The ORF3 protein of porcine circovirus type 2 promotes secretion of IL-6 and IL-8 in porcine epithelial cells by facilitating proteasomal degradation of regulator of G protein signalling 16 through physical interaction

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Received 15 September 2014
Accepted 6 January 2015

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Porcine circovirus type 2 (PCV2) is the main aetiological agent of postweaning multisystemic wasting syndrome. The mechanism of pathogenicity associated with PCV2 infection is still not fully understood. Nevertheless, the fact that large amounts of proinflammatory cytokines within lymphoid tissues are released during the early stage of PCV2 infection may induce chronic inflammatory responses followed by the destruction of lymphoid tissues. However, how PCV2 infection causes an excessive inflammatory response in the host immune system during the early stage of PCV2 infection has still not been elucidated. In this study, we show that direct interaction between the PCV2 ORF3 and regulator of G protein signalling 16 (RGS16) within the cytoplasm of host cells leads to ubiquitin-mediated proteasomal degradation of RGS16. Facilitated degradation of the RGS16 by PCV2 ORF3 further enhances NFκB translocation into the nucleus through the ERK1/2 signalling pathway and increased IL-6 and IL-8 mRNA transcripts. Consequently, more severe inflammatory responses and leukocyte infiltration occur around host cells. This evidence may be the first clue explaining the molecular basis of how excessive amounts of proinflammatory cytokines within lymphoid tissues are released during the early stage of PCV2 infection.

INTRODUCTION

Porcine circovirus (PCV) belongs to the genus Circovirus in the family Circoviridae, which includes non-enveloped, icosahedral small viruses with a single-stranded, closed-circular DNA as their genome (Segalés et al., 2013). Two types of porcine circoviruses have been identified; PCV1 fails to induce disease in pigs following several experimental infections, whereas PCV2 is considered the primary aetiiological agent that induces porcine circovirus-associated diseases, including postweaning multisystemic wasting syndrome (PMWS) (Tischer et al., 1995; Allan et al., 1999).

PMWS has become one of the largest reasons for economic loss in the swine industry. Mortality from PMWS varies from 1 to 30%, depending on immunocompetency and genetic predisposition of individual pigs (Rodriguez-Arrioja et al., 2002). The initial signs of PCV2 infection are enlargement of peripheral lymph nodes followed by depletion of lymphocytes (Opriessnig et al., 2007). The lymphopaenia mediated by PCV2 infection causes secondary infections with other pathogens in pigs. Pigs develop PMWS after infection by several other pathogens, with multiple symptoms, including progressive weight loss, respiratory dysfunction and diarrhoea (Allan & Ellis, 2000; Segalés et al., 2005).

The mechanism of pathogenicity associated with PCV2 infection is not fully understood. During the early stage of
PCV2 infection, large amounts of proinflammatory cytokines within lymphoid tissues are released and may induce chronic inflammatory responses followed by destruction of lymphoid tissues (Darwich et al., 2003). However, how PCV2 infection causes an excessive inflammatory response in the host immune system has still not been elucidated.

Among PCV2 ORFs, ORF3 and ORF4 are not essential for replication of PCV2, but are associated with PCV2 pathogenicity (Liu et al., 2005, 2006; He et al., 2013). Previously, the interaction of PCV2 ORF3 with regulator of G protein signalling 16 (RGS16) has been reported (Timmusk et al., 2006). RGS proteins regulate many biological activities, such as hormone secretion, neurotransmission and inflammation, by blocking heterotrimeric G protein signalling (Xie & Palmer, 2007). Especially, RGS16 can modulate lymphocyte trafficking and inflammatory responses by inhibiting proinflammatory cytokine production (Cho & Kehrl, 2009; Druey, 2009; Shankar et al., 2012).

In the present study, we investigated the mechanism and biological consequence of the physical interaction between the PCV2 ORF3 protein and porcine RGS16. As a result, this study may provide the first evidence showing how PCV2 infection facilitates the expression of proinflammatory cytokines and creates a more severe inflammatory response during the early stages of PCV2 infection.

