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A nonstructural and antigenic glycoprotein is encoded by ORF3 of the IAF-Klop strain of porcine reproductive and respiratory syndrome virus

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Summary. Open reading frame 3 (ORF3) of the genome of porcine reproductive and respiratory syndrome virus (PRRSV), Quebec strain IAF-Klop, was reverse-transcribed and cloned into the procaryotic expression vector pGEX-4T-1, then subcloned into the eucaryotic expression vector pAdCMV5 which was used as a shuttle vector to generate a replication-defective recombinant adenovirus. The procaryotic GST-ORF3 recombinant fusion protein was used to raise a monospecific antiserum in rabbits. By Western-immunoblotting with PRRSV-infected cell extracts, the ORF3 encoded protein had an estimated molecular mass (M_r) of 42 kDa, similar to that of the protein expressed by the adenovirus vector. Endoglycosidase F digestion showed that the ORF3 encoded protein occurs in an highly glycosylated form (GP_3) in the infected MARC-145 cells. Pulse-chase and radioimmunoprecipitation experiments revealed that the GP_3 protein was present in amounts equivalent to those of the N, M, and GP_5 proteins in the infected cells, whereas no GP_3 could be detected in purified virions. During the first 30 min of chase, the GP_3 undergoes a gradual downward shift of its apparent M_r, thought to result from trimming of the mannose-rich glycan structures. Tested convalescent pig sera that were found to be seropositive to PRRSV by indirect immunofluorescence reacted positively with the recombinant GST-ORF3 fusion protein by immunoblotting. Data indicated that the ORF3 protein of the Quebec reference strain of PRRSV is a highly glycosylated and antigenic protein, which is nonstructural.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is responsible for reproductive failure in sows and gilts (late-term abortions, increased numbers of stillborns, mummified and weak-born pigs), and respiratory problems in pigs of all ages [12]. PRRSV is a member of a new group of small enveloped positive-strand RNA viruses, presently classified within the family *Arteriviridae*, order *Nidovirales*, which also includes equine arteritis virus (EAV), simian hemorrhagic fever virus (SHFV), and lactate dehydrogenase elevating virus (LDV) [3, 6]. The viral genome is approximately 15 Kb in length and contains eight open reading frames (ORFs) which are transcribed in the cells as a nested set of subgenomic mRNAs [25, 26]. The major structural proteins consist of a 25 kDa envelope glycoprotein (GP_5), an 18–19 kDa unglycosylated membrane protein (M), and a 15 kDa nucleocapsid (N) protein, encoded by ORFs 5, 6 and 7, respectively [21, 22, 27]. Recent findings on characterization of the structural proteins of Lelystad virus (LV), the European prototype strain of PRRSV, indicate that expression products of the ORFs 2 and 4 are also incorporated in virus particles as additional minor membrane-associated glycoproteins designated as GP_2 and GP_4, respectively [28, 35]. The ORF3 product of LV has been also characterized as a highly glycosylated structural protein (GP_3) of the virion [35, 37]. However, it is noteworthy that i) the ORF3 product of LDV has been recently characterized as a nonstructural protein [8], and that ii) the ORF3 of North American strains of PRRSV has been reported to encode for a highly glycosylated protein (6 potential-linked glycosylated sites) that presents less than 55% amino acid (aa) sequence identity with that of its European counterpart and lacks at least 10 aa residues at its C-terminal region [16, 21]. The latter region of the GP_3 is hypervariable and carries epitopes responsible for antigenic differences between European and North American strains [7, 16]. Further, there is no evidence that the homologous protein of EAV is structural [6].

In the present study, the nature of the ORF3 encoded-product of a Quebec reference strain of PRRSV was further investigated. A monospecific antiserum was raised in rabbits against recombinant *E. coli*-expressed glutathione S-transferase (GST)-ORF3 fusion protein which was used in immunoblotting and radioimmunoprecipitation (RIPA) experiments with both PRRSV-infected cell extracts and purified extracellular virus. A replication-defective recombinant adenovirus was also constructed to confirm the nature of the ORF3 encoded-product. Data indicated that the ORF3 of the Quebec reference strain encodes a 42 kDa glycosylated and antigenic protein, which is not incorporated into the viral particles.

Materials and methods

Viruses and cells

The Quebec cytopathic strain IAF-Klop of PRRSV was propagated in MARC-145 cells, a clone of MA-104 cells highly permissive to PRRSV [17]. Sequencing analyses of the ORFs 3 to 7 of the Quebec isolate revealed a close genomic relationship with reference North American strains [9, 21]. Ad/CMVlacZ, a replication-defective E1- and E3-deleted human adenovirus [1], as well as the AdCMV5/ORF3 recombinant adenovirus, were propagated in

293 cells (ATCC CRL-1573), an adenovirus-transformed human embryonic cell line [14]. COS-7 cells (ATCC CRL-1651) were also used for transient expression experiments.

