Regulator of G protein signalling 16 is a target for a porcine circovirus type 2 protein

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Interaction studies have suggested that the non-structural protein encoded by open reading frame 3 (ORF3) of porcine circovirus type 2 (PCV2) binds specifically to a regulator of G protein signalling (RGS) related to human RGS16 (huRGS16). The full-length clone of RGS16 was generated from porcine cells and sequence analysis revealed a close relationship to huRGS16 and murine RGS16. In vitro pull-down experiments verified an interaction between porcine RGS16 (poRGS16) and ORF3 from PCV2. Using GST-linked ORF3 proteins from three different genogroups of PCV2 and from porcine circovirus type 1 (PCV1) in the pull-down experiments indicated that there were differences in their ability to bind poRGS16. Quantitative RT-PCR demonstrated that the expression of poRGS16 mRNA could be induced by a number of cell activators including mitogens (LPS and PHA), interferon inducers (ODN 2216 and poly I : C) and the neurotransmitter norepinephrine. Immunofluorescence labelling confirmed the induced expression of poRGS16 at the protein level and suggested that the PCV2 ORF3 protein co-localized with poRGS16 in LPS-activated porcine PBMC. Furthermore, poRGS16 appeared to participate in the translocation of the ORF3 protein into the cell nucleus, suggesting that the observed interaction may play an important role in the infection biology of porcine circovirus.

INTRODUCTION
Circoviruses, belonging to the family Circoviridae and encompassing avian and porcine pathogens (Todd et al., 2001), are non-enveloped, single-stranded, circular DNA viruses, with similarities to the anelloviruses, viruses with unknown pathogenicity that are not yet assigned to any family (Biagini, 2004; Todd et al., 2005). Porcine circovirus type 1 (PCV1) is not known to cause any disease, whereas infection with porcine circovirus type 2 (PCV2) is associated with a number of disease syndromes (Allan et al., 1999; Harding, 2004; Segalés et al., 2004; Opriessnig et al., 2007). Of these, it is generally accepted that post-weaning multisystemic wasting syndrome (PMWS) is caused by PCV2, but only if other, still not fully specified, infectious or environmental co-factors are present. The lymphoid organs of pigs with severe PMWS are depleted of lymphoid cells and infiltrated by cells of the myeloid lineage. Consequently, PMWS is associated with a suppression of the host immune response and affected animals are more susceptible to secondary infections (Chae, 2005; Segalés et al., 2005).

The genome of PCV2 is among the smallest (1769 nt) of all known viruses and appears to have only four major open reading frames, ORFs 1–4 (Meehan et al., 1998). ORF1 encodes two replicase proteins (Rep and Rep9), corresponding to two different splicing products, and ORF2 encodes a structural protein forming the viral capsid (Cap). The proteins encoded by ORF3 and 4 have to date not been assigned any clear function and it has not been fully proven that these putative polypeptides are expressed in infected cells. However, their overall presence and conserved amino acid composition indicate that they are functional and a
possible role in apoptosis has been assigned to the PCV2 ORF3 protein (Liu et al., 2005, 2006; Karuppannan et al., 2009).

To clarify the function of proteins from circoviruses further, their localization and interaction with viral components or cellular proteins have been studied. The Rep and Cap proteins of both PCV1 (Finsterbusch et al., 2005) and PCV2 (Meerts et al., 2005a) co-localize in the nucleus of infected or transfected porcine kidney (PK) cells. Similarly, the Cap protein of the avian circovirus beak and feather disease virus (BFDV) localizes to the nucleus and can interact with both single- and double-stranded DNA as well as with the Rep protein (Heath et al., 2006). The Cap protein of the human anellovirus torque teno virus (TTV) suppresses the activation of NF-kB and thereby hinders the production of a number of pro-inflammatory cytokines and chemokines (Zheng et al., 2007). In a yeast two-hybrid system, ORF3 of PCV2 was indicated as an interaction partner for ubiquitin ligase (Liu et al., 2007). Using a bacterial two-hybrid screening of a porcine expression library from PK-15 cells, we have previously demonstrated that Rep of PCV2 interacts with Cap and with cellular proteins corresponding to syncoilin and c-myc (Timmusk et al., 2006). Among the proteins that interact with ORF3 in this BacterioMatch system, a protein with homology to the murine and human regulator of G protein signalling 16 (RGS16) was detected.

Regulators of G protein signalling (RGSs) constitute a family of proteins that modify signalling via G protein-coupled receptors (GPCRs). As reviewed by Xie & Palmer (2007) and Jean-Baptiste et al. (2006), the conserved, approximately 120 aa, RGS domain of the RGS proteins binds to the activated Gz subunit of G-proteins and thereby terminates the G-protein signalling. Most RGS proteins can thereby affect the cellular response to a variety of stimuli, including hormones, neurotransmitters and chemokines. RGS16 might also indirectly affect the interplay between the nervous system and the immune system through deactivation of G proteins stimulated by, for example, epinephrine or norepinephrine (NE) via δ2A-adrenoreceptors (Hoffmann et al., 2001).