RESULTS

PCV2 ORF3 physically interacts with the N-terminus of host RGS16 within cytoplasm

Previously, interaction between PCV2 ORF3 and RGS16 was reported in a bacterial two-hybrid system (Timmusk et al., 2006). To confirm this result in a mammalian system, cell lysates from ORF3-Flag expressing PK15 cells were subjected to immunoprecipitation (IP) using anti-RGS16 antibody. Immunoprecipitates were immunoblotted using anti-Flag antibody. As shown in Fig. 1(a), RGS16 was co-immunoprecipitated with PCV2 ORF3 (Fig. 1a, upper panel), whereas no interaction was detected between control Ig and PCV2 ORF3 (Fig. 1a, upper panel). Immunoblotting using the anti-RGS16 antibody indicated that the same amounts of RGS16 were present in total cell lysates (Fig. 1a, middle panel). Equivalent protein from both samples was verified by immunoblotting using anti-β-actin antibody (Fig. 1a, lower panel). These results confirmed that RGS16 and PCV2 ORF3 interacted with each other at the physiological level in PK15 cells.

Haemagglutinin (HA)-tagged ORF3 protein (ORF3-HA) was expressed in PK15 cells to determine the subcellular localization of the interaction between RGS16 and PCV2 ORF3. The results indicated that the RGS16 and ORF3-HA were mainly expressed in the cytoplasm. The combined results indicate that PCV2 ORF3 and RGS16 interacted with each other within the cytoplasm of host cells (Fig. 1b).

As shown in Fig. 1(c), cDNA constructs containing three RGS16 deletion mutants were designed to examine whether these domains are essential for the interaction of RGS16 with the ORF3 protein. In the yeast two-hybrid system, the full-length PCV2 ORF3 cDNA and either a plasmid-containing gene encoding a full-length RGS16 (Fig. 1c, full) or plasmids containing genes encoding three truncated mutant forms (Met1-Phe55, Ser56-Leu180, Ala181-Thr202, Fig. 1c) of cDNAs were co-transformed into EGY48 yeast cells. Yeast cells transformed with full-length RGS16 and one deletion mutant (Met1-Phe55) containing the RGS16 N-terminal domain were grown in synthetic medium (Ura−, His−, Trp−), whereas yeast cells transformed with the other deletion mutants (Ser56-Leu180 and Ala181-Thr202) failed to grow (data not shown). To confirm this result, we determined the binding activity of these constructs by measuring the relative β-galactosidase expression level. As shown in Fig. 1(d), the β-galactosidase assay results confirmed that the RGS16 N-terminal domain bound to PCV2 ORF3 and neither of these mutants (Ser56-Leu180 and Ala181-Thr202) bound to PCV2 ORF3.

Interaction between PCV2 ORF3 and host RGS16 facilitates ubiquitin-mediated proteasomal degradation of host RGS16

Immunoblotting was performed to investigate RGS16 protein expression in PK15 cells that expressed each PCV2 ORF protein. PK15 cells expressing empty vector were used as a control. The results showed that the amount of RGS16 was reduced markedly in only PCV2 ORF3-expressing PK15 cells, and not in those of other ORF-expressing cells (Fig. 2a). The ORF3-expressing PK15 cells were then treated or not treated with lipopolysaccharide (LPS) and poly(I:C) for 48 h. After treatment, the RGS16 protein expression level was checked in stimulated cells and compared with the levels in unstimulated cells by Western blot analyses. The RGS16 protein level was reduced in ORF3-expressing cells treated with LPS or poly(I:C) for 48 h, but not in empty vector transfectants treated with LPS or poly(I:C) (Fig. 2b). These results indicate that the decreased level of the RGS16 protein in ORF3-expressing PK15 cells was consistent and remained unchanged under unstimulated or stimulated conditions.

To confirm these results, RGS16 protein expression was measured in WT PCV2-infected PK15 cells or the ORF3 deletion mutant of PCV2-infected PK15 cells by Western blot analyses. The results showed that RGS16 expression was markedly reduced in cell extracts of WT PCV2 infected cells but not in those of uninfected cells and ΔORF3 PCV2-infected cells (Fig. 2c).

