RESEARCH REPORT

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SWINE HEALTH

Title: Structural characterization of the PRRSv glycan shield - **NPB #09-227**

Investigator: Michael P. Murtaugh

Institution: University of Minnesota

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Industry Summary: Knowing the composition and structure of PRRSV is an important prerequisite for development of immunological prevention and control strategies, just as knowing what a spark plug looks like is important when you need to replace it. The surface of a PRRSV virion is covered with a small amount of envelope proteins, and a large amount of carbohydrate sugars (glycan) that are attached to the proteins. The glycan shield is the primary structure encountered by antibodies, other host defense molecules, and the surface of permissive cells. Since the interaction of a virus and the host is based on interactions of molecular structures, it is important to know what these structures are, and to determine what roles they play. Here, we used several approaches to dissect the glycan shield of PRRSV and deduce the significance for interactions with cells that are permissive for infection. The glycans are primarily on the major envelope protein, GP5, and have complex structures dominated by terminal glucosamine, lactosamine and sialic acid sugars. Proteins that bind to these specific terminal sugars reduce viral infectivity. However, it appears that the interference may be due to steric hindrance, suggesting that the sugars themselves do not have a direct role in virus-host cell interactions.

Keywords: porcine reproductive and respiratory syndrome, swine, pig, pathogenesis, immunity

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For more information contact:

National Pork Board • PO Box 9114 • Des Moines, IA 50306 USA • 800-456-7675 • Fax: 515-223-2646 • pork.org

Scientific Abstract: The infectious virion of porcine reproductive and respiratory syndrome virus (PRRSV) consists of a protein-encapsidated RNA, enveloped in a lipid bilayer membrane studded with glycosylated proteins and nonglycosylated matrix protein. The surface of the virion is comprised primarily of protein-linked carbohydrates (glycans) since very little amino acid sequence projects out of the lipid envelope. The abundance of glycans on the virion surface suggests they may have important roles in virus-cell interactions, infection of permissive cells, and as targets of viral neutralization. Thus, we characterized the glycan structures by physical, biochemical and cell biological methods. The glycans of highly purified PRRSV virions are located primarily on major envelope glycoprotein 5 (GP5), are structurally complex, and contain terminal sugars that are primarily sialic acid, glucosamine and lactosamine. Pokeweed mitogen (PWM) and *Lycopersicon esculentum* agglutinin (LEA), lectins that have a specific affinity for N-acetyl-β-D-glucosamine (GlcNAc) oligomers, bound to virions and decreased PRRSV infection of porcine macrophages and MARC-145 cells in a dose-dependent manner. Poly(GlcNAc)-lectins were slightly less inhibitory than lectins specific for sialic acids, but co-operated to inhibit infection to a higher level. The mechanism of inhibition may have been due to interference with protein-protein interactions, since enzymatic removal of glycans on the viral envelope did not alter viral infectivity. These findings not only advance the current knowledge of the molecular mechanisms of PRRSV infection in permissive cells but also provide an improved understanding of PRRSV structure that will aid in design of improved vaccines.

Introduction: Infection of permissive cells by viruses occurs through molecular interactions between protein and carbohydrate structures on the interacting surfaces. Glycoproteins in the membrane of enveloped viruses commonly are the key viral structure mediating infection. However, in arteriviruses, including porcine reproductive and respiratory syndrome virus (PRRSV), structural determinants of viral infectivity have not been elucidated definitively. The interaction of PRRSV with its host cell, the porcine macrophage is poorly understood, thus severely limiting our understanding of pathogenic processes in a critically important disease agent of swine. Although envelop glycoprotein 5 (GP5) is generally assumed to be essential for infection in the Arteriviridae, genetic and structural evidence is not strongly supportive. In particular, cryo-electron microscopic analysis indicates a paucity of protein structure on the virion surface. By contrast, complex carbohydrate

structures are predicted to decorate ectodomain portions of envelope proteins at high mass ratios. Thus, it is likely that permissive cells encounter a “glycan shield” on the spherical virions.

