RESEARCH REPORT
SWINE HEALTH

Title: Identification of the viral protein that mediates PRRSV attachment to the sialoadhesin receptor on primary macrophages, and determination of the minimal epitope on this protein needed for receptor interaction. **NPB Project #05-191.**

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Date Submitted: February 15, 2007

Abstract:

To identify potential PRRSV ligands that mediate virus binding with the macrophage-specific PRRSV receptor sialoadhesin, we constructed a soluble form of sialoadhesin. This soluble sialoadhesin consists of the first 4 IgG domains of Sn, fused to the human IgG1 Fc domain. The soluble sialoadhesin was excreted in the medium of transfected HEK-293T cells upon transfection of coding DNA. Further characterization revealed that the recombinant receptor had the same conformation as the native sialoadhesin and that it was processed similarly to the native sialoadhesin. The soluble sialoadhesin was also shown to be functional by red blood cell binding assays and in PRRSV infection blocking experiments. The soluble receptor was then used in a modified immunoprecipitation reaction. Soluble sialoadhesin was coated on protein A coated beads and mixed with a lysate of semi-purified virus. Afterwards, proteins bound to the soluble sialoadhesin were separated from non-bound proteins. Analysis of the obtained fractions on Western blot with PRRSV specific monoclonal antibodies revealed that the viral M-GP5 complex was bound to the soluble sialoadhesin, while GP4 and GP3 did not bind. Together, these results suggest that the M, or the GP5 protein mediate PRRSV attachment to sialoadhesin.

Introduction:

The Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is currently one of the most important diseases in swine, having devastating economical effects on both pig production and breeding. The major challenge to combat this disease is the development of a highly effective PRRSV (subunit) vaccine that is both effective against the heterogeneous array of circulating PRRSV isolates and safe for use in both production and breeding units. Current vaccines do not fulfill these requirements resulting in recurrent infections in vaccinated farms. For most viruses, subunit vaccines are developed on basis of the viral proteins (ligands) that interact with the receptors on target cells.

Antibodies that are directed towards these viral receptor-binding proteins neutralize the virus, since they block interaction with the cellular receptor, thereby blocking viral entry and subsequent infection. For PRRSV however, the viral protein that binds to the receptor on macrophages (sialoadhesin) has not yet been identified, and this lack of knowledge seriously hampers directed development of a new vaccine.

Objectives:

1. Construction of a soluble, Fc-tagged porcine sialoadhesin

This work package is necessary to be able to produce large amounts of recombinant sialoadhesin that will be needed in the other work packages. Since this form of the sialoadhesin is without transmembrane domain and thus soluble, it will be excreted in the medium and this will allow, together with the Fc tag, straightforward purification.

2. Small scale production and functional characterization of the soluble porcine sialoadhesin

A small amount of Fc-tagged soluble porcine sialoadhesin will first be purified and the functionality of this soluble form will be compared to that of the native sialoadhesin.

3. Intermediate scale production and purification of the Fc-tagged soluble sialoadhesin

During this part of the research, large amounts of the soluble sialoadhesin will be produced and purified.

4. Identification of the sialoadhesin-binding PRRSV protein using the Fc-tagged soluble sialoadhesin

By using the purified soluble sialoadhesin in a modified immunoprecipitation reaction, we will pick up the PRRSV protein that mediates virus attachment to the sialoadhesin receptor from a lysate of viral proteins.

Materials & Methods:

1. Construction of a soluble, Fc-tagged porcine sialoadhesin

The extracellular domain of porcine sialoadhesin was PCR amplified with gene-specific primers and Pfx polymerase (Invitrogen) from the porcine sialoadhesin cDNA we previously cloned and subcloned into the human IgG1-Fc containing vector pEE14-Fc, in frame with the Fc coding region. The presence of the Sn4D domain in the vector was confirmed by restriction digest analysis and sequencing and the obtained vector was designated pEE14-Sn4D-Fc. To obtain a non-sialic acid binding Sn4D-Fc protein, a point-mutation was introduced in the sialic acid binding domain (R_{97} to E) in the pEE14-Sn4D-Fc using the Quickchange site directed mutagenesis kit (Stratagene), resulting in pEE14-Sn4D_{RE}-Fc. The presence of the specific mutation and the absence of other non-specific mutations were confirmed by restriction digest analysis and sequencing.