We tested stability of the RGS16 protein in PCV2 ORF3-expressing PK15 cells, compared with that of empty vector transfectants following treatment with cycloheximide (CHX), to investigate whether the interaction between PCV2 ORF3 and RGS16 destabilized the RGS16 expression in host cells. The RGS16 protein in PCV2 ORF3-expressing
PK15 cells degraded much faster than that of the empty vector transfectants (Fig. 2d). We treated PCV2 ORF3-expressing PK15 cells with MG132, an inhibitor of proteasome complex, or left them untreated, and monitored the RGS16 protein expression pattern to determine whether the interaction between PCV2 ORF3 and RGS16 leads to degradation of RGS16 in a proteasome-dependent manner. The results clearly showed that the MG132 treatment...
**Fig. 2.** Interaction between PCV2 ORF3 and RGS16 facilitates degradation of RGS16 through a ubiquitin-mediated proteasome-dependent pathway. (a) RGS16 protein expression levels in empty vector transfectants (Mock) and PCV2 ORF1-, ORF2- or ORF3-expressing PK15 cells (ORF1, ORF2, ORF3). Total cell lysates from each transfectant were analysed by immunoblotting using anti-RGS16 or anti-β-actin antibodies. All results are shown as mean±SE. *P<0.05; **P<0.01; ***P<0.001.
rescued RGS16 from proteasomal degradation in PCV2 ORF3-expressing PK15 cells, compared with that in untreated cells (Fig. 2e). To confirm this result, we also treated or did not treat MG132 WT PCV2-infected PK15 cells and ΔORF3 PCV2-infected cells with MG132 (Fig. 2e). As expected, the MG132 treatment in WT-infected PK15 cells increased the RGS16 level compared with that in cells without treatment (Fig. 2e). The RGS16 levels in both empty vector transfectants and ΔORF3 PCV2-infected cells were not significantly affected by the treatment of MG132 (Fig. 2e). Therefore, these results indicate that the interaction between PCV2 ORF3 and RGS16 facilitates proteasome-dependent degradation of RGS16.

The ubiquitination assay was performed in ORF3-expressing PK15 cells in the absence or presence of MG132 to determine whether the direct binding of PCV2 ORF3 to RGS16 promotes ubiquitination of RGS16. Each cell lysate was subjected to IP using the anti-RGS16 antibody followed by immunoblotting using the anti-β-actin antibody. Mouse IgG was used as an isotype control for IP (Fig. 2f, upper panel, lane 1). We could not detect differences in the level of RGS16 ubiquitination in either empty vector transfectants or ORF3-expressing PK15 cells without MG132 (Fig. 2f, upper panel, lanes 2 and 3). However, an increased level of RGS16 ubiquitination was observed when ORF3-expressing PK15 cells were treated with MG132 (Fig. 2f, upper panel, lane 5), compared with that of empty vector transfectants (Fig. 2f, upper panel, lane 4). Consistent with this result, RGS16 protein expression partially recovered from ubiquitin-mediated proteasomal degradation in the presence of MG132 (Fig. 2f, middle panel, lane 5), compared with that in the absence of MG132 (Fig. 2f, middle panel, lane 3). The amounts of total protein from total cell lysates were equal, as shown by Western blot analyses using β-actin antibody (Fig. 2f, lower panel). To further confirm that RGS16 is ubiquitinated upon ORF3 expression, exogenous HA-tagged ubiquitin was expressed in mock or PCV2 ORF3-expressing PK15 cells. Then, each cell lysate was subjected to IP using the anti-RGS16 antibody followed by immunoblotting using the anti-HA antibody. Consistent with results shown in Fig. 2(f), an increased level of RGS16 ubiquitination was observed in ORF3-expressing PK15 cells (Fig. 2g, upper panel, lanes 4 and 5), compared with those of mock (Fig. 2g, upper panel, lanes 3 and 5) in the absence or presence of MG132. These data clearly indicate that the interaction between PCV2 ORF3 and RGS16 facilitates degradation of RGS16 through ubiquitin-mediated proteasomal degradation.

**Decreased expression of RGS16 enhances NFκB translocation into the nucleus through the ERK1/2 signalling pathway in host cells**