PRRSV is highly host restricted, growing in vivo in cells of the monocyte/macrophage lineage. In vitro, PRRSV grows in primary cultures of PAM, as well as in MA-104, the African green monkey kidney epithelial-like cell line, and its derivative, MARC-145 cells, the latter have been routinely used for in vitro propagation of wild-type and vaccine strains. Our goal is to understand the structural basis of virus tropism and the role of carbohydrates. Here, we used structural, biochemical and cell biologic approaches to characterize glycan structures on the virion surface and the role they play in permissive cell infection.

Objectives:

1. Determination of the types of glycans that are present on PRRSv strains with different predicted GP5 glycosylation patterns. The research question is: What is the effect of predicted GP5 glycosylation pattern on glycan composition of the PRRSV virion? The intended outcome is basic knowledge of all possible glycan types on PRRSv virions. The results will also indicate the amount of variation in structural composition of each type, which will help to focus the work in objective 2.
2. Characterization of the sugar composition and molecular structure of individual glycans in virus strains with different predicted GP5 glycosylation patterns. The research question is: What is the effect of predicted glycosylation pattern on the molecular characteristics of virion glycans? The intended outcome is basic knowledge of specific glycan structures and their variation in genetically diverse PRRSv isolates that may help explain variation in cross-protective immunity.

Materials & Methods: *Viruses.* The PRRSV isolate VR-2332 (GenBank number PRU87392) was propagated in MARC 145 cells maintained in EMEM medium (Invitrogen Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS). Cells were infected and incubated at 37°C, 5% CO_2. Infected cell supernatant was harvested at 48 h after infection, before cytopathic effect was observed. Cellular debris was removed by centrifugation at 17,000 x g for 1 h, and supernatants were mixed with 10% polyethylene glycol-8000 (Fisher Scientific, Fair Lawn NJ) at 4°C overnight. Virus was pelleted at 22,000 x g for 2 h, resuspended in Tris-buffered saline (TBS), and pelleted twice through a 0.5 M sucrose (Fisher Scientific) cushion at 110,000 x g for

3 h. The final pellet was re-suspended in a 20% iodixanol (Sigma-Aldrich, St. Louis, MO) solution in TBS and banded by ultracentrifugation at 250,000 x g for 9 h to achieve self-generating gradient. The purified virus band was removed with a sterile needle and stored in aliquots at -80°C.

Infection assays. MARC 145 cells or alveolar macrophages cultured under standard conditions in 6-well plates were incubated with 10^6 $TCID_{50}$/ml PRRSV for1 hour, washed with warm saline, and cultured for 24 hr. Infection was determined by qRT-PCR of culture media, and FACS analysis of cells. Cells were washed, trypsinized, fixed, permeabilized and stained for 1 h with fluorescein-labeled SR30 anti-nucleocapsid monoclonal antibody (Rural Technologies, Brookings SD) and analyzed. For lectin blocking experiments, virus preparations were incubated with lectins for 1 hour before addition to cells.

Biochemical procedures. Endoglycosidase digestion with N-glycosidase F (PNGase), and Endo-β-N-acetylglucosaminidase H (endoH) was performed at 37°C for 1 h. For determination of glycan composition, purified virus was resolved on SDS polyacrylamide gels and the GP5 band was excised. Glycans were released with PNGase and purified on a Sep Pak C18 column. Eluted glycans were permethylated to protect sialic acid residues, and analyzed by LC-MS/MS mass spectrometry. The masses of all released glycans, represented by peaks of mass and charge ratio were used to determine the oligosaccharide structures based on arithmetic computation from the known masses of mannose, galactose, N-acetylglucosamine, fucose, sialic acid and other sugars. Mass spectrometry procedures were performed in the laboratory of Ron Orlando, Complex Carbohydrate Research Center, University of Georgia, Athens.

