminate the IFN-γ responses of vaccinated animals. Secondly, previous studies have demonstrated that the expressed Cap protein alone is capable of forming virus-like particles (VLPs) (Nawagitgul et al., 2000). Theoretically, VLPs have better immunogenicity and this has emerged as an effective strategy to develop subunit vaccines against virus infections. Indeed, the poor immunogenicity of the native Cap protein was observed in previous reports (Blanchard et al., 2003; Kamstrup et al., 2004) and in this study. The particular factor that affects the immunogenicity of the Cap protein is not known. Additionally, the PCV2 Cap protein is a nuclear protein directed by the N-terminal domain (amino acids 1–41) (Liu et al., 2001). A large number of studies have demonstrated that directing a transgenic antigen to different subcellular compartments can affect the processing and presentation of an antigen, which, in turn, can alter the character of the immune response to the antigen (for example, Higgins et al., 2000). Whether the nuclear-targeted PCV Cap protein affects antigen presentation to the host immune system and consequently influences the elicited immune response is not clear. Based on the above considerations, we have investigated the immunogenicity of Cap protein targeted to different subcellular locations. Our results have clearly shown that targeting the PCV2 Cap protein to different subcellular compartments does indeed influence both humoral and cellular immune responses.

Meerts et al. (2006) have demonstrated that the absence of PCV2-neutralizing antibodies is correlated with high PCV2-replication and with PCV2-related disease (PMWS), indicating that it would be worthwhile to improve the level of PCV2-neutralizing antibodies induced by the new generation of vaccines against PCV2. In the present study, mice immunized with Sc-ORF2 or M-ORF2 developed significantly higher PCV2-specific neutralizing antibody titers than those inoculated with pc-ORF2 encoding native Cap protein. Furthermore, this difference was more pronounced with Cy-ORF2. The DNA constructs Sc-ORF2 and M-ORF2 encode secreted and membrane-anchored Cap protein, respectively, whereas Cy-ORF2 and pc-ORF2 encode cytoplasm- and nuclear-targeted Cap protein, respectively. Based on the neutralizing antibody titers induced by Cap proteins with different cellular localization, we conclude that intracellular Cap protein has poor immunogenicity. A possible explanation for this is that the membrane-anchored as well as the secreted antigens are produced and processed intracellularly and then presented on or secreted from the cell surface, resulting in easy recognition by antigen-presenting cells (APCs), which subsequently leads to efficient antigen processing and presentation of the expressed antigens.

We also evaluated the PCV2-specific cellular immune responses after restimulation in vitro with recombinant Cap protein. Enhanced cell-mediated immune responses were observed in mice immunized with M-ORF2, compared with pc-ORF2 encoding native Cap protein. Relatively lower levels of IFN-γ responses were observed in mice immunized with Sc-ORF2 and Cy-ORF2. Considering that a high level of IFN-γ seems to have an adverse effect on protective immunity, the secreted Cap protein should be a better antigen for vaccine development than M-ORF2 and pc-ORF2, because immunization with Sc-ORF2 induced lower IFN-γ responses, as well as higher neutralizing antibody.

Based on the data from the ELISA and serum neutralization assays, it is noteworthy that there was only poor correlation between ELISA antibody titers and neutralizing antibody titers. The ELISA antibody titers obtained from mice immunized with pc-ORF2 or M-ORF2 were similar, while significantly higher PCV-2-specific neutralizing antibodies were observed in mice immunized with M-ORF2 than those given pc-ORF2. Likewise, there were significant differences in ELISA antibody titers between mice immunized with pc-ORF2 and Cy-ORF2, but no significant difference in neutralizing antibody titers between the two groups. The antigen used for ELISA detection was Cap protein lacking the 20 N-terminal amino acid residues, whereas the 41 N-terminal amino acid residues of Cap protein were deleted in the DNA constructs Cy-ORF2 and M-ORF2. On the basis of this observation, we speculate that there is a non-neutralizing immunodominant epitope in the 20–41 amino acid region of Cap protein. Efforts to confirm this immunodominant epitope will be beneficial to vaccine development and clinical diagnosis.

In summary, our data have demonstrated that the immunogenicity of the PCV2 Cap protein can be significantly enhanced by a molecular design that alters the cellular localization of the expressed transgenic antigen. DNA construct Sc-ORF2 appears to be the best candidate for DNA vaccination, and the secreted Cap protein should be an ideal antigen for use in development of vaccines against PCV2. Our present data only demonstrate the potential of Sc-ORF2 in a small animal model. It will be necessary to further study the potential of this vaccine in the natural host system before comprehensive evaluation and practical application can be suggested.

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