Cloning and procaryotic expression of the ORF3 gene

Viral genomic RNA was extracted from infected MARC-145 cells by the one-step guanidinium isothiocyanate-acid phenol method [4]. The ORF3 gene was then reverse-transcribed and amplified by polymerase chain reaction (PCR) using the following primers: ETS3 (5′-ATGGCTAATAGCCGTACA-3′) which comprises the first ATG codon of the ORF3 gene and, ETR3 (5′-CTATCGCCGTGCGGCACT-3′) which includes the C-terminal stop codon of the viral gene. The PCR amplified product was purified using the Geneclean II nucleic acid purification kit (BIO 101, LaJolla, CA) then, cloned into the pGEX-4T-1 (Pharmacia) plasmid vector to generate the synthesis of GST-ORF3 recombinant fusion protein in BL21 (DE3)-*Escherichia coli* cells (Novagen), as previously described [22].

Monospecific antisera to PRRSV individual proteins

For the production of a monospecific antiserum to IAF-Klop ORF3 product (α3), two New-Zealand albino rabbits were inoculated by the intradermal route with 300 μg of the electroeluted GST-ORF3 recombinant protein emulsified with complete Freund adjuvant (Gibco BRL). The rabbits were boosted three times at 2-week-intervals by the intramuscular route with the same amount of protein emulsified in incomplete Freund adjuvant. Reactivity of the antisera was assessed by indirect immunofluorescence (IIF) assay [20] on acetone-fixed PRRSV-infected MARC-145 cells. Monospecific antisera to ORF5 (α5), ORF6 (α6) and ORF7 (α7) products were obtained from a previous study [22].

Eucaryotic transient expression assays and generation of recombinant adenovirus

The PCR-amplified ORF3 gene was also subcloned into the *Bam*HI unique site of the pAdCMV5 plasmid vector [24]. For this purpose, *Bam*HI recognition sites were added at the 5′ end of both ETS3 and ETR3 primers, and in the case of ETS3, the ATG initiator codon was preceded by a triple GCC motif in order to provide an optimal Kozak consensus sequence for efficient translation [18]. In this shuttle vector, the foreign gene is driven by an optimized human cytomegalovirus (CMV) promoter, and the expression cassette is flanked on one end by the encapsidation and packaging signals of the human adenovirus type 5, and on the other end, by an adenovirus sequence allowing recombination and generation of replication-defective recombinant adenovirus in which E1 gene is replaced by the expression cassette [24].

For transient expression assays of pAdCMV5/ORF3, COS-7 cells, as well as 293 cells, in 10 cm^2-tissue culture plates were transfected with 5 μg of plasmid DNA by calcium phosphate co-precipitation [13]. For IIF, cells were incubated at 37 °C for 48 h, fixed with 80% acetone for 20 min at 4 °C and reacted for 30 min with either rabbit anti-ORF3 (α3) monospecific serum or hyperimmune pig anti-PRRSV serum, as previously described [20]. For the generation of a recombinant adenovirus, pAdCMV5/ORF3 was linearized at the unique *Cla* I site and rescued into the genome of Ad/CMVlacZ by homologous recombination in 293 cells, as described elsewhere [15]. Recombinant AdCMV5/ORF3 viruses were identified by PCR using the ETS3 and ETR3 oligonucleotide primers.

Serological tests

The IIF test, using acetone fixed PRRSV-infected MARC-145 cells, as well as the virus neutralization (VN) test, were performed as previously described [19, 20]. The IIF antibody titers were expressed as the reciprocal of the highest dilution of tested pig or rabbit sera giving

specific cytoplasmic fluorescence. For Western immunoblotting, concentrated "crude antigen preparation" of PRRSV was subjected to 12% SDS-PAGE and electrotransferred onto nitrocellulose membranes (45 μm pore size, Schleicher and Schuëll) [19]. Immunological identification of viral proteins was confirmed following incubation of the saturated nitrocellulose membranes in the presence of 1:50 dilution of the rabbit α3 monospecific antiserum or porcine anti-PRRSV positive or negative sera, as previously described [20, 22].

Virus purification and metabolic labeling of PRRSV proteins

For Western immunoblotting, extracellular virions were concentrated and semi-purified as follows. Confluent monolayers of MARC-145 cells in 150-cm^2 tissue culture flasks (Falcon) were infected with PRRSV IAF-Klop strain at a m.o.i. of 1 $TCID_{50}$ of virus per cell. When 25% of the cells were affected by cytopathic changes (CPE), cells and supernatants were subjected to two freeze-thaw cycles. The cellular suspension was then clarified by low speed centrifugation at 5000 **g** for 20 min. The clarified supernatant fluids (100–200 ml) were subsequently ultracentrifuged at 100 000 **g** (rotor SW40Ti, Beckman) for 3 h through a 30% (W/V) sucrose cushion in TBS buffer. The viral pellet was finally resuspended in 100 to 200 μl of TBS buffer and kept at −80 °C until used. Such viral antigenic preparation was referred as "crude antigen".