2. Small scale production and functional characterization of the porcine sialoadhesin

pSn4D-Fc and pSn4D_{RE}-Fc were purified with standard protein A sepharose column chromatography from a supernatant of HEK-293T cells transfected with pEE14-Sn4D-Fc or pEE14-Sn4D_{RE}-Fc.
The amount of soluble protein in supernatant and after purification were determined with an an Fc-based Elisa.
The soluble sialoadhesins were characterized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel, after which the proteins were transferred to a PVDF membrane (Membrane Hybond-P, Amersham Biosciences) via Western Blotting. Membranes were blocked overnight in PBS + 0.1% Tween 20 + 5% skimmed milk. Detection was done by incubation of

the blot with HRP-labelled goat-anti-human Fc antibodies, followed by visualisation using ECL (Amersham Biosciences).
Biological activity of the soluble protein was determined using a red blood cell binding ELISA Previously, we have shown that porcine sialoadhesin, like mouse and human sialoadhesins specifically binds to sialic acids. This sialic-acid binding capacity is dependent on the correct confomation of sialoadhesin. ELISA plates were first coated with goat-anti-human IgG-Fc, followed by serial dilutions of Fc-tagged pSn4D-Fc or pSn4D$_{RE}$-Fc. Human RBC were then added, the plates were incubated for 30 min at room temperature and non bound RBC were removed from the plates by gentle washing. As a negative control, Fc-tagged soluble proteins were either treated with pSn specific mAb 41D3 or RBC were pretreated with neuraminidase to remove sialic acids. Quantitative analysis of RBC binding was based on the presence of large amounts of peroxidases in RBC. The ELISA plates were dried after RBC binding, fixed with methanol and a peroxide substrate (Substrate Reagent Pack; R&D systems) was added to the ELISA wells following the manufacturers recommendations, and the absorbance was measured at 450 nm (OD450) with a Thermo Labsystems Multiskan RC.
Effect of the purified Fc-tagged soluble sialoadhesin on PRRSV infection of macrophages – PRRSV will be purified by gradient ultracentrifugation as previously described, mixed with serial dilutions of purified soluble sialoadhesin and inoculated on primary alveolar macrophages. If PRRSV can bind to the soluble sialaodhesin, we expect to see a strong reduction or a full block of the infection.

3. Intermediate scale production and purification of the Fc-tagged soluble sialoadhesin

HEK-293T cells (cultivated in 24-well plates) were transfected with the pEE14-pSn4D-Fc or the pEE14-pSn4D$_{RE}$-Fc plasmid using calcium phosphate
Batch cultures of HEK-293T cells (40 flasks per week = 2 liters of supernatant) were transfected with calcium phosphate following standard techniques (Amersham Biosciences). Five days after transfection, soluble sialoadhesin was purified from the supernatant using standard protein A chromatography. Purified protein was dialyzed to PBS, the quantity was determined and the quality was checked using red blood cell binding assays as described in part 2, and finally the protein was stored at 4°C untill used.

4. Identification of the sialoadhesin-binding PRRSV protein using affinity chromatography with immobilized Fc-tagged soluble sialoadhesin

Viral proteins were solubilized from semipurified PRRSV LV by a 1 h incubation in Tris buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA) containing 1% NP-40 (BDH Chemicals Ltd., Poole, England) and insoluble material was pelleted at 10000 g for 30 min. 100 µl of Dynabeads protein A (Invitrogen) were coated with 25 µg Sn4D-Fc or Sn4D$_{RE}$-Fc and incubated with virus lysate at 37°C. After 90 min incubation, the unbound lysate fraction was collected, beads were washed 4 times with PBS and bound material was eluted with 0.1 M citrate buffer [pH 3.1]. Following addition of non-reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer to the unbound lysate fraction and the eluate, samples were boiled for 5 min and resolved on 12% SDS-PAGE, after which the proteins were transferred to a PVDF membrane (Membrane Hybond-P, Amersham Biosciences) via Western Blotting. Membranes were blocked overnight in PBS + 0.1% Tween 20 + 5% skimmed milk. Detection was done by subsequent incubations of the blot with monoclonal antibodies directed against the structural proteins of PRRSV LV, biotinylated sheep-anti-mouse polyclonal antibodies (GE Healthcare) and streptavidin-biotinylated HRP complex (GE Healthcare), followed by visualisation using ECL (Amersham Biosciences).

Results:

1. Construction of a soluble, Fc-tagged porcine sialoadhesin

The PRRSV receptor porcine sialoadhesin (pSn) is a type I transmembrane protein. To allow the production and purification of large amounts of pSn, a soluble (without transmembrane domain) pSn was made, consisting of the first 4 IgG domains of pSn, linked to the Fc part of human IgG (Sn4D-Fc). The first 4 domains were chosen since previous experiments with mouse Sn showed that the first 4 domains are capable of binding with sialic acids and since PRRSV attachment to pSn is most likely dependent on the interaction between sialic acid on PRRSV and the sialic acid binding domain of pSn, which is located in the first IgG domain. A schematic overview of porcine sialoadhesin and the soluble Sn4D-Fc is given in Figure 1. Due to the human IgG Fc domain, the soluble pSn (Sn4D-Fc) is expressed as a disulfide linked dimer, similarly to the structure of antibodies. A control, sialic acid binding mutant of the soluble sialoadhesin that contains a mutation in the sialic acid binding domain of sialoadhesin was generated by site-directed mutagenesis, resulting in pEE14-pSn4D$_{RE}$-Fc (Fig. 2).

