Arch Virol (2003) 148: 951–972
DOI 10.1007/s00705-002-0943-y

Archives of Virology
Printed in Austria

Adenoviral-expressed GP_5 of porcine respiratory and reproductive syndrome virus differs in its cellular maturation from the authentic viral protein but maintains known biological functions

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Received April 8, 2002; accepted October 15, 2002
Published online March 3, 2003

Summary. The ORFs 5, 6 and 7, encoding for the three major structural proteins, GP_5, M and N, of the IAF-Klop strain of PRRSV were cloned and expressed in 293 cells using replication-defective human type 5 adenoviral vectors (hAdVs). Although the M protein gene could be cloned into hAdVs and expressed constitutively in 293 cells under the control of the hCMV immediate early promotor/enhancer, hAdVs expressing N and GP_5 proteins, which appeared to be toxic or interfered with adenovirus replication, could only be generated by inclusion of a tetracycline-regulatable promotor in the transfer vector pAdTR5. The recombinant (rec) proteins appeared similar to the authentic viral proteins in regards to their M_rs and antigenicities. However, the recGP_5 apparently possesses different N-linked oligosaccharides residues. Its sensitivity to endo-β-galactosidase digestion indicates that poly-N-acetyllactosamine is present on the individually-expressed protein, but not on the authentic GP_5 anchored into the virion envelope. The recGP_5 apparently accumulates within the ER compartment as a glycoprotein that possesses high-mannose N-linked oligosaccharide side chains sensitive to endo-β-N-acetylglucosaminidase H treatment, by contrast to its viral counterpart for which N-linked oligosaccharide side chains are of both high-mannose and complex types. Coinfection of 293 cells with hAdVs expressing the M and GP_5

*In memoriam. The authors wish to offer their condolences to the family and friends of Dr. Serge Dea, who died on January 3, 2003, and acknowledge his tremendous accomplishment in the field of Nidoviruses.

did not lead to M-GP_5 heterodimer formation, as demonstrated in PRRSV-infected cells. Moreover, cells infected with inducible hAdV/ORF5 showed that GP_5 of the North American strain is proapoptotic. Indeed, when the expression cassette was turned-on, caspase 3 activity in hAdV/ORF5 infected cells was enhanced and DNA fragmentation could be detected by TUNEL assays. Pigs intradermally injected twice with hAdV/ORF5 developed antibody titers to the authentic viral GP_5 as soon as 10 days following challenge with the homologous virulent PRRSV strain, as revealed by Western blot and virus neutralization tests, suggesting the establishment of a specific immune memory.

Introduction

Porcine respiratory and reproductive syndrome virus (PRRSV) is the causative agent of an economically important pig disease, with a worlwide distribution, characterized by reproductive failure in sows of any parities and respiratory problems in unweaned and growing pigs [10, 16]. The virus is morphologically, structurally and genomically similar to other members of the genus *Arterivirus*, which also includes *Equine arteritis virus* (EAV), murine *Lactate dehydrogenase-elevating virus* (LDV) and *Simian hemorrhagic fever virus* (SHFV) [10, 29, 39]. These viruses, as well as PRRSV, were recently classified in the order *Nidovirales*, which includes members of the *Arteriviridae* and *Coronaviridae* families [4]. The viral genome consists in a positive single-stranded polyadenylated RNA molecule of approximately 15 kb in length and is composed of nine open reading frames (ORF1a, ORF1b, ORF2a, ORF2b, and ORF3-7) which are expressed as a nested set of sub-genomic mRNAs [29, 30, 39, 46]. Sequence analysis revealed that European and North american strains represent two distinct genotypes, with less than 55% amino acid (aa) identities for proteins encoded by ORFs 2, 4, and 5 [10]. The ORF1, representing nearly 75% of the viral genome, encodes for proteins with replicase and polymerase activities [39]. The ORFs 5 to 7 encode for the three major structural proteins of the virion: 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) [23, 24, 30]. These structural proteins are closely associated both in the infected-cells and in the virion, the association of GP_5 and M into disulfide-linked heterodimers being essential for authentic maturation and post-translational modification of GP_5 [24], as previously demonstrated for the homologous G_L and M envelope proteins of EAV [9].

Circulating antibodies in PRRSV-infected pigs responsible for viral neutralization (VN) in cell cultures are mainly directed against GP_5 [15], and neutralizing monoclonal antibodies have been obtained following immunization of Balb/c mice with *E. coli*-expressed GST-ORF5 rec fusion protein [33]. More recently, genetic immunization of pigs with plasmidic DNA encoding the ORF5 gene not only triggered the immune system for the production of anti-PRRSV neutralizing antibodies, but also conferred protection against development of clinical disease and lung lesions following intratracheal challenge with the homologous virulent strain [34]. However, DNA immunization was apparently not sufficient to inhibit

virus persistence and shedding in the respiratory tract of challenged pigs. On the other hand, when *E. coli*-expressed GST-ORF5 rec fusion protein was used as immunogen prior to a challenge with pathogenic virus, the disease was more severe despite the development of high titers (> 2048) of non-neutralizing antibodies to GP_5 [34]. Data obtained suggest that the immune response to unglycosylated linear epitopes of *E. coli*-expressed ORF5 product is not sufficient to protect pigs [34], and that the establishment of an effective mucosal immunity against glycosylated and conformational dependent epitopes is probably crucial, particularly to control virus persistence and shedding. However, this aspect of immunity against PRRSV has not been investigated so far. Consequently, the use of viral vectors with the ability to infect pig cells of the intestinal or respiratory tract, such as adenoviral and poxviral vectors, could be preferable to produce large amounts of recGP_5 that have conserved the major characteristics of the authentic viral glycoprotein [45].

Live human adenovirus type 5 (hAd5) has been shown to be an excellent delivery system for vaccine immunogens [17, 45, 47]. Both replication-defective and replication-competent hAd5 vectors (hAdV) have been used for insertion of foreign genes [17]. However, replication-defective viruses, which have been deleted for the E1 genomic region (ΔE1) that is known to be crucial for virus replication, are often preferred to replication-competent vectors for their biosafety. Indeed, under those conditions, the vaccinated animals are expected to be unable to shed infectious viruses that could dissiminated to unvaccinated pigs kept in the same building. Therefore, there is no risk for human of being infected by a replicative hAdV either from direct contact, meat or animal by-products. Although unable to replicate *in vivo*, ΔE1 hAdVs can induce an immune response to inserted gene products after systemic or intranasal administration [12, 14, 31]. More specifically, the efficacy in swine of hAdV as recombinant vaccine carrying genes of a variety of swine pathogens was demonstrated [3, 43, 44]. The main objectives of the present study were to construct replication-defective hAdVs expressing the three major structural proteins of PRRSV in a vaccine strategy overview and to compare the adenoviral-expressed recGP_5 protein to the authentic PRRSV protein in terms of cellular processing and final maturation, antigenicity, immunogenicity and proapoptotic activity.

Materials and methods

Viruses, cells and antisera

The Québec cytopathogenic IAF-Klop strain of PRRSV was propagated in MARC-145 cells, as previously described [23]. Ad/CMVlacZ [1], a replication-defective E1- and E3-deleted hAd5, as well as rec hAdVs, were propagated in 293 cells (ATCC CRL-1573). Infectivity titers of hAdVs stocks were determined by calculation of plaque forming units (PFU) per ml on 293 cell monolayers [18]. AdCMV/tTA, which permits the constitutive expression of the tetracycline transactivator (tTA) under the control of the constitutive human CMV immediate-early promoter/enhancer was used in co-infection to allow expression of rec proteins in cells infected with hAdVs expressing the transgenes under the control of the TR5 promoter. Doxycycline (Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada) (1 μg/ml)

was used to inhibit expression of the transgenes in hAdV-infected cells [26]. The 293 TetOn cells (Clontech Laboratories Inc., Palo Alto, California, U.S.A.), that constitutively express the reverse tetracycline transactivator (rtTA), were cultivated in the presence of 1 μg/ml of doxycycline to induce the expression of the transgenes driven by the tetracycline-regulatable promoter (TR5) [26]. BMAdE1 cells, an A549 cell line expressing AdE1 proteins from a vector designed to eliminate the generation of replication competent adenoviruses [25], were propagated in similar conditions as 293 cells [1]. Hyperimmune rabbit monospecific antisera α5, α6 and α7, directed respectively against GP_5, M and N proteins of the homologous IAF-Klop PRRSV strain expressed in *E. coli*, were obtained from previous studies [24].