For radiolabeling of PRRSV proteins, confluent monolayers of MARC-145 cells in 75-cm^2 tissue culture flasks (Falcon) were infected at a m.o.i. of 1 to 5 $TCID_{50}$ of virus per cell. At 24 h post-infection, infected cultures were rinsed twice with PBS and incubated in methionine-free DMEM for 1 h at 37 °C. Following this starvation period, [^{35}S] methionine (specific activity of 1,120 Ci/mmol, Amersham Searle Co, Oakville, Ontario) was added at a final concentration of 50 μCi/ml. Incubation was continued for 2 h at 37 °C for the preparation of radiolabeled infected cell lysates [22]. For the preparation of highly purified viral antigen, extracellular virions were labeled in a minimal volume (3–4 ml), and freezing and thawing of the cell monolayers was omitted to avoid large aggregates of viral and contaminant cellular proteins. Following clarification of the supernatant fluids by low speed centrifugation and isopycnic ultracentrifugation on continuous 15 to 30% (W/V) CsCl gradient [20], fractions with the highest infectivity titers ($10^{10}-10^{11}$ $TCID_{50}$/ml) were collected, diluted 1/10 in TBS and subjected to a final ultracentrifugation at 100,000 **g** for 2 h (rotor SW50, Beckman). The highly purified radiolabeled virus in the pellet was resuspended in RIPA buffer (100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% Triton X-100, 2 mM phenylmethylsulfonide fluoride (PMSF)) and used for subsequent immunoprecipitation experiments.

Immunoprecipitation and endoglycosidase F treatment

Aliquots of [^{35}S] methionine labeled- PRRSV proteins from purified virus preparations or infected-cell lysates were adjusted with RIPA buffer to a final volume of 500 μl, then incubated overnight at 4 °C with 5–10 μl of the rabbit monospecific antisera. The immune complexes were then adsorbed for 2 h to protein A-Sepharose CL4B beads (Pharmacia), washed five times in RIPA buffer and dissolved in electrophoresis sample buffer containing 5% (V/V) β-mercaptoethanol, as previously described [20]. Treatment with endoglycosidase F/N-glycosidase F (glyco F) (Boehringer Mannheim) was performed, as previously described [22].

Results

Identification of the PRRSV ORF3-encoded protein and generation of a recombinant adenovirus

Sequencing analyses revealed that the predicted ORF3 protein of the IAF-Klop strain of PRRSV (EMBL/GenBank accession AF003344) is very similar to that

of ATCC VR-2332 and other North American isolates [21]. Following cloning of the enzymatically amplified ORF3 in the pGEX-4T-1 vector, a recombinant ORF3 protein fused to GST (GST-ORF3) was synthesized by transformed *E. coli* cells. The latter accumulated within the cells in the form of inclusion bodies, which could be solubilized in the presence of lysosyme, Triton X-100 and 8M urea, then separated by SDS-PAGE. The electroeluted GST-ORF3 was tested by Western immunoblotting for its reactivity with serum of a pig that has been experimentally-infected with the homologous IAF-Klop strain [19]. A protein with an approximate M_r of 52.3 kDa was revealed (data not shown), in agreement with the M_r of the ORF3 product estimated from the aa sequence (26.3 kDa) in addition to the M_r of GST which is about 26 kDa. Using acetone-fixed MARC-145 cells that have been infected with the autologous virus, IIF antibody titer of 1:1024 was determined for the rabbit α3 monospecific antiserum. However, this antiserum failed to neutralize CPE induced in MARC-145 cells by 100 $TCID_{50}$ of the virus. When reacted by Western immunoblotting with proteins of the concentrated crude viral preparation (contaminated by cellular proteins), the α3 monospecific antiserum specifically recognized a 42 kDa protein (Fig. 1C, lane 1), which was also weakly recognized by the autologous anti-PRRSV pig

