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GP3 is a structural component of the PRRSV type II (US) virion

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Abstract
Glycoprotein 3 (GP3) is a highly glycosylated PRRSV envelope protein which has been reported as being present in the virions of PRRSV type I, while missing in the type II PRRSV (US) virions. We herein present evidence that GP3 is indeed incorporated in the virus particles of a North American strain of PRRSV (FL12), at a density that is consistent with the minor structural role assigned to GP3 in members of the *Arterivirus* genus. Two 15aa peptides corresponding to two different immunodominant linear epitopes of GP3 derived from the North American strain of PRRSV (FL12) were used as antigen to generate a rabbit monospecific antiserum to this protein. The specificity of this anti-GP3 antiserum was confirmed by radioimmunoprecipitation (RIP) assay using BHK-21 cells transfected with GP3 expressing plasmid, MARC-145 cells infected with FL12 PRRSV, as well as by confocal microscopy on PRRSV-infected MARC-145 cells. To test if GP3 is a structural component of the virion, ^{35}S-labelled PRRSV virions were pelleted through a 30% sucrose cushion, followed by a second round of purification on a sucrose gradient (20–60%). Virions were detected in specific gradient fractions by radioactive counts and further confirmed by viral infectivity assay in MARC 145 cells. The GP3 was detected in gradient fractions containing purified virions by RIP using anti-GP3 antiserum. Predictably, the GP3 was less abundant in purified virions than other major structural envelope proteins such as GP5 and M. Further evidence of the presence of GP3 at the level of PRRSV FL12 envelope was obtained by immunogold staining of purified virions from the supernatant of infected cells with anti-GP3 antiserum. Taken together, these results indicate that GP3 is a minor structural component of the PRRSV type II (FL12 strain) virion, as had been previously described for PRRSV type I.

Keywords: GP3, PRRSV, sucrose gradient, structural protein, purified virions

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive failure in pregnant sows and respiratory distress in piglets and growing pigs, being considered one of the most important viral diseases of pigs in countries with extensive swine industry (Neumann et al., 2005). The disease is caused by PRRS virus (PRRSV), which was first isolated in Europe and then in the US (Collins et al., 1992). PRRSV is a single stranded RNA virus belonging to the family *Arteriviridae*, genus *Arterivirus* (Meulenberg, 2000). Two distinct serotypes of PRRV are recognized, type I (European) and type II (US), both exhibiting a significant degree of genetic and antigenic heterogeneity while maintaining identical pathobiological phenotypes (Snijder and Meulenberg, 2001). The viral genome is approximately 15.0 kb in length and contains nine open reading frames flanked by untranslated regions (UTRs) at the 5′- and 3′-end (Meulenberg, 2000). ORFs 1a and 1b encode for non-structural polypeptides with replicase-associated activities whereas ORFs 2–7 are translated from a nested set of subgenomic RNA (sgRNA) encoding the structural proteins (Snijder and Meulenberg, 2001). The major structural proteins include glycoprotein 5 (GP5), M and N (encoded by ORFs 5, 6 and 7) while the minor structural proteins include GP2, GP3 and GP4 (encoded by ORFs 2a, 3 and 4 respectively) and the small E protein (encoded by ORF 2b) (Snijder and Meulenberg, 2001; Wu et al., 2005). Analyses of purified virions of European strains of PRRSV have confirmed that six of these PRRSV proteins are authentic structural proteins, these are: the four glycoproteins: GP2a, GP3, GP4 and GP5; a nonglycosylated membrane protein M and the nucleocapsid protein N (Dea et al., 2000). On the other hand, recent findings have demonstrated that 2b protein, expressed from a second ORF entirely contained within ORF2, is also a minor structural component of PRRSV (Wu et al., 2001, 2005). The structural nature of GP3 is still controversial, as there is conflicting data regarding its presence as a constituent of the envelope of virus particles. It has been convincingly demonstrated that GP3 is a 45- to 50-kDa structural protein of the PRRSV LV (type I or European) strain (Meulenberg et al., 1995). However, the GP3 has been reported as being a non-structural protein of the PRRSV type II IAF-Klop strain, with a subset of viral GP3 being released into the cell culture medium as a non-virion associated and membrane-free form (Gonin et al., 1998; Mardassi et al., 1998). In this context,

