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Expression of Open Reading Frame 5 Protein of Porcine Reproductive and Respiratory Syndrome Virus Using Semliki Forest Virus Expression System

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ABSTRACT

The ORF5 gene encodes a major envelope glycoprotein (GP5), which is one of the three major proteins of porcine reproductive and respiratory syndrome virus (PRRSV). The GP5 protein has been known to be a 24.5-26kDa N-glycosylated envelope protein. The GP5 is involved in inducing neutralizing antibodies. For this reason, the GP5 is primary candidate for the PRRSV subunit vaccine. To produce the native form of GP5 in mammalian cells, we have cloned the ORF5 gene from PRRSV CNV-1 into the Semliki Forest virus (SFV)-based expression vector, resulting in recombinant pSFV-ORF5. By the infection with recombinant pSFV-ORF5 to BHK-21 cells, the GP5 expression was confirmed by immunocytochemistry and immunoblotting assay. The recombinant virus particle harboring ORF5 gene was infectious to BHK-21 and MARC-145. The RNA synthesis and expression of GP5 in the infected cell was also confirmed by RT-PCR.

Key words : PRRS virus, ORF5 gene, GP5, Semliki Forest virus-based expression vector, recombinant SFV particle.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a disease found in swine farms worldwide and it is characterized by reproductive failures such as late-term abortions in sows and by respiratory illness and high mortality in young pig[1,2,5,6,7,8,10,11]. PRRSV are related to those of a group of small, enveloped, positive-strand RNA viruses, including murine lactate dehydrogenase elevating virus (LDV), equine arteritis virus (EAV) and simian haemorrhagic fever virus, which are presently classified within the family *Arteriviridae*, order *Nidovirales*[12,13,14,15,16,17]. The genome of PRRSV is about 15kb in length and contains eight open reading frames (ORFs) designed ORF1a, ORF1b, and ORFs 2 to 7. According to sequence data, ORFs 1a and 1b represent nearly 75% of the viral genome and code for functional proteins associated with virus replication[14,15,18,19,20]. The virion contains three major structure proteins, 24.5-26kDa enveloped glycoprotein (GP5), 18-19kDa unglycosylated membrane (M) protein and 15kDa nucleocapsid (N) protein, encoded by ORFs 5, 6 and 7, respectively[19,20,27]. The GP5 is involved in inducing neutralizing antibodies, antigenic variability, apoptosis and possibly antibody-dependent enhancement phenomena[21,22,23,24,25,26]. For this reason GP5 has been thought a primary candidate for the subunit vaccine.

The SFV is a small enveloped alphavirus that contains a 42S RNA genome replicating in the cytoplasm of the infected cells without the involvement of the nucleus. Infection with SFV results in a suppression of host cell protein synthesis and enhances the production of viral proteins. This expression system is based on a genomic SFV cDNA inserted into an SP6 promoter plasmid and subsequently modified by deletion of the SFV structural genes to allow insertion of a heterologous gene as part of the SFV replicon. The helper RNA encoding the structural proteins of SFV is necessary for assembly and packaging of recombinant RNAs. The major advantages of this system, as comparing with other expression systems, are a broad range of susceptible host cells including those of insect, avian and mammalian origin and the high levels of RNA and protein produced in transfected cells by self- amplifying system[28,29].

In this study, Semliki Forest Virus (SFV)- based expression system to express GP5 in mammalian cells was adopted. We constructed pSFV-ORF5 plasmid vector by cloning ORF5 gene into the SFV replicon. Expression of GP5 and recombinat SFV particle harboring GP5 were demonstrated in the transfected BHK-21. The RNA synthesis and GP5 expression were also identified in the infected BHK-21 and MARC-145 cells with recombinant virus particles.

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MATERIALS AND METHODS

Cell and virus culture

The BHK-21 cells and MARC -145 cells were cultured in minimum essential medium (α-MEM)(Sigma, U.S.A.) supplemented with 10% tryptose phosphate broth, 5% fetal bovine serum, 20 mM HEPES, 2 mM glutamine , penicillin (0.1 IU/ml) and streptomycin(0.1 μg/ml). The PRRSV CNV-1 was inoculated onto MARC-145 cell monolayers in cell culture plate[3,30]. When cytopathic effect was observed on 70% of cell monolayer, the virus culture medium was harvested and stored at -70℃.

