# Biochemical Properties and Processing of the Three Major Structural Proteins of PRRS Virus Expressed by Recombinant Adenoviruses

*Structural, functional and community aspects*

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## 1. INTRODUCTION

The ORFs 5 to 7 encode for the three major structural proteins of porcine reproductive and respiratory syndrome virus (PRRSV) : the envelope glycoprotein $GP_5$ (25-26 kDa), the non-glycosylated membrane protein M (18-19 kDa) and the nucleocapsid protein N (14-15 kDa), respectively (Mardassi et al., 1995; Meulenberg et al., 1993). The latter structural proteins of PRRSV are closely associated both in the infected-cells and in the virion, the $GP_5$ and M proteins being associated in the form of heterodimers (Mardassi et al., 1996). The $GP_5$, which is highly glycosylated (Mardassi et al., 1995), have been found to play an important role in the induction of a protective immune response (Pirzadeh and Dea, 1998). Immunization experiments of mice with *E. coli*-expressed GST-ORF5 recombinant fusion protein, as well as with purified PRRSV, induced specific anti-$GP_5$ neutra-lizing MAbs (Pirzadeh and Dea, 1997). The antibodies in PRRSV-infected pigs antisera responsible for the viral neutralisation in cell culture have been also determined to be $GP_5$ specific (Gonin et al., 1999). In DNA immunization experiments, pigs injected with a plasmidic vector expressing the ORF5 gene not only produced neutralizing antibodies to PRRSV, but were also protected against development of clinical disease and lung lesions following an intratracheal challenge with a high

The Nidoviruses (Coronaviruses and Arteriviruses).
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infectious dose of PRRSV. On the other hand, parenteral inoculation of SPF piglets with *E. coli*-expressed GST-ORF5 recombinant fusion protein rather resulted in an increased severity of lung lesions, despite the development of high titers (> 2048) of non-neutralizing antibody titers to $GP_5$ (Pirzadeh and Dea, 1998). Consequently, the use of an eucaryotic expression system should be preconized to produce large amounts of a recombinant protein that would have to preserve the major characteristics (glycosylation, conformational epitopes) of the native major envelope glycoprotein of PRRSV. Recently, a replication defective human type 5 adenovirus (Ad) has been used successfully for the eucaryotic expression and characterization of the ORF3 product of PRRSV (Mardassi et al., 1998). Previous investigators have also demonstrated that recombinant Ad (recAd) carrying the structural genes of different viruses, mainly the envelope or spike glycoproteins of murine and porcine coronaviruses, were effective for the induction of antibodies to the expressed rec glycoproteins that conferred protection (Both et al., 1993; Torres et al., 1995; Wesseling et al., 1993).

The main objectives of the present study were to construct recAds expressing the three major structural proteins of PRRSV and to assess if the recombinant (rec) expressed proteins have conserved the antigenicity, glycosylation properties and biological functions (cytotoxicity, proapoptotic phenotype, immunogenicity) of the native major structural proteins of PRRSV.

## 2. METHODOLOGY AND RESULTS

The IAF-Klop PRRSV N, M and $GP_5$ coding sequences (ORF7, ORF6 and ORF5, respectively) (EMBL/Genbank accession No. U64928) were inserted into the unique *Bam*H1 site of the adenovirus transfer vectors pAdCMV5 or pAdTR5 (Massie et al., 1998) so that the ORFs 5, 6 and 7 coding sequences would be under the control of the constitutive human CMV immediate-early (IE) promoter/enhancer (pAdCMV5) or the tetracyclin regulatable promotor (pAdTR5). Rec plasmids were rescued into the genome of Ad/CMVlacZ by homologous recombination in 293 cells, as described elsewhere (Ascadi et al., 1994) and the following replication-defective recAds were generated: AdTR5/ORF5, AdCMV5/ORF6 and AdT/R5/ORF7. The recAdCMV/tTA permitted the constitutive expression of tTA in infected cells which is required for expression in recAd-infected cells of the foreign gene that has been cloned dowstream and under the

control of the TR5 promotor. In the presence of doxycyclin (1 μg/ml), the expression of the foreign gene is inhibited in recAd-infected cells (Massie et al., 1998). Immunolo-gical identification of the native viral proteins, as well as of that of the 293- expressed rec proteins was confirmed by Western blot (WB) and radioimmuno-precipitation (RIPA) using the homologous hyperimmune porcine anti-PRRSV serum, rabbit monospecific antisera (α5, α6 and α7) and MAbs. The fact that the rec proteins were recognized by antibodies specific for each of the three major structural proteins of PRRSV in both WB and RIPA, was indicative that at least the major antigenic determinants of the native viral structural proteins have been conserved (data not shown). Also the rec proteins displayed the same electro-phoretic profiles than that of the native proteins of PRRSV (data not shown).