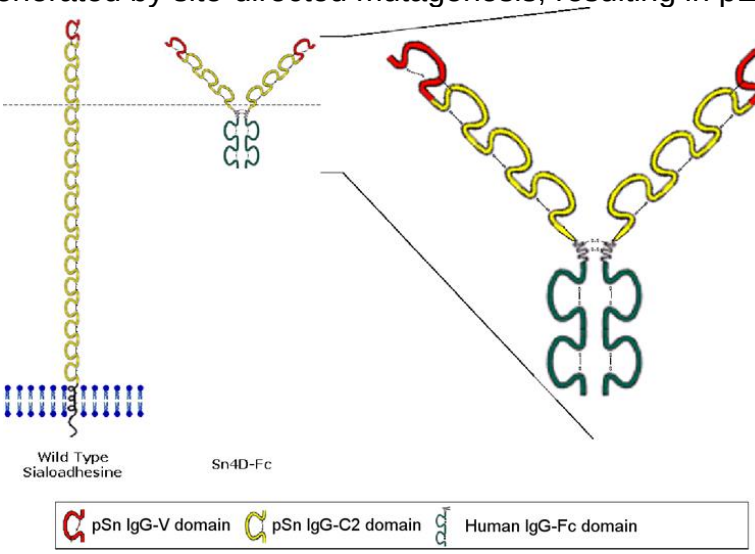

Figure 1. Schematic overview of porcine sialoadhesin (pSn) and the soluble, Fc-tagged sialoadhesin (Sn4D-Fc) that was made in the project.

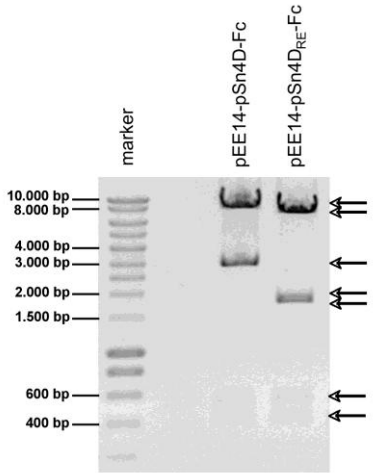

Figure 2. Restriction digest analysis of pEE14-pSn4D-Fc and pEE14-pSn4D$_{RE}$-Fc. Plasmid DNA was cleaved with BstBI and NgomIV and separated on a 1% agarose gel. The pEE14-pSn4D-Fc is only cleaved by the BstBI enzyme and shows 4 bands after cleavage. The point mutation introduced in the pEE14-pSn4D-Fc also introduced a restriction site for NgomIV and this results in 5 bands after cleavage, with two bands between 1500 and 2000 bp of approximately the same size.

2. Small scale production and functional characterization of the soluble porcine sialoadhesin

To evaluate functional expression of the soluble Fc-tagged pSn, HEK-293T cells were transfected with the pEE14-pSn4D-Fc or the pEE14-pSn4D$_{RE}$-Fc plasmid. As a positive control, cells were transfected with the pcDNA3.1/pSn plasmid, which contains the full length pSn. Cells were stained 24 h post transfection with FITC-labeled goat-anti-human IgG-Fc (Fc detection) and with pSn-specific mAb 41D3 in combination with TexasRed-labeled goat-anti-mouse (pSn detection) (Figure 3). From these results, we could conclude that both the Fc part and the sialoadhesin part were expressed in HEK-293T cells and that the conformation dependent epitope of pSn that is involved in PRRSV attachment and that is recognized by mAb 41D3 is still present in the soluble Sn constructs.