Generation of replication-defective hAdVs expressing PRRSV N, M and GP_5 proteins

The ORF5, 6 and 7 coding sequences of the IAF-Klop strain of PRRSV were amplified by RT-PCR using specific sets of oligonucleotide primers, which were designed from the nt sequence determined in a previous study (EMBL/Genbank accession No. U64928) [15]. All primers contained two *Bam*HI restriction sites at their 5′ end, and in the case of sense primers, the ATG initiator codon was preceded by a triple GCC motif in order to provide an optimal Kozak consensus sequence for efficient translation [20]. For each reaction, the amplified product was inserted into the unique *Bam*HI site of the pAdCMV5 or pAdTR5 transfer vectors [26], to ensure that the transgenes would be under the control of the constitutive hCMV immediate-early promoter/enhancer (pAdCMV5) or the tetracycline-regulatable promotor (pAdTR5). The rec plasmids were rescued into the genome of Ad/CMVlacZ by homologous recombination in 293 cells, as described elsewhere [18]. Upon cotransfection, virus plaques were isolated, amplified in 293 cells, and analyzed for the expression of the rec proteins (recN, recM and recGP_5) either by Western blot or by radioimmunoprecipitation assays (RIPA). The hAdVs AdTR5/ORF5, AdCMV5/ORF6 and AdTR5/ORF7, which expressed the recombinant GP_5, M and N proteins, respectively, were subjected to three consecutive rounds of plaque purification on BMAdE1 clone 78 cells. Subsequently, selected viral clones were amplified on BMAdE1 clone 220 cells, as described elsewhere [27].

Western blot

Extracellular PRRSV virions produced in MARC-145 cells were concentrated by ultracentrifugation at 100,000 g (rotor SW40Ti, Beckman) for 3 h through a 30% (W/V) sucrose cushion in 50 mM Tris-buffered saline (TBS), pH 8.0. After denaturation by boiling in the presence of 5% (V/V) β-mercaptoethanol, viral proteins were subjected to 12% SDS-PAGE and electrotransferred onto nitrocellulose membranes (45 μm pore size, Schleicher and Schuëll, Xymotech Biosystems, Mount Royal, Québec, Canada) [22]. Immunological detection of the authentic GP_5 protein was performed following incubation of the saturated nitrocellulose membranes in the presence of 1:500 dilution of the rabbit α5 monospecific antiserum (positive control) or 1:50 dilution of pig sera immunized with recombinant adenoviruses, as previously described [23, 24].

Metabolic labelling, immunoprecipitation and endoglycosidases treatment of PRRSV authentic or recombinant proteins

Radiolabelling with [^{35}S]-methionine (specific activity 1,120 Ci/mmole, Amersham Searle Co., Oakville, Ontario) of viral proteins synthesized in PRRSV-infected MARC-145 cells, as well as rec proteins synthesized in 293 or 293 TetOn cells infected with hAdVs, was carried out essentially as described elsewhere [23, 24]. The precipitated immune complexes were adsorbed for 2 h to protein A-sepharose CL4B beads (Amersham Pharmacia Biotech, Baie

d'Urfé, Québec, Canada) and dissolved directly in electrophoresis sample buffer containing 5% β-mercaptoethanol. Overnight treatments at 37 °C of eluted immunoprecipitated proteins with 4, 200 and 3 mU of endoglycosidase (endo-β-N-acetylglucosaminidase H or Endo H, endoglycosidase F/N-Glycosidase F or Glyco F, endo-β-Galactosidase or Endo β) were done, as previously described [24]. Thereafter, immunoprecipitates were analyzed by 12% SDS-PAGE and fluorography [23].

In vitro inhibition of N-linked glycosylation and protein transport

For N-linked glycosylation and protein transport inhibition experiments, infected 293 cells were pretreated for 1 h with 5 μg/ml of brefeldin A (BFA; Sigma-Aldrich Canada Ltd, Oakville, Ontario Canada), in methionine-deprived DMEM prior to labelling with [^{35}S]-methionine in the presence of the aforementioned drugs. When labelling was carried out overnight in the presence of BFA, the latter was added freshly every 4 h, since BFA is unstable during incubation [24].

Evaluation of the cytotoxicity and proapoptotic activity of recombinant proteins

Monolayers of MARC-145 cells were infected with PRRSV IAF-Klop strain at a MOI of 0.4 $TCID_{50}$ per cell or coinfected with AdTR5/ORF5 and AdCMV/tTA at a MOI of 100 PFU per cell of both viruses, in the presence or absence of 1 μg/ml of doxycycline. Cellular changes associated with intracellular synthesis of the $recGP_5$ were visualized at 70 h pi under a light microscope (Leitz, Leica Microsystems Inc., Richmond Hill, Canada). A commercial TUNEL assay (*In situ* cell death detection kit, fluorescein, Roche, Laval, Québec, Canada) was used for the detection of DNA fragmentation in PRRSV and hAdVs-infected cells. Apoptosis was also assessed by detecting the activation of procaspase 3. At 72 h pi, MARC-145 cells that have been coinfected with AdTR5/ORF5 and AdCMV/tTA or infected with IAF-Klop virus (MOI: 0.04 $TCID_{50}$/cell) were disrupted in a lysis solution from the ApoAlert Caspase® Fluorescent Assay Kit (Clontech Laboratories Inc., Palo Alto, California, U.S.A.). Five μl (corresponding to 60 to 75 μg of protein) of the cell lysates were added to 90 μl of a solution containing 50 mM HEPES, pH 7.0, 10% glycerol, 0.1% CHAPS, 2 mM EDTA and 5 mM DTT. Then, 10 μM of a specific substrate for caspase 3, the DEVD-AMC fluorogenic substrate (Biomol Research Laboratories Inc., Plymouth Meeting, PA, U.S.A.), was added and the rate of fluorescence released was monitored with a 96-well plate fluorometer (Cytofluor, Perseptive Biosystems, Foster City, CA, U.S.A.). The results were expressed as fluorescence released (fluorescence units or FU) per second per μg of cell lysates.

Pig immunization

Nine crossbred F1 (Landrace × Yorkshire) castrated specific pathogen-free (SPF) piglets, four to five weeks of age, were obtained from a breeding farm located in Southern Québec. The breeding stock and piglets were tested and proven to be seronegative for PRRSV, encephalomyocarditis virus (EMCV), porcine parvovirus (PPV), haemagglutinating encephalomyelitis virus (HEV), swine influenza virues H1N1 and H3N2, transmissible gastroenteritis virus (TGEV) and *Mycoplasma hyopneumoniae*. The piglets used in this study were from two different litters and were randomly divided into one control group of three pigs and one experimental group of six pigs kept in facilities equipped with a microorganism-free, filtered in-flowing and out-flowing air system. The animals were fed commercial feed and water *ad libitum*. The six experimental pigs were inoculated with 1×10^9 PFU of the hAdV AdTR5/ORF5 and 5×10^9 PFU of the hAdV AdCMV/tTA in a mixture containing 100 μL of PBS and 100 μL of the poloxamer SP1017 0,02% (Suprateck Pharma Inc., Laval, Qc,

Canada) [21]. The viral mixture was administered intradermally under the right ear using a 30 gauge needle. The three control pigs received by the same route of immunization a mixture of 100 μL of the hAdV AdCMV/tTA in PBS and 100 μL of the SP1017 poloxamer. The animals received a booster of the appropriate antigenic mixture at day 32, and were challenged intranasally at day 60 with 10^5 $TCID_{50}$ of the IAF-Klop strain in 5 ml of clarified cell culture supernatant fluid. Pigs were bled at post-challenge days 0, 10 and 21.