RGS16 inhibits G protein-coupled receptor (GPCR)-mediated signal transduction by binding and blocking activity of the Gz subunit, which has the ability to hydrolyse GTP (Xie & Palmer, 2007). The signalling pathway mediated by GPCR leads to translocation of NFκB from the cytoplasm into the nucleus through AKT (protein kinase B) or ERK1/2 (Anger et al., 2007). We examined AKT and ERK1/2 expression patterns to investigate whether decreased expression of RGS16 affects GPCR-mediated signal transduction. Fig. 3(a) shows that ORF3-expressing PK15 cells had increased expression levels of phospho-AKT (pAKT) and phospho-ERK1/2 (pERK1/2) relative to those of empty vector transfectants, whereas no changes were detected in the level of total AKT or ERK1/2 in either cell type. Additionally, inhibiting AKT or ERK1/2 phosphorylation with LY294002 (pAKT) or with U0126 (pERK1/2) had no effect on RGS16 protein expression in either cell type (Fig. 3a).
**Fig. 3.** Degradation of RGS16 mediated by PCV2 ORF3 enhances the translocation of NFκB into the nucleus via the ERK1/2 pathway. (a) Empty vector transfectants (Mock) or PCV2 ORF3-expressing PK15 cells (ORF3) were left untreated or were treated with LY294002 (50 μM) and U0126 (20 μM) for 1 h, harvested, and analysed by immunoblotting using each relevant antibody. pAKT, Phosphorylated AKT; pERK, phosphorylated ERK1/2. All results are shown as mean ± se. *P<0.05; **P<0.01; ***P<0.001. (b) Uninfected PK15 cells (Uninfected), WT PCV2-infected PK15 cells (WT PCV2), or ΔORF3 PCV2-infected PK15 cells (ΔORF3 PCV2) were analysed by immunoblotting using each relevant antibody. pAKT, Phosphorylated AKT; pERK, phosphorylated ERK1/2. All results are shown as mean ± se. *P<0.05; **P<0.01; ***P<0.001. (c) Empty vector transfectants (Mock) or PCV2 ORF3-expressing PK15 cells (ORF3) were treated as described above, harvested, and analysed by immunoblotting using each relevant antibody. TCL, Total cell lysates: Cytosol, cytosolic fractions; Nucleus, nuclear fractions. (d)
Uninfected PK15 cells (Uninfected), WT PCV2-infected PK15 cells (WT PCV2) or ΔORF3 PCV2-infected PK15 cells (ΔORF3 PCV2) were analysed by immunoblotting using each relevant antibody. All results are representative of three independent experiments.

To further confirm whether decreased RGS16 protein levels resulted in the increased level of pAKT or pERK1/2, total cell extracts from uninfected PK15 cells, WT PCV2-infected PK15 cells or ΔORF3 PCV2-infected PK15 cells were subjected to Western blot analyses. As a result, expression of pAKT or pERK1/2 increased markedly in WT PCV2-infected PK15 cells, compared with those of uninfected PK15 cells and ΔORF3 PCV2-infected PK15 cells (Fig. 3b). Therefore, these results indicate that RGS16 is an upstream molecule that inhibits both the AKT and ERK1/2 signalling pathways and that decreased expression of the RGS16 protein in PCV2 ORF3-expressing cells leads to increased AKT or ERK1/2 activity.

Many reports indicate that NFκB is frequently activated by several cellular signal transduction pathways upon viral infection (Bonizzi & Karin, 2004). Activation of NFκB is required for its translocation into the nucleus upon proteasomal degradation of IkBα (Proost et al., 1996). To investigate whether increased AKT or ERK1/2 phosphorylation affects NFκB translocation into the nucleus, proteins from total cell lysates, the cytosolic fraction or nuclear fraction of empty vector transfectants, or PCV2 ORF3-expressing PK15 cells were purified, and the NFκB and IkBα protein expression patterns were analysed by immunoblotting. Expression of the NFκB protein clearly increased in the nuclear fraction of ORF3-expressing PK15 cells compared with that of the empty vector transfectants (Fig. 3c). However, no significant changes in NFκB protein expression were observed in total cell lysates or cytosolic fractions of ORF3-expressing PK15 cells compared with expression levels of empty vector transfectants (Fig. 3c). In accordance with the increased translocation of NFκB into the nucleus, the expression level of the IkBα protein was decreased in total cell lysates or cytosolic fractions of ORF3-expressing PK15 cells, compared with those of empty vector transfectants (Fig. 3c). To define which molecule mediated NFκB translocation, either LY294002 or U0126 was added to empty vector transfectants and ORF3-expressing PK15 cells and the expression patterns of the NFκB and IkBα proteins were analysed. The results clearly indicated that inhibiting ERK1/2 phosphorylation by U0126 blocked both IkBα degradation and NFκB translocation into the nucleus in ORF3-expressing PK15 cells, although inhibiting AKT phosphorylation by LY294002 did not block NFκB translocation into the nucleus or degradation of IkBα in ORF3-expressing PK15 cells (Fig. 3c).

