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Short communication

The GPRLQPY motif located at the carboxy-terminal of the spike protein induces antibodies that neutralize *Porcine epidemic diarrhea virus*

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Abstract

The spike protein of *Porcine epidemic diarrhea virus* is the main surface glycoprotein involved in virus attachment and entry and therefore is the target of neutralizing antibodies. Here, the immunogenicity of a novel antigenic domain found on the carboxy-terminal of the spike protein characterized by the peptide motif GPRLQPY, was evaluated. A synthetic peptide whose linear sequence is identical to the 24 a.a. carboxy-terminal portion of the spike protein (S-CT24) elicited a strong antibody response in BALB/c mice that had specific reactivity against the S-CT24 and PEDV. These antibodies were shown to have a specific affinity to the GPRLQPY motif, as demonstrated by non-reactivity with a peptide that lacks this motif. In addition, antiS-CT24 antibodies exhibited neutralizing activities against KPEDV-9 in focus reduction neutralization tests suggesting that the GPRLQPY motif induces neutralizing antibodies against PEDV.

Keywords: *Porcine epidemic diarrhea virus*; Spike protein; Neutralizing epitope; Synthetic peptide; Focus reduction neutralization test

Porcine epidemic diarrhea virus (PEDV) is a swine coronavirus that attacks the gastrointestinal tract and cause the destruction of villous enterocytes and villous atrophy within the jejunum and ileum, resulting in induced watery diarrhea (Ducatelle et al., 1981). This virus is pathologically similar to another swine coronavirus, *Transmissible gastroenteritis virus* (TGEV), making it difficult to differentiate the two based on clinical symptoms alone (Pritchard et al., 1999).

PEDV was first discovered in 1971 and rapidly spread across Europe and parts of Asia. Although the incidence of PED outbreaks in Europe has diminished significantly by 1990, it remains as one of the most important viral pathogens in swine among Asian countries (Pensaert, 1999). In Korea, seasonal outbreaks resulting in high mortality of neonatal piglets as well as weight loss in fattening pigs cause serious economic losses in the swine industry (Chae et al., 2000).

Due to the economic importance of PEDV, several cell-adapted strains of the virus have been developed as candidate live attenuated vaccines (Kweon et al., 1999; Song et al., 2003; Park et al., 2007). Development of other vaccine candidates have focused on the CO-26K fragment equivalent (COE), a domain found on the spike protein that is reported to contain the neutralizing epitope against PEDV (Chang et al., 2002; Kang et al., 2006). Recently, another B-cell epitope was reported to induce neutralizing antibodies against PEDV (Cruz et al., 2006). This epitope, characterized by the GPRLQPY motif, was mapped to the carboxy-terminal region of the spike protein using phage–peptide library.

The location of this antigenic motif on the cytoplasmic tail of the spike protein is of great interest since it has important implications on the possible involvement of this domain in virus attachment and entry. In this study, the antigenicity and neutralizing activity of antibodies against the GPRLQPY motif was investigated. Antibodies were generated by immunizing BALB/c mice with a peptide having a linear sequence identical to the 24 a.a. carboxy-terminal region of the PEDV spike protein then characterized by ELISA and focus reduction neutralization test. The properties of these antibodies were compared with those of polyclonal antisera and 2C10 monoclonal antibodies to PEDV.

Cell-adapted strain of PEDV (KPEDV-9) was propagated in African green monkey kidney cells (Vero, CCL-81) following

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the procedure described by Hofmann and Wyler (1988). Briefly, 175 cm^2 TC flasks (Nunc) containing a confluent monolayer of Vero cells were inoculated with KPEDV-9 and cultured in minimal essential medium (MEM, Gibco Life Science) containing 10 $\mu g\,ml^{-1}$ trypsin at 37 °C for 24 h. Infected cells were harvested and placed in 100 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA buffer (STE buffer, pH 7.4). Progeny virions trapped in intracellular vesicles were released by repeated freezing/thawing and harvested in the supernatant by centrifugation at 10,000 × *g* for 10 m. Harvested virus was titrated by focus formation assay as described previously (Cruz and Shin, 2007). Briefly, two-fold dilutions of the virus stock were inoculated to Vero cells grown to confluence in 96-well TC plate (Nunc). After adsorption at 37 °C for 2 h, the inoculum was removed and the monolayer was overlaid with MEM containing 0.5% methyl cellulose and 10 $\mu g\,ml^{-1}$ trypsin then kept at 37 °C, 5% CO_2 for 12 h. After fixing with 5% formaldehyde and permeating with 1% Nonidet P-40, the cell monolayer was probed with mouse anti-PEDV polyclonal antisera followed by biotin-conjugated anti-mouse IgG (Vector Laboratories). Foci of virus-infected cells were visualized by addition of avidin-biotinylated horseradish peroxidase (HRP, Vector Laboratories) and 3,3′-diaminobenzidine tetrahydrochloride dihydrate (DAB, Vector Laboratories) in the presence of NiCl and H_2O_2. The number of foci, indicated by clusters of dark gray-stained cells were observed and counted under an inverted light microscope (Zeiss).