RGS16 belongs to a subfamily of RGS proteins (R4/B) that is characterized by a 30 aa helix structure at the N-terminal end of the RGS domain. The amphipathic nature of this helix targets the proteins to plasma membranes or acidic phospholipid vesicles in the cytosol (Heximer et al., 2001). Thus, RGS16 can be localized to cellular compartments known to harbour PCV2 proteins in infected cells (Vincent et al., 2003, 2005, 2007). Furthermore, RGS16 is upregulated in immature dendritic cells (DCs) when exposed to inflammatory stimuli such as lipopolysaccharide (LPS) or the synthetic RNA analogue polyI:C (Shi et al., 2004). In vitro studies have demonstrated that PCV2 persists in porcine DCs (Vincent et al., 2003) and seems to impair their maturation in response to signalling via Toll-like receptor (TLR)9 (Vincent et al., 2005). Consequently, the putative interaction between a PCV2 ORF3-encoded protein and a porcine homologue to RGS16 deserves further studies.

In the present paper, the expression of RGS16 was studied in porcine peripheral blood mononuclear cells (poPBMCs) exposed to different activators. Using plasmids expressing ORF3 of PCV2 or porcine RGS16 (poRGS16), the co-localization of these proteins was studied in porcine cells. A better understanding of the role of RGS16 during the infection and in particular the possible interaction with ORF3 could help to clarify the role of ORF3 in PCV2-induced pathogenesis.

METHODS

Cell cultures and activators used for cell stimulation. A porcine kidney cell line, free of PCV, (PK-15/A) was kindly provided by Dr Francis McNeilly, Veterinary Science Division, Department of Agricultural and Rural Development for Northern Ireland, Belfast, UK. The PK-15/A cells were cultured in Dulbecco’s minimal essential medium (DMEM) supplemented with penicillin, streptomycin and 5 % fetal calf serum (FCS; Gibco) at 37 °C in a humid atmosphere with 7 % CO₂. For transfection studies, COS-1 cells were cultured as described above. Blood was collected from conventionally reared Yorkshire pigs in heparinized tubes (143 USP heparin; B-D Vacutainer) and poPBMCs were isolated by density-gradient centrifugation (45 min, 500 g) on Ficoll-Paque (Amersham Pharmacia Biotech). After three washes in PBS, poPBMCs were suspended in RPMI 1640 medium (BioWhittaker) supplemented with 20 mM HEPES, 2 mM L-glutamine, 200 IU penicillin ml⁻¹, 100 μg streptomycin ml⁻¹, 0.5 μM 2-Mercaptoethanol and 5 % FCS. When indicated, poPBMC were incubated (37 °C, 7 % CO₂) in the presence of LPS serotype 0111:B6 (2.5 μg ml⁻¹; Sigma), phytohaemagglutinin A (PHA; 1 μg ml⁻¹; Sigma), concanavalin A (ConA; 5 μg ml⁻¹; Amersham Pharmacia Biotech), polyriboinosinic-polyribycytidylid acid (polyI:C; 5 μg ml⁻¹; Amersham Pharmacia Biotech) or the oligodeoxynucleotide (ODN) 5’-ggGAGCGATCGTGCCCgggG-3’ (ODN 2216; 5 μg ml⁻¹; Cybergene AB). The poly I:C preparation was preincubated with Lipofectin (3 μg ml⁻¹; Invitrogen) as described previously (Wikström et al., 2007). Parallel cultures with poPBMC cultured in plain growth medium served as controls and in some cases the growth medium was supplemented with NE (100 μM; Sigma).

Isolation and sequencing of poRGS16. Full-length cDNA was isolated by 5’- and 3’-rapid amplification of cDNA ends (5’- and 3’-RACE) using the SMART RACE cDNA Amplification kit (Clontech), according to the manufacturer’s instructions. Universal primers (Clontech) were used in combination with RGS16 primers (Supplementary Table S1, available in JGV Online), designed from the partial sequence obtained from the BacterioMatch interaction study using PCV2 ORF3 as a bait (Timmusk et al., 2006). Two full-length clones of poRGS16 were isolated, one from the PK-15/A cell-derived template and one from LPS-activated poPBMC. The PCR products obtained (658 bp) were cloned into pGEM-T-Easy vector (accession no. EU271873).