Results: *Glycan characterization*. Purified PRRSV was digested with increasing concentrations of PNGase or EndoH and fractionated on SDS polyacrylamide gels. The appearance of bands was substantially unchanged in samples digested with EndoH. In samples digested with PNGase, however, a band corresponding to glycosylated GP5 disappeared, and a new band appeared at about 18kD, corresponding to the predicted molecular weight of mature, nonglycosylated GP5. Based on this result, showing that the glycan shield was comprised primarily, if not wholly, by N-linked carbohydrate structures on GP5, purified virions were fractionated on SDS polyacrylamide gels and the GP5 band was isolated for determination of glycan carbohydrate composition by mass spectrometry.

Fifty-eight potential N-glycan structures were determined of which 47 were complex type, eight were hybrid type, and 3 were high-mannose type. The terminal sugars present on glycan structures are most important for molecular interactions. Among the 47 potential complex type glycan structures, 20 contained 3 to 5 N-acetyl glucosamine units, 22 contained terminal sialic acid, and 8 contained 2 to 4 N-acetyl lactosamine units. Terminal sialic acid residues had been predicted previously, but the potential involvement of N-acetyl glucosamine and N-acetyl lactosamine oligomers was novel and suggested that additional carbohydrate binding specificities might be important in PRRSV infection of host cells.

Lectin binding to virus. The preceding results suggested that lectins specific for glycans displaying multiple terminal copies of N-acetyl glucosamine or N-acetyl lactosamine would bind to PRRSV. To test this possibility, Sepharose beads coated with lectins of various specificities were incubated with PRRSV VR2332, then collected by centrifugation. Bound PRRSV was quantified by qRT-PCR. The results showed that Pokeweed mitogen (PWM) and *Lycopersicon esculentum* lectin (LEA) specifically bound PRRSV virions, whereas uncoated beads did not. These two lectins are specific for N-acetyl glucosamine oligomers.

Lectin inhibition of viral infectivity. To determine if lectin binding inhibited viral infection, lectins were incubated with PRRSV then used to infect cells. PMW and LEA significantly inhibited PRRSV binding to MARC 145 cells in a dose-dependent manner, and also blocked viral infection of macrophages and MARC 145 cells, with inhibition of infection being greater for macrophages than for MARC 145 cells. Interestingly, order of incubation of lectin with virus first, then addition to cells, versus incubation of lectin with cells followed by addition of virus, gave results that depended on the host cell used. Addition of lectin to cells first gave greater inhibition on macrophages, whereas incubation of virus with lectin first gave greater inhibition on MARC 145 cells. To further investigate the role of glycans in viral infection, experiments were carried out on MARC 145 cells to determine if N-acetyl glucosamine dimers and trimers that mimic the oligomeric termini of GP5 glycans competed for binding to PWM and thus suppressed PWM inhibition of PRRSV infectivity. There was no effect of dimeric or trimeric N-acetyl glucosamine on PWM inhibition of viral infectivity under any condition tested. Similarly, co-incubation of dimers or trimers and virus on MARC 145 cells showed no effect on viral

infectivity. In various experiments there was either no effect or a dose-dependent increase in the percentage of infected MARC 145 cells.

Effect of endoglycosidases on infectivity. Treatment of PRRSV VR2332 with N-acetylglucosaminidase, which specifically removes terminal N-acetyl glucosamine, did not reduce the level of infection of MARC 145 cells. However, incubation of cells with enzyme prior to addition of virus significantly reduced the rate of infection. The endoglycosidases PNGase and EndoH, which cleave broader ranges of N-linked glycans, also did not reduce infectivity of PRRSV on MARC 145 cells when incubated with virus or with cells. In the case of alveolar macrophages, the general endoglycosidases moderately reduced infection when added to virus or to cells. Importantly, the effects that were observed were due to the treatments themselves. Controls included incubation of virus at 37°C in the absence of enzyme to account for the possibility of temperature-dependent inactivation of PRRSV. The incubation conditions at 37°C reduced viral titers insignificantly at 1 h, although longer incubation times substantially reduced infectivity.