Results

Generation of the hAdVs

Preliminary attempts to generate replication-defective hAdVs expressing in mamalian cells the products of ORFs 5 and 7 genes of the North American IAF-Klop strain of PRRSV were unsuccessful. Apparently, high level of intracellular synthesis of the GP_5 and N proteins interfered with replication or assembly of the hAdVs. Indeed, replication-defective hAdVs could be generated by substitution of the constitutive expression cassette of the pAdCMV5 transfer vector by an inducible vector in which the tetracycline-regulatable promoter was used to control the level of transcription of the transgenes in the cells. On the other hand, hAdVs expressing constitutively the recM protein could be obtained, but viral stocks had only infectivity titers of 10^7 PFU per ml. Usually, infectivity titers of recombinant hAdV viral stocks vary between 10^8-10^{10} PFU/ml and even more following virus purification by isopycnic ultracentrifugation. RIPA experiments, in which lysates of hAdVs-infected 293 cells were incubated with specifc rabbit hyperimmune sera raised to GST-ORF5 (α5), -ORF6 (α6) and -ORF7 (α7) rec fusion proteins of the homologous virus, permitted visualization of immunoprecipitated polypeptides co-migrating with the authentic major viral structural proteins, GP_5, M and N (Fig. 1). Data suggested that hAdV-expressed rec proteins conserved most antigenic determinants of the authentic viral structural proteins. The $recGP_5$ of the IAF-Klop strain was also recognized by heterologous monospecific rabbit hyperimmune serum raised toward GST-ORF5 recombinant fusion proteins generated for five other prototype PRRSV strains, either from North America or Europe, a further evidence that antigenicity of the $recGP_5$ was most entirely conserved [35]. As previously reported by Mardassi et al. (1996), in RIPA experiments the three major structural proteins synthesized in PRRSV-infected MARC-145 cells coprecipitated (Fig. 1) because of their proteinic interactions, such as the presence of disulfide bonds.

Glycosylated nature of the $recGP_5$

The reactivity profiles of the monospecific rabbit α6 and α7 hyperimmune sera, when tested by RIPA with lysates of 293 cells infected with either AdCMV5/ORF6 or AdTR5/ORF7, and deduced M_rs of the recM and recN proteins, were indistinguishable from those obtained with the authentic viral M and N proteins. However, reactivity profiles obtained with monospecific rabbit α5 hyperimmune serum, when tested with lysates of PRRSV-infected MARC-145 and 293 cells,

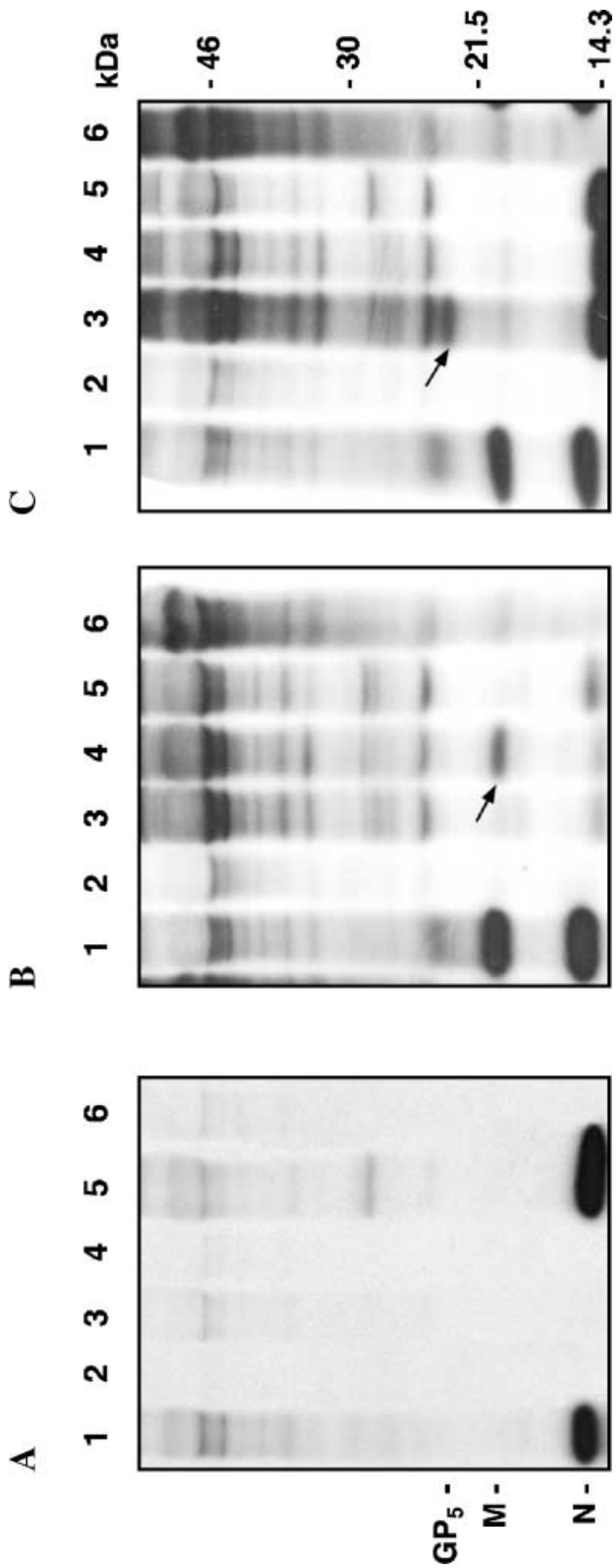


Fig. 1. Expression in 293 cells of the PRRSV recombinant major structural proteins (*N*, *M* and *GP*$_5$) by hAdVs. Radio-immunoprecipitation profiles obtained using lysates of MARC-145 cells infected with PRRSV (lane *1*) and lysates of 293 cells infected with hAdVs: AdTR5/ORF5 (lane *3*), AdCMV5/ORF6 (lane *4*) and AdTR5/ORF7 (lane *5*) or mock-infected MARC-145 and 293 cells (lanes *2* and *6*). **A** Reactivity of α7 monospecific anti-N serum; **B** Reactivity of α6 monospecific anti-M serum; **C** Reactivity of α5 monospecific anti-GP_5 serum. In panel **B** and **C**, the arrows indicate the recM and recGP_5 respectively

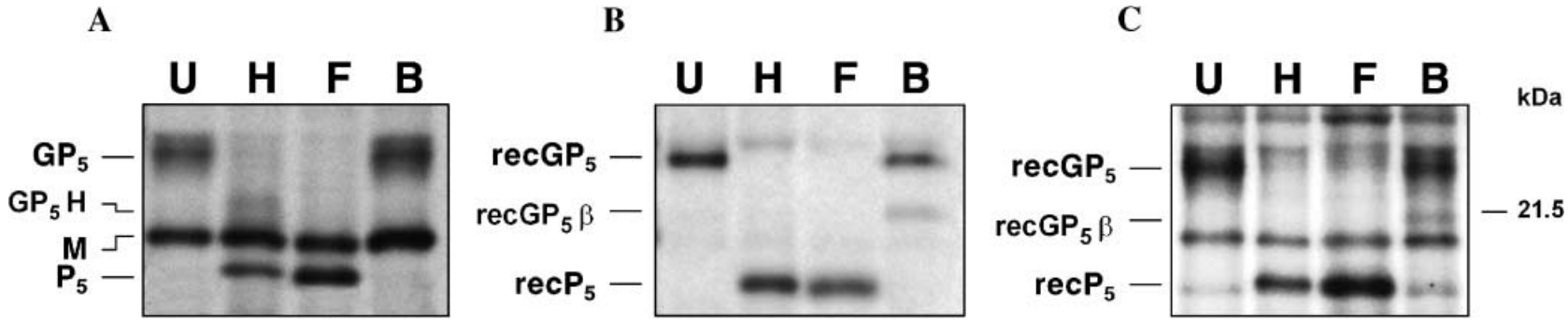


Fig. 2. Glycosylation nature of the recGP$_5$ in 293 and MARC-145 cells. **A** Immunoprecipitation profiles obtained with lysates of PRRSV-infected MARC-145 cells. **B** Immunoprecipitation profiles obtained with lysates of AdTR5/ORF5-infected 293 cells. **C** Immunoprecipitation profiles obtained with lysates of AdTR5/ORF5-infected MARC-145 cells. Authentic and recombinant proteins were immunoprecipitated using α5 monospecific rabbit antiserum. Immunoprecipitated proteins were untreated (lane *U*) or treated with endoglycosidases Glyco F (lane *F*), Endo H (lane *H*) and Endo β (lane *B*)

