Title: Physical Characterization of the PRRSV Virion - **NPB #05-202**

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Date Submitted: March 23, 2007

Abstract

Current research strategies for development of better methods of PRRSV diagnosis, prevention and control are based primarily on the assumption that PRRSV behaves in pigs like related viruses behave in their own host species. However, PRRSV does not behave like other viral diseases. The 4-5 week course of acute, viremic infection and the months-long persistence in lymphoid tissues are exceptional. Here, we expanded on a prior NPB project to further investigate the molecular composition of the PRRSV virion to better understand the nature of viral interaction with host cell membranes and neutralizing antibodies. We determined that PRRSV is secreted from infected cells, both porcine macrophages and simian MA-104 cells, within 48 hr as a single population with respect to density, infectivity, RNA composition, and protein profile. Direct assessment of viral protein composition by two independent direct physical methods (mass spectrometry) indicated the presence of nucleocapsid (N), membrane protein (M), and envelope glycoproteins (GP) 5, 3, 2a, and 2b. We did not detect GP4 but detected a novel open reading frame peptide within GP5. We established the specific amino acid sequences of the mature forms of GP5, M and N and discovered that GP5 exists in a number of distinct glycosylation states. Host cell proteins also were identified in the virion. Growth characteristics of PRRSV were similar in macrophages and MA-104 cells. The results will help us determine the effect of GP5 glycosylation on antibody neutralization, a key concept in anti-viral immunity, and will provide a better foundation for development of effective vaccines.

These research results were submitted in fulfillment of checkoff funded research projects. This report is published directly as submitted by the project's principal investigator. This report has not been peer reviewed

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III. Introduction

Current research strategies for development of better methods of PRRSV diagnosis, prevention and control are based primarily on the assumption that PRRSV behaves in pigs like related viruses behave in their own host species, and that the pig immune response to PRRSV is fundamentally the same as a mouse or human immune response to viral infection. However, PRRSV does not behave like many other viral diseases of pig. The 4-5 week course of acute, viremic infection and the months-long persistence in lymphoid tissues are exceptional. Therefore, a better approach to understanding and solving the problem of PRRS in pigs requires concrete knowledge about the virus. Here, we determined that GP5 and M are major viral proteins that comprise the virion, that GP5 exists in several distinct glycosylated forms, that GP2 and GP3 are minor viral proteins, and GP4 is not present in the virion. Thus, contrary to one published report, the viral structure that binds cells to initiate infection is not a trimer of GP2, GP3 and GP4. We currently are determining if glycosylation of GP5 affects infectivity and antibody neutralization of the virion.

IV. Objectives

Objective 1. To determine the relationship between protein composition of PRRSV virions and viral infectivity. Research Question 1. What are the similarities and differences in protein structure of the virions of highly and lowly infective viral preparations?

Objective 2. To determine the effect of virus strain on protein composition of the virion. Research Question 2. What are the differences in protein composition of virions of North American PRRSV strains that give virion subpopulations of different physical densities?

Objective 3. Determine the effect of host cell on protein composition of the virion. Research Question 3. Are the virions produced in MA-104 cells the same as virions produced in porcine macrophages?

V. Materials & Methods

PRRSV strains VR2332 and JA142 were grown on MA-104 cells to titers of 10^6-10^7. These lab-adapted strains grew poorly on porcine alveolar macrophages. Cell supernatants collected at 48 hours after infection were concentrated by pelleting two times through a sucrose cushion, and banding by density gradient centrifugation in cesium chloride. Purified virus, visible as a opalescent blue band on white light illumination, was recovered,

and dialyzed against saline.

Viral proteins were separation by gel electrophoresis and subjected to mass spectrophotometry analysis directly or after trypsin digestion. The methods for gel electrophoresis, in-gel protease digestion, and mass spectrometry (including ion trap, MALDI-TOF, ESI quadrupole TOF) are described and detailed protocols are available through the U of MN MSCLS website (http://www.cbs.umn.edu/mass_spec/). Resulting molecular weights were compared to a custom database of all possible viral proteins to putatively identify the presence of individual viral proteins in virions.

Electron microscopy was performed on MA-104 cell or alveolar macrophage monolayers at 12-18 hr after infection. Cells were fixed, embedded, sectioned, stained and viewed in a Phillips CM12 electron microscope. Purified virus preparations were coated on carbon-coated grids, fixed in glutaraldehyde, negative stained and viewed in the Phillips CM12 electron microscope.

Immunofluorescence was performed on infected monolayers of macrophages or MA-104 cells with SDOW17 monoclonal antibody or affinity purified porcine antibodies to PRRSV nsp2, GP5, and M.