To confirm that the increased translocation of NFκB into the nucleus is dependent on ORF3 of PCV2, NFκB and IkBα protein expression patterns were analysed in uninfected PK15 cells, WT PCV2-infected PK15 cells, or ΔORF3 PCV2-infected PK15 cells. As shown in Fig. 3(d), the amount of NFκB translocation into the nucleus clearly increased in WT PCV2-infected PK15 cells, whereas the amount of NFκB translocation into the nucleus in both uninfected PK15 cells and ΔORF3 PCV2-infected PK15 cells remained unchanged. Additionally, the amount of IkBα in WT PCV2-infected PK15 cells decreased significantly compared with those of uninfected PK15 cells and ΔORF3 PCV2-infected PK15 cells (Fig. 3d). These combined results clearly suggest that degradation of RGS16 by the interaction between PCV2 ORF3 and RGS16 in host cells leads to increased translocation of NFκB into the nucleus through the ERK1/2 signalling pathway.

**Increased IL-6 and IL-8 expression in PCV2-infected cells leads to increased migration of neutrophils to host cells**

To examine the phenotypic change due to increased NFκB translocation into the nucleus in PCV2-infected host cells, the IL-6 and IL-8 mRNA transcript expression levels in empty vector transfectants or PCV2 ORF3-expressing PK15 cells were measured by real-time PCR analyses. The results showed that IL-6 and IL-8 mRNA levels increased markedly in ORF3-expressing PK15 cells, compared with those of empty vector transfectants (Fig. 4a). To confirm this result, the amounts of secreted IL-6 and IL-8 from both empty vector transfectants and ORF3-expressing PK15 cells were measured by ELISA. As expected, IL-6 and IL-8 secretion clearly increased in ORF3-expressing PK15 cells, compared with those of empty vector transfectants (Fig. 4b).

To confirm the increased IL-6 and IL-8 secretion in ORF3-expressing PK15 cells, IL-6 and IL-8 expression patterns were further analysed by real-time PCR analyses and ELISAs from uninfected PK15 cells, WT PCV2-infected PK15 cells, and ΔORF3 PCV2-infected PK15 cells. As shown in Fig. 4(c, d), the amounts of IL-6 and IL-8 mRNA transcripts and the secretion of IL-6 and IL-8 increased significantly in WT PCV2-infected PK15 cells, compared with those of uninfected PK15 cells and ΔORF3 PCV2-infected PK15 cells. Therefore, these results indicate that decreased RGS16 expression by interaction with PCV2 ORF3 leads to increased expression and secretion of proinflammatory cytokines such as IL-6 and IL8 in host cells.

IL-8 is a well-known chemoattractant stimulating the migration of granulocytic neutrophils (Baggioni & Clark-Lewis, 1992). To test whether increased secretion of IL-8 in PCV2-infected cells affects migration of neutrophils to host cells, pig neutrophils were isolated and the transwell migration assay was conducted with supernatants from empty vector transfectants or ORF3-expressing PK15 cells. Culture medium without cells was used as a negative control.
control. As shown in Fig. 4(e), migration of neutrophils into the lower chamber increased distinctly when we used supernatants from ORF3-expressing PK15 cells, compared with those from empty vector transfectants. To confirm this result, we also conducted transwell migration assays with supernatants from uninfected PK15 cells, WT PCV2-infected PK15 cells, and ΔORF3 PCV2-infected PK15 cells. The results clearly indicated that the migration of neutrophils increased when we used supernatants from WT PCV2-infected PK15 cells, compared with those from empty vector transfectants (Mock) or PCV2 ORF3-expressing PK15 cells (ORF3). (a) Real-time PCR analyses were performed to detect the IL-6 and IL-8 mRNA transcripts in empty vector transfectants (Mock) or PCV2 ORF3-expressing PK15 cells (ORF3). (b) ELISAs were performed to detect soluble IL-6 and IL-8 secreted by empty vector transfectants (Mock) or PCV2 ORF3-expressing PK15 cells (ORF3). (c) Real-time PCR analyses were performed to detect IL-6 and IL-8 mRNA transcripts in uninfected PK15 cells (Uninfected), WT PCV2-infected PK15 cells (WT PCV2), or ΔORF3 PCV2-infected PK15 cells (ΔORF3 PCV2). (d) ELISAs were performed to detect soluble IL-6 and IL-8 secreted by uninfected PK15 cells (Uninfected), WT PCV2-infected PK15 cells (WT PCV2), or ΔORF3 PCV2-infected PK15 cells (ΔORF3 PCV2). (e) Transwell migration assays were performed with pig neutrophils using culture supernatants from empty vector transfectants (Mock) or PCV2 ORF3-expressing PK15 cells (ORF3). (f) Transwell migration assays were performed with pig neutrophils using culture supernatants from uninfected PK15 cells (Uninfected), WT PCV2-infected PK15 cells (WT PCV2), or ΔORF3 PCV2-infected PK15 cells (ΔORF3 PCV2). All results are shown as mean ± SE. *P<0.05; **P<0.01; ***P<0.001.