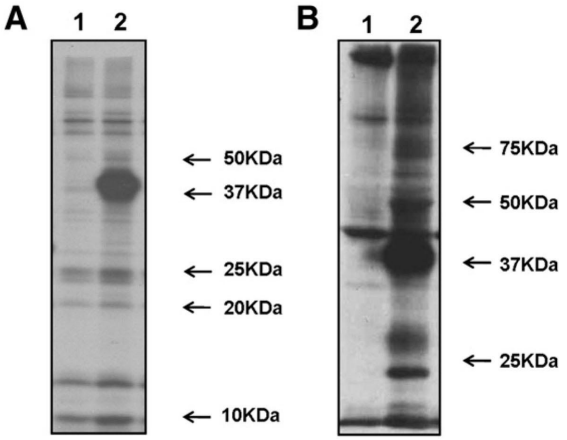

Figure 1. Radioimmunoprecipitation with a rabbit monospecific GP3 antiserum. A) Immunoprecipitation analysis of GP3 expressed in BHK-21 cells (lane 2). Cells transfected with an empty vector (lane 1). B) MARC145 cells infected with PRRSV FL12 (lane 2). Mock-infected cells (lane 1).

the observed in vivo immunogenicity of GP3 has been explained by its ability to be secreted from infected cells as previously described (Mardassi et al., 1998). However, a structural role for GP3 in PRRSV type II has been suggested by recent findings reporting its ability to induce neutralizing antibodies (Cancel-Tirado et al., 2004; Jiang et al., 2008). In addition, partial protective immunity was observed in vivo by immunization of animals with a recombinant baculovirus expressing ORF3 (Plana Duran et al., 1997) or with recombinant fowlpox expressing GP3/GP5 (Shen et al., 2007). Overall, it is evident that according to the available data, the structural nature of the GP3 in PRRSV type II virions is still unclear and remains a matter of controversy.

Glycoprotein 3 is the second most heterogeneous protein of PRRSV with approximately 54 to 60% amino acid identity between North American and European isolates (Meng et al., 1995). The protein encoded by ORF3 is also the most glycosylated PRRSV protein with seven putative N-linked glycosylation sites that are well conserved among strains from both PRRSV serotypes (Gonin et al., 1998). Thus, the predicted molecular mass (M_r) of the ORF3 protein (27–29 kDa) is smaller than its apparent M_r estimated by gel electrophoresis (42–50 kDa) (Dea et al., 2000; Gonin et al., 1998).

We herein present evidence that GP3 is incorporated into virus particles of a North American strain of PRRSV (vFL12). Our results indicate that GP3 represents just a small fraction of the structural proteins of PRRSV, which is consistent with the notion that GP3 is one of the minor structural glycoproteins of the *Arteriviridae* (Meulenberg, 2000; Snijder and Meulenberg, 2001).

Results

Generation and characterization of GP3 monospecific antiserum

In order to confirm the identity of GP3, we prepared an anti-GP3 monospecific antiserum by injecting rabbits with two synthetic peptides conjugated to KLH. Both 15 amino acid peptides within ORF3 protein (Q^{61}AAAEVYEPGRSLWC^{75} and S^{83}EDDHDDLGFMVPG^{96}C) were shown to be highly immunogenic in our previous experiment as measured by its reactivity with serum from pigs experimentally infected with a North American strain of PRRSV (de Lima et al., 2006). An emulsion containing both KLH-conjugated peptides emulsified in Freund's adjuvant were injected in two rabbits at 2 weeks intervals. Specific anti-GP3 antiserum was collected at 77 days after the first injection, further characterized and used in all experiments. Specificity of the anti-GP3 antiserum was further confirmed by an indirect immunofluorescence staining of MARC 145 cells at about 48 h post infection with a PRRSV FL12 strain. A cytoplasmic pattern of staining was observed when the anti-GP3 antiserum was used as a primary antibody followed by a secondary anti-rabbit IgG antibody conjugated with Alexa Fluor 488 and observed under confocal microscopy with nuclei stained with DAPI (data not shown). No staining was observed following incubation of PRRSV-infected MARC-145 cells with rabbit pre-immune serum (data not shown).