At 18 to 24 h post infection (pi), following a starvation period of 60 min in methionine-deprived DMEM, the PRRSV-infected MARC-145 cells or the 293 recAds-infected cells were pulse-labelled with 250μCi of [$^{35}$S]methionine (sp activity of 1,120 Ci/mmole, Amersham Searle Co., Oakville, Ontario) for 5 h. Cells lysates were incubated with the α5 rabbit monospecific antiserum and the immune complexes were collected by addition of protein A-sepharose CL4B beads (Pharmacia) and analyzed by 12% SDS-PAGE, as previously described (Mardassi et al., 1996). The immune complexes were then incubated with either Endo-β-N-acetylglucosaminidase H (Endo H), Endoglycosidase F/N-Glycosi-dase F (Glyco F) or Endo-β-Galactosidase (Endo β) to characterize the N-glycosylation type of the $GP_5$ (Fig. 1). The rec$GP_5$ appeared different from the native protein in regards of its glycosylation process. The rec$GP_5$ apparently possessed poly-*N*-acetyllactosamine residues because of its sensitivity to Endo β digestion (Fig. 1a and b, lane b). Also the rec$GP_5$ did not possess oligosacharides of the complex type since it was totally sensitive to Endo H (Fig. 1b, lane h) contrary to its native counterpart (Fig.1a, lane h). Differences observed in the N-glycosylation was independent of the cell substrate since AdTR5/ORF5 propagated in MARC-145 cells have the same endoglycosidases digestion profiles than AdTR5/ORF5 propagated in 293 cells (Fig. 1b and c).

Coexpression of the three major structural proteins in recAds-infected 293 cells did not changed the fate of the glycosylation process of $GP_5$ (Fig. 2). Also, even if the N, M and $GP_5$ proteins were present in 293 cells, no formation of viral particles could be observed in the recAds-infected cells by electron microscopy and no formation of M-$GP_5$ heterodimers was observed in RIPA experiments (data not shown).

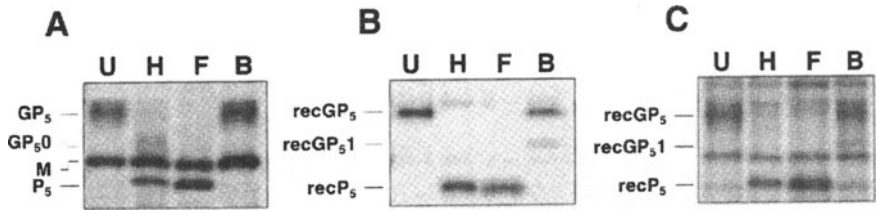


*Figure 1.* Glycosylation nature of the recGP$_5$ in 293 and MARC-145 cells. Immunoprecipitation profiles obtained with: A) lysate of PRRSV-infected MARC-145 cells; B) lysate of AdTR5/ORF5-infected 293 cells; C) lysate of AdTR5/ORF5 + AdCMV/tTA infected MARC-145 cells. In all cases, viral proteins were immunoprecipitated using α5 monospecific antiserum. The immuno-precipitated proteins were untreated (lane U) or treated with endoglycosidases; Glyco F (lane F), Endo H (lane H) and Endo β (lane B).

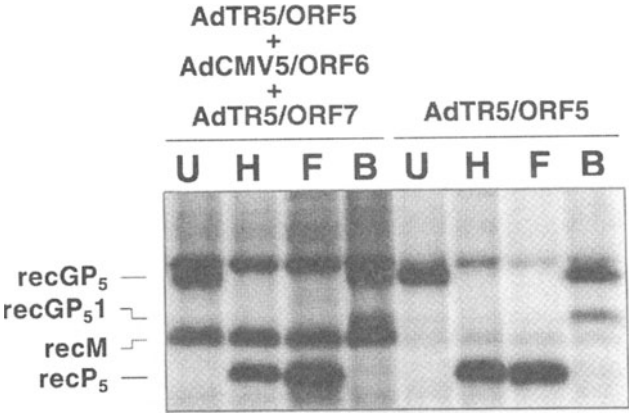


*Figure 2. Glycosylation nature of the coexpressed recN, recM and recGP$_5$ proteins in 293 cells.* RIPA experiments were conducted with cell lysates of recAds-infected 293 cells. Expressed rec proteins were immunoprecipitated with α5, α6 and α7 monospecific antisera, then electrophoresed after no treatment (U) or treatment with endoglycosidases Glyco F (lane F), Endo H (lane H) and Endo β (lane B).