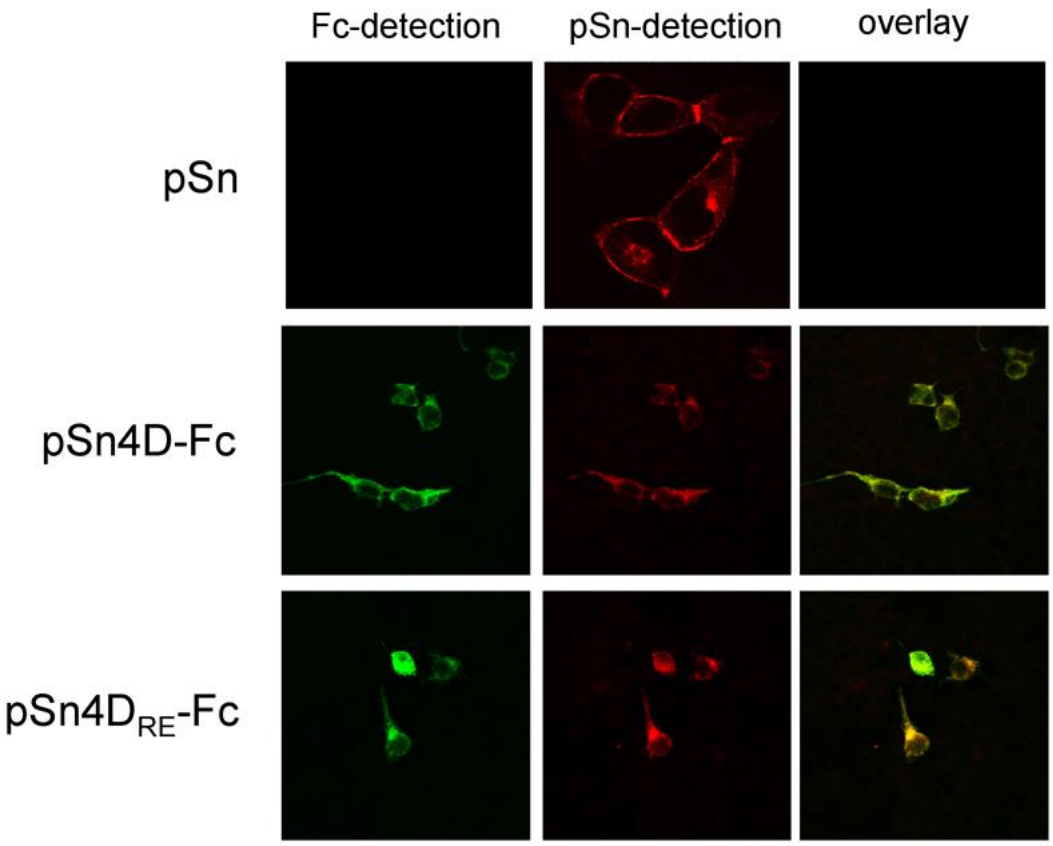

Figure 3. Immunofluorescence staining of pSn, pSn4D-Fc and pSn4D$_{RE}$-Fc transfected HEK-293T cells with FITC-labeled goat-anti-human IgG-Fc (Fc detection) and with pSn-specific mAb 41D3 in combination with Texas Red-labeled goat-anti-mouse (pSn detection). The overlay shows a superposition of both the green and red signal and results in a yellow color when the green and red signal co-localize.

To analyze if the soluble sialoadhesin was secreted in the medium, supernatant was collected at 2, 3, or 5 days post transfection and analyzed for the presence of Fc-tagged sialoadhesin with an Fc-ELISA. Maximal amounts of soluble protein were detected in the supernatant at 5 days post transfection for both constructs (ranging from 0.2 to 1 µg/ml supernatant; data not shown). Supernatants of pEE14-pSn4D-Fc transfected cells were also analyzed by SDS-PAGE and Western Immunoblotting with both the pSn-specific mAb 41D3 and anti-Fc antibodies, followed by detection with ECL (Fig. 4). In non-reducing conditions, a band of approximately 190 kDa was detected with both antibodies, while a band of approximately 80 kDa was detected in reducing conditions with the anti-Fc antibodies. Detection of the pSn4D-Fc with mAb 41D3 in reducing conditions was not possible since this mAb detects a conformational epitope that is sensitive to reducing conditions.

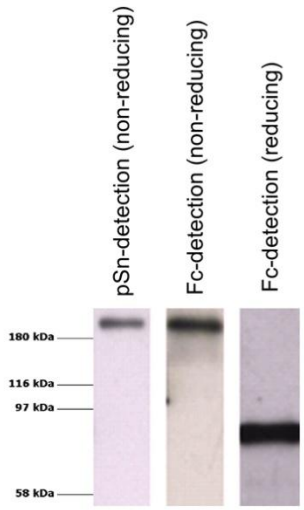

Figure 4. SDS-PAGE and Western Immunoblotting analysis of supernatant of pEE14-pSn4D-Fc transfected cells with the pSn-specific mAb 41D3 or anti-Fc antibodies, followed by detection with ECL.

For purification of both the soluble, Fc-tagged sialoadhesins, supernatants of transfected HEK-T cells were first clarified by high speed centrifugation and filtration through a 0.22µm pore filter. The soluble proteins were then purified from the cleared supernatants by their Fc tag using standard protein A sepharose chromatography which is used for purification for human antibodies. When the soluble Fc-tagged sialoadhesins were purified from supernatants containing FBS, some other, non-Fc tagged proteins were present in the purified fractions (Figure 5). Therefore, a modified transfection protocol was used to produce the recombinant proteins in medium without serum but with the serum substitute ITS (Insulin-Selenium-Transferrin supplement; Invitrogen). This modification of the protocol resulted in highly pure soluble, Fc-tagged proteins after protein A chromatography with a concentration of ± 30 µg/ml and ± 100 µg/ml for respectively pSn4D-Fc and pSn4D$_{RE}$-Fc (Figure 6).