Cloning and expression of poRGS16 and ORF3 from PCV1 and PCV2. To produce glutathione S-transferase (GST) ORF3 fusion proteins, the entire ORF3 was cloned from two Swedish isolates of
PCV2 (GenBank accession nos. EU408780 and EU386606), from PCV2 Stool (PCV2-St; GenBank accession no. AF055392), from PCV1 (GenBank accession no. AY193712) and from the corresponding N-terminal part encoding 104 aa of the ORF3 region from PCV1 (GenBank accession no. AY193712). The various ORF3-coding regions were inserted into the EcoRI and XhoI sites of the pGEX-5T plasmid (Stratagene) for expression in Escherichia coli BL21 (DE3) as described previously (Timmusk et al., 2006). Briefly, for the GST pull-down studies, the PCV2 full-length ORF3 proteins from SG1, SG2, SG3, the full-length ORF3 protein from PCV1 and the partial ORF3 from PCV3, as well as negative control proteins, were expressed by using the pGEX-5T system (Berg et al., 1998). All proteins were expressed in E. coli BL21 (DE3) and induced with 1 mM IPTG at room temperature overnight. Bacterial pellets were resuspended in 1× PBS with 1 mg lysozyme ml⁻¹ according to the manufacturer’s instructions (GE Healthcare) and sonicated on ice with short bursts. After sonication, 20% Triton X-100 was added to a final concentration of 1% and incubated on ice for 30 min to aid solubilization of the fusion protein. The lysates were cleared by centrifugation (10 min at 16,000 g at 4 °C) and the fusion proteins were purified using Sepharose 4B (GE Healthcare) using the batch purification system. The bound GST proteins were eluted with elution buffer (50 mM Tris/HCl, 10 mM reduced glutathione, pH 8.0) and aliquots were frozen in liquid nitrogen. The amount of recovered proteins were quantified by using nanodrop and the purity of the proteins were observed by SDS-PAGE.

The coupled transcription–translation system (TNT; Promega) was used for generation of radiolabelled poRG16. The luciferase gene, provided by the TNT kit, and poRG16 were expressed as 35S-labelled proteins from pcDNA3 plasmids (Invitrogen) according to the manufacturer’s instructions. For generation of fluorescent proteins, the entire coding region (711 bp) of mCherry was excised from the vector pBSK-mCherry E3 (the plasmid originated from Dr Roger Tsien, Howard Hughes Medical Institute, and was a generous gift from Dr Nora Ausmees, Department of Cell and Molecular Biology, Uppsala University, Sweden) and inserted into pcDNA3 XhoI–XbaI sites (pcDNA3–mCherry). The coding regions for ORF3 from various PCV isolates (see above) were inserted in the same reading frame at the NotI site on the N-terminal side of mCherry, using a universal forward primer in combination with specific reverse primers that were unique for each genotype of PCV2/ORF3 (Supplementary Table S1). The recombinant proteins generated from these plasmid constructs approached an additional 16 aa (RGSTSPSILSSDDAPAT) in between ORF3 and mCherry. A plasmid for the expression of GFP–poRG16 fusion proteins was generated by inserting 606 bp of poRG16 into the XhoI–EcoRI sites of the pEFP plasmid (BD Biosciences). A plasmid for the expression of histidine-tagged ORF3 protein (His–ORF3) was generated by cloning PCR-amplified ORF3 protein from PCV2 (EU408780) into the EcoRV–XhoI sites of pcDNA3.1–His (Invitrogen). All constructs were validated by sequencing.

GST pull-down analysis. A GST pull-down assay was used to estimate protein interactions, as described previously (Timmusk et al., 2006). In brief, 5 μg GST fusion proteins were mixed with 35S-labelled proteins, both the protein of interest and an internal negative control (luciferase), in PBS. The proteins were incubated together for 30 min at room temperature. Thereafter, the volume was increased to 150 μl and GST–Sepharose beads (GE Healthcare) were added to the mixture. The pull-down incubation was performed for 3 h at room temperature with slow but continuous rotation. The GST fusion proteins were recovered by a brief centrifugation of the GST–Sepharose beads. The pellet was washed four times in PBS and the last wash was divided into two equal aliquots. Each aliquot was boiled in SDS loading buffer. The two identical sets of samples were then analysed on two parallel SDS-PAGE gels. Unrelated proteins (ORF2 of PCV2 and bornavirus B33 protein) fused with GST (Timmusk et al., 2006; Berg et al., 1998) were used as negative controls. One of the gels was dried and the binding of ORF3 from various PCV isolates to poRG16 was measured by phosphoimager analysis.

Western blot analysis. Gels were transferred electrophoretically (16 V, 15 min) to nitrocellulose membrane (Hybond-C Amersham Pharmacia) by semi-dry electroblothing. The nitrocellulose membrane was blocked with 2% casein in TBS plus 0.05% Tween 20 (TBS-T) solution for 1 h at room temperature and washed three times for 5 min with TBS-T, then the membrane was incubated for 3 h with anti-GST monoclonal antibody (mAb) (Amersham Pharmacia), washed three times with TBS-T solution (5 min each) and incubated with goat anti-mouse polyclonal antibody (pAb) (1:10000 in TBS-T; DAKO). The membrane was washed three times for 5 min with TBS-T solution, once for 5 min with 1× TBS and rinsed with water. The chemiluminescence reaction was then developed using the Western blot detection kit (Pierce Protein Research Products Thermo scientific kit).