The specificity of the anti-GP3 monospecific antiserum was further investigated by radioimmunoprecipitation assays. The gene encoding for GP3 protein of FL12 strain of PRRSV was PCR amplified and cloned into pGEM3 expression vector using EcoRI and XhoI restriction sites. The entire coding region was confirmed by nucleotide sequencing. Transfection followed by radioimmunoprecipitation using GP3 antibody revealed a clear protein species of ~ 42–45 kDa (Figure 1A, lane 2). This particular signal was absent in the control lane which was processed in a similar way, but in this case transfected with pGEM3 only. This data clearly indicates specific reactivity of generated GP3 antibody against PRRSV GP3 protein. In addition the anti-GP3 monospecific antiserum efficiently immunoprecipitated the GP3 from lysates of PRRSV-infected MARC-145 cells (Figure 1B, lane 2). In the same experiments several other distinct signals (27–29 kDa) were also observed which most likely correspond to either partially glycosylated forms of GP3 or co-immunoprecipitation of other viral proteins i.e. GP2 and/or GP5.

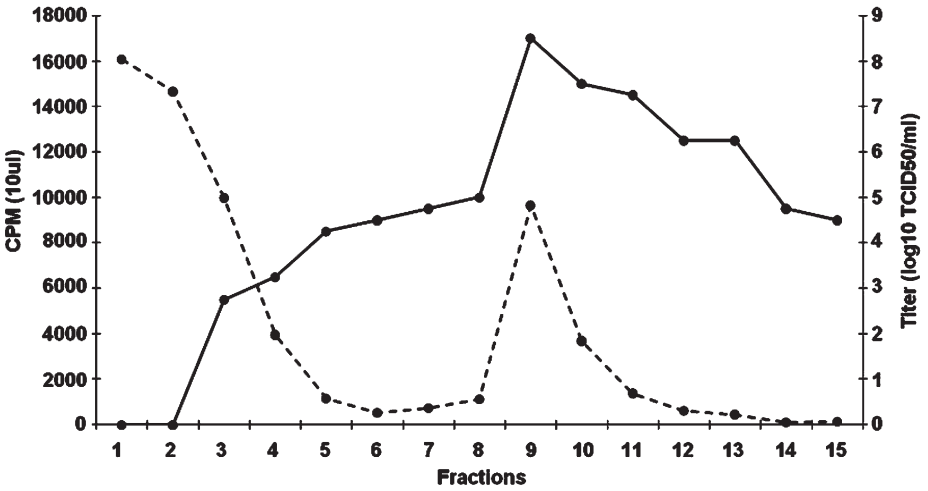

Figure 2. Radiolabeled viral suspension previously pelleted through a 30% sucrose cushion was layered on a continuous sucrose gradient (20–60%) in TEN buffer. Virus titer and CPM/tube (determined in a scintillation counter) in the different fractions was determined. Fractions 8–11 which correspond to the peak infectivity and CPM counts were pooled, centrifuged and used for subsequent radioimmunoprecipitation experiments.

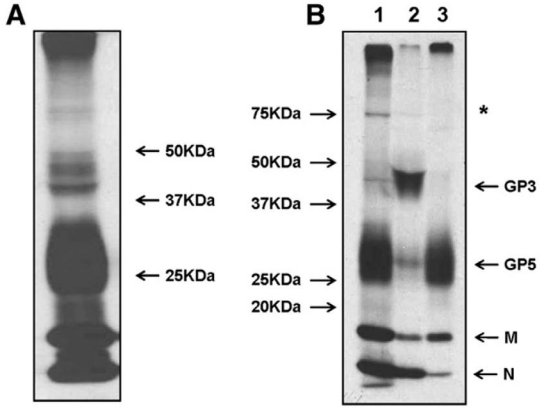

Figure 3. A) Total protein derived from purified virus collected from a sucrose gradient (fractions 8–11) were solubilized in RIPA buffer and analyzed by reducing SDS-PAGE (12% gel). B) Radiolabeled and purified virions were subjected to immunoprecipitation with GP3 monospecific antiserum (lane 2 — 70% of the lysate) or with a polyclonal antibody anti-GP5 and a monoclonal antibody to M protein (lane 3 — 20% of the lysate). 10% of the lysate was loaded onto lane 1. Immunoprecipitates were analyzed by reducing SDS-PAGE (12% gel). Position of the size markers are indicated in the left and positions of the PRRSV envelope proteins in the right side of the fluorograms. (*) Unidentified protein.