Reverse transcription-polymerase chain reaction (RT-PCR) of PRRSV RNA and cloning

The RNA of PRRSV was obtained from the PRRS virus CNV-1 culture supernatant by using viral RNA extraction kit (Qiagen, U.S.A.). The cDNA of the PRRSV ORF5 gene was synthesised by reverse transription (RT) and amplified by PCR. The reverse oligonucleotide primer for ORF5 gene(5'-TTCCCGGGCTAAGGACGACCCCATTG -3') was used for priming RT reaction. The *Sma* I restriction site was incorporated at 5' end of primer. 10 $\mu\ell$ of RT mixture consisting of 3$\mu\ell$ of RNA template, 2$\mu\ell$ of 5X buffer, 3$\mu\ell$ of dNTP mixture(Roche, Germany), 1$\mu\ell$ of reverse primer, 1$\mu\ell$ of RNase inhibitor (Takara, Japan) and 1$\mu\ell$ of Molony murine leukemia virus (M-MLV) reverse transcriptase (Promega, U.S.A.) was prepared in PCR tubes. After heat denaturation at 70℃ for 5min, RT was performed for 60min at 42℃ in a DNA thermal cycler (Perkin-Elmer, U.S.A.). For the PCR, the forward primer for ORF5 gene (5'-TTGGATTCCATGTTGGAGA AATGCTTG -3') and reverse primer were used. The forward primer specific for ORF5 gene contained *BamH* I restriction site at 5' end. After heat denaturation at 98℃ for 10 min, 88$\mu\ell$ of PCR mixture consisting of 66$\mu\ell$ of DEPC water, 10$\mu\ell$ of 10X PCR buffer with $MgCl_2$, 4$\mu\ell$ of forward primer, 4$\mu\ell$ of reverse primer, 2 $\mu\ell$ of dNTP mixture, and 2$\mu\ell$ *Taq* polymerase (Roche, Germany) were added to PCR mixture. The PCR condition contained 40 cycles (composed of 1 min at 94℃ for denaturation, 1 min at 50℃ for annealing, and 1 min at 72℃ for extension) and l cycle (composed of 10 min) at 72℃ for extension. The reaction was carried out in GeneAmp PCR system 2400 (Perkin Elmer, U.S.A.). The PCR product was cloned into the pSFV-1 plasmid which was digested with *BamH* I and *Sma* I and treated with calf intestine alkaline phosphatase (Promega, U.S.A) for 1hr at 37℃. The resulting recombinant pSFV plasmid was designated as pSFV-ORF5 and put into *E. coli* DH5α.

RNA synthesis and transfection of mammalian cells

Recombinant pSFV-ORF5 plasmid and pSFV-helper were linearized with *Spe* I and capped RNA was synthesized *in vitro* at 37℃ for 1 hr in a total volume of 50$\mu\ell$ containing 40 mM Tris-HCl(pH7.5), 6 mM $MgCl^2$, 2 mM spermidine, 5 mM DTT, 1 mM each of ATP, CTP and UTP, 0.5 mM GTP, 1 mM Cap analogue m7G (5')PPP(5')G, 40 units RNase inhibitor, and 50units SP6 RNA polymerase. Baby hamster kidney-21 (BHK-21) cells were trypsinized and washed twice with PBS and resuspended in PBS at concentration of 1×10^7/ml. Cell suspension was mixed with 100$\mu\ell$ of RNA transcripts (50$\mu\ell$ of pSFV-helper RNA transcripts and 50$\mu\ell$ of pSFV-ORF5 RNA transcripts). The mixture was used to electroporate 1×10^7 cells by using Gene Pulser Ⅱ(BioRad, U.S.A.). The cells were pulsed twice at 830v / 25 μF at room temperature. The transfected cells were resuspended in 12 ml of culture medium(α-MEM) supplemented with 10% tryptose phosphate broth, 5% fetal bovine serum, 20 mM HEPES, 2 mM glutamine, penicillin (0.1IU/ml) and streptomycin(0.1 μg/ml) and plated onto 100mm tissue culture dishes(Nunc, Denmark). The cells were incubated for 2 days in an atmosphere of 5% CO_2 at 37℃. The culture fluid was harvested and clarified by centrifugation for 15 min at 3000 $\times$g. The supernatant was transferred to a fresh ultracentrifuge tube and centrifuged at 35,000 $\times$g (SW41 Ti rotor; Beckman, U.S.A.) for 3 hr to collect virus particles. The virus pellet was resuspended in TNE buffer (50 mM Tris-HCl [pH7.4], 100 mM NaCl and 0.5 mM EDTA) and stored quickly in aliquots at -70℃.