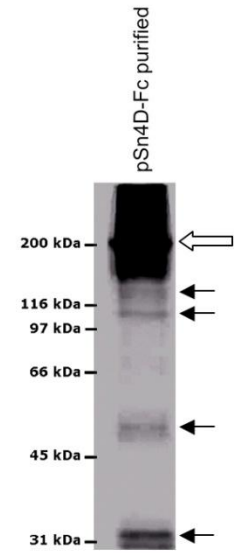

Figure 5. Coomassie blue analysis of pEE14-pSn4D-Fc purified from supernant containing fetal bovine serum. The open arrow indicates the location of the pSn4D-Fc protein, the black arrows indicate contaminating bands after purification.

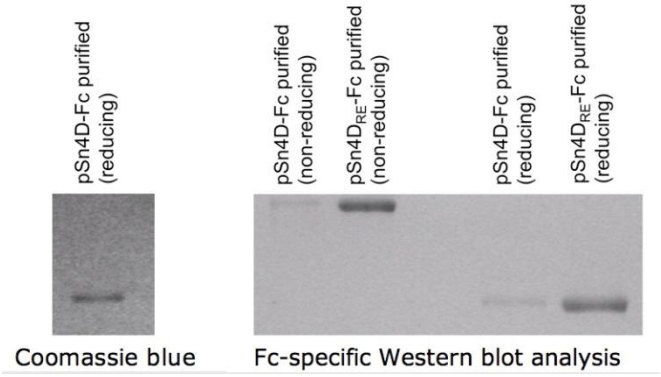

Figure 6. Coomassie blue and Western Immunoblotting analysis of pEE14-pSn4D-Fc and pEE14-pSn4D$_{RE}$-Fc purified from serum-free medium by protein A chromatography.

Purified pSn4D-Fc was also detected on Western Immunoblotting after treatment with various glycosidases to analyze intracellar processing (Figure 7). Treatment with N-glycosidase F, which removes all N-linked glycans, resulted in a clear shift of the size of the protein, showing that the protein contains N-linked glycosylations, similar to what we previously observed for full length porcine sialoadhesin. Since endoglycosidase H, which removes high mannose N-linked glycans, and neuraminidase, which removes sialic acids, had no effect on the size of pSn4D-Fc, it was concluded that the N-linked glycans were of the complex type that do not contain sialic acid.

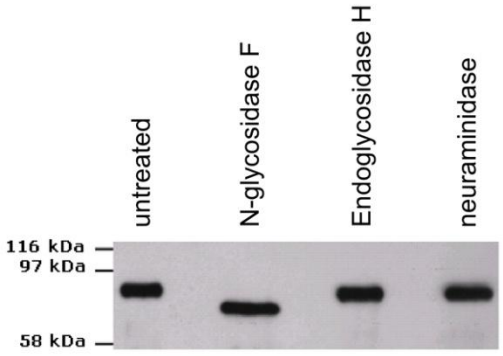

Figure 7. Western Immunoblotting analysis of pEE14-pSn4D-Fc after treatment with various glycosidases.

Previously, we have shown that porcine sialoadhesin, like mouse and human sialoadhesins specifically binds to sialic acids. This sialic-acid binding capacity, which is very much dependent on the correct conformation of sialoadhesin, was demonstrated by showing that sialoadhesin can bind to red blood cells that contain sialic acids on their cell surface. Purified pSn4D-Fc and pSn4D$_{RE}$-Fc were after purification analyzed for their functional sialic acid binding capacity using a solid phase red blood cell (RBC) binding assay. ELISA plates were first coated with goat-anti-human IgG-Fc, followed by serial dilutions of Fc-tagged pSn4D-Fc or pSn4D$_{RE}$-Fc. Human RBC were then added, the plates

were incubated for 30 min at room temperature and non bound RBC were removed from the plates by gentle washing. As a negative control, Fc-tagged soluble proteins were either treated with pSn specific mAb 41D3 or RBC were pretreated with neuraminidase to remove sialic acids. Qualitative analysis of RBC attachment using light microscopy showed strong RBC binding to both SiglecE-Fc and pSn4D-Fc coated ELISA wells (Figure 8), while neuraminidase treatment of RBC, which removes sialic acids, completely abolished binding. Pre-treatment of coated ELISA wells with pSn-specific mAb 41D3 blocked RBC binding to pSn4D-Fc coated wells, but had no effect on SiglecE-Fc coated wells. Quantitative analysis of RBC binding was based on the presence of large amounts of peroxidases in RBC. The ELISA plates were dried after RBC binding, fixed with methanol and a peroxide substrate (Substrate Reagent Pack; R&D systems) was added to the ELISA wells following the manufacturers recommendations, and the absorbance was measured at 450 nm (OD450) with a Thermo Labsystems Multiskan RC. Qualitative analysis confirmed the light microscopic analysis and showed that pSn4D-Fc bound RBC to similar levels as SiglecE-Fc (Figure 9). RBC attachment was not observed when RBC were treated with neuraminidase or when pSn4D-Fc was pretreated with pSn-specific mAb 41D3. RBC attachment to the $pSn4D_{RE}$-Fc was also not detected.