Analysis of RGS16 mRNA expression. Real-time PCR and/or semi quantitative RT-PCR assays were used to determine the expression of RGS16 mRNA in poPBMC activated by PHA, polyclonal C, LPS, ODN 2216 or NE.

Total RNA was isolated by combining the recommended protocols for the Trizol reagent (Invitrogen) and the RNeasy mini (Qiagen) kits. In brief, the RNA-containing aqueous phase, obtained after phase separation in the Trizol reagent, was mixed with an equal amount of 70% ethanol and transferred to an RNeasy mini kit spin column for RNA purification. The isolated RNA was treated with DNase (Promega ) using an extended incubation time of 30 min, but otherwise according to the manufacturer’s instructions, and first strand cdNA was synthesized using 2 μg RNA as template, oligo dT primer and SuperScript II reverse transcriptase (Invitrogen), according to the manufacturer’s recommended protocol.

TaqMan real-time PCR was performed for RGS16 and a housekeeping gene Cyclophilin A, using published primers (Supplementary Table S1) (http://www.ars.usda.gov/Services/docs.htm?docid=6065) and 5’-6-carboxylfluorescin (FAM)- and 3’ black hole quencher 1 (BHQ1)-labelled probes (cyclophilin A, FAM-TGC CAG GGT GGT GAC TTC ACA CGC C-BHQ1; RGS16, FAM-ATC GCC GTC TGA CTG GTC ATT ACA GTA GCT-BHQ1 (both 5’-3’)) (Duvigneau et al., 2005; Dawson et al., 1999). The cdNA was diluted five times and added in 1 μl volumes to triplicate 25 μl reactions containing 1× Absolute QPCR Mix (ABgene), 0.4 μM each primer and 0.2 μM 5’-FAM- and 3’-BHQ1-labelled probe. The samples were amplified and analysed in an IQ5 real-time PCR machine (Bio-Rad) using an initial 15 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 61 °C for 30 s. The FAM fluorescence signal was measured at the 61 °C step for each cycle. Relative expression of RGS16 in the different samples used as comparators in the medium control is expressed as 2-DDCt, calculated by subtracting the cycle threshold (Ct) values of RGS16 from the Ct values of cyclophilin A (the housekeeping gene) for each sample and then subtracting this ΔCt value for each sample with the ΔCt value of the medium control to generate the ΔΔCt value (Livak & Schmittgen, 2001).

Semi quantitative PCR amplification was performed using RGS16- or cyclophilin A-specific primers (Supplementary Table S1) and the following cycling parameters: 30 cycles of 95 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min. After the reaction, PCR products were analysed on 1% (w/v) agarose gels.

Indirect immunofluorescence (IF) labelling. The endogenous expression of RGS16 and ORF3 in poPBMC and PK-15/A was determined by indirect IF labelling using a pAb to human (hu)RGS16 (rabbit anti-human RGS16; AVIVA Systems Biology) and a mouse...
mAb to 6× His targeting the histidine-tagged ORF3 construct (Clontech), respectively. The cells were fixed in a mixture of 2% glutaraldehyde and 2% formaldehyde for 15 min before incubation with an appropriate dilution of the primary antibody. After blocking with 0.5 or 2% BSA in PBS (1 h at room temperature), the cells were washed four times with PBS containing 0.02% Tween (PBS-T), and stained with an FITC-labelled antibody to rabbit IgG (DAKO) or with anti-mouse IgG mAb coupled to Texas red (Serotech). After five washes with PBS-T, nuclei were stained by incubating in Hoechst 33258 dye (Sigma-Aldrich) for 15 min at room temperature.

**Cell transfection.** The co-localization of RGS16 and ORF3 was studied in poPBMC activated by LPS and transfected with pcDNA3–ORF3–mCherry. In addition, COS-1 cells or PK15/A cells were transfected with pcDNA3–ORF3–mCherry alone or in combination with pEGFP–RGS16. The cells were seeded on glass slides placed in the bottom of six-well plates (Nunc) and incubated for 1 h before transfection with the indicated expression vector (pcDNA3–ORF3–mCherry, pcDNA3–His–ORF3, pEGFP–RGS16 or pcDNA3–mCherry as a control) using Fugene reagent (Roche Diagnostics), following the manufacturer’s recommendations. Using either control plasmids or plasmids with the ORF3 insert, the transfection efficiency was rather low, around 5% of the PBMCs. After an additional 24–48 h incubation, cells were washed once with PBS and fixed in a mixture of 2% glutaraldehyde and 2% formaldehyde for 15 min. The fixed cells were washed with PBS once and labelled by indirect IF as described above. All fluorescence and phase-contrast microscopy was performed using an Axiosplan II imaging fluorescence microscope equipped with appropriate filter sets, an Axioscan charge-coupled device camera and Axiovision software (Carl Zeiss Light Microscopy). Digital images were processed using Adobe Photoshop version 7.0 software.