Fig. 4. Increased secretion of IL-6 and IL-8 increased recruitment of neutrophils to PCV2-infected cells. (a) Real-time PCR analyses were performed to detect the IL-6 and IL-8 mRNA transcripts in empty vector transfectants (mock) or PCV2 ORF3-expressing PK15 cells (ORF3). (b) ELISAs were performed to detect soluble IL-6 and IL-8 secreted by empty vector transfectants (Mock) or PCV2 ORF3-expressing PK15 cells (ORF3). (c) Real-time PCR analyses were performed to detect IL-6 and IL-8 mRNA transcripts in uninfected PK15 cells (Uninfected), WT PCV2-infected PK15 cells (WT PCV2), or ΔORF3 PCV2-infected PK15 cells (ΔORF3 PCV2). (d) ELISAs were performed to detect soluble IL-6 and IL-8 secreted by uninfected PK15 cells (Uninfected), WT PCV2-infected PK15 cells (WT PCV2), or ΔORF3 PCV2-infected PK15 cells (ΔORF3 PCV2). (e) Transwell migration assays were performed with pig neutrophils using culture supernatants from empty vector transfectants (Mock) or PCV2 ORF3-expressing PK15 cells (ORF3). (f) Transwell migration assays were performed with pig neutrophils using culture supernatants from uninfected PK15 cells (Uninfected), WT PCV2-infected PK15 cells (WT PCV2), or ΔORF3 PCV2-infected PK15 cells (ΔORF3 PCV2). All results are shown as mean ± SE. *P<0.05; **P<0.01; ***P<0.001.
uninfected PK15 cells (Fig. 4f). Neutrophil migration decreased significantly when we used supernatants from ΔORF3 PCV2-infected PK15 cells, compared with those from WT PCV2-infected PK15 cells (Fig. 4f). Taken together, these results show that enhanced expression and secretion of IL-8 leads to increased migration of neutrophils to PCV2-infected cells.

**DISCUSSION**

Immunosuppression caused by PCV2 infection in pigs initiates increased susceptibility against several other infectious diseases, which often develops into porcine circovirus-associated diseases, including PMWS (Allan et al., 1999; Opriessnig et al., 2007). During the early stages of PCV2 infection, innate immune cells secrete excessive amounts of proinflammatory cytokines, which often leads to a chronic inflammatory response (Darwich et al., 2003). Then, the chronic inflammatory response substantially depletes immune cells by apoptosis within lymphoid tissues (Dvorak et al., 2013). Therefore, defining the mechanism of why severe inflammation occurs during the early stage of PCV2 infection is the key to developing a method to prevent the initiation of porcine circovirus-associated diseases.

Four major ORFs have been identified in the PCV2 genome. Among them, PCV2 ORF1 encodes a gene for virus replication, and ORF2 encodes a gene for capsid formation (Mankertz et al., 1998; Nawagtitul et al., 2000). ORF3 and ORF4 may regulate host immune responses by interacting with host proteins (Liu et al., 2005, 2006, 2007; He et al., 2013). The function of PCV2 ORF3 may be to activate host cell signalling, whereas the function of PCV2 ORF4 may be to downregulate host cell signalling (Liu et al., 2007; He et al., 2013). In particular, a previous experiment demonstrated that PCV2 infection in host cells activates NFκB by IκB degradation (Wei et al., 2008). As NFκB signalling is also involved in initiating inflammatory responses (Baldwin, 1996; Bonizzi & Karin, 2004), PCV2 ORF3 may be responsible for enhanced secretion of proinflammatory cytokines by host cells after PCV2 infection.