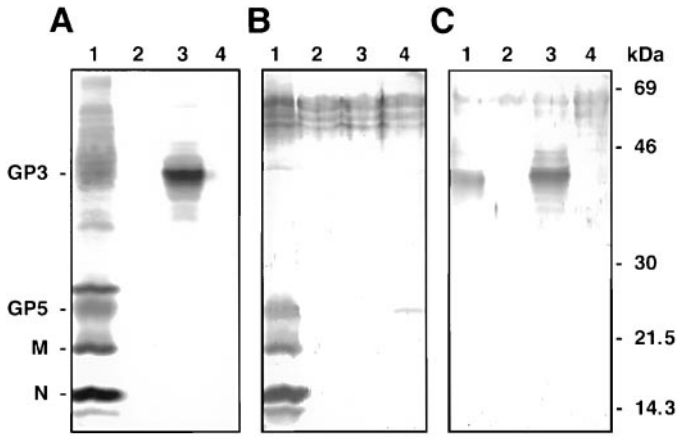

Fig. 1. Identification of the ORF3-encoded protein in crude antigenic preparation of PRRSV (IAF-Klop strain) and lysates of 293 cells infected with AdCMV5/ORF3. Concentrated extracellular virions recovered from clarified supernatant fluids of PRRSV-infected MARC-145 cells, following two freeze-thaw cycles and concentration through a cushion of 30% sucrose solution (*A1, B1, C1*), as well as extracts from 293 cells infected with the AdCMV5/ORF3 (*A3, B3, C3*), were solubilized in sample buffer, fractionated in a 12% SDS-polyacrylamide gel, and electrotransferred to nitrocellulose membrane. Individual nitrocellulose strips were incubated with the α3 monospecific antiserum (*C1* to *C4*), the serum from a pig that was experimentally-infected with the homologous PRRSV strain (*A1* to *A4*) and, a pool of α5, α6 and α7 monospecific antisera (*B1* to *B4*). No reactivity of the antisera was observed towards lysates of mock-infected MARC-145 (*A2, B2, C2*) and 293 (*A4, B4, C4*) cells. Positions of the size marker proteins are indicated in the right, and positions of the three PRRSV major structural proteins N, M and GP_5, and that of the putative ORF3-encoded protein (GP_3) are indicated in the left

serum (Fig. 1A, lane 1), but not by the pre-immune rabbit and pig sera (data not shown). As expected, the autologous anti-PRRSV pig serum also revealed the three major structural proteins of PRRSV, N, M and GP_5 from the crude viral preparation (Fig. 1A, lane 1), their identity being confirmed using a pool of the α5, α6 and α7 monospecific antisera (Fig. 1B, lane 1).

To further demonstrate the identity of the ORF3 product with that of the 42 kDa viral protein, a recombinant adenovirus (AdCMV5/ORF3) carrying the ORF3 gene of the IAF-Klop strain of PRRSV was constructed. In transient expression experiments, synthesis of the recombinant protein in COS-7 and 293 cells transfected with the transformed transfer vector pAdCMV5/ORF3 was revealed by IIF with the α3 monospecific antiserum and the autologous anti-PRRSV pig serum. Using both antisera, an intense cytoplasmic fluorescence could be observed in mostly 15 to 20% of the cells, and the expressed OFR3 protein appeared to accumulate near the perinuclear region (Fig. 2). A similar fluorescence pattern was observed following infection of 293 cells with the generated AdCMV5/ORF3 recombinant virus (data not shown). Lysates from 293 cells that have been infected with AdCMV5/ORF3, as well as lysates from mock-infected cells, were