Synthetic peptides S-CT24 and S-CT17 were designed and purchased from Anygen, Korea. These peptides were based on the putative neutralizing epitope identified previously using a seven-mer phage–peptide library (Cruz et al., 2006). The S-CT24 peptide (ACFSGCCR*GPRLQPY*EAFQKVHVQ) and S-CT17 peptide (ACFSGCCREAFQKVHVQ) were reconstituted in 10 mM phosphate-buffered saline (PBS, pH 7.4) and kept at −20 °C until use.

Groups of six 6-weeks-old BALB/c mice (Samtako, Korea) were immunized intraperitoneally with 100 μg S-CT24 peptide, S-CT17 peptide or 1×10^5 ffu KPEDV-9 whole virus. The antigens were mixed with Freund's complete adjuvant (Sigma–Aldrich) for the initial immunization, then with Freund's incomplete adjuvant (Sigma–Aldrich) on succeeding immunizations administered every 2 weeks for 6 weeks. Blood samples were collected from the retro-orbital sinus prior to immunization and 1 week after final immunization b. Mice sera were extracted by centrifugation at 1500 × *g*, 10 m and kept at −20 °C prior to use. Euthanasia was performed by cervical dislocation.

Antibody responses of BALB/c mice against the S-CT24, S-CT17 peptides and whole virus antigen were evaluated by ELISA following the procedure described by Bundo-Morita et al. (1986) for Japanese encephalitis virus and dengue virus, with some modifications. The peptide antigens (S-CT24 and S-CT17) were used as coating antigens to evaluate the antigenicity of the GPRLQPY motif in mice while whole virus antigen was used to compare the reactivity of the antibodies elicited by the peptide antigens and KPEDV-9. Five-hundred nanograms of the peptide antigens or 100 ng of whole virus antigen in 0.1 M sodium bicarbonate buffer (pH 9.6) were coated onto a 96-well Maxisorp immunosorbent plate (Nunc) and kept at 4 °C for 15 h followed by blocking with 100 μl of 4% skim milk (DIFCO) in PBS for 1 h at room temperature. Coating and blocking solutions were removed and the wells were washed three times with 300 μl of PBS containing Tween 20 (PBS-T) at 3 m intervals. Preimmune and immune sera collected from BALB/c mice were diluted at 1:100 and dispensed in corresponding wells for 1 h at 37 °C then washed three times with PBS-T. Antigen–antibody reaction was probed with HRP-anti-mouse IgG (Vector Lab) for 1 h at 37 °C followed by 100 μl of 0.5 $mg\,ml^{-1}$ ō-phenylenediamine substrate (OPD, Calbiochem) in the presence of 0.03% H_2O_2. The peroxidase reaction was quenched with 100 μl 2 N H_2SO_4. The color reaction was measured at 490 nm (OD_{490}) using a Thermomax ELISA plate reader (Molecular Devices).

The virus neutralizing activity of the sera from mice immunized with different PEDV antigens were measured by focus reduction neutralization test (FRNT) following the procedure described by Okuno et al. (1985), with some modifications. The immune sera were heat-treated at 56 °C for 30 m then four-fold serially diluted in PBS starting at 1:400. Pre-titered KPEDV-9 (1×10^3 ffu ml^{-1}) was mixed with equal volume of diluted sera and incubated at 37 °C for 30 m then chilled on ice for 10 m. Fifty microliters of the serum–virus mixture was inoculated in quadruplicate wells of a 96-well tissue culture plate (Falcon Laboratories) containing monolayer of Vero cells and incubated at 37 °C for 2 h. Unbound virus and excess antibodies were aspirated and the cell monolayer was overlaid with MEM containing 0.5 $mg\,ml^{-1}$ methyl cellulose and 10 $\mu g\,ml^{-1}$ trypsin then placed at 37 °C for 12 h. The succeeding steps were performed according to the focus formation assay as mentioned above. The number of resulting foci were measured and compared.