Infection with recombinant virus particles

BHK-21 cells were seeded onto 24-well tissue culture plate and tissue culture dishes. Prior to infection, the cells were washed twice with PBS. The frozen recombinant virus particles were thawed quickly at room temperature. The virus particles were treated with 500 μg/ml chymotrypsin (Roche, Germany) and 0.5 mM $CaCl_2$, and then incubated on ice for 30 min. To inactivate chymotrypsin activity, 0.5 volume of aprotinin (2 ㎎/ml, Sigma, U.S.A.) was added. The activated vrus particles were diluted in MEM containing 1% FBS and inoculated into BHK-21 cells. After 90 min incubation at 37℃ in an atmosphere of 5% CO_2, the inoculum was removed and fresh medium was added. At 24 hr postinfection, cells were washed three times with PBS and lysed with cell lysis buffer (1% NP-40, 50 mM Tris-HCl [pH7.6], 150 mM NaCl, 2 mM EDTA, 1 μg/ml PMSF) on ice for 1 hr. The cell lysates were centrifuged at 12,000$\times$g at 4℃ for 5 min to remove insoluble materials and used as samples for sodium dodecyl sulfate-polyacrlyamide gel electrophoresis (SDS-PAGE) and RT-PCR.

Immunocytochemistry

The cell monolayers were rinsed twice with PBS and fixed with ice-cold methanol at 4℃ for 15 min. The fixed cell monolayers were washed three times with PBS and incubated at room temperature for 1 hr with PBS containing 1% gelatin to block nonspecific bindings. The PRRSV antibody positive pig sera (1:500 dilution) were added and incubated at room temperature for 3 hr. The cell monolayers were washed with PBS three times and then treated with rabbit

anti-pig IgG conjugated with peroxidase (Sigma, U.S.A) at room temperature for 1 hr. The cells were finally washed with PBS and visualized by adding H_2O_2 solution containing DAB (3,3'- diaminobenzidine), (Vector, U.S.A.).

Western immunoblotting

The SDS-PAGE was performed by the method of Laemmli[4,31]. Cell lysates were mixed with SDS-PAGE sample buffer (62.5 mM Tris-Cl [pH6.8], 10% glycerol, 2% sodium dodecylsulfate [SDS], 5% 2-mercaptoethanol, 0.05% bromophenol blue) and then boiled at 100℃ for 5 min and chilled on ice. The denatured protein samples were electrophoretically separated on 12% polyacrylamide SDS- gels and transferred onto polyvinylidine difluoride (PVDF) membrane (Roche, Germany). The membrane was blocked for 1 hr with blocking buffer (50 mM Tris-Cl, 5% skimmed milk, pH8.0). The blot was incubated for overnight at room temperature with PRRSV antibody positive pig sera(1:250 dilution) in shaking chamber. After washing three times in washing buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20), the blot was treated with rabbit anti-pig IgG conjugated with peroxidase (Sigma, U.S.A.) at room temperature for 2 hr. After rinsing blot in washing buffer, the specific protein band was visualized using DAB solution. The reaction was stopped by immersing the membrane in distilled water.

RT-PCR from infected cell with recombinant virus particles

The RNA from recombinant virus particles was obtained from cell culture media by using RNA extraction kit (Qiagen, U.S.A.). The cDNA synthesis of ORF5 gene and its amplification by RT-PCR were conducted by the method as described previously [30].