**RESULTS**

**Identification of poRGS16 as a partner for the PCV2 ORF3 protein**

Using the ORF3 protein of PCV2 as bait, bacterial two-hybrid screening of a porcine expression library from PK-15/A cells revealed an interaction with a peptide of 65 nt similar to the C-terminal part of huRGS16 (Timmusk et al., 2006). To complete the sequence of this putative interaction partner, 5’- and 3’-RACE were performed on RNA isolated from the PK-15/A cell line. The full-length cDNA generated encoded a 202 aa protein with a typical RGS domain (116 aa) spanning over residues 62–178. Multiple alignments (Fig. 1) confirmed a high similarity at the RGS domain (87.1%) RGS16 and the sequence was consequently designated poRGS16 (GenBank accession no. EU271873). Furthermore, poRGS16 contained a classical basic nuclear localization signal (NLS) at residues 5–13 (LAAFPTTCL) and a motif similar to the nucleus export signal (KKLR) in the middle of the RGS domain, as described in mice and humans (Chatterjee & Fisher, 2000). Similar RACE–PCR experiments also amplified poRGS16 from RNA isolated from poPBMC stimulated by LPS which only differed at one amino acid (position 57).

The interaction between poRGS16 and ORF3 was further validated in a GST pull-down assay using first a construct encoding the ORF3 protein of PCV2-St fused to GST. A clear interaction between this ORF3–GST construct and 35S-labelled poRGS16 was revealed (data not shown), confirming that these proteins interact with each other. As a control, a construct with GST–ORF2 from PCV2 (Timmusk et al., 2006) was included. Neither this fusion protein nor luciferase, which was also used as an internal control for the specificity of the interaction, interacted with the 35S-labelled poRGS16.

**ORF3 from different PCV2 genogroups bind RGS16**

PCV2 can be divided into genogroups according to differences in the ORF2 sequence encoding the structural Cap (Olvera et al., 2007). Accordingly, six variable positions in the ORF3 region have been identified by Timmusk et al. (2008), comparing PCV2 from different Swedish genogroups corresponding to PCV2a and PCV2b in Segalés classification (Segalés et al., 2008). To examine the possibility that ORF3 from different genogroups of PCV2 possess variable binding properties to RGS16, GST pull-down assays were performed using GST–ORF3 from PCV2a (SG1: EU386606), PCV2-St (SG2: AF055392) and PCV2b (SG3: EU408780). The first genotype predominates in pigs in healthy Swedish farms and the two others in farms affected by PMWS. For further comparison, a full-length PCV1 GST–ORF3 construct and a construct based on the 104 aa at the N-terminal part of ORF3 from the non-pathogenic PCV1 were included. Five micrograms from each recombinant GST–ORF3 protein was incubated with an equal amount of TNT–poRGS16 and TNT–luciferase, loaded to GST beads. TNT–luciferase was used instead of TNT–poRGS16 as an internal control. The pull-down products were divided into two aliquots and the two sets of samples were separated on two parallel SDS-PAGE gels. The first gel was analysed using a Phosphorimager, showing that ORF3 from all PCV2 genotypes precipitated the RGS16 protein (Fig. 2a). The GST–ORF3 fusion proteins representing different genogroups of PCV2 varied in their capacity to bind RGS16: the proportion of RGS16 that was precipitated by PCV2a ORF3 was about two times lower than that precipitated by PCV2b ORF3 (Fig. 2a). In contrast, the N terminus of PCV1 ORF3, as well as the full-size protein, failed to precipitate a significant amount of RGS16 protein. A Western blot analysis was performed with the second gel to provide a control for the amount of GST–ORF3 protein loaded (Fig. 2b).

**Activation of poRGS16 by inflammatory stimuli**

To study the regulation of poRGS16 expression, poPBMC were stimulated for 20 h in vitro with PHA, LPS, poly I:C and the oligonucleotide ODN 2216. Because huRGS16 is involved in signal delivery via the alpha 2 adrenoreceptor (2AR) to which the neurotransmitter NE binds, the influence of NE treatment on the expression of poRGS16 was included. Semiquantitative RT-PCR analyses (Fig. 3) indicated that all these treatments upregulated the
expression of RGS16 in poPBMC compared with the unstimulated control and the housekeeping gene cyclophilin A. An upregulation of RGS16 in poPBMC was further supported by double-checking the RGS16 mRNA expression level after PHA or LPS stimulation using TaqMan real-time PCR, normalizing against cyclophilin A and comparing with the unstimulated control by the $2^{-\Delta\Delta Ct}$ method (Fig. 4a). The effect of ODN 2216 and NE on the expression of RGS16 mRNA was further examined by real-time PCR using PBMCs from six pigs (Fig. 4b). The results demonstrated a great individual variation in the upregulation of RGS16 mRNA in response to these inducers. The expression of mRNA for RGS16 was upregulated (two- to eight-fold) in PBMC from three of the six pigs tested after stimulation with ODN 2216 or NE; PBMCs from one pig had increased RGS16 mRNA levels after stimulation with ODN 2216 but not after stimulation with NE; and PBMCs from the other two pigs did not change their levels of RGS16 mRNA expression after stimulation with either ODN 2216 or NE.