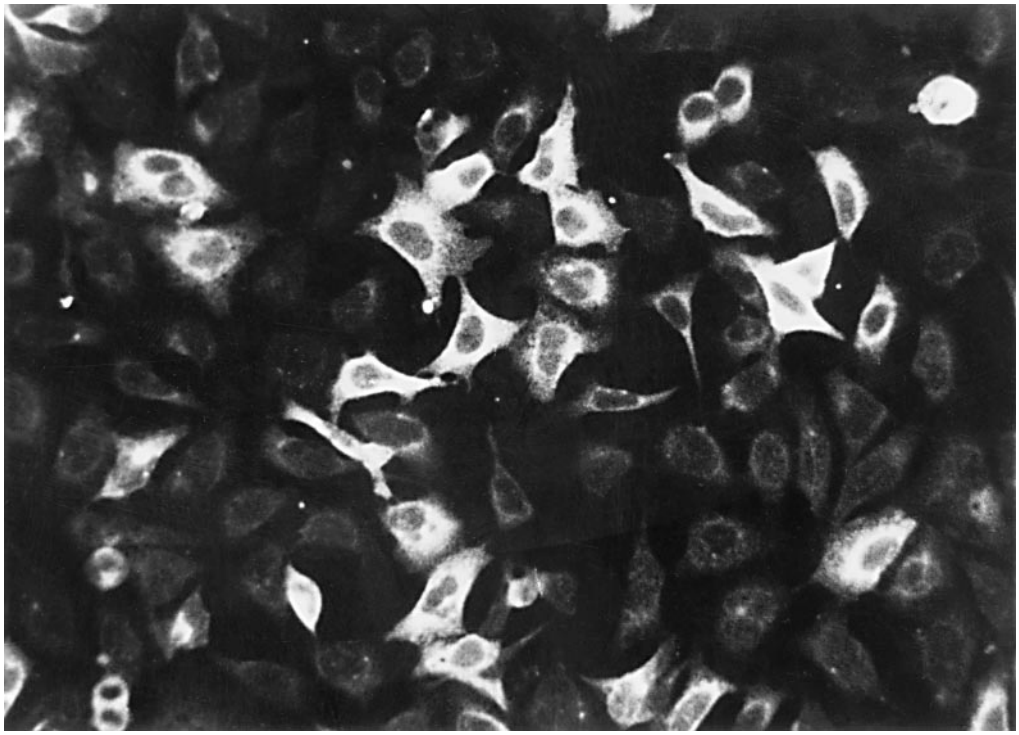

Fig. 2. Immunofluorescent staining of COS-7 cells at 48 h post-transfection with pAd CMV5/ORF3 plasmid. Expression of GP_3 of PRRSV (IAF-Klop strain) was confirmed by IIF following incubation in the presence of the rabbit α3 monospecific antiserum. A similar fluorescent profile was obtained following incubation with the autologous anti-PRRSV porcine hyperimmune serum. Expressed GP_3 protein was mostly confined to the perinuclear region

also tested to assess reactivities by Western immunoblotting of the rabbit α3 monospecific antiserum and the anti-PRRSV pig serum. Under the experimental conditions used, the rabbit α3 monospecific antiserum also recognized a 42 kDa protein band present in lysates of AdCMV5/ORF3 infected cells (Fig. 1C, lane 3), co-migrating with a protein present in the semi-purified or crude virus preparation (Fig. 1C, lane 1). The adenovirus-expressed recombinant ORF3 protein could also be detected by the anti-PRRSV pig serum (Fig. 1A, lane 3). Both antisera failed to recognize such a 42 kDa protein from lysates of mock-infected cells (Fig. 1 A and 1 C, lanes 2 and 4).

The ORF3 product of the IAF-Klop strain is highly glycosylated

To further characterize the ORF3-encoded protein expressed in PRRSV-infected cells, RIPA experiments were conducted with lysates of [^{35}S] methionine-labeled infected MARC-145 cells after treatment or not with endoglycosidase F. Following incubation in the presence of a pool of α5, α6 and α7 rabbit antisera, the three major structural proteins N, M and GP_5 could be immunoprecipitated from PRRSV-infected cell lysates (Fig. 3A, lane 1). An additional protein with an approximate M_r of 42 kDa was immunoprecipitated by the homologous anti-PRRSV porcine serum (data not shown); the latter was strongly precipitated by the rabbit α3 monospecific antiserum (Fig. 3A, lane 3; Fig. 3B, lane 1), together with other protein bands of lower intensity corresponding to the M protein and a 31 kDa protein.

Furthermore, following digestion of the PRRSV-infected cell lysates with endoglycosidase F, a protein with an estimated M_r of 27 kDa (Fig. 3B, lane 2), rather than the 42 kDa protein, was immunoprecipitated by the rabbit α3 monospecific antiserum, which confirmed the glycosylated nature of the ORF3 protein expressed in the infected cells. Assuming that the acquisition of one N-linked

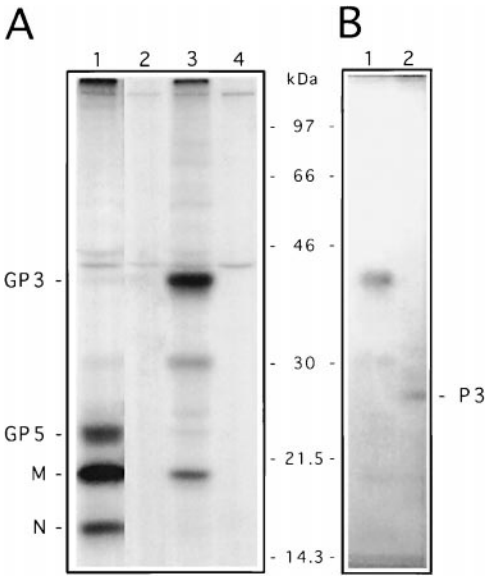

Fig. 3. Identification of PRRSV (IAF-Klop strain) ORF3 protein in the infected cells and evidence of its glycosylated nature. PRRSV-infected MARC-145 cells were radiolabeled with [^{35}S]methionine, disrupted in the lysis buffer, and incubated in the presence of monospecific anti-PRRSV sera after no treatment (*A1– A4*, *B1*) or treatment (*B2*) with endoglycosidase F. The figure represents the immunoprecipitation profiles obtained following incubation in the presence of a pool of monospecific α5, α6, and α7 antisera (*A1, A2*), or α3 antiserum alone (*A3, A4, B1, B2*). *A2* and *A4* represent the absence of reactivity with mock-infected cells

