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Antimicrobial Host Defense Peptides in an Arteriviral Infection: Differential Peptide Expression and Virus Inactivation

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Abstract

Antimicrobial host defense peptides (AHDPs) are effective against a wide range of microbes, including viruses. The arteriviral infection caused by porcine reproductive and respiratory syndrome virus (PRRSV) is a devastating pandemic that causes the most economically significant disease of swine. We sought to determine if the expression of AHDPs was influenced by infection with PRRSV, and if porcine AHDPs have direct antiviral activity against PRRSV. Because pulmonary alveolar macrophages (PAMs) are primary targets of PRRSV infection, gene expression of porcine AHDPs was evaluated in lungs from fetal and 2-wk-old congenitally infected pigs. In PRRSV-positive lungs and PAMs, gene expression of most porcine AHDPs showed little upregulation. However, gene expression of porcine β-defensin-1 (pBD-1), pBD-4, pBD-104, pBD-123, and pBD-125 were downregulated more than threefold in 2-wk-old congenitally infected pig lungs. Incubation of PRRSV with pBD-3 or PG-4 significantly inhibited viral infectivity in MARC-145 cells. Using nine protegrin or protegrin-derived peptides, we determined that a cyclic analog of PG-4 increased anti-PRRSV activity, and that substitution of phenylalanine with valine eliminated most PG-4 antiviral activity. In PAMs, pBD-3 and PG-4 at 5–40 μg/mL consistently suppressed PRRSV titers. Collectively, these findings suggest a potential role for some porcine AHDPs as innate antiviral effectors in PRRSV infection. Moreover, modulation of porcine innate immune mechanisms with AHDPs may be one means of limiting the impact of this costly pandemic viral disease.

Introduction

An important defense component of an animal's innate immune response is constitutive or inducible production of antimicrobial host defense peptides (AHDPs). Many AHDPs have broad-spectrum antimicrobial activity against bacteria, fungi, protozoa, and viruses. Although antiviral activity of AHDPs was noted in early studies, it has only been recently that research in this area has intensified (3,4,12,14). Using direct inactivation assays, several studies have shown that LL-37, the only cathelicidin AHDP in humans, significantly reduces infectivity of several viruses (1,9,10,11). Similarly, constitutive expression of β-defensins and combinations of other AHDPs in oral epithelia and vaginal fluids has been shown to form an effective innate immune barrier against HIV infection (22,29). Human β-defensin-3 (hBD-3) inhibited influenza virus infection through direct interaction with virion surface glycoprotein, preventing virus fusion to the cell membrane, and suppressed HIV entrance into cells by competition for viral co-receptors on immunocompetent cells (6,15). Retrocyclins, circular θ-defensins based on human sequences, also have antiviral activity. Retrocyclin (RC)-1 protected human cells from infection by numerous primary HIV-1 isolates, and RC-2 effectively suppressed infection by several viruses (2,7,33,34). To our knowledge, protegrin (PG)-1 is the only porcine AHDP that has been reported to have antiviral activity (30,31). To date, about 30 porcine AHDPs, including 13 β-defensins (pBDs) and 12 cathelicidins, have been identified (28). α-Defensins have not been identified in pigs, and it has been suggested that some porcine cathelicidins, specifically protegrins, substitute for the functions of α-defensins found in other species (3,8).

The porcine arterivirus, porcine reproductive and respiratory syndrome virus (PRRSV), is an enveloped, positive-sense, single-stranded RNA virus that has been an immunological challenge and a devastating pathogen for the swine industry (17,19). We reasoned that some porcine AHDPs may be antiviral effectors during PRRSV infection. Accordingly, we examined the gene expression profile of several porcine AHDPs in lungs from PRRSV-negative and PRRSV-positive pigs, and evaluated the direct inactivation activity of a group of synthetic porcine AHDPs. Here we

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report that PRRSV infection induces changes in porcine AHDP expression in lungs, and that pBD-3 and PG-4 directly suppress PRRSV infectivity.

Materials and Methods

Virus strains and titration

The PRRSV strains used to infect pigs (SDSU-23983-P6 [P6]) and cells (SDSU-23983-P7 [P7]) have been previously described (27). In addition, a full-length cDNA infectious clone with expression of green fluorescent protein (GFP) in the region of nonstructural protein 2 (Nsp2) of PRRSV (GFP-PRRSV) was used to facilitate fluorescent microscopic examination of virus infection. The GFP-PRRSV was generated from the North American type 1 PRRSV isolate, SD01-08, and maintained growth properties similar to those of the parental virus in cell cultures (5). The tissue culture 50% infectious dose ($TCID_{50}$) of P7 and GFP-PRRSV stocks were $10^{7.25}$ and $10^{7.14}$ PFU/mL, respectively. Cells were infected at a multiplicity of infection (MOI) of 0.1 $TCID_{50}$/mL (22,23). MARC-145 cells were used for virus titration as previously described (27). P7-virus-infected monolayers of MARC-145 cells in 96-well tissue culture plates were fixed with 80% cold acetone and incubated with fluorescent-labeled monoclonal antibodies to PRRSV nucleocapsid protein N (SDOW17; Rural Technologies, Inc., Brookings, SD). PRRSV-positive cells were identified by fluorescent microscopy (23,24,27). All animal and virus procedures were approved by the Kansas State University Institutional Animal Care and Use, and Biosafety Committees.