or infected with AdTR5/ORF5 after treatment with various endoglycosidases, suggested that the oligosaccharide residues of the recGP_5 differ from those of the authentic major envelope GP_5 glycoprotein. Consistent with previous findings [24], the monospecific α5 antiserum coprecipitated the M protein being associated with the GP_5 as disulfide-linked heterodimers in PRRSV-infected cells (Fig. 2a). Also, the authentic GP_5 synthesized in PRRSV-infected MARC-145 cells appeared as a highly glycosylated protein with estimated M_r of 25 kDa that was converted to a single species of 16,5 kDa (P_5) following treatment with Glyco F, an endoglycosidase known to cleave all N-linked oligosaccharides side chains [24]. However, it was totally resistant to Endo β (Fig. 2a), indicating that it lacks *N*-linked glycans modified by poly-*N*-acetyllactosamine [24]. On the other hand, treatment with Endo H (Fig. 2a), which cleaves high-mannose and hybrid oligosaccharides [24], demonstrated that the authentic viral GP_5 is partially sensitive to this glycosidase giving rise to at least two protein species of 16,5 kDa (P_5) and 21–22 kDa (named GP_5H for sensitivity to Endo H), suggestive

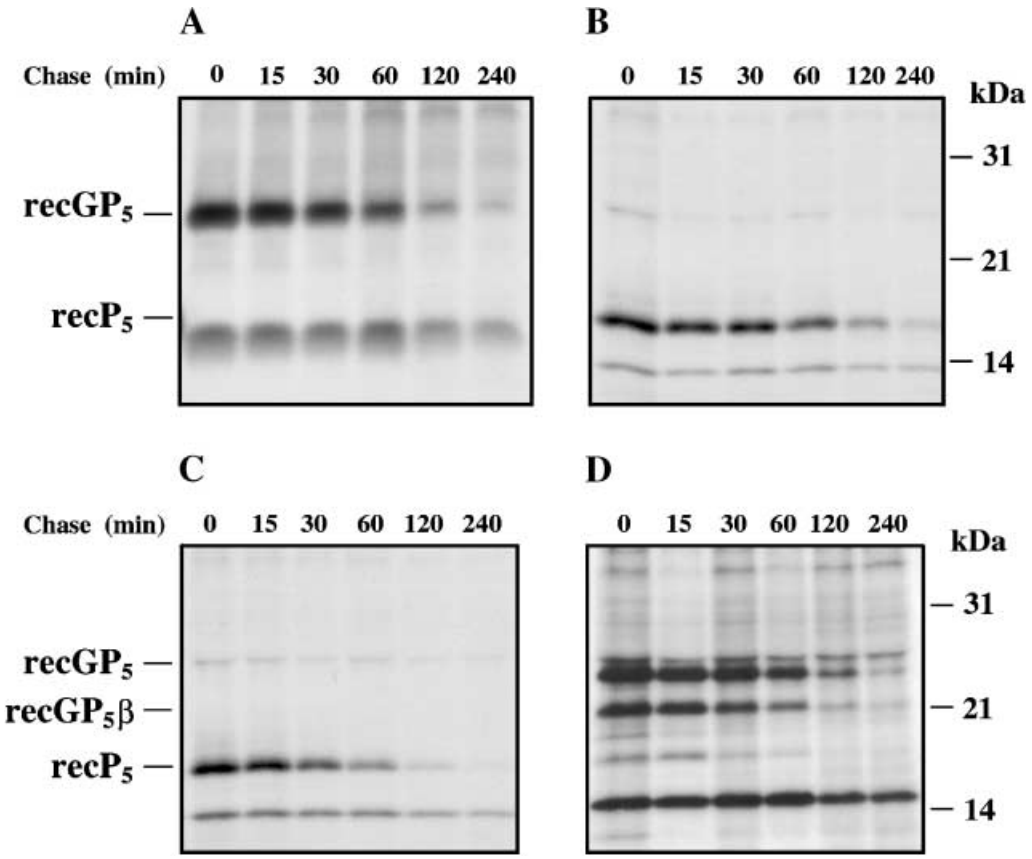


Fig. 3. Processing of the recGP_5 in AdTR5/ORF5-infected 293 cells. AdTR5/ORF5-infected 293 cells were pulse-labelled with [^{35}S] methionine for 30 min, then washed and chased with DMEM containing 5 mM of cold L-methionine. RIPA were performed with cell lysates prepared after the indicated chase period and the immunoprecipitated proteins were untreated (**A**) or treated with endoglycosidases; Glyco F (**B**), Endo H (**C**) and Endo β (**D**)

of the presence of N-linked oligosaccharides of the complex type. Consequently, data indicated that recGP$_5$ lacks N-linked oligosaccharides of the complex type since it was totally sensitive to Endo H (Fig. 2b). The recGP$_5$ was rather partially sensitive to digestion with Endo β, giving rise to two protein species of 20–22 kDa (named recGP$_5$ β for sensitivity to Endo β) and 25 kDa (recGP$_5$), respectively. In order to certify that differences in the glycosylation nature of recGP$_5$ were independent of the cell type used to propagate the hAVds, MARC-145 cells were coinfected with AdCMV5/tTA and AdTR5/ORF5. Upon individual expression in these cells, the recGP$_5$ appeared to be similarly glycosylated than after propagation of the hAdVs in 293 cells (Fig. 2c).

Processing of recGP$_5$ individually expressed in 293 cells

Pulse-chase experiments were performed to examine in greater detail the fate of recGP$_5$ upon expression in 293 cells (Fig. 3). Sensitivity to Endo H and partial resistance to Endo β glycosidases were maintained during the 240 min chase period (Fig. 3c,d), suggesting that individually expressed recGP$_5$ in 293 cells probably accumulated in the ER, being subjected to partial modification by poly-N-acetyllactosamine. In further experiments, the transport of GP$_5$ in PRRSV-infected MARC-145 cells was shown to be blocked by the addition of BFA in the culture medium (Fig. 4), a chemical which inhibits translocation of proteins from the ER to the Golgi compartment [19]. In the presence of this inhibitor, GP$_5$ synthesized in the context of a PRRSV-infection did not acquire any Endo H resistance (Fig. 4). The results obtained indicate that during the maturation cycle of PRRSV, GP$_5$ first accumulated in the ER in the form of P$_5$,