The response to ODN 2216 was evaluated further by measuring the amount of alpha interferon (IFN-α) induced by an immunoassay (Artursson et al., 1995). Also, the amount of IFN-α produced in response to stimulation with ODN 2216 varied considerably between pigs (588, 751, 820, 979, 3146 and 438 U IFN-α ml$^{-1}$) when determined in the cell culture medium collected after 20 h. Similarly, pigs that did not upregulate RGS16 following exposure to ODN 2216 were responsive to the ODN.

Taken together, poRGS16 expression appeared to be activated by substances that induce cell proliferation (PHA), production of type-1 IFNs (ODN 2216 and poly I:C) or other pro-inflammatory cytokines (LPS) as well as by the adrenoreceptor agonist NE but at least in the case of ODN 2216 and NE, the ability to upregulate RGS16 mRNA expression and the level of upregulation varied considerably between individuals.

**Expression of the RGS16 protein in porcine cells**

In order to study the regulation of RGS16 at the protein level, IF labelling was performed on poPBMC exposed to PHA or LPS stimulation using TaqMan real-time PCR, normalizing against cyclophilin A and comparing with the unstimulated control by the $2^{-\Delta\Delta Ct}$ method (Fig. 4a). The effect of ODN 2216 and NE on the expression of RGS16 mRNA was further examined by real-time PCR using PBMCs from six pigs (Fig. 4b). The results demonstrated a great individual variation in the upregulation of RGS16 mRNA in response to these inducers. The expression of mRNA for RGS16 was upregulated (two- to eight-fold) in PBMC from three of the six pigs tested after stimulation with ODN 2216 or NE; PBMCs from one pig had increased RGS16 mRNA levels after stimulation with ODN 2216 but not after stimulation with NE; and PBMCs from the other two pigs did not change their levels of RGS16 mRNA expression after stimulation with either ODN 2216 or NE.

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various stimuli in vitro. These IF labelling experiments confirmed that after 20 h exposure to ODN 2216, LPS, PHA or NE, the expression of RGS16 was clearly increased compared with poPBMC cultured in plain growth medium (Fig. 5) that only showed a faint labelling. In poPBMC activated by ODN 2216, the RGS16 staining was concentrated to cytoplasmic granulae. Activation by ODN 2216 also induced a morphological change in the cells characterized by elongated extensions. Granulae labelled for RGS16 were observed in these extensions as well as in the central cytoplasm, occasionally close to the nucleus. RGS16 protein expression was also upregulated in LPS-stimulated poPBMC but with a different staining pattern. A strong staining was then observed in a small area of the cell’s outer membrane and at cytoplasmic structures, possibly related to intracellular filaments. Also, cells kept their round shape when stimulated with LPS and did not show any elongations. Cells activated with PHA or NE also showed an induced expression of RGS16 and tended to stick to each other, but did not display any elongated extensions. Thus, an endogenous production of RGS16 can be induced in poPBMC by diverse inflammatory stimuli, including IFN inducers, at both the mRNA and protein level.

**PCV2 ORF3 protein co-localizes with RGS16 in activated PBMC cells**

In order to study the interaction between ORF3 from PCV2 and poRGS16 in situ, poPBMCs were activated by LPS for 20 h before being transfected with an expression plasmid for ORF3-mCherry fusion protein (pcDNA3–ORF3–mCherry) and labelled for endogenous RGS16 by indirect IF. When LPS-activated poPBMC were transfected with a control expression vector (pcDNA3–mCherry), the red fluorescence was distributed in the whole cytoplasmic area (data not shown). In contrast, the red fluorescence became concentrated to cytoplasmic granulae located in the perinuclear region when LPS-activated poPBMC were transfected with pcDNA3–ORF3–mCherry. Interestingly, this ORF3 staining was found in areas also labelled for endogenous RGS16 expression, suggesting that both proteins were localized to the same cellular compartment (Fig. 6). Although the transfection rate was rather low, this pattern was observed consistently in cells expressing ORF3–mCherry, and did not appear as an exceptional artefact. Additional examples of cells with overlapped signal for RSG16 and ORF3 are shown in Supplementary Fig. S1 (available in JGV Online).

To further document the co-localization of poRGS16 and PCV2–ORF3, COS-1 cells were co-transfected with expression vectors for GFP–RGS16 and pcDNA3–His–ORF3. The GFP–RGS16 showed a perinuclear localization with an additional diffuse cytoplasmic expression at 24 h and His–ORF3 protein (visualized by staining using an anti-His, Texas Red-coupled mAb) was mainly found in the perinuclear region concentrated in cytoplasmic granulae (Fig. 7). This observation was consistent with the intracellular distribution of RGS16 and ORF3 in PBMCs. However, His–ORF3 was translocated into the nucleus 48 h post-transfection in cells co-transfected with the GFP–RGS16-encoding plasmid (Fig. 7). We never observed this translocation in cells transfected only with pcDNA3–His–ORF3, suggesting that it depends on the co-expression of ORF3 and RGS16.