GP3 is incorporated into PRRSV type II virion in a lesser amount than the major envelope proteins

In order to show that the GP3 was associated with the PRRSV type II virion, we utilized a two-step purification process of extracellular virus. The first round consisted of pelleting a clarified supernatant from PRRSV-infected MARC-145 cells containing radiolabeled virions through a 30% sucrose cushion followed by a 20–60% (W/V) sucrose gradient purification of ^{35}S-labeled virus. Fractions were carefully collected from the top of the tube and used for titration, radioactivity counting and immunoprecipitation experiments. Fractions containing the highest infectivity titers and radioactive counts (fraction nos. 8–11, Figure 2) were used for subsequent experiments.

Initially, total proteins derived from the pooled fractions containing purified virus collected from a sucrose gradient were solubilized in RIPA buffer and analyzed by reducing SDS-PAGE without addition of antibody. We observed well defined bands with molecular weights corresponding to those reported for the major PRRSV structural proteins (Figure 3A). On the other hand, a small amount of a protein with a molecular weight of approximately 42–45 kDa was also clearly observed in the lysate containing virion proteins.

In order to confirm the identity of the above-mentioned 42–45 kDa protein, the pooled fractions containing purified virions were immunoprecipitated by incubation with anti-GP3 antibody and with a pool of a polyclonal antibody anti-GP5 and a monoclonal antibody to M protein (generated in our laboratory against a synthetic peptide corresponding to a highly immunogenic linear epitope of M protein). However, due to the small amount of 42–45 kDa protein visualized in the gel (Figure 3A), we used the majority of the lysate (70%) for immunoprecipitation with anti-GP3 antibody, 20% of the lysate with an anti-GP5 and anti-M antibody and the remaining 10% of lysate used for loading as total protein (Figure 3B). Using this strategy, a specific band corresponding to GP3 was detected in pooled gradient fractions containing purified virions by RIP with anti-GP3 antiserum (Figure 3B, lane 2). In addition, a faint band, most likely corresponding to GP2 and/or GP5 (27–29 kDa), was also co-precipitated by the anti-GP3 antibody.

Further evidence of the presence of GP3 at the level of PRRSV FL12 envelope was obtained by immunogold staining of the envelope of purified virus present in the supernatant of infected cells with anti-GP3 antiserum (Figure 4). Purified virus was incubated with either pre-immune rabbit serum (Figure 4, panels A and B; as a negative control), anti-GP3 monospecific rabbit antiserum (Figure 4, panels C to G) and anti-PRRSV rabbit polyclonal antibody (Figure 4, panels H to J; as a positive control) followed by an anti-rabbit IgG conjugated with gold particles. No staining was observed when purified virions were incubated with a PRRSV negative serum whereas a strong reactivity was observed in virions incubated with a polyclonal antibody against GP5 as shown by the number of gold particles surrounding the virus envelope (Figure 4, panels H to J). On the other hand, the number of gold particles that were found associated with virions was clearly reduced when incubated with the anti-GP3 monospecific antiserum (Figure 4, panels C to G).