carbohydrate side chain results in an increase of approximately 2.6 kDa of the total M_r of a protein [2], the observed difference of approximately 16 kDa between the glycosylated and unglycosylated forms of the ORF3-encoded protein of the IAF-Klop strain of PRRSV is compatible with the effective in vivo glycosylation of all seven potential N-linked glycosylation sites (an additional one being present in the putative signal peptide sequence), previously deduced from its amino acid sequence. As the protein expressed by the recombinant adenovirus displayed exactly the same electrophoretic profile (Fig. 1A and 1C, lanes 3), it can be assumed that the overall glycosylation process of the recombinant protein in 293 cells is very similar to that occurring in MARC-145 cells.

The ORF3 product of the IAF-Klop strain is not packaged into extracellular virions

Pulse-chase experiments were performed to examine in greater detail the intracellular synthesis and fate of the IAF-Klop ORF3-encoded protein in comparison to that of major N, M and GP_5 viral structural proteins. At 24 h PI, PRRSV-infected MARC-145 cells were pulse labeled with [^{35}S]methionine for 15 min and the fate of intracellular (*cell lysates*) and extracellular (*medium*) viral proteins were monitored during a chase period that varied from 15 to 240 min. Viral proteins were solubilized from extracellular virions or infected cell lysates, then analysed by RIPA using a cocktail of rabbit α3, α5, α6 and α7 monospecific antisera (Fig. 4A).

At 0 min of chase (following pulse), the three major viral proteins N, M and GP_5 were efficiently precipitated from infected cell lysates and migrated in the gel with the expected mobility. The ORF3 product was first detected in the infected cell lysates as a major band of 42 kDa which underwent a gradual decrease to a 39 to 40 kDa protein by the end of the 240 min-chase period. At that time, most of the GP_3 has been lost from the cells, but there was also a decrease in the amounts of other proteins that co-precipitated, namely a protein with an approximated M_r of 30 kDa which was also detected throughout the 240 min-chase period. In further experiments, this additional intracellular protein was identified as GP_4 on the basis of its reactivity with a rabbit monospecific anti-GST-ORF4 serum [10]. On the other hand, when RIPA was performed with the concentrated extracellular virions (Fig. 4A, *medium*) of chased cells, N, M and GP_5, but not the 39 to 42 kDa protein, were obviously precipitated all together after 60 min, and increased as the chase period continued. Only small amounts of the 42 kDa protein were visible at 120 and 240 min of chase.

To confirm the above observation, RIPA was performed using an highly purified fraction that contained at least 10^{10} $TCID_{50}$ of virus. As shown in Fig. 4B (lane 3), the N, M and GP_5 proteins were precipitated at very high level consistent with the high infectivity titer of the CsCl gradient fraction. However, the 39 to 42 kDa protein immunoprecipitated from the infected cell lysates by the rabbit α3 monospecific antiserum (Fig. 4B, lane 1) could not be revealed in this highly concentrated virus preparation, thus confirming unambigeously that the latter is not packaged into the virion.