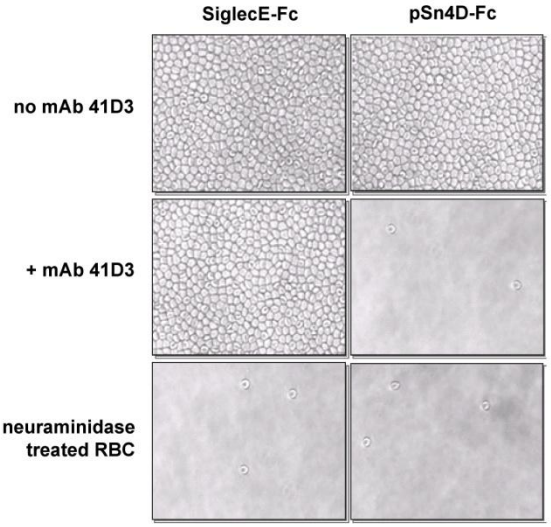

Figure 8. Analysis of human RBC binding to ELISA plates coated with Fc-tagged soluble proteins.

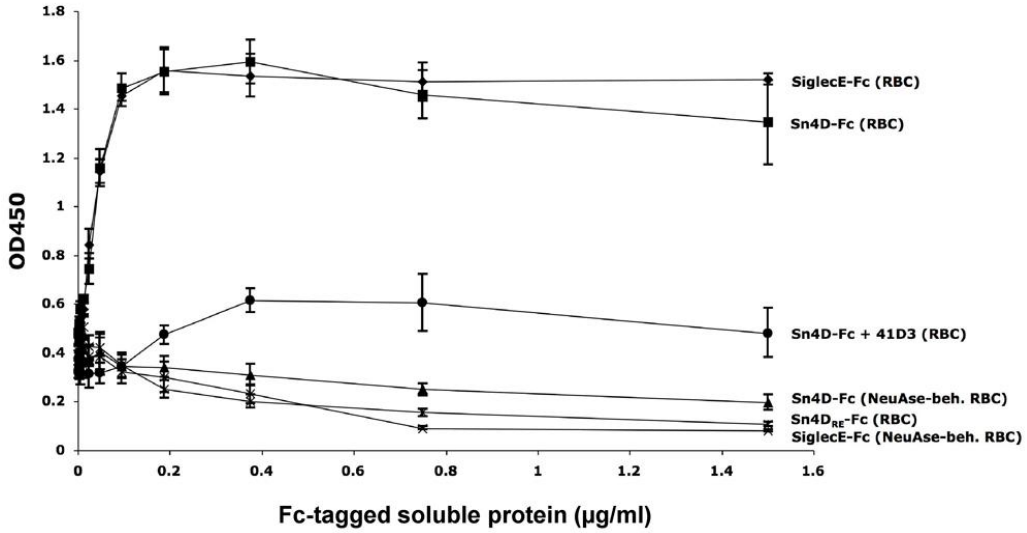

Figure 9. Quantitative analysis of human RBC binding to ELISA plates coated with Fc-tagged soluble proteins.

To analyze the effect of the purified Fc-tagged soluble sialoadhesin on PRRSV infection of macrophages, PRRSV was incubated with purified pSn4D-Fc in medium (30 µg/ml) for 1 h, or with medium alone, and the mixtures were inoculated on primary alveolar macrophages for 1 h, either at 4°C or at 37°C. The virus was then removed, fresh medium was added and the cells were further incubated at 37°C. Ten hours after inoculation, the cells were fixed and the number of infected cells was quantified after staining with nucleocapsid-specific mAb P3/27 and HRP-labeled goat anti-mouse. Incubation of PRRSV at 4°C or 37°C with pSn4D-Fc clearly reduced infection of macrophages (Figure 10), showing that pSn4D-Fc is capable of binding to PRRSV and thereby blocking infection of macrophages.