Discussion

In the present study, we confirmed the structural nature of GP3 in a North American strain of PRRSV (vFL12). PRRSV vFL12 is recovered from MARC-145 cells transfected with an infectious full-length cDNA clone of PRRSV which has been extensively used in our laboratories (Ansari et al., 2006; de Lima et al., 2006, 2008; Kwon et al., 2006, 2008; Truong et al., 2004). In previous studies, GP3 of PRRSV has been shown to be incorporated into virions of European-type strains (van Nieuwstadt et al., 1996), however such

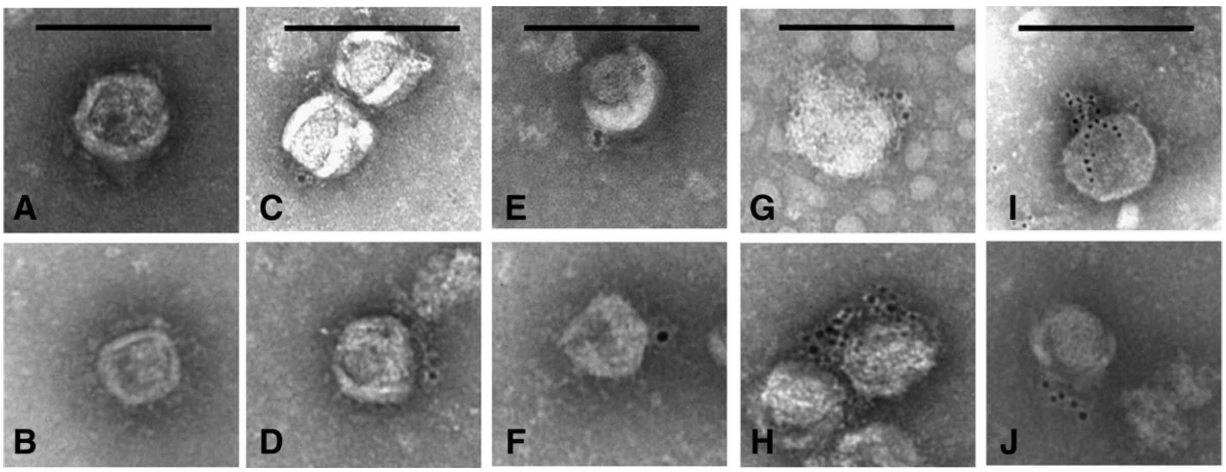

Figure 4. Immunogold labeling of purified PRRSV particles with different antibodies. Purified virus was incubated with either pre-immune rabbit serum (A and B; negative control), anti-GP3 monospecific rabbit antiserum (C to G) or, anti-PRRSV rabbit polyclonal antibody (H to J) followed by an anti-rabbit IgG conjugated with gold particles. Black bars shown correspond to 100 nm.

occurrence in PRRSV type II strains has been questioned by some publications (Gonin et al., 1998; Mardassi et al., 1998). Since then, the notion that PRRSV type II virions lacked GP3 prevailed.

In our study, RIP experiments confirmed the specificity of the anti-GP3 monospecific antiserum as measured by its reactivity with ORF3 protein either expressed by plasmid-transfected BHK-21 cells or by PRRSV FL12-infected MARC-145 cells. Likewise, the GP3 was also detected by indirect immunofluorescence assay in the cytoplasm of methanol/acetone-fixed PRRSV-infected MARC-145 cells using the antiserum as a primary antibody (data not shown). These findings unequivocally demonstrated the ability of the anti-GP3 antibody to recognize the ORF3 protein derived from the North American strain of PRRSV (FL12).

In order to demonstrate that association of the GP3 with the PRRSV type II virion, we used a purified preparation of ^{35}S-labeled virus from the supernatant of infected MARC-145 cells. By using a scintillation counter, we observed a peak of radioactivity which corresponded to the fractions 8–11 collected from the sucrose gradient. Interestingly, viral titration of all fractions revealed the highest infectious titers in these fractions, thus confirming the presence of highly purified virions. Pooled fractions were directly analyzed by SDS-PAGE or subjected to RIP experiments using GP3 antibody. From these experiments, we concluded that the GP3 was less abundant in purified virions than other known structural proteins such as GP5 and M (Figure 3). The appearance of GP5 as a broad smear is consistent with its incorporation into virions as a mature glycoprotein with heterogeneous glycan moieties. The co-precipitation of the M protein in these experiments appears to be an inconsistent event and could possibly be related to the experimental conditions or due to some interaction between the M and GP3 protein. This type of unexpected co-precipitations amongst PRRSV proteins were also reported by Wu et al. (2005), who described some interaction occurring between N and 2b proteins.