Modification of infected cells with glycan-modifying drug. The preceding studies do not provide a mechanistic model for the role of glycans, if any, in PRRSV infection of macrophages and MARC 145 cells. To help determine if the presence of complex glycan structures on PRRSV, regardless of the exact composition containing sialic acid, N-acetyl glucosamines or N-acetyl lactosamines, contributed importantly to the infectivity of PRRSV, host MARC 145 cells were cultured in the presence of swainsonine, a drug that inhibits glycoside hydrolases, resulting in the production of hybrid-type glycans instead of complex glycans. Therefore, it was expected that a role for complex glycans in PRRSV infectivity would result in reduced growth if absence of complex glycans on virus early in the growth phase reduced subsequent infection rates. However, there was no difference in progeny virus secreted into the media at 6, 12, 24, 48 and 72 hr from cultures containing no swainsonine or drug at 1 or 5 ug/ml.

Results with MN184 strain of PRRSV. Experiments proposed in the project proposal comparing glycan structures on strains VR2332 and MN184 were not completed due to the unexpectedly intense investigations that were needed to understand and explain the results of infection inhibition experiments using lectins, N-

acetyl glucosamine oligomers, endoglycosidases, and swainsonine. These studies raise significant issues discussed below that need to be resolved before comparative studies are initiated.

Additional results information. Structural analysis of N-linked glycans on GP5 were carried out in collaboration with the Ron Orlando laboratory at the University of Georgia in two visits approximately 11 months apart. The data reported here were obtained in the first visit, in which the range of potential glycan structures, containing terminal sialic acids, N-acetyl glucosamines, and N-acetyl lactosamines, was determined. This information facilitated the development of numerous hypotheses that were addressed in subsequent experiments that helped elucidate the role of the glycan shield in PRRSV interaction with permissive cells. A second visit to the Orlando laboratory, in May, 2011, was for the purpose of specifically determining the range of glycan structures and the extent of glycosylation on each of the candidate N-linked glycosylation sites on VR2332. We expected that the findings would help explain how common glycosylation actually was at each site and if there were correlations with lectin binding, viral binding to cells, and infection. The experiments necessary for success involved large scale virus purification, preparative isolation of GP5, digestions with nonstandard proteinases required to isolate peptides containing each putative glycosylation site, and MALDI-TOF mass spectrometric identification of glycans on individual peptides as well as the frequency of glycosylation at each site. Virus purification and protein isolation were accomplished, but we failed to recover peptides following proteolytic digestion in the Orlando laboratory or to identify glycan structures. Detailed post-mortem analysis revealed possible explanations and provided opportunities for repeated the study so that a successful outcome can be achieved.

Discussion: The key findings of our investigation were that the glycan shield of PRRSV is composed of complex carbohydrates decorating GP5 that contain, in addition to terminal sialic acid, terminal N-acetylglucosamine and N-acetyllactosamine. These findings were confirmed by independent methods. Subsequent experiments were unable to unequivocally establish that the glycans directly facilitated infection of permissive cells, since various methods to remove or eliminate glycans on intact virions did not reduce infectivity. Thus, the results were consistent with the possibility that binding of lectins to the surface of virions caused steric hindrance that interfered with viral-host cell interactions. The implication of this finding may be

substantial. If glycans are not intimately involved in PRRSV infection, then the current multi-step model of the infectious process is unnecessarily complicated and may slow progress in elucidation of the key molecular events that could be targeted for pharmacological or immunological intervention. Importantly, if glycans are only peripheral to viral infection, then GP5 might not play a central role in mediating infection. Such a possibility has been raised now that strong evidence has been presented that a multimeric protein structure containing GP2, GP3, and GP4 is required for infection of permissive cells. If true, then the substantial investment of research funds in development of new vaccines that is focused on GP5 may be unlikely to yield a benefit.