Several host proteins were identified as binding with PCV2 ORF3 in a bacterial two-hybrid system (Timmsuk et al., 2006). Among them, RGS16 is the most relevant molecule to explain modulation of host cell signalling affected by PCV2 ORF3 because RGS proteins have emerged as key modulators of many biological activities, including lymphocyte trafficking and inflammatory disorders (Cho & Kehrl, 2009; Druey, 2009). Indeed, previous reports have demonstrated that RGS16 is a negative regulator of heterotrimeric G protein signalling (Dohlman & Thorner, 1997; Berman & Gilman, 1998) and that inhibiting RGS16 reverses downregulation of NFκB expression (Vasilatos et al., 2013). Recent observations using RGS16-deficient mice indicate that RGS16 attenuates inflammatory responses by suppressing the secretion of proinflammatory cytokines (Shankar et al., 2012). Therefore we hypothesized that the interaction between PCV2 ORF3 and host RGS16 may affect host cell signalling related to inflammatory responses during PCV2 infection.

To prove this hypothesis, we first demonstrated that the N terminus of host RGS16 physically interacted with PCV2 ORF3 within the cytoplasm (Fig. 1). The N-terminal 33 aa, which are required for the association with the plasma membrane and RGS protein activity, are conserved within RGS4, RGS5 and RGS16 (Chen et al., 1999). Interestingly, our results indicate that an interaction between PCV2 ORF3 and RGS16 facilitates degradation of RGS16 through a ubiquitin-mediated proteasome-dependent pathway (Fig. 2). A previous report also showed that PCV2 ORF3 decreases Pirh2 expression through physical interaction (Liu et al., 2007). Therefore, our results combined with previous results suggest that PCV2 ORF3 may facilitate ubiquitin-mediated proteasomal degradation of its binding partner in host cells. A recent observation provides additional evidence supporting the idea that some viral proteins facilitate degradation of their binding partner in host cells through a ubiquitin-mediated proteasome-dependent pathway (Wang et al., 2013).

As we found decreased host RGS16 expression through the interaction with PCV2 ORF3, we analysed changes in the RGS16 downstream signalling pathway. The results indicated that the decreased expression of RGS16 causes increased translocation of NFκB into the nucleus through increased degradation of cytosolic IκBα by increasing the ERK1/2 signalling pathway in host cells (Fig. 3). These results were further confirmed by analyses using ΔORF3 PCV2-infected cells and cells treated with a pAKT inhibitor (LY294002) or a pERK1/2 inhibitor (U0126) (Fig. 3). Similar to our results, previous observations indicate that PCV2 ORF3 activates NFκB through IκBα degradation (Wei et al., 2008).

Because transcription of proinflammatory cytokine mRNA is dependent on activating NFκB signalling (Baldwin, 1996; Bonizzi & Karin M, 2004), we examined IL-6 and IL-8 expression patterns to confirm whether PCV2 ORF3 increased IL-6 and IL-8 expression in host cells. The results clearly indicated that PCV2 ORF3 expression leads to increased expression and secretion of IL-6 and IL-8 in host cells and increased migration of neutrophils to host cells (Fig. 4). These results were further confirmed by analyses using ΔORF3 PCV2-infected cells (Fig. 4).

In conclusion, the mechanism of how PCV2 ORF3 facilitates inflammatory responses in host cells was defined in this study. PCV2 ORF3 physically interacts with host RGS16, leading to degradation of the RGS16 protein. Degradation of RGS16 further enhances NFκB translocation into the nucleus through the ERK1/2 signalling pathway and increases mRNA transcripts of the proinflammatory cytokines IL-6 and IL-8. Consequently, more severe inflammatory responses and leukocyte infiltration occur around infected cells. This evidence may be the first clue explaining the molecular basis of how large amounts
of proinflammatory cytokines are released within lymphoid tissues during the early stages of PCV2 infection.

METHODS

Reagents. Purified RGS16, ubiquitin, NFκB/p65, IκBα, lamin B antibodies and mouse IgG were purchased from Santa Cruz Biotechnology. Purified β-actin and Flag antibodies were purchased from Sigma. Purified PCV2 ORF2 antibody was purchased from Jenobiotech. Purified Akt, p-Akt, Erk1/2, p-Erk1/2 and HA antibodies were purchased from Cell Signaling Technology. Alexa Fluor 594-conjugated goat anti-mouse IgG, Alexa Fluor 594-conjugated goat anti-rabbit IgG, and Alexa Fluor 488-conjugated goat anti-mouse IgG antibodies were purchased from Invitrogen. LYS294002 and U0126 were purchased from Cell Signaling Technology. LPS and poly(I : C) were purchased from Sigma.