The amount of GP3 incorporated into PRRSV type II virions is clearly lower when compared to the three major structural proteins N, M and GP5. It could be speculated that only a small fraction of the protein is incorporated into virions while other fraction(s) is (are) released into the culture medium. A comparison of the staining intensities suggests that the well-known major structural proteins such as M and GP5 predominate in the virion, while GP3 would be incorporated in significantly lower amounts. Thus, in order to clearly visualize the GP3, a large amount of the lysate containing purified virions was required whereas a large amount of GP5 was precipitated by the anti-GP5 polyclonal antibody in a fraction of just a smaller percentage of the lysate. Similarly, analyses of the structural nature of GP3 and GP4 of Lelystad virus required a large amount of virus to visualize these proteins by immunostaining on the nitrocellulose strips (van Nieuwstadt et al., 1996). According to this data, we demonstrated that a higher amount of lysate derived from purified virus is necessary to visualize GP3 than the amount required to detect the major envelope proteins like GP5 or M protein. This circumstance may, at least partially, explain the unsuccessful detection of ORF3 protein incorporated into virions in a previous study reporting that GP3 from a North American strain of PRRSV would not be part of the virion structure (Gonin et al., 1998). Our results are also consistent with the detection of a much fainter band corresponding to the ORF 2b protein when compared to the amounts detected for GP5, M or N protein (Wu et al., 2001). Collectively, this data indicates that the 42–45 kDa protein is an integral but minor structural component of the PRRSV type II virion. Immunogold staining of purified virus present in the supernatant of infected cells with anti-GP3 antiserum provided further evidence of the presence of GP3 at the level of PRRSV FL12 envelope (Figure 4). While no staining was observed in purified virions incubated with a PRRSV negative serum, a strong reactivity was observed in virions incubated with an anti-PRRSV polyclonal antibody as shown by the number of gold particles surrounding the virus envelope (Figure 4, panels H to J). On the other hand, the number of gold particles that were found associated with virions was clearly reduced when incubated with the anti-GP3 monospecific antiserum (Figure 4, panels C to G). This finding might be explained by the small amount of GP3 incorporated into PRRSV virions.

The structural role for GP3 was suggested by recent findings reporting the ability of the PRRSV GP3 to induce neutralizing antibodies either alone or in combination with GP4 and GP5 (Jiang et al., 2008). In addition, Cancel-Tirado et al. (2004) have demonstrated that a monoclonal antibody to Gp3 was associated with inhibition of PRRSV replication in porcine alveolar macrophages, thus providing further evidence for a possible presence of neutralizing epitopes in GP3. Recently the same laboratory has provided additional evidence, by chimeric reverse genetics studies of PRRSV US type II, that ORF3 contributes to the induction of neutralizing activity in serums, together with ORF5 and 6 (Kim and Yoon, 2008). In addition, partial protective immunity against PRRSV challenge was reported in pregnant sows immunized with a recombinant baculovirus expressing ORF3 from a Spanish strain of PRRS as measured by the number of piglets born alive and healthy at the time of weaning (Plana Duran et al., 1997). Pigs immunized with recombinant fowlpox expressing GP3/GP5 developed a specific immune response against the PRRSV proteins and most importantly, a degree of protection was also conferred against challenge with a pathogenic strain of PRRSV (Shen et al., 2007). Furthermore, it has also been reported that ORF3 encodes a structural protein of Equine Arteritis virus (EAV). An additional study showed that the minor envelope glycoproteins GP2b, GP3, and GP4 of EAV form a heterotrimeric complex providing further evidence that the GP3 homologous protein of EAV is structural (Wieringa et al., 2004). Considering the homology among arteriviruses, other studies have indicated that all seven structural proteins of EAV are required for the production of infectious progeny virus (Molenkamp et al., 2000; Snijder et al., 1999). Similar observations were also reported for PRRSV LV strain. Some authors (Wissink et al., 2005) have shown that all of PRRSV minor proteins interact with each other and they are assembled into virions as a multimeric complex, thus being essential for virus infectivity. Overall, this set of data substantiates our observations indicating that GP3 is a minor structural component of the PRRSV type II virions.