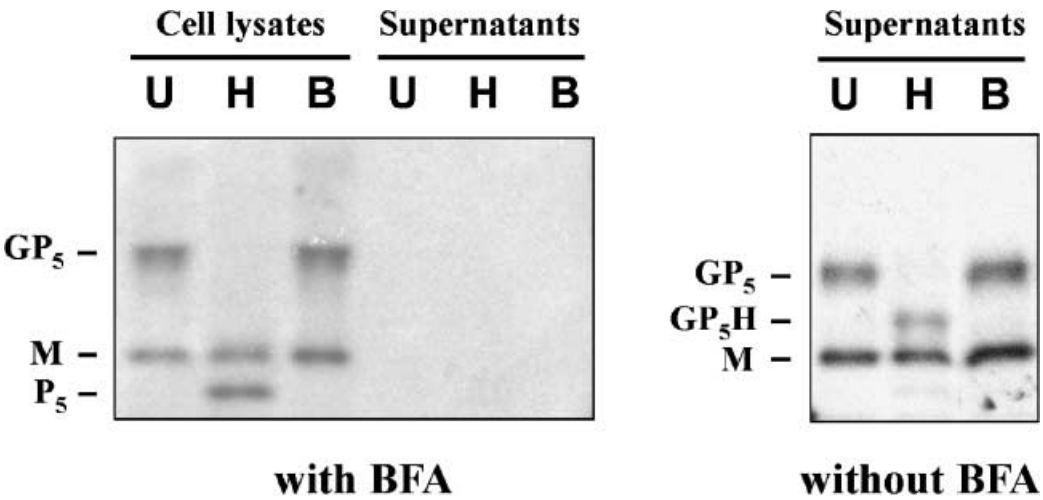


Fig. 4. Transport inhibition of the GP$_5$ major envelope-associated glycoprotein. PRRSV-infected MARC-145 cells were incubated with or without 5 μg/ml of BFA and RIPA were done on cell lysates and supernatant of infected cells using the monospecific α5 antiserum. Immunoprecipitated proteins were untreated (*U*) or treated with endoglycosidases: Endo H (lane *H*) and Endo β (lane *B*)

as demonstrated with the individually expressed $recGP_5$ (Fig. 2b). Neither the three major viral structural proteins (Fig. 4), nor assembled viral particles (data not shown), could be found in the supernatant fluids of PRRSV-infected cells in the presence of BFA, contrarily to the situation observed in the absence of BFA (Fig. 4). Therefore, through its transport from the ER to the Golgi apparatus, the GP_5 apparently acquires oligosaccharide side chains of the complex type, giving rise to a 21–22 kDa protein species (GP_5H) (Fig. 4). As previously described [24], GP_5H was the protein species incorporated in the mature virions. Contrarily to the modifications of $recGP_5$ observed in cells that have been infected with AdTR5/ORF5, the authentic GP_5 synthesized following PRRSV infection remained resistant to Endo β glycosidase even if its transport was blocked in the ER (Fig. 2b and 4). As a constituent of the envelope of mature virions, GP_5 has been previously found to be associated with M in the form of disulfide-linked heterodimers [24]. However, coinfection of 293 cells with hAdVs expressing individually the recN, recM or $recGP_5$ protein did not change the fate of the $recGP_5$ (Fig. 5). Although the simultaneous expression of the recM and $recGP_5$ could be detected in the infected 293 cells (Fig. 5), no heterodimer recM-$recGP_5$ formation could be demonstrated (data not shown). Furthermore, no coprecipitation of the $recGP_5$ and recM was demonstrated following incubation of the monospecific rabbit α6 hyperimmune serum with hAdV/ORF5 and hAdV/ORF6-coinfected cell lysates (data not shown), contrarily to the situation observed with lysates of

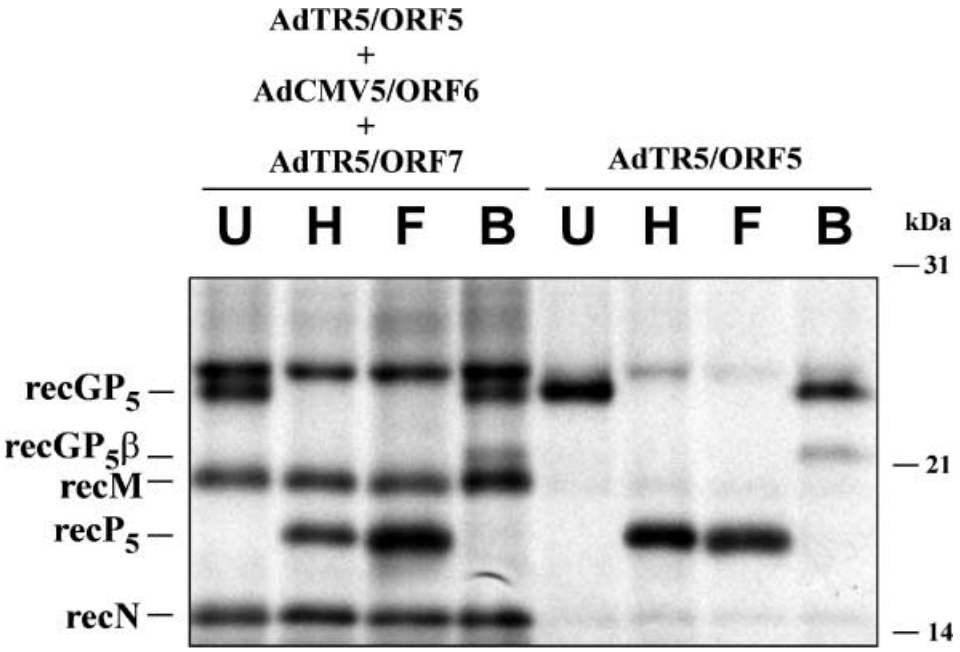
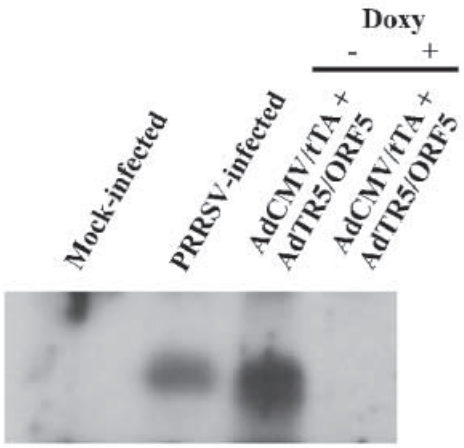
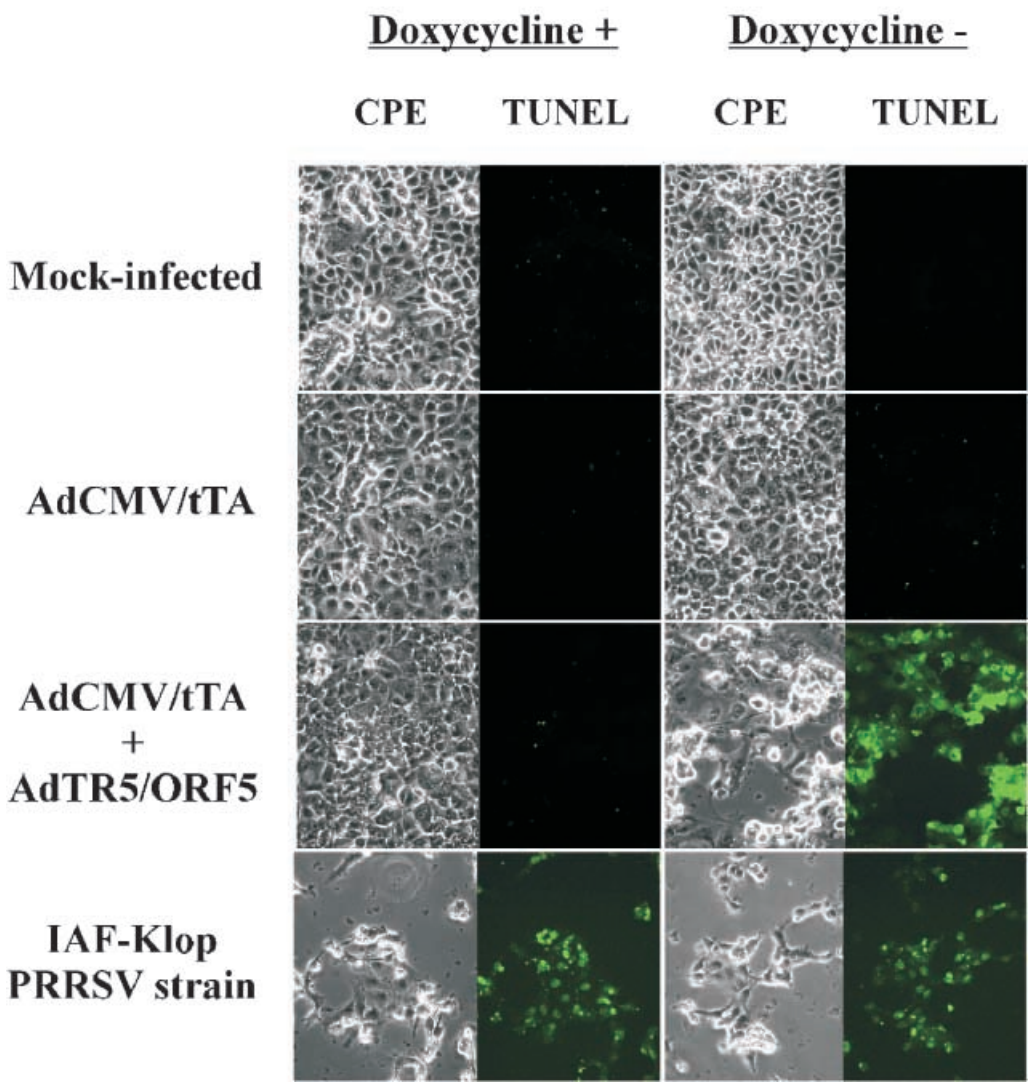