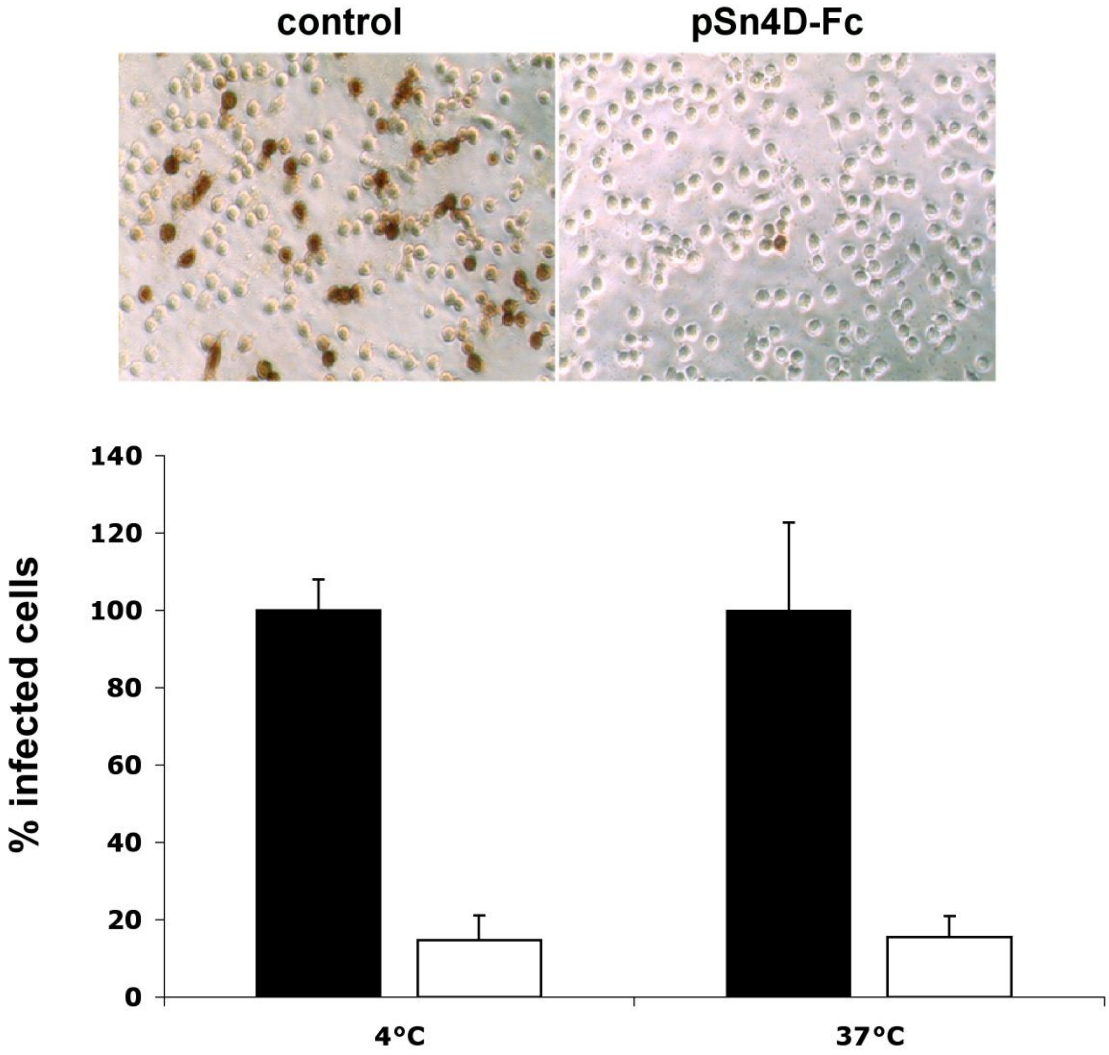

Figure 10. Effect of pSn4D-Fc on PRRSV infection. PRRSV was incubated with pSn4D-Fc (open bars) or PBS (black bars) for 1 h at 4°C or 37°C and then inoculated on macrophages for 1 h at 4°C or 37°C. Virus was then removed and the cells were further incubated for 10 h at 37°C, fixed and stained to detect infected macrophages with an immunoperoxidase staining with the nucleocapsid-specific mAb P3/27 and HRP-labeled goat-anti-mouse.

3. Intermediate scale production and purification of the Fc-tagged soluble sialoadhesin

During this part of the research, large amounts of the soluble sialoadhesin were produced by batch culture of transfected HEK-293T cells (2 liters per week). Soluble, Fc-tagged proteins were purified from 20 liters culture supernatant and used in part 4 of the project.

4. Identification of the sialoadhesin-binding PRRSV protein using the Fc-tagged soluble sialoadhesin

To identify viral proteins that bind with sialoadhesin, we used the soluble sialoadhesin in a modified immunoprecipitation reaction. Analysis of original, bound and non-bound fractions on Western blot with PRRSV specific monoclonal antibodies revealed that the viral M-GP5 complex was bound to the soluble sialoadhesin, while GP4 and GP3 did not bind. Together, these results suggest that the M, or the GP5 protein mediate PRRSV attachment to sialoadhesin.