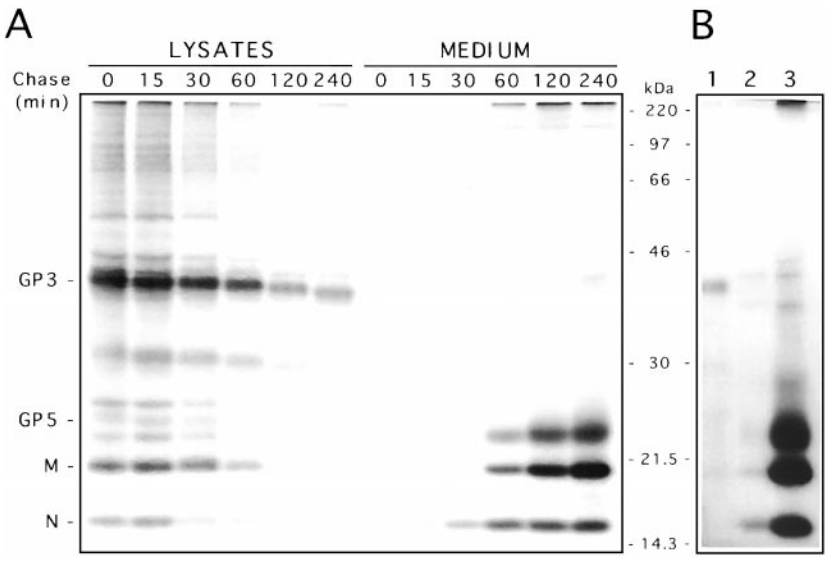

Fig. 4. Synthesis and fates of IAF-Klop PRRSV GP_3 protein comparatively to the three major structural proteins N, M and GP_5. **A** IAF-Klop-infected MARC-145 cells were labeled with 50 μCi/ml of [^{35}S]methionine at 24 h post-infection for 15 min (*0*) and chased for various times (15, 30, 60, 120, and 240 min). Labeled proteins from infected cell lysates were solubilized in RIPA buffer and subjected to radioimmunoprecipitation with a pool of monospecific α3, α5, α6 and α7 antisera. Immunoprecipitates were analysed by reducing SDS-PAGE (12% gel). Released virions derived from each of the above labelling time periods were collected from the culture medium after centrifugation at 100 000 × **g**. Proteins from extracellular virus were also immunoprecipitated with a pool of monospecific α3, α5, α6 and α7 antisera and analyzed by reducing SDS-PAGE (12% gel). Positions of the size marker proteins are indicated in the right, and positions of the three PRRSV major structural proteins N, M and GP_5, and that of the GP_3 protein are indicated in the left. **B** *B1* represents the GP_3 protein immunoprecipitated by the monospecific α3 antiserum from infected cells lysates. *B2* and *B3* correspond to the immunoprecipiation profiles revealed following incubation of CsCl gradient-purified [^{35}S]methionine-labeled extracellular virions with the monospecific α3 antiserum alone (*B2*) and a pool of monospecific α3, α5, α6 and α7 antisera (*B3*)

Reactivity of convalescent pig sera to ORF3 protein of the IAF-Klop strain of PRRSV

In order to determine if the ORF3 product of the IAF-Klop strain of PRRSV elicits antibody response in PRRSV-infected pigs, clinical sera obtained from pig farms that have experienced acute or chronic outbreaks of typical PRRSV infection were tested by ELISA towards recombinant GST-ORF3 fusion protein. From a total of 19 pooled sera tested (all of which had approximate IIF antibody titres of 1:1024), 9 reacted positively by ELISA with titers ranging from 1:50 to 1:1600 (data not shown). By Western immunoblotting, most of the convalescent pig sera reacted positively to the recombinant GST-ORF3 fusion protein, no reactivity being observed with serum from a SPF pig (Fig. 5).

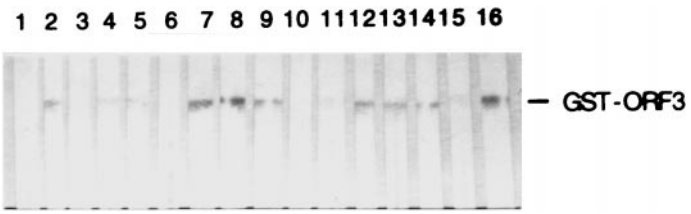

Fig. 5. Reactivity of PRRS convalescent pig sera toward recombinant GST-ORF3 fusion protein as determined by immunoblotting. Following IPTG induction, bacterial lysates were clarified and expressed fusion protein were purified by electroelution, separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. Half of the convalescent pig sera (1:100 dilution) tested reacted positively to the recombinant GST-ORF3 protein. *16* represents the reactivity of the homologous anti-PRRSV porcine hyperimmune serum. The seronegative SPF pig serum demonstrated no reactivity to the recombinant fusion protein (*1*)

Discussion

The aim of the present study was to further characterize the ORF3 encoded protein of a North American strain of PRRSV, the Quebec reference IAF-Klop strain. The use of a monospecific polyclonal antiserum raised against *E. coli*-expressed recombinant protein usually overcomes the problems related to low affinity binding, and thus was preconized for serological identification, rather than antipeptide or monoclonal antibodies which have been used by previous investigators for the identification and characterization of the ORF3 product of LV, the prototype European strain [7, 27]. Moreover, a recombinant adenovirus was constructed to further characterize the nature and site of intracellular synthesis of the ORF3 encoded protein of the North American strain of PRRSV, rather than in vitro transcription and translation experiments as recently reported by others for the study of the ORF3 protein of the murine arterivirus [8].

Data obtained with the Quebec reference strain of PRRSV indicated that i) the ORF3 encoded protein is synthesized at reasonable amounts in PRRSV-infected cells, ii) the apparent M_r of the protein gradually decreases in size most probably due to modifications of its sugar residues, iii) recombinant adenovirus-expressed ORF3 product has exactly the same electrophoretic profile than that of the GP_3 detected in the PRRSV- infected cell lysates and crude viral preparations, and iv) the GP_3 of PRRSV is not packaged into extracellular viral particles. To confirm the validity of the above findings, extracellular virus was highly purified, and only the most infectious fraction of the isopycnic CsCl density gradient was used for immunoprecipitation. Since GP_3 could not be detected immunologically from highly purified and concentrated virus (Fig. 4B), but only from concentrated crude viral preparations contaminated by cellular membranes, we postulated that GP_3 of PRRSV is most probably a non-structural glycoprotein, as recently reported for the ORF3 encoded protein of LDV [8]. This finding is in contrast to previous conclusion by others that the GP_3 of the reference European strain