Fig. 5. Glycosylation nature of the coexpressed recN, recM and $recGP_5$ proteins in 293 cells. Radiolabelled recombinant proteins were immunoprecipitated with α5, α6 and α7 antisera and untreated (lane *U*) or treated with endoglycosidases; Glyco F (lane *F*), Endo H (lane *H*) and Endo β (lane *B*)

A


Doxy
-
+
Mock-infected
PRRSV-infected
AdCMV/tTA + AdTR5/ORF5
AdCMV/tTA + AdTR5/ORF5

B


Doxycycline +
Doxycycline -
CPE
TUNEL
CPE
TUNEL
Mock-infected
AdCMV/tTA
AdCMV/tTA + AdTR5/ORF5
IAF-Klop PRRSV strain

PRRSV-infected cell cultures wherein the formation of GP_5-M heterodimers was previously found to occur [24].

Proapoptotic activity of hAdV-expressed recGP$_5$ protein in MARC-145 cells

Coinfection of MARC-145 cells with AdCMV/tTA and AdTR5/ORF5 permitted the expression of $recGP_5$ which was completely inhibited in the presence of doxycycline (Fig. 6a). Interestingly, when $recGP_5$ was expressed individually in MARC-145 cells, cellular degenerescence was observed (Fig. 6b). These degenerative changes were neither observed when doxycycline was added to the medium (Fig. 6b, left panel) nor in cells expressing the recN protein alone (data not shown). The cellular changes observed with $recGP_5$ appeared almost identical to those observed in the PRRSV-infected cell cultures [23]. Indeed, scattered and enlarged cells with very small granular inclusions in their cytoplasm usually started to be detectable by 24 h pi, then infected cells showed a tendency to clump into foci that could be well delineated from the unaffected monolayer by 36 to 48 h pi. Many of the infected cells eventually detached from the surface of the culture flask, more than 75% of the cell monolayer being severely damaged by 72 h pi. To test whether the observed cytotoxicity was due to apoptosis, cells were assayed for the presence of DNA fragmentation by a TUNEL fluorescent assay, 3 days after infection. As illustrated in Fig. 6b, positive fluorescent cells were detected only when GP_5 expression was turned on and only in areas affected by degenerative damages. DNA fragmentation was also detected in PRRSV-infected cells (Fig. 6b). Additional evidence that apoptosis was occurring in AdTR5/ORF5-infected cells was obtained by the demonstration of procaspase 3 activation. As depicted in Fig. 7, an important increase in caspase 3 activity was detected only in cells expressing the $recGP_5$. Addition of doxycycline to inhibit $recGP_5$ expression did repress the procaspase 3 activation which was detected at the same level than in control cell cultures which had been infected with AdCMV/tTA alone. The levels of caspase 3 specifically induced by $recGP_5$, that were evaluated from three separate experiments, were at least 39 and 10 fold higher than those detected in the control uninfected cell monolayers and AdCMV/tTA infected cells, respectively. By comparison, PRRSV infection also resulted in procaspase 3 activation, but to a lower extent than AdTR5/ORF5 infection where $recGP_5$ was individually

Fig. 6. Induction of apoptosis in MARC-145 cells by regulatable expression of the $recGP_5$ protein. **A** Detection of the $recGP_5$ by Western blot in lysates of MARC-145 cells infected with PRRSV IAF-Klop strain or with both AdCMV/tTA and AdTR5/ORF5 in presence or absence of doxycycline using the anti-GP_5 rabbit monospecific antisera (α5). **B** Visualization with a light microscope of the cytopathic effect (*CPE*) of MARC-145 cells infected with PRRSV IAF-Klop strain and with AdCMV/tTA alone or in combination with AdTR5/ORF5, in presence or absence of doxycycline at 70 h pi. Visualization of the same hAdV infected-cells with a fluorescence microscope after treatment with a fluorescein TUNEL assay for the detection of DNA fragmentation

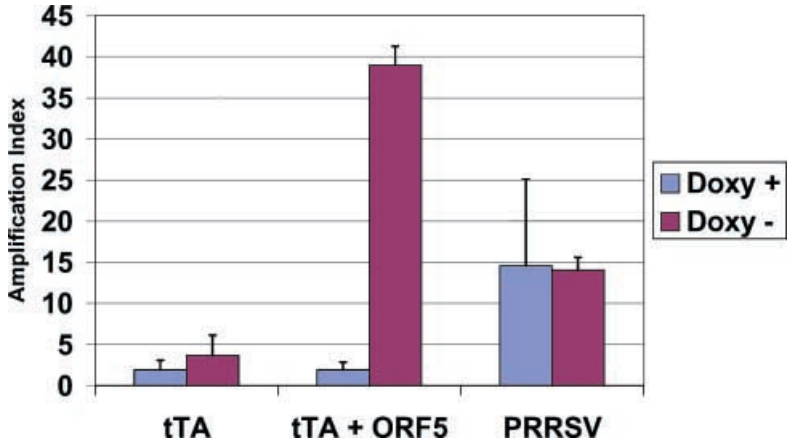


Fig. 7. Procaspase 3 activation. Confluent monolayers of MARC-145 cells were infected with either AdCMV/tTA (*tTA*) alone or with both AdCMV/tTA and AdTR5/ORF5 (*ORF5*) at a MOI of 50 PFU/cell, in the presence or absence of doxycycline. Control MARC-145 cells were mock-infected or infected with IAF-Klop PRRSV strain. Measurement of the procaspase 3 activation was done by quantifying the release of fluorescence and the results were expressed as FU/(sec $\times$ μg). The amplification indexes represent the level of procaspase 3 activation in infected cells divided by the level of procaspase 3 activation in uninfected cells