We also tested the co-localization of GFP–RGS16 and ORF3–mCherry on PK-15/A cells that were co-transfected with expression vectors for these labelled proteins. The GFP–RGS16 also showed a perinuclear localization in the PK-15/A cells 24 h after transfection (Supplementary Fig. S2) and ORF3–mCherry was also found in the perinuclear region, as observed in COS-1 cells. Again, the red fluorescent ORF3 was translocated to the nucleus 48 h
post-transfection. In contrast, we never observed this translocation in cells transfected with ORF3–mCherry only, further suggesting that poRGS16 contributes to a translocation of the PCV2 ORF3 protein to the nucleus (Supplementary Fig. S2).

**DISCUSSION**

An interaction between poRGS16 and ORF3 from PCV2, as previously indicated by the BacterioMatch assay (Timmusk et al., 2006), was verified in the present study by pull-down experiments. The full-length RGS gene sequences that were generated by 3′- and 5′-RACE from porcine LPS-stimulated PBMC and from a PK cell-line were highly similar to human and murine RGS16, and a phylogenetic analysis of these sequences confirmed that the porcine RGS was grouped with RGS16 (data not shown), indicating that it was most probably a true orthologue. A survey of the RGS16 genomic location in cow, dog, mouse and human sequences revealed that this gene is systematically encoded in the neighbourhood of RNaseL, an important element of

![Fig. 4. Quantitative real-time PCR analysis of RGS16 mRNA expression in poPBMC. RGS16 mRNA levels were determined by TaqMan real-time PCR in PBMC obtained from one pig and stimulated with PHA or LPS for 20 h (a) or in PBMC obtained from six pigs stimulated with NE or ODN 2216 for 20 h (b). The obtained data were normalized against cyclophilin A levels and compared with the corresponding unstimulated control according to the 2−ΔΔCt method. Results are expressed as the mean ± SD of the PCR triplicate for (a) and as the mean ± SD of each of the six pigs for (b).](http://vir.sgmjournals.org)
the IFN-induced 2',5'-oligoadenylate synthetase pathway. Thus, the viral infections that activate production of type I IFNs might thereby also indirectly activate transcription of a region of the genome harbouring RGS16.

The interaction between poRGS16 and ORF3 varied for ORF3 proteins from the three different genogroups of PCV2 (Segalès et al., 2008; Timmusk et al., 2008) and was barely detectable for either the corresponding region of ORF3 from PCV1 (315 bp) or the full-length PCV1 ORF3 (612 bp). Interestingly, PCV1 is regarded as non-pathogenic, whereas several studies suggest that genogroups of PCV2 can differ in their contribution to the development of PMWS (Carman et al., 2008; Gagnon et al., 2007; Timmusk et al., 2008). It was recently shown that mutations within the genome of PCV2 were silent in the ORF coding for the replicase but induced amino acid substitutions in the ORF3 in the opposite transcription orientation (Timmusk et al., 2008). Thus, PCV2 could theoretically retain the unmodified Rep and Rep’ proteins despite alterations in the ORF3 protein. Using a yeast two-hybrid system, ORF3 of PCV2 was indicated as an interaction partner for a ubiquitin ligase that facilitates the expression of p53, supporting a role for the ORF3-encoded protein in apoptosis (Liu et al., 2007). Indeed, recent in vitro studies show that mutations in the genome of PCV2 that generate amino acid substitutions in ORF3 but remain silent in ORF1 can affect the virus-induced cell death without altering the rate of viral replication (Karuppannan et al., 2009). In another yeast two-hybrid analysis, no protein interaction with ORF3 of PCV1 or PCV2 was found, but a number of interaction partners for Rep and Cap were identified (Finsterbusch, et al., 2009).

Thus, the function of PCV2 ORF3 remains uncertain but the data presented here, that ORF3 of PCV2 interacts with poRGS16, indicate that the virus can interfere with G-protein coupled signalling within the cell.