Both genotypes of PRRSV have been reported to induce apoptosis in the infected cells (Suarez et al., 1996; Sur et al., 1997). On the other hand, only the GP$_5$ of the European genotype has been reported to induce apoptosis (Suarez et al., 1996). Since, there is only a 52% aa identity between the European and the North American genotypes, the apoptotic phetotype of the GP$_5$ of North American IAF-Klop strain was verified by infecting MARC-145 cells with the recAd AdTR5/ORF5. The cytopathic changes observed following expression of the recGP$_5$ in MARC-145 cells appeared almost identical to those observed in the case of a PRRSV infection (Kim et al., 1993). Indeed, scattered and enlarged cells with very small granular

inclusions in their cytoplasm usually started to be detectable by 24 h pi, than infected cells showed a tendency to clump by foci that could be well delineated from the non-affected monolayer by 36 to 48 h pi. Numerous infected cells eventually detached from the plates, more than 75% of the cell monolayers being severely damaged by 72 h pi (data not shown). No effect on MARC-145 cells could be observed at 72 h pi when doxycyclin was added to the medium. To verify if the cytopathic effect was due in part to apoptosis, the level of procaspase 3 activation was measured by incubating cell lysates in the presence of a specific subtrate for caspase 3, the DEVD-AMC fluorogenic substrate (BIOMOL Research Laboratories, Inc.). When caspase 3 is present in the cell lysates, the AMC substrate is cleaved and the fluorescence increases with time. The results obtained were reported in Fig. 3 and expressed as constant fluorescence released (fluorescence units or FU) per second per μg of cell lysates. Fig. 3 demonstrated that the recGP$_5$ induced apoptosis in MARC-145 cells and that degenerative effects were not due to Ad itself because in the presence of doxycyclin the inhibition of the GP$_5$ synthesis correlated with inhibition of the procaspase 3 activation, the level of caspase 3 being 11 times higher when the GP$_5$ was expressed in the cells. Also, PRRSV infection induced an activation of the procaspase 3.

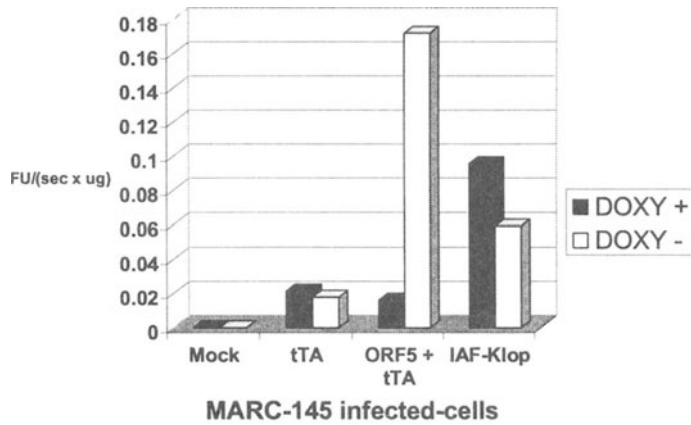


*Figure 3.* Procaspase 3 activation. MARC-145 cells were infected with only AdCMV/tTA or with both AdCMV/tTA and AdTR5/ORF5 at a MOI of 100 PFU/cell in the presence or absence of 1 μg/ml of doxycyclin.

Our main objective is to construct an efficient recombinant vaccine against PRRSV infection. Consequently , to study the immunogenicity of recAds, mice have been immunized with AdTR5/ORF7 and AdTR5/ORF5. The mice develop-ped specific antibodies against the N and GP$_5$ proteins,

antibody titers of 64 to 128 being detected in their sera by indirect immunofluorescence (data not shown) 55 days post-inoculation.

## 3. DISCUSSION

We are not yet able to tell if the differences observed concerning the glycosylation type and processing of the $GP_5$ expressed individually by recAd may alter its biological and immunological functions. Nevertheless, recAds were established as useful tools to study the structural proteins of PRRSV *in vitro* and *in vivo*. Furthermore, since genetic immunization was previously found to trigger the immune system of pigs to produce neutralizing antibodies to PRRSV and induced protection against development of the clinical disease and histopathological lesions, but failed to eliminate the presence of the virus in lungs of infected animals, data suggested that mucosal immunity may have a major role to play in the protection against this infection. The use of human or porcine adenoviruses as viral vectors for the induction of mucosal immune reponses against enteric or respiratory viral diseases in pigs has been proposed by several investigators and found to be very effective because of their tropism for the BALT (bronchoalveolar-associated lymphoid tissues) and GALT (gut-associated lymphoid tissue). Such viral vector, either replicative-defective or replicative, may proved very efficient against PRRSV infection. However, more studies are needed in order to identify the viral proteins carrying antigenic determinants involved in the effective humoral and cellular immune response and whether these determinants are well preserved among different field strains.

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