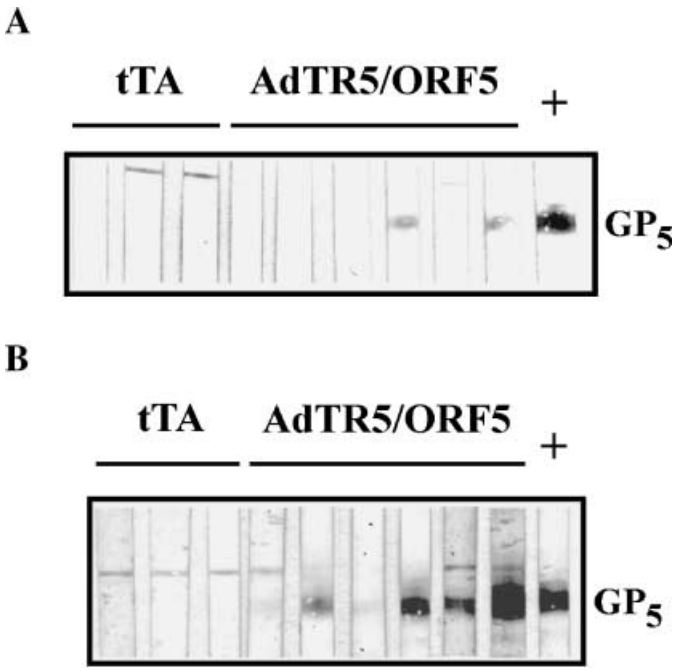


Fig. 8. Serological reactivities of AdTR5/ORF5-immunized pigs toward the authentic viral GP_5 protein. Lysates of IAF-Klop PRRSV-infected MARC-145 cells were prepared in RIPA buffer, clarified and electrophoresed on 12% SDS-polyacrylamide gels. Proteins were electrotransferred onto a nitrocellulose membrane and individual strips were incubated in the presence of serum from the pigs (diluted 1/50) that have been injected twice with AdTR5/ORF5 + AdCMV/tTA (immunized pigs) or AdCMV/tTA (control pigs) alone, and collected at day 10 (**A**) and day 21 (**B**) after intranasal challenge with virulent homologous PRRSV strain. tTA: sera of control pigs; AdTR5/ORF5: sera of pigs pre-immunized with AdCMV/tTA plus AdTR5/ORF5; +: reactivity of anti-GP_5 rabbit monospecific antisera (α5)

expressed. This was probably due to the lower number of cells expressing the GP_5 protein, since an MOI of only 0,04 $TCID_{50}$ per cell could be used for PRRSV, as compared to 50 PFU per cell for the hAdV AdTR5/ORF5.

Immune response induced in pigs by hAdV AdTR5/ORF5

Following two intradermal injections of the mixture AdCMV/tTA and AdTR5/ORF5, none of the immunized piglets developed significant antibody titers (> 16), as demonstrated either by indirect immunofluorescence (IIF) on PRRSV-infected MARC-145 cells or virus neutralization (VN) [22, 32]. Furthermore, none of the hyperimmunized pigs reacted positively to the authentic GP_5 viral protein by Western blot. However, following a challenge with a low dose of virulent virus given intranasally 14 days after the booster, the six AdTR5/ORF5-immunized pigs produced high antibody titers to the authentic viral GP_5 protein, a clear signal being obtained by Western blot as soon as 10 days post-challenge (Fig. 8). In comparison, pigs that were vaccinated with the AdCMV/tTA vector alone were still negative by Western blot at day 10 and day 21 post-challenge. Only the pigs that were vaccinated with the AdTR5/ORF5 developed VN antibody titers. Indeed, at day 10 post-challenge, two of the pre-immunized pigs had VN antibody titers of 128–256, and one had a VN antibody titer of 16. The control pigs were still negative by VN at day 21 post-challenge.

Discussion

In the present study, generation of replication-defective hAdVs expressing constitutively ORFs5 and 7 of a North American strain of PRRSV under the control of the hCMV immediate-early promoter/enhancer was unsuccessful, despite that such hAdV was obtained for the ORF6. However, infectivity titers of the replication-defective hAdV/ORF6 propagated in 293 cells, complementing the E1 functions of the hAd5, were at least 10 to 100 fold lower than those usually expected for such recombinant adenoviruses [18]. Further characterization of the authentic GP_5 of IAF-Klop strain of PRRSV demonstrates that, as the homologous protein of European strains [40], it induces cell death by apoptosis (Fig. 6b and 7) and appears to be toxic for 293 cells. Both characteristics could explain the failure to generate hAdVs that constitutively express this major envelope glycoprotein of PRRSV. Whether similar phenomena can also interfere with generation of hAdVs that constitutively express the N protein should be further investigated, as no cytotoxicity in 293 cells transfected with pAdCMV5/ORF7 could be observed (data not shown). However, previous investigators [36] identified the nucleolus of PRRSV-infected cells as the primary site for N protein localization within the nucleus and have observed some toxicity. Even if cell death did not appear to correlate with this early localization of the N protein during PRRSV replication cycle, it may interfere with some cellular functions required for the generation of hAdVs. Consequently, to generate hAdVs that could express the GP_5 and N proteins, an inducible system was required to be able to control the synthesis

of both proteins to a threshold level above which they become toxic for the cells and interfere with replication and assembly of hAdVs. This was done by using the tetracycline-regulated promoter contained in the pAdTR5 transfer vector which has been already reported to permit the construction of hAdVs expressing toxic proteins [26, 27]. In MARC-145 cells, coinfection of hAdVs expressing the tTA transactivator (AdCMV/tTA) with hAdVs expressing the PRRSV structural genes under the control of the tetracycline-regulated promoter (AdTR5/ORF5 and AdTR5/ORF7) was essential to allow efficient expression of PRRSV rec proteins. Addition of doxycycline in the culture medium also completely suppressed expression of the transgenes (Fig. 6a), thus confirming that the tetracycline-regulated expression system was very efficient for controlling the expression levels of both viral proteins, to allow generation of hAdVs carrying the ORFs 5 and 7.

The antigenicity of the recN, recM and recGP_5 expressed by hAdVs (AdTR5/ORF7, AdCMV5/ORF6 and AdTR5/ORF5) was conserved since the rec proteins could be detected by the homologous monospecific rabbit antiserum raised against the corresponding viral protein expressed in *E. coli*. [24], by the homologous porcine anti-PRRSV hyperimmune serum, as well as heterologous monospecific rabbit hyperimmune sera raised toward GST-ORF5 recombinant fusion proteins of five other prototype PRRSV strains, either from North America or Europe [35]. Data also suggested that both, linear and conformational epitopes of PRRSV major structural proteins were conserved following expession by the rec hAdVs, despite that glycosylation of the recGP_5 protein was found to be different from that of the authentic GP_5. Indeed, whereas the authentic viral GP_5 of the IAF-Klop strain was resistant to treatment with Endo β glycosidase [24], the individually expressed recGP_5 was partially sensitive to digestion by this endoglycosidase (Fig. 2). Consequently, by contrast to the authentic viral GP_5, the recGP_5 expressed individually by hAdVs possesses N-acetyllactosamine side chain oligosaccharides. Several structural features on the authentic protein (fucose, branching, galactose sulfate residues) may be responsible for its resistance to Endo β glycosidase [37]. The presence of such oligosaccharide residues (N-acetyllactosamine side chains) have been previously reported in the case of the G_L protein of EAV, the counterpart of the GP_5 of PRRSV [8]. The sensitivity of the recGP_5 protein to Endo H glycosidase is an indication that the individually expressed protein is associated with intracellular membranes or accumulates within the ER. This correlates with the fact that in PRRSV-infected MARC-145 cells incubated with BFA, which inhibits translocation of the proteins to the Golgi apparatus [19], the authentic GP_5 remained sensitive to Endo H, as in case of the recGP_5 (Fig. 4). However, even after treatment with BFA, the authentic GP_5 still differs from the recGP_5 in regards to their distinct reactivity to Endo β digestion. Further studies are required to identify whether this difference can be attributed to interactions with proteins synthesized during the replication of hAdVs or if interactions with other unidentified PRRSV proteins are required for final maturation of GP_5 occuring in the Golgi apparatus. The glycosylation state of the GP_5 may have an important role to play in its biological activity, as previously demonstrated for LDV in mice for which the glycosylation state of the major envelope structural protein is

implicated in the neuropathogenicity of the various strains, as well as inactivation by neutralizing antibodies [5].