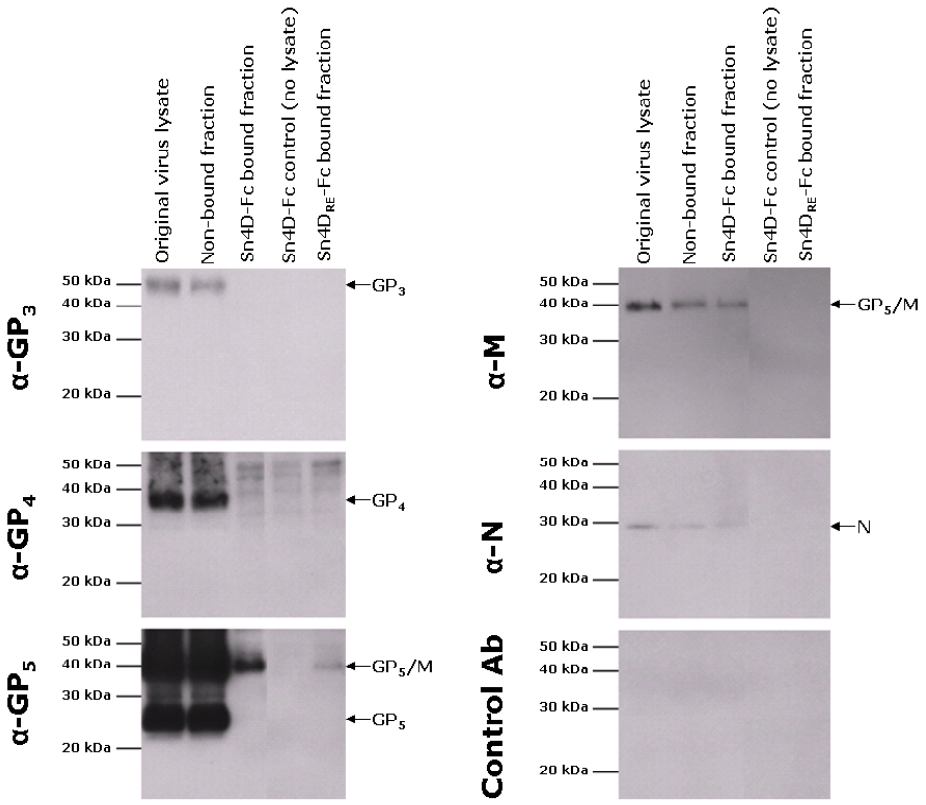

Figure 11. Identification of the viral protein(s) that bind with the sialoadhesin receptor. Protein A beads were coated with the soluble, Fc-tagged proteins and incubated with a PRRSV lysate for 90min at 37°C. The bound and non-bound fraction were collected and subjected to Western Blot analysis. Viral proteins were detected using virus-specific monoclonal antibodies

Discussion:

Natural infections with PRRSV are known not to induce a robust protective immune response and consequently persistent infections and re-infections of infection-immune pigs with genetically and antigenically differerent PRRSV isolates are frequently observed in the field. An abundant, strain-specific and non-protective PRRSV-specific antibody response can be detected starting from 5 to 9 days post-inoculation, but major variations have been observed in both the kinetics of appearance

and the levels of virus-neutralizing (VN) antibodies in individual pigs, ranging from pigs with VN antibodies detected only from 4 to 10 weeks after inoculation, to pigs that fail to develop detectable VN antibodies. Although VN antibodies are in general induced only at low levels and late in infection, several reports have described that these VN antibodies can confer protection towards PRRSV infection when they are present at sufficient high levels, indicating that a vaccine that is capable of inducing VN antibodies would confer protection towards PRRSV infection. Although PRRSV has now been studied for more than 15 years and several groups have tried to develop new vaccines, no current vaccine is both effective towards the broad array of genetically divergent PRRSV isolates that is currently circulating and safe to use in pregnant sows and boars. The major problem in developing new vaccines is the lack of fundamental knowledge on the entry process of PRRSV. Identification of the viral protein that mediates PRRSV attachment to porcine sialoadhesin, which we identified as the receptor on macrophages for both American and European PRRSV strains, will allow future development of an effective and safe subunit vaccine, one of the aims of the PRRS initiative.
We have identified a PRRSV protein complex, M-GP5, that binds to sialoadhesin, the macrophage-specific internalization receptor for this virus. Several reports have already shown that protection of pigs from PRRSV infection depends on the presence neutralizing antibodies that are mostly directed towards the GP5 protein. Together, these data suggest that GP5 is the viral ligand that mediates PRRSV attachment to sialoadhesin.

Lay Interpretation:

Protection of pigs towards PRRSV infection mainly depends on the presence of antibodies in the pig that block virus infection of macrophages. Those neutralizing antibodies are directed against the part of the virus that mediates attachment to the receptor sialoadhesin, which is used by the virus to enter the primary target cell, the macrophage. We have identified a viral protein that binds to sialoadhesin. Identification of the GP5 protein as the putative PRRSV ligand for sialoadhesin clearly opens perspectives for subunit vaccine development.

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http://www.vetvirology.ugent.be/research/prrs/prrs.html