Fig. 5. IF analysis of RGS16 expression in activated poPBMC. PoPBMCs were stimulated for 20 h with LPS (a), PHA (b), NE (c), ODN 2216 (d) or cultured in plain growth medium (e) before the expression of poRGS16 was visualized by indirect IF using a pAb to huRGS16 in combination with an FITC-labelled secondary antibody. Cell nuclei were counterstained blue. These are representative pictures from stimulation experiments that were repeated on six different PBMC preparations.
The expression of RGS16 is inducible and treatment of human immature DCs with poly I:C or LPS increased the expression of RGS16 mRNA (Shi et al., 2004). Gene expression profiling of human monocyte-derived DCs demonstrated that RGS16 was induced when the cells were treated with LPS in combination with interleukin (IL)-10 (Perrier et al., 2004). These observations are interesting in relation to PCV2 because IL-10 is a cytokine that has been repeatedly associated with the development of PMWS (Darwich et al., 2008) and in vitro studies have indicated that IFN (Meerts et al., 2005b) and LPS (Chang et al., 2006) can upregulate the replication of PCV2. In the present study, ODN 2216 or polyI:C was used as inducer of type I IFNs, according to protocols previously established for porcine cells (Wikström et al., 2007). Both ODN 2216, which activates IFN-α production via TLR9, and polyI:C, which mimics double-stranded RNA and is described to activate production of type 1 IFN via TLR3 (Uenishi & Shinkai, 2008), induced an increased RGS16 mRNA expression as determined by RT-PCR. The IFN-α response to stimulation with ODN 2216 showed a substantial variation between individuals, which could be due to either a genetic variation in the IFN-α-producing capacity (Edfors-Lilja et al., 1998) or to a polymorphism in the ligand-binding or signal-transducing domains of TLR9 (Uenishi & Shinkai, 2008). Accordingly, the quantitative RT-PCR confirmed that ODN 2216 induced RGS16 expression in poPBMC but also revealed that the level of expression varied considerably between pigs. Since it was recently demonstrated that inter-individual differences in the immune response to LPS were dependent on the level of TLR4 expression (Jaekal et al., 2007), it is likely that a similar differential expression of TLR3 and TLR9 was responsible for the large inter-individual differences observed in IFN-α and RGS16 upregulation. Nevertheless, it is notable that PCV2 establishes non-productive infections in porcine DCs and that the viral DNA apparently affects cytokine responses induced by TLR7 and TLR9 agonists in these cells (Perrier et al., 2004).

The present results indicate that signalling via TLR9 can induce the expression of RGS16 in poPBMC as previously shown for LPS-mediated signalling via TLR4 in murine monocyte-derived DCs (Shi et al., 2004). To study the effects of signalling via a known GPCR known to be regulated by RGS16 (Hoffmann et al., 2001), poPBMC were cultured in the presence of NE. Variable levels of mRNA for RGS16 were detected by quantitative RT-PCR and indirect IF labelling using a pAb to huRGS16 confirmed the translation to protein. Social stress of pigs induces the release of catecholamines and can have long-term effects on their antiviral immunity, favouring IL-10 production (de Groot et al., 2001). It is now well established that stress hormones induce a Th2 shift in the cytokine balance, characterized by IL-10 production (Elenkov & Chrousos, 2006), and NE modulates the cytokine response of human DC towards a Th2 profile (Goyarts et al., 2008). Thus, a number of factors in conventional pig husbandry that have been associated with PMWS, such as environmental stress and/or co-infections, are likely to elicit signals that induce the expression of RGS16 in poPBMC.

The interaction between PCV2 ORF3 and poRGS16 that was visualized in the pull-down experiment was further supported by fluorescence labelling of RGS16 in cells that were LPS-stimulated and transfected with the mCherry-
ORF3 plasmid. According to the results from the bacterial two-hybrid screening (Timmusk et al., 2006), the RGS16 region necessary for the binding to ORF3 constituted aa 1–197 at the N terminus, which is also the region responsible for direct and specific recognition of GPCRs (Xie & Palmer, 2007). Although the RGS16 domain interacting with G proteins and ORF3 do not strictly overlap, they contain four residues in common. Binding of ORF3 to RGS16 could thus generate a steric hindrance for RGS–G protein interactions. Consequently, ORF3 binding to RGS16 may, for instance, affect the signalling of pathways triggered by epinephrine–α2AR (Chen et al., 2001; Hoffmann et al., 2001). RGS16 is also involved in the regulation of signalling via several GPCRs, including chemokine receptors, and could thereby influence the migration of T cells (Lippert et al., 2003). An even more intriguing observation, indicating that RGS16 contributed to the translocation of ORF3 to the cell nucleus, was made in cells co-transfected with RGS16 and ORF3. However, improved methods to trace ORF3 in cells that support replication of PCV2 are needed for closer examination of these proposed roles for PCV2 ORF3.

NOTE ADDED IN PROOF

During the submission process of this paper, results were published showing that point mutations that cause amino acid substitutions within the ORF3 but are silent in ORF1 seem to affect the pathogenicity of PCV2 when administrated to 3-week-old specific pathogen-free piglets (Karuppannan et al., 2009).

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Fig. 7. Co-localization of poRGS16 and PCV2–ORF3 in COS-1 cells. The co-localization of poRGS16 and PCV2–ORF3 was studied in COS-1 cells transfected with pcDNA3–His–ORF3 and a plasmid encoding GFP–RGS16, then stained with anti-HIS Texas red mAb. The intracellular localization of poRGS16 and PCV2–ORF3 expressed from the plasmids was examined by fluorescence microscopy after 24 h (left panel) and 48 h (right panel). The upper row of pictures represent the superposition of green fluorescence (RGS16) and red fluorescence (ORF3 expression), the middle row represent the anti-His–ORF3 staining and the bottom row shows the GFP–RGS16 expression. These pictures are representative of four different COS cell preparations.
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