As for the autologous proteins of EAV, the GP_5 and M proteins of PRRSV form heterodimers prior to the final processing of the N-oligosaccharide side chains of GP_5 [9, 24]. Consequently, the formation of these heterodimers play a key role in the maturation and infectivity of the PRRSV virions [9, 13, 24]. However, in the present study, such heterodimers could not be demonstrated by co-infection experiments with hAdVs-expressed recGP_5, recM and/or recN proteins (Fig. 5), done in two different cell lines (data not shown). Also, no virion-like particles could be observed in the supernatant medium by negatively stained electron microscopy. Since such heterodimers could not be obtained after transient expression assays of the M and GP_5 proteins in 293 cells co-transfected with the corresponding recombinant transfer vectors (data not shown), it seems unlikely that the hAdVs interfered with the heterodimers formation. It is plausible that other PRRSV proteins are essential for the appropriate folding and glycosylation of the GP_5.

Recently, expression of the two major envelope proteins of EAV as heterodimers has been reported to be necessary for induction of neutralizing antibodies in mice immunized with recombinant Venezuelan equine encephalitis (VEE) virus replicon particles [2]. Data obtained in this study can not be generalized for arteriviruses infecting other animal species. Indeed, in previous studies, the production of VN antibodies could also be recovered in pigs, but not in mice, following genetic immunization with plasmidic DNA carrying the PRRSV ORF5 in the absence of M protein [34]. In contrast, VN antibodies to PRRSV could be recovered from the serum of mice following their immunization with *E. coli*.-expressed ORF5 protein [33]. Similarly, in the case of EAV, the presence of VN antibodies could be demonstrated in the sera from horses and mice immunized with synthetic peptides deduced from the aa sequence of the G_L protein, or with a bacterial fusion protein [6]. Consequently, the ability of the individually-expressed PRRSV GP_5 protein to induce neutralizing antibodies in pigs was not surprising. The fact that neutralizing antibodies were induced only in mice immunized with the G_L/M heterodimers of EAV expressed by recombinant VEE indicates that in the context of the recombinant viral infection, the presence of the M protein is critical to the expression of neutralizing epitopes, which are apparently conformation-dependent. In the case of the PRRSV recombinant GP_5 protein individually expressed by hAdVs, data obtained indicate that at least some of the epitopes involved in VN are available to the antigen presenting cells, and probably that other neutralizing conformation-dependent epitopes may require the interaction with the M protein to become available to cells involved in the immune response.

In the present study, it has been further demonstrated that immunization (vaccination) of pigs with adenovirus that are able to express the GP_5 of PRRSV in the absence of M protein triggers their immune system for the production of VN antibodies, which however could only be detected 10 days after they have been challenged with a low dose of the virulent homologous virus. These serological data differ significantly from those obtained following natural infection with

PRRSV, since infected pigs usually do not produce VN until 3 to 4 weeks post-infection [22, 32]. However, two weeks after the first injection of the rec hAdVs, pigs had developed high specific indirect immunofluorescence (IIF) antibody titers (>160) to adenovirus structural proteins (data not shown), which may have interfere with an efficient expression of the recGP$_5$ and subsequently delayed the establishment of a specific immune response against the GP$_5$. To demonstrate the effectiveness of PRRSV challenge, collected sera were tested for the presence of anti-PRRSV antibodies by IIF, by using a commercial ELISA (IDEXX) and by using an anti-N blocking ELISA [11]. At the day of challenge, all tested sera were negative for antibodies to PRRSV, whereas at 10 days post-challenge, all sera tested positive using those three diagnostic tests, thus confirming the seroconversion to PRRSV (data not shown). Since the latter serological tests were reported to permit detection of antibodies directed only (blocking ELISA) or mainly (IIF and IDEXX ELISA) to the N protein of PRRSV [11], they were expected to be negative or only weakly positive before challenge, since the vaccination was against the GP$_5$ only. In spite of the demonstration that authentic GP$_5$ specific antibodies in experimentally infected pigs sera are only detected no earlier than 28 days post-infection by western blot [22], GP$_5$ specific antibodies could be detected as early as 10 days post-challenge in AdTR5/ORF5 vaccinated pigs. Data obtained suggest that vaccination of pigs with adenovirus expressing recGP$_5$ at least pre-sensibilized cells from the lymphoid organs and established an immune memory, which following a subsequent PRRSV infection, results in a rapid clonal expansion of memory cells to the neutralizing epitopes of the authentic viral GP$_5$ protein. Since AdCMV/tTA, which permits the constitutive expression of the tetracycline transactivator (tTA), had to be co-inoculated to the pigs with AdTR5/ORF5 to obtain expression of recGP$_5$, the *in vivo* expression of the GP$_5$ may have been less efficient than in cell cultures. Indeed, the cells had to be infected by both rec hAdVs to express the recGP$_5$. Therefore, the vaccinated animals probably did not produce enough antibodies to reach the threshold required to be detected by the serological tests used in the present study. With the type of inducible vector used in this study to control the expression level of recGP$_5$, the hAdV strategy was less successful than previously described DNA vaccine [34], since *in vivo* co-infection of the same cells by AdCMV/tTA and AdTR5/ORF5 could not be controlled. This may explain why only a specific memory immune response was established in vaccinated pigs (Fig. 8).

Recently, an adenovirus expressing both the gene of interest and the tTA has been constructed and proven to be efficient for *in vivo* expression of the transgene [7]. Other promoters can also be used to repress the expression of a toxic transgene in cells constitutively expressing a repressor protein (like the lac repressor protein). Such promoters allow the generation of hAdVs carrying foreign genes encoding toxic proteins and are efficient to permit high expression of a transgene in other cell lines [28]. In the context of vaccine development using recombinant adenoviruses, those strategies and other alternatives are being studied.

European, as well as North American, strains of PRRSV were reported to induce apoptosis in the infected cells both *in vitro* and *in vivo* [40-42]. The

use of recombinant vaccinia virus expressing the GP_5 of the European genotype demonstrated that the major envelope glycoprotein is involved in this pathological phenomenon [40]. In the present study, the proapoptotic phenotype of the IAF-Klop GP_5 was demonstrated in cells infected with the replication-defective hAdVs using two different approaches: firstly, the demonstration of DNA fragmentation by TUNEL assays (Fig. 6), and secondly, the demonstration of procaspase 3 activation (Fig. 7). Despite only 53% aa identity between the GP_5 of North American and European strains [35], both GP_5 display proapoptotic activity. Further studies should permit to identify the GP_5 region or aa residues involved and conserved amongst strains from both genotypes. Recently, it has been demonstrated, *in vivo* and *in vitro*, that apoptosis occured in PRRSV-uninfected bystander cells [38]. This phenomenon could not be demonstrated in the present study since a MOI of 50 PFU was necessary to obtain sufficient expression of the recGP_5 by the replication-defective adenovirus vectors. Studies are in progress to further characterize the proapoptotic activity of the GP_5 of the IAF-Klop strain and determine which caspase(s) cascade(s) is activated. Previous studies with the Spanish OLOT strain of PRRSV demonstrated that the GP_5-induced apoptotic activity could not be prevented by using a cell line permanently expressing the anti-apoptotic bcl-2 gene, suggesting the use of an alternative and/or unknown apoptotic pathway [40].

Acknowledgements

The authors wish to thank Louise Wilson, Marcel Desrosiers, and François Bouthillier for their excellent technical assistance. This report was taken in part from a dissertation to be submitted by C.A. Gagnon to the INRS-Institut Armand-Frappier, in partial fulfillment of the requirements for the PhD degree. This work was supported by the Natural Sciences and Engineering Research Council of Canada (Strategic grant STP02002083) and Biovet Inc., St-Hyacinthe, Quebec, Canada. C.A. Gagnon was a recipient of a fellowship from the Medical Research Council of Canada. This is a NRC publication 46145.

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