RESULTS

Construction of pSFV-ORF5

To express PRRSV GP5 in mammalian cells, we used the SFV-based expression system. Genomic RNAs of the PRRSV CNV-1 were extracted from virus culture media. The ORF5 gene was amplified by RT-PCR and cloned into the pSFV-1 vector. This cloning step resulted in pSFV-ORF5. The schematic diagram of pSFV-ORF5 and SFV- helper were illustrated in Fig.1.

Expression of the GP5 of PRRSV

The SFV expression system is provided with a packaging-deficient helper plasmid, pSFV-helper, which produces the structural proteins of SFV. When these proteins are produced in the same cells as RNA from pSFV 1-derived plasmids, the recombinant RNA is packaged into SFV particles that can be used for large scale infection of various mammalian cells.

The capped RNAs (Fig.2) transcribed *in vitro* from the SP6 promoter on the linearized pSFV-ORF5 and pSFV- helper were cotransfeced into BHK-21 cells by electroporation.

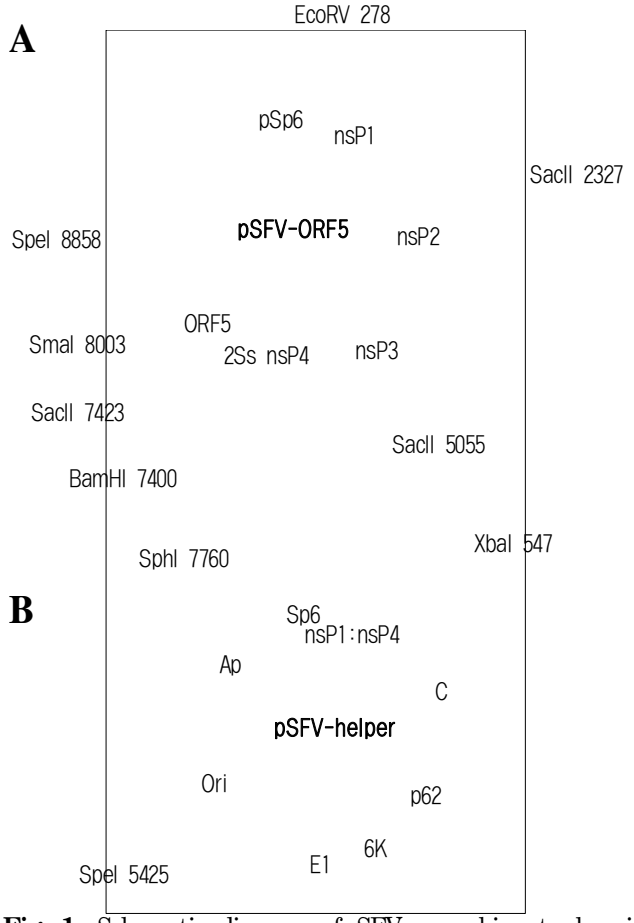

Fig. 1. Schematic diagram of SFV recombinant plasmid pSFV-ORF5(A) and pSFV-helper(B). The black bar in panel A indicates ORF5 gene. The location of *Spe* I restriction site used for linearization of the plasmids prior to RNA synthesis from the SP6 promoter is indicated.

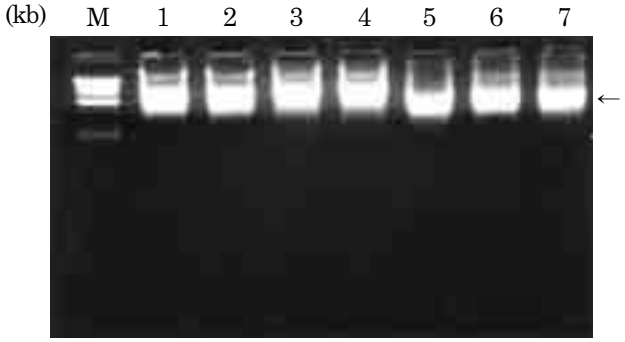

Fig. 2.. *In vitro* RNA transcripts from the linearized recombinant plasmid DNAs (M : 1kb DNA ladder, lanes 1-4 : pSFV-ORF5, lanes 5-7 : pSFV-helper). The arrow indicates the RNA transcripts.