Reactivity of the different polyclonal antisera from BALB/c mice against the S-CT24, S-CT17 peptide or whole PEDV antigen were evaluated and compared with the 2C10 mAb. As shown in Fig. 1A, mice immunized with the S-CT24 peptide demonstrated very strong reactivity against the S-CT24 peptide (2.064 ± 0.162), suggesting that the S-CT24 peptide induced a high antibody response. In contrast, mice immunized with the S-CT17 peptide and whole PEDV antigen showed no reactivity against the S-CT24 peptide (0.044 ± 0.007 and 0.050 ± 0.008, respectively). When the antisera were reacted with whole PEDV antigen mice immunized with whole virus antigen showed high antibody reactivity (0.642 ± 0.056) while mice immunized with the S-CT24 peptide showed moderate reactivity (0.113 ± 0.011). However, mice immunized with the S-CT17 peptide still did not exhibit any antibody reactivity (0.050 ± 0.009) (Fig. 1B). In Fig. 1C, an ELISA using the S-CT17 peptide as coating antigen revealed that the S-CT17 peptide failed to induce detectable antibody response in mice unlike that of the S-CT24 peptide. In addition, sera from mice immunized with the S-CT24 peptide did not show any antibody reactivity with the S-CT17 peptide, indicating that there was no antibody cross-reactivity with the S-CT17 peptide. Another observation is the similarity in the antibody profiles of the S-CT24 antisera and 2C10 mAb. The 2C10 mAb strongly reacted with the S-CT24 peptide (1.085 ± 0.042) while showing moder-