Materials and methods

Virus, cells and antibodies

FL12 strain is the PRRSV virus recovered from MARC-145 cells transfected with RNA transcripts produced *in vitro* from the full-length infectious cDNA clone (vFL12) derived from PRRSV NVSL 97-7895 type II strain (GenBank accession no. AY545985) (Truong et al., 2004). MARC-145 cells (Kim et al., 1993) were propagated in Dulbecco's Modified Eagle's Medium (DMEM) containing 5–10% fetal bovine serum (FBS) and antibiotics (100 U/ml of penicillin, 20 μg/ml of streptomycin, and 20 μg/ml of kanamycin) and were used for viral multiplication and titration and for viral protein expression. The BHK-21 cells were maintained in MEM containing 5% fetal bovine serum (FBS) and antibiotics and used for transient expression of GP3. Indirect immunofluorescence assays (IFA) were performed as previously described (Kwon et al., 2006) using PRRSV N protein-specific monoclonal antibodies (MAbs): SDOW17 (National Veterinary Services Laboratories – NVSL, Ames, IA) and SR30 [kindly provided by Dr. Eric Nelson (South Dakota State University, SD, USA)]. The secondary antibody used in both cases consisted of a goat antimouse IgG antibody (Alexa Fluor – 488, Molecular Probes, Eugene, OR). For RIP experiments we used an anti-GP5 polyclonal antibody (kindly provided by Dr Carl Gagnon, University of Montreal) and an anti-M monoclonal antibody raised against a synthetic peptide corresponding to a highly immunogenic B-cell linear epitope of M protein (de Lima et al., 2006) and previously characterized in our lab (data not shown).

Selection and peptide synthesis

Based on previous studies in our laboratory (de Lima et al., 2006) we selected two highly immunogenic 15-mer peptides based on the glycoprotein 3 (GP3) amino acid sequence of a North American strain of PRRSV (NVSL 97-7895). The peptides were synthesized and conjugated to a carrier protein (KLH) either by adding a cysteine residue or via activated EDC chemistry to its N-terminal region. The amino acid residues of the selected peptides were Q^{61}AAAEVYEPGRSLWC^{75} (cysteine originally found within the amino acid sequence of GP3) and S^{83}EDDHDDLGFMVPG^{96}C (cysteine residue added in order to facilitate its conjugation with KLH). These peptides were selected for the antiserum preparation based on their observed antigenicity and immunodominance in vivo (de Lima et al., 2006). While no definitive knowledge exists about the topology of PRRSV GP3, we would anticipate, based on previous knowledge of other multi-glycosylated viral envelope proteins, that these peptides are likely located in the ectodomain of PRRSV GP3.

Production of anti-GP3 monospecific rabbit antiserum

Two rabbits were used for this immunization; each receiving both KLH-conjugated peptides emulsified in complete (once, first shot) and incomplete (several ensuing and successive shots) Freund's adjuvant. Animals were co-immunized (solution containing both peptides) at days 0, 14, 28, 42, 56 and 70. Bleedings were performed on days 49, 63 and 77 post immunization and serum samples tested for the presence of anti-GP3 antibodies. Specific anti-GP3 monospecific antiserum was collected at 77 days after first injection, characterized and used in all experiments.

Expression of GP3 in BHK-21 cells

For transient expression of GP3, the BHK-21 cells were plated in six well plates at 80% confluency. The following day the cells were washed with PBS and infected with recombinant vaccinia virus (VTF7-3) at a MOI of 3.0. Forty-five minutes after inoculation of the cultures, the cells were washed with PBS again and DNA-Lipofectamine complex (2.0 µg of plasmid DNA: 5.0 µl of Lipofectamine) was added to cells according to manufacturer's protocol. The supernatant was replaced with complete growth medium after 4 h post-transfection. At 16 h post-transfection, the cells were washed with PBS and incubated with Methionine- and Cysteine-free DMEM media to induce starvation. After one hour, 50 µCi of S^{35} (Express Protein Labelling Mix, [35S], Perkin Elmer) was mixed with 600 µl of Cys-Met free media, added onto cells and further incubated for 4 h. Immunoprecipitation was performed as described below.