The helper RNA that encoded the viral structural proteins of the wild type SFV, but lack the packaging signal found in the non-structural protein. As a result, only SFV recombinant RNA was packaged within a amphotropic viral

coat, and a recombinant virus that was capable of one round of infection was generated. Expression of the PRRSV GP5 was detected by immunocytochemistry using PRRSV antibody positive pig sera. The dark brown staining in cell cytoplasm indicated the expression of GP in the transfected cells. (Fig.3).

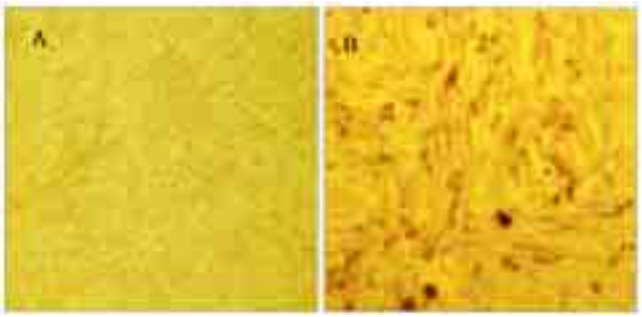

Fig. 3. Immunocytochemistry of BHK-21 cells expressing GP5. The BHK-21 cells were transfected with the RNA transcripts of pSFV-ORF5 and pSFV-helper. The cells expressing GP5 were visualized at 48 hr post transfection by immunocytochemistry (A : control cells, B : transfected cells).

Infection of recombinant virus particles into mammalian cells.

To investigate whether the SFV-derived system could be used to reconstitute assembly of infectious viral particles carrying a target foreign gene, we infected the recombinant virus particles into BHK-21 cells and MARC-145 cells. The medium containing recombinant virus particle was harvested at 48 hr posttransfection and was used for infection of BHK-21 cells and MARC-145 cells. After 48 hr post infection, infected cells were deteriorated and died significantly, but didn't in control cells. The evidence of infection of recombinant virus and expression of GP5 in the infected cells were demonstrated by immunocytochemistry (Fig.4), immunoblotting (Fig. 5) and RT-PCR (Fig. 6).

Immunocytochemistry using PRRSV antibody positive pig sera indicated that recombinant virus particles containing ORF5 gene were able to infect cells and GP5 was efficiently expressed in the cytoplasm of BHK-21 cells Fig.4: A,B) and MARC-145 cells (Fig.4: C,D). The dark brown staining in cell cytoplasm indicates the expression of GP5 in the infected cells. Immunoblot analysis showed a molecular mass of 20-25kDa (Fig. 5, lane 2), but many other cellular protein bands were also reacted with pig sera due to not using PRRSV monoclonal antibody. The RT-PCR using ORF5 primer set was carried out. The expected size, 603bp of ORF5 gene, was visualized by ethidium bromid staining in 1.0% agarose gel (Fig. 6). This data showed that recombinant virus particles were infectious to cells and RNA were synthesised in infected cells.

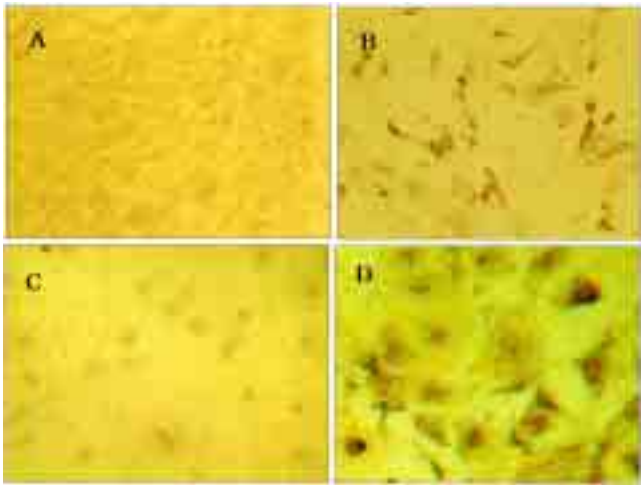

Fig. 4. Immunocytochemistry of GP 5 of PRRSV expressed in BHK-21 cells (A and B) and MARC-145 cells (C and D) infected with the recombinant SFV virus harboring ORF5 gene of PRRSV (A and C : control cells, B and D : infected cells).