VI. Results

Objective 1. To determine the relationship between protein composition of PRRSV virions and viral infectivity. This question arose from observations that virus bands observed at various densities in a sucrose gradient also varied in infectivity. We eventually determined through further purification of virus preparations on cesium chloride gradients, electron microscopy, analysis of cells and culture media of infected cells, and examination of cell cultures at various times after infection that there is only one form of the virus and contains all of the infectivity of the sample. Virus is secreted from the cell after its assembly, so that very little infectivity is cell-associated at any time. Cultures that are left for more than two days accumulate significant proportions of viral capsid, without and envelope, that appears as a more dense band on cesium chloride gradients but is not infectious. Thus, intact PRRSV virions are complete with capsid and envelope and are infectious. Incomplete virions with a distinctly higher density, containing RNA as determined by RT-PCR, and only nucleocapsid protein as determined by SDS gel electrophoresis, appear after cells begin to lyse and are noninfectious. This indicates that incomplete viral capsids without envelopes are being released into the media. There was no

evidence of defective interfering virus or unusual heteroclite virions, although heteroclite RNA was present in infectious virions.

Objective 2. To determine the effect of virus strain on protein composition of the virion. Since we discovered that there were no viral subpopulations with different physical densities, the original rationale of this objective was rendered moot. However, since there is genotypic and pathogenic variation in PRRSV strains, we examined the protein composition of two strains, VR2332 and JA142 by to mass spectrophotometric methods. Cesium chloride-purified viruses were separated on SDS acrylamide gels, bands excised, and digested with trypsin; or proteins were separated by liquid chromatography, and labeled with I-TRAQ reagents (Applied Biosystems). The resulting fragments were subjected to mass spectrometry for direct identification of viral proteins in the virion. Conclusive presence of predicted structural proteins N, M, GP5, GP3, GP2a and GP2b was demonstrated. GP4 was not present. Interestingly, no peptides were observed in the N-terminal half of the putative GP5 and M. These sequences are predicted to encode the host cell attachment domain and the primary antibody neutralization domain blocking viral infectivity. Thus, viral proteins were separated on SDS gels and individual bands were cut out and submitted to the Mayo Clinic for N-terminal amino acid sequencing. These results showed conclusively that mature GP5 begins at amino acid 32 (serine). A predicted glycosylation site at amino acid 30, hence, is not present in the protein. In addition, M and N were both shown to begin at amino acid 2. Neither contains in the mature form the initiator methionine. It presumably is removed by a cellular aminopeptidase which carries out this function on a wide variety of cellular proteins.

Objective 3. Determine the effect of host cell on protein composition of the virion. At this point, we had determined that PRRSV is monomorphic and that there appears to be no effect of host cell on structural characteristics of the virus. However, mass spectrometry consistently identified about 8 host cell proteins associated with highly purified virions. They included poly A binding protein, annexins A2 and VI, apolipoprotein A1, heat shock proteins 70 and 90α, and moesin.

VII. Discussion

Previous research indicated that PRRSV separates as three bands of different densities in sucrose gradients, suggesting that a variety of forms are produced in infection (Yuan et al. 2004). Our results indicate that only a

single viral species is produced in infected cells, and accounts for all of the infectivity of MA-104 cells infected in vitro. Virions purified on a cesium chloride density gradient are all the same, and contain GP2b and five of the six classically predicted structural proteins, excepting only GP4 (Wu et al. 2005). GP2b had not been observed at the time of the final report of the previously funded project, NPB 04-122. Electron microscopy confirmed that purified virions, which contain all of the infectivity of an infected cell monolayer, are monomorphic. Thus all of the evidence to date indicates that PRRSV infection yields infectious particles of a single type, contrary to a previous report (Yuan et al. 2004). An unexpected observation was the lack of GP4 in purified virions. The protein is predicted to form a trimeric structure with GP2a and GP3 that is required for viral infectivity (Wissink et al. 2005). Our data suggest that presence of a trimer is impossible, since GP2a and GP3 peptides were detected reproducibly, and there was no evidence from any replicate of GP4. Establishment of the exact N-terminus of mature GP5, starting at amino acid 32, will provide a solid foundation for design of peptides and other molecular probes intended to explore the role of GP5 in immunological reactions and infection processes. Lastly, the host cell proteins associated with viral particles include moesin, a plasma membrane protein involved in a wide variety of cellular interactions, poly A binding protein, which presumably binds the poly A tail of the viral RNA genome, and two heat shock proteins, which may be important chaperones in viral assembly. Identification of cellular components of the PRRSV virion provides candidate targets for treatment or prevention strategies targeted to disruption of viral growth in pigs.

References:

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VIII. Lay Interpretation

The research here shows that PRRSV grows in cells as a population of viruses with the same shape, the same RNA molecules, and the same protein composition. There was no evidence for multiple forms that varied in infectivity or in other properties. This finding implies that variation in the behavior of PRRSV isolates in the laboratory is primarily determined by the viral genetic material rather than external factors like culture conditions. It also means that instrinsic features of PRRSV, such as protein composition of the virion, are probably shared by all viruses regardless of biological differences in virulence or immune reactivity. One possible exception would be glycosylation patterns, which we are beginning to study. Our data are also the first direct demonstration of the protein composition of the virion. This information is essential to understanding the antigens that are present on the surface of the PRRSV that might contribute to viral neutralization and to cellular infection. This information is needed because the current model of viral neutralization and immune response, i.e. antibodies to GP5 neutralize the virus and are key to control of infection and resistance to challenge, have so far not been helpful in the development of effective strategies for controlling PRRS in swine herds.

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