Viral infection and titration. All PK15 cells (pig kidney epithelial cells, ATCC) used in this study were PCV1-free cells (data not shown). PK15 cells were maintained at 37 °C and 5% CO2 in DMEM (Welgene) supplemented with 10% (v/v) FBS and penicillin/streptomycin (Welgene). The WT PCV2 type a (GenBank accession no. FR823451) was isolated from a pig spleen and propagated in PK15 cells. Then, each ORF1, ORF2 and ORF3 PCV2 cDNA was subcloned into the pLNCX2-HA vector. The cDNA encoding each expression vector was introduced into PK15 cells as described previously using an immunofluorescence assay (Fenaux et al., 2002). The PCV2 WT or ΔORF3 virus was used to infect PK15 cells at an m.o.i. of 1 TCID50 (Liu et al., 2005).

Cloning and expression of each PCV2 ORF gene. Each PCV2 ORF region was isolated by PCR from genomic DNA of pig spleen tissue. The primer sequences used for PCR are given in Table S1. Then, each ORF1, ORF2 and ORF3 PCV2 cDNA was subcloned into the pLNCX2 (Invitrogen) retroviral vector through the Xhol and NotI restriction enzyme sites. To generate the ORF3-Flag cDNA-expressing vector, the cDNA encoding PCV2 ORF3 was subcloned into the pLNCX2-Flag vector through the SalI restriction enzyme site. The cDNA encoding ORF3-Flag HA-expressing vector was constructed by inserting PCV2 ORF3 cDNA into the pLNCX2-HA vector. The cDNA encoding each expression vector was introduced into PK15 cells as previously described (Kim et al., 2011).

Quantification of the interaction between RG16s and PCV2 ORF3 in a yeast two-hybrid system. Three RG16s deletion mutants (Met1-Phe55, Ser56-Leu180 and Ala181-Thr202) were isolated by PCR. Then, PCR products spanning each fragment were cloned into the EcoRI and Xhol or the BamHI and Xhol restriction enzyme sites of the pGilda vector to generate each Lex A-RG16 fusion protein for bait construction containing Lex A in a yeast two-hybrid system. PCV2 ORF3 was also isolated by PCR. The PCR product spanning the fragment was cloned into the EcoRI and Xhol restriction enzyme sites of the pG4-5 vector to generate the B42-PCV2 ORF3 fusion protein. The primer sequences used for each PCR are described in Table S1. The primers used for each PCR are described in Table S1. The primers used for each PCR are described in Table S1. The primers used for each PCR are described in Table S1. The primers used for each PCR are described in Table S1.

Confocal microscopy, Western blot analyses, co-immunoprecipitation (co-IP) and ubiquitination assays. Confocal microscopy, Western blot analyses, co-IP and endogenous ubiquitination assays were performed as described previously (Kim et al., 2011). To measure stability of RG16s protein, cells were treated or not treated with 100 μg ml−1 CHX (Sigma) for 6, 12, 18 and 24 h to measure RG16s stability. Cells were treated or not treated with 20 μM proteasome inhibitor MG132 (EMD Chemicals) for 4 h in culture medium. After treatment, the cells were harvested, washed in PBS, centrifuged and resuspended in a cell lysis solution. To perform exogenous ubiquitination assays, the cDNA encoding HA-ubiquitin was introduced via transfection into stable PCV2 ORF3 or empty vector expressing PK15 cells. After transfection, cells were used to perform ubiquitination assays.

Measurement of IL-6 and IL-8 expression and neutrophil transwell migration assay. Pig IL-6 and IL-8 mRNA transcripts in PK15 cells were quantitatively measured by real-time PCR analyses as previously described (Hyun et al., 2010). The primer sequences used for each PCR are described in Table S1. IL-6 and IL-8 secretion from PK15 cells was measured by sandwich ELISA using pig IL-6 (Abcam) and IL-8 ELISA (Invitrogen) kits. Pig neutrophils were isolated from peripheral blood according to a method described previously (Wittamer et al., 2005; Sroussi et al., 2007).

Statistical analyses. Mean independent variable values were compared using Student’s t-test.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Next-Generation BioGreen 21 programme (no. PJ011130), Rural Development Administration, Republic of Korea.

REFERENCES