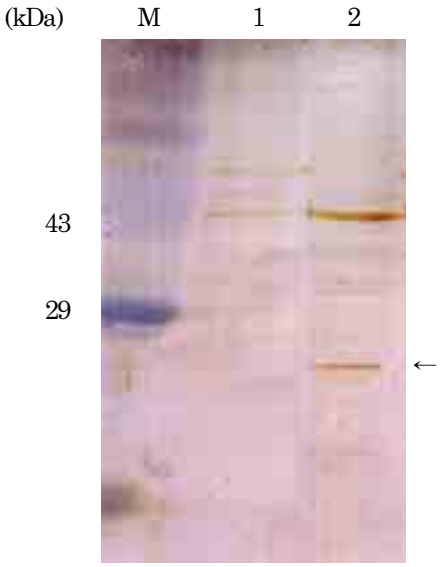

Fig. 5. Western immunoblotting of GP5 of PRRSV expressed in BHK-21 cells. The BHK-21 cell lysates were electrophoretically separated on 12% SDS-PAGE and transferred onto polyvinylidine difluoride (PVDF) membrane. The blot was treated with PRRSV antibody positive swine sera (M: marker, lane 1: mock infected BHK-21 cell lysates, lane 2: infected BHK-21 cell lysates with recombinant virus particles).

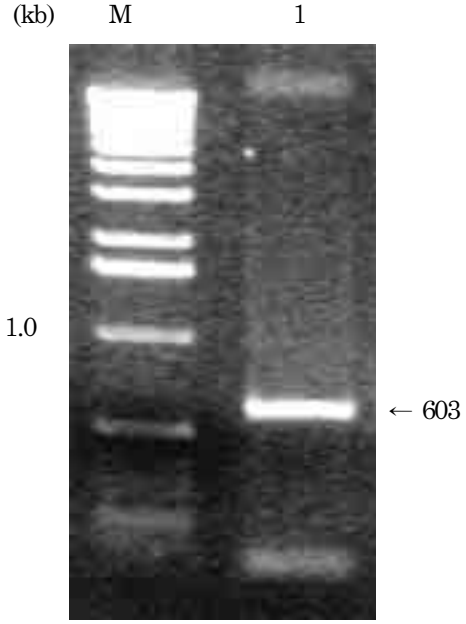

Fig. 6. The RT-PCR product of ORF 5 gene from the infected BHK-21 cell lysates with recombinant virus particles. The cell lysates were prepared at 24 hr postinfection. (M: 1kb DNA ladder, lane 1: RT-PCR product).

DISCUSSION

The GP5 of PRRSV is the major structural protein and play an important role in the immune responses[27]. The GP5 contains most of the epitopes involved in virus neutralization. The monoclonal antibodies that recognized the GP5 of PRRSV were reported to neutralize virus infectivity and at least two types of neutralizing determinants are associated with the GP5 of PRRSV[23,24,25,26,27]. The SFV has been adapted as an expression system by generating a plasmid containing the entire genome of the virus, except the gene for structural proteins (the capsid and three component of the enveloped protein). Replacement of these structural protein genes with foreign DNA results in high level expression of the heterologous proteins[28,29,30]. In this study, we have cloned and expressed the GP5 of PRRSV in mammalian cells by the SFV-based expression system. The ORF5 gene was inserted into individual SFV1 expression plasmid that served as templates for *in vitro* synthesis of recombinant RNA. The capped RNA transcription of both SFV-ORF5 and SFV-helper plasmid were conducted *in vitro* and introduced into the cytoplasm of BHK-21 cells by electroporation. This cotranfection of BHK-21 cells with RNA transcripts resulted in the infectious recombinant viral particles. The results of RT-PCR, immunocytochemistry and immunoblotting assay demonstrated that recombinant virus particles were infectious to cells and RNA were synthesised and GP5 was expressed in infected cells , BHK-21 and MARC-145. Because the native form of heterologus protein such as GP5 of PRRSV could be produced in this SFV-derived expression system, the GP5 expressed in the this study can be used for PRRSV vaccine antigen. The immunogenicity of the recombinant virus particles will be investigated in the future study.

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