LV of PRRSV is another putative envelope glycoprotein of the virion. The data obtained were unexpected since the ORF3 proteins of all the arteriviruses possess similar structure, notably the lack of a potential transmembrane segment, except for a signal peptide [8, 34]. Although the overall charges of the N-terminal region of the GP_3 of LV and IAF-Klop strains are different, they possess the same number of hydrophobic peaks [21]. Since sequence analyses did not reveal notable differences in the aa sequences of the GP_3 of the IAF-Klop strain, the discrepancy noted with the LV strain is most probably due to the procedure used in the present study to avoid contamination of purified viral preparation with cellular membranes to which may be associated envelope viral glycoproteins. One may hypothesize that failure of the α3 monospecific antiserum to precipitate GP_3 from purified extracellular virions is possible because the latter was raised using an unglycosylated *E. coli* expression product, but this is unlikely since pulse chase experiments demonstrated that it precipitated efficiently GP_3 from PRRSV-infected cell lysates despite modifications of its sugar residues. This antiserum also immunoprecipitated the GP_3 expressed by the recombinant adenovirus and the nonglycosylated product (P3) obtained following treatment of PRRSV-infected cell lysates with glyco F, an enzyme mixture which hydrolyzes N-glycans of the high mannose- and complex-type. The P3 protein displayed a molecular mass of approximately 27 kDa, consistent with that predicted from the aa sequence of the ORF3 apoprotein [21].

It is noteworthy that almost half of the clinical sera tested reacted positively towards the recombinant GST-ORF3 fusion protein. Although a serological survey using an unglycosylated *E. coli* expression product is relatively meaningless, data obtained indicate that GP_3 contains linear antigenic determinants, which are independent of the glycosylation process that trigger the immune system of PRRSV-infected pigs to produce specific antibodies. On the other hand, the monospecific α3 antiserum raised in rabbits was not neutralizing, in agreement with previous findings by others on the biological activities of anti-GP3 monoclonal antibodies to the Lelystad virus [7], and the demonstration that the carboxyterminal portion of PRRSV ORF3 encodes a non-neutralizing peptide [16]. This is also consistent with the fact that antibody titers of naturally-infected pigs to GP_3, as determined by ELISA using *E. coli*-expressed recombinant GST-ORF3 fusion protein as antigen, seemed not to correlate with neutralization titers [11]. At present, only antibodies to either the GP_4 or the GP_5 of PRRSV have been described as neutralizing [29, 30]. In view of recent findings by other that baculovirus-expressed ORF3 protein may confer partial protection in pregnant sows against reproductive disorders [31] in the absence of a noticeable neutralizing humoral response, the exact role of anti-GP_3 antibody response and the role of GP_3 in the induction of a cellular immunity remain to be determined.

Even if the exact roles and functions of the ORF3 product of *Arteriviruses* are still to be determined, it is noteworthy that i) the ORF 3 product of EAV has not been characterized as a structural protein [34], and ii) a recent report indicates that the LDV ORF3 protein is a highly glycosylated, nonstructural protein that most likely is produced in a soluble and membrane-bound glycoprotein that elicits a

strong antibody response in infected mice [8], properties that seem to be shared by the GP_3 of North American strains of PRRSV. Such findings have been already described for few positive-stranded RNA viruses. In the case of *Flaviviruses*, the NS1 proteins of tick-borne encephalitis virus [5] and Dengue virus [32] are known as secreted non-structural proteins that are highly glycosylated and antigenic, and able to induce protective immunity [33]. Furthermore, the membrane-associated E3 protein of Sindbis virus, a member of the *Alphavirus* genus, is also another example of a nonstructural, soluble glycoprotein encoded by an RNA virus [36]. In pulse-chase experiments, an intracellular reduction of GP_3 of the IAF-Klop strain was observed during the 240 min chase period which may suggest loss of cellular integrity (due to CPE) or release from the cells in soluble form into the medium. At the time when the pulse-chase experiments were performed (24 h post-infection), trypan blue staining of PRRSV-infected cell monolayers revealed not more than 1 to 2% of cell death, a level which was similar to that obtained with mock-infected cells (data not shown). This finding ruled out the possibility that the loss of ORF3 protein during the 240-min chase period was due to intensive cell damages. Data obtained from recent studies on the intracellular processing and fates of the GP_3 expressed by the AdCMV5/ORF3 virus substantiated the hypothesis that the GP_3 must be released from the infected cells in a soluble form [23].

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