Viral infection and metabolic labeling

Viral proteins derived from MARC-145 cells infected with PRRSV FL12 strain and extracellular virions were labeled as follows: for labeling of viral expressed intracellular GP3, the MARC-145 cells in six well plates were infected with PRRSV FL12 strain at a M.O.I. of approximately 1. At 18 h post infection (pi), cells were washed twice with PBS and subjected to starvation in methionine/cysteine-free DMEM for one hour. The starved cells were radiolabeled with 0.6 ml of methionine/cysteine-free DMEM containing 100 µCi of S^{35} (Express Protein Labelling Mix, [35S], Perkin Elmer) for 4 h and RIP was performed as described below. For radiolabeling of extracellular virions, confluent monolayers of MARC-145 cells in 100 mm plates were infected with PRRSV FL12 strain and, after 18 h, the infected cells were labeled with 6.0 ml of 100 µCi of l-[^{35}S] methionine/ml. In this case the final medium was composed of 90% of Cys-Met-free medium and 10% of complete growth medium. Virus particles were labeled overnight and the supernatant was collected and clarified by low speed centrifugation. Radiolabeled PRRSV proteins and extracellular virus were used in subsequent immunoprecipitation experiments.

Virus purification

Above radiolabeled PRRSV virions were subjected to two rounds of purification. The clarified supernatant from PRRSV FL12-infected MARC 145 cells containing radiolabeled virus was first pelleted through a 30% sucrose cushion and then submitted to a 20–60% (W/V) sucrose gradient overnight at 4 °C (100,000 *g*). Fractions were carefully collected from the top of the tube and used for titration, radioactivity counting and immunoprecipitation experiments.

Radioimmunoprecipitation and SDS-PAGE analysis

Immunoprecipitation experiments were performed with 1) BHK-21 cells transfected with GP3 expressing plasmid vector, 2) radiolabeled proteins derived from MARC-145 cells infected with PRRSV; and 3) radiolabeled extracellular virions as described elsewhere (Ansari et al., 2006). Fractions with the highest infectivity and CPM counting were pooled and used for subsequent RIP experiments. Briefly, radiolabeled purified virus or cell extracts were resuspended in RIPA buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% Sodium deoxycholate, and 1× protease inhibitor). The clarified supernatants were incubated with anti-GP3 polyclonal antibody or with a pool of anti-GP5 and anti-M antibodies (1:100 dilutions) for 16 h at 4 °C with shaking. Approximately 3.0 µg of constituted Protein A Sepharose (Protein A Sepharose™ CL-48, GE Health care Bio-science AB) was added per tube and further incubated at 4 °C for two hours. The immune complexes were centrifuged at 10,000 rpm for 30 s and washed with RIPA buffer three times with five minute incubation, each time keeping the samples in a rotator at 4 °C. Finally the immune complexes were resuspended in 2× sample buffer and boiled for five minutes. The samples were again centrifuged briefly and the supernatants were analyzed by SDS-12% PAGE gel. The gel was fixed with 10% acetic acid for 15 min, washed three times with distilled water, treated with 0.5 M sodium salicylate for 30 min, dried and finally exposed to X-ray film at − 70 °C.

Immunogold staining

Briefly, after two rounds of low speed centrifugation, the clarified supernatant of PRRSV-infected MARC-145 cells was pelleted through a 30% sucrose cushion by ultracentrifugation (25,000 rpm) for 2 h. Purified virus suspension was placed onto a formvar-carbon coated copper grid (duplicate grids were used for each experiment) for approximately 1 min. Each grid was then placed on a drop of 5%BSA in PBS for 10 min. After, the samples were incubated with an anti-GP3 monospecific rabbit antiserum for 30 min. Pre-immune rabbit serum was used as a negative control and anti-PRRSV rabbit polyclonal hyperimmune serum (obtained by repeated immunizations of rabbits using supernatant and lysates of PRRSV-infected MARC-145 cells) as a positive control. All antibodies were diluted in PBS with 5% BSA. The samples were then washed three times of five minutes each with PBS and incubated with an anti-rabbit IgG conjugated with 10 nm gold particles. After another washing step, the samples were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, negative-stained on a drop of 1% sodium silicotungstate and observed under electron microscopy.

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