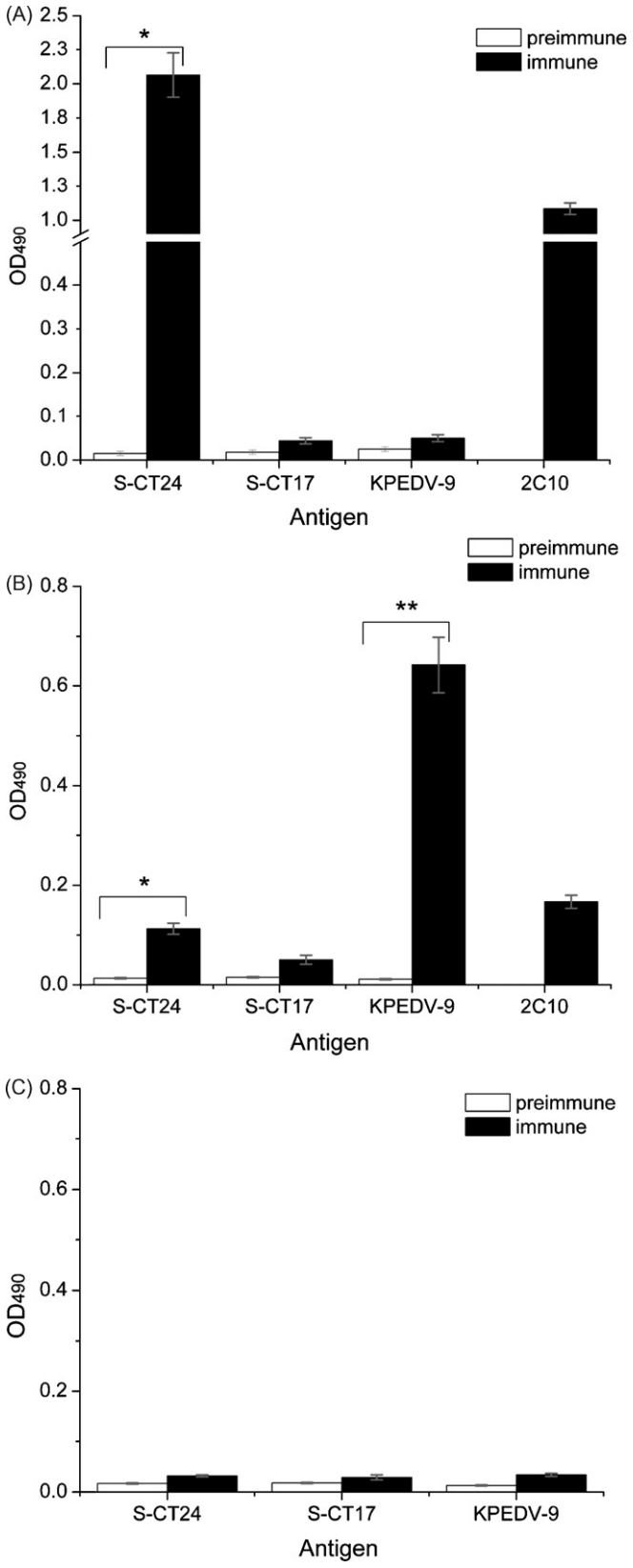

Fig. 1. Antibody response of BALB/c mice immunized with S-CT24 peptide, S-CT17 peptide and KPEDV-9 whole virus as well as 2C10 mAb. The sera and 2C10 mAb were reacted with (A) S-CT24 peptide antigen, (B) KPEDV-9 whole virus antigen and (C) S-CT17 peptide antigen coated on ELISA plate. (*Note*: immune sera were taken 1 week after final immunization)

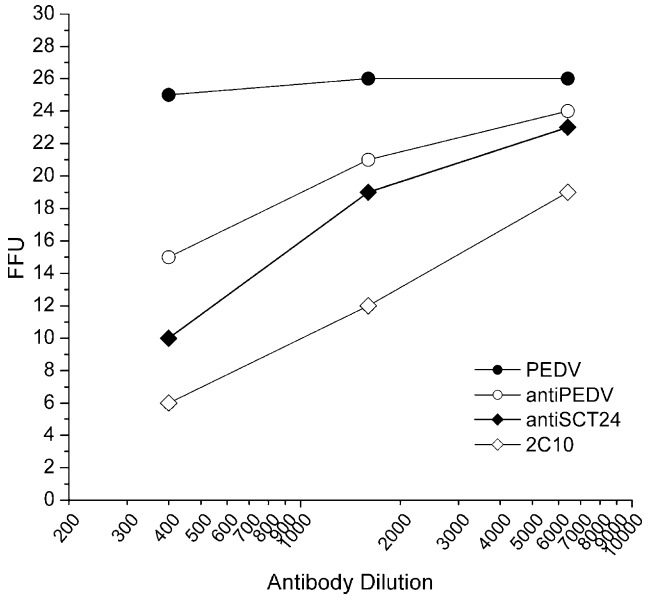

Fig. 2. Antibodies from S-CT24 immunized mice neutralize PEDV in vitro. FRNT of KPEDV-9 using S-CT24 antisera shows a significant decrease in virus infectivity compared to KPEDV-9 strain antisera. (PEDV:KPEDV-9 treated with normal mouse serum, anti-PEDV:KPEDV-9 treated with PEDV polyclonal antisera, antiS-CT24:KPEDV-9 treated with S-CT24 antisera and 2C10:KPEDV-9 treated with 2C10 mAb)

ate reactivity against whole PEDV antigen (0.167 ± 0.013) and very low reactivity towards the S-CT17 peptide (0.049 ± 0.002), comparable with the reactivities of antiS-CT24 antibodies against the same antigens. From these results, it is interesting to note that although the S-CT24 peptide is only 24 amino acids long, it can induce a high antibody response in mice. The S-CT24 peptide varies from the S-CT17 peptide only by the insertion of seven amino acids (GPRLQPY). These findings suggest that the GPRLQPY motif found within the C-terminal region of the spike protein is highly antigenic and capable of inducing an antibody response that has measurable reactivity against PEDV.

The neutralizing activities of sera from mice immunized with KPEDV-9 and S-CT24 peptide were compared with the neutralizing activity of the 2C10 mAb. Fig. 2 shows the result of FRNT with KPEDV-9 in the presence of anti-PEDV polyclonal antisera, antiS-CT24 antisera, and 2C10 mAb at various dilutions. KPEDV-9 showed an infectivity titer of 25 ± 3.3 ffu per well when mixed with diluted normal mouse serum (1:400). When mixed with antiS-CT24 antisera or 2C10 mAb, the infectivity of KPEDV-9 was decreased by more than 50% under similar conditions (10 ± 1.6 ffu per well or 60% reduction and 6 ± 2.5 ffu per well or 76% reduction, respectively). Interestingly, the polyclonal antisera from mice immunized with KPEDV-9 only showed a 40% reduction in virus infectivity (15 ± 1.9 ffu per well), comparably lower than that of the antiS-CT24 antisera and 2C10 mAb under similar conditions. These observations imply that antibodies generated by the S-CT24 peptide have neutralizing activities against PEDV comparable to that of the 2C10 mAb. It is important to note that lower dilution (1:100) of the antisera reduced the number of foci (<3 ffu per well). How-

ever, the number of foci in the virus control was also reduced drastically (<15 per well) (data not shown), suggesting that other components in the serum may already affect the infectivity of the virus.

Several studies have identified the spike protein as the primary surface glycoprotein involved in virus attachment and entry of coronaviruses (Gallagher and Buchmeier, 2001). The amino-terminal half (S1) is involved in virus attachment with host cellular receptors (Delmas and Laude, 1990; Cavanagh, 1995), while the carboxy-terminal half (S2) undergoes conformational changes after S1 attachment to facilitate fusion of the virus envelope with the host membrane and allowing entry of the viral genome into the host (Yoo et al., 1991). Because of the important role of the spike protein in initiating viral entry, development of vaccines that aims to prevent virus entry must be directed against this protein (Duarte and Laude, 1994). In the case of PEDV, recombinant vaccines currently being studied have focused solely on the COE epitope (a.a. 422–638), based on the findings of Chang et al. (2002) which identified this core neutralizing epitope by comparing the sequence homology of the PEDV spike protein with the CO-26K fragment of TGEV (Kang et al., 2006).

Recently, a putative neutralizing epitope located at the carboxy-terminal region of the PEDV spike protein, characterized by the ^{1368}GPRLQPY^{1374} motif, was reported (Cruz et al., 2006). Phages displaying this motif have been observed to block 2C10, a monoclonal antibody that exhibits neutralizing activities against PEDV in vitro. Although it is known that the carboxy-terminal endodomain is involved in the assembly of spike protein and progeny virions by interacting with the matrix protein (Godeke et al., 2000; Ye et al., 2004), it is still unclear what role this domain has in viral entry, and how antibodies that recognize this domain reduce virus infectivity in Vero cells. However, an antigenic motif on the endodomain of a surface glycoprotein of viruses exists like the ^{746}ERDRD^{750} motif on HIV gp41 (Broliden et al., 1992). The exact membrane topology and location of this motif is still controversial, and it has been suggested that the antibodies that antibodies against this motif on the gp41 glycoprotein cross reacts with an antigenically similar epitope on the ectodomain of p17 matrix protein (Buratti et al., 1997). Nonetheless, the antibodies that recognize such motif have neutralizing activities against the virus (Boucher et al., 1990).

To investigate the GPRLQPY motif, two peptides representing the carboxy-terminal endodomain of the spike protein were synthesized. The antigenicity of the peptide which carried the GPRLQPY motif (S-CT24) induced high antibody production in mice while the other peptide which had the GPRLQPY motif removed (S-CT17) did not elicit detectable antibody response in mice. The antibodies induced by the S-CT24 peptide was shown to be specific for the GPRLQPY motif as it did not react to the S-CT17 peptide in ELISA. Antibody specificity may be evaluated using linear peptides of 10 amino acids in length and that certain antibodies only require about two to three residues for recognition (Das and Hari, 1994; Van Regenmortel, 1992). Hence, our findings clearly suggest that antibodies directed against the GPRLQPY motif on the C-terminal endodomain of the spike protein of PEDV are capable of neutralizing PEDV.

In summary, we demonstrated the high antigenicity of the GPRLQPY motif found on the carboxy-terminal region of the S protein as shown by the strong antibody response against the peptide identical to the 24a.a. carboxy-terminal region which also reacted with whole virus. Furthermore, we found that the antibodies generated by this motif had neutralizing properties similar to that of the 2C10 mAb.

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