PRRSV infection and tissue sample collection

Infection of animals and cells with PRRSV, and collection of tissue samples have previously been described (23,24,27). Briefly, pregnant sows obtained from a closely monitored PRRSV-negative herd were infected intranasally at 90 d of gestation with a sixth-passage isolate of SDSU-23983, a typical North American field isolate (23). Methods for the preparation of the PRRSV inoculum by cultivation on MARC-145 cells and infection of pigs have been previously described (24). Fetuses were obtained at 108 and 111 d of gestation, or animals were allowed to give birth and live-born pigs were euthanized at 14 d of age. The pigs were periodically checked for porcine circovirus 2 (PCV2) by PCR and found to be negative. Routine microhistological examination from all control and infected fetuses and pigs showed no evidence of PCV2 infection. Tissue samples were immediately placed in RNA*later* (Ambion, Inc., Austin, TX) and stored at −20°C until use (23,24). Two days before treatment or infection with PRRSV, pulmonary alveolar macrophages (PAMs) were thawed from stocks and plated in 24-well tissue culture plates (7×10^4 cells/well) in supplemented RPMI 1640 medium and cultured in a humidified 5% CO_2 + 95% air atmosphere at 37°C. After one change of fresh medium 2 h later to remove non-adherent cells, the PAMs were cultured with replenished medium containing synthetic AHDPs. The PAMs used for viral infection were infected with PRRSV-P7 at a MOI of 0.1 $TCID_{50}$/mL for 18 h. Supernatants were collected for viral titration on MARC-145 cells.

Real-time RT-PCR assay

Real-time, RT-PCR arrays in a 96-well microplate format (iCycler 5.0; BioRad, Hercules, CA) were performed using the validated primers. The reactions were conducted with an SYBR Green RT-PCR system (Qiagen, Valencia, CA) using 150 ng of total RNA in a 25-μL reaction mixture and RT-PCR conditions as recommended (27). The specific optic detection was set at 78°C for 15 sec after each amplification cycle of 95°C for 15 sec, 56–57°C for 30 sec, and 72°C for 40 sec. Critical threshold (C_t) values and melt curves were monitored

Table 1. Porcine Cathelicidin Primer Sequences for RT-PCR Analysis

Cathelicidin	*Primer sequence (5′ to 3′)*	*GenBank accession number*	*Location in cDNA (nt)*
PR-39			
Sense	CGGAGCTGTGTGACTTCAAGGAGAA	L23825	295–319
Antisense	ATGGGTATGTTATCAGCCACTCCAT		560−534
PF-1/2			
Sense	CGGAGCTGTGTGACTTCAAGGAGAA	X75438	280–304
Antisense	AAAGGTGGAGGCGGAGGGAACCA		643−621
PMAP-23			
Sense	CGGAGCTGTGTGACTTCAAGGAGAA	L26053	291–315
Antisense	AAATTTGGGTTTCTGTGGCCGACG		454–431
PMAP-36			
Sense	CGGAGCTGTGTGACTTCAAGGAGAA	L29125	291–315
Antisense	ACCCAAGGGTATTGAGCCGACAAT		505−482
PMAP-37			
Sense	CGGAGCTGTGTGACTTCAAGGAGAA	L39641	291–315
Antisense	TCCGACCACGATCACTGAGGAAAT		449–426
PG-4			
Sense	CGGAGCTGTGTGACTTCAAGGAGAA	X79868	278–302
Antisense	GTCCGACACAGAAGCAGATCCAACC		442–466
PG-1–5			
Sense	CGGAGCTGTGTGACTTCAAGGAGAA	X79868	278–302
Antisense	TGCCGTCGCAACCGTCATCCT		464−444

and collected by the iCycler 5.0 software, and the final products after 40 PCR cycles were analyzed on agarose gels. The C_t values of AHDP genes were normalized against C_t values of the housekeeping gene (GAPDH) amplified from the same RNA samples to obtain $2^{-\Delta Ct}$. Values of $2^{-\Delta Ct}$ from PRRSV-infected tissues were plotted against $2^{-\Delta Ct}$ values from control tissues and used to calculate a relative expression index ($2^{-\Delta\Delta Ct}$) (16). Generation and standardization of pBD primers have been previously described (26), and gene-specific primers for porcine cathelicidins are shown in Table 1.

Peptide synthesis and preparation

The C-terminal peptides of pro-pBD-1, pro-pBD-2, and pro-pBD-3 were chemically synthesized by solid phase peptide synthesis (Abgent Inc., San Diego, CA), as were PR-39 and an analogue of pBD-3 whose six cysteines were replaced with alanines (pBD-3ΔC). The material was eluted as a single peak by reverse-phase HPLC, and peptide identity was confirmed by mass spectroscopy. Final purity of the peptides was >95%. Protegrins, protegrin-related peptides, and RC-2 were synthesized by solid-phase peptide synthesis, using fluorenylmethoxycarbonyl chemistry. Peptides were purified by HPLC, and concentration was determined by measuring absorbance at 280 nm. PG-1, PG-4, and PG-5 were derived from the C-terminal 19 amino acids of their respective endogenous precursors. *In vivo*, these precursors lose their final glycine post-translationally during the process that amidates their C-termini. The cyclic versions of PG-4 and PG-5 had cyclic backbones, as will be illustrated. Protegrin peptides were oxidized to form two inter-strand disulfide bonds. The other two analogs of PG-4 include PG-4F-V, in which phenylalanine at position 14 was replaced with valine, and cyclic PG-4NCP, which contains 1-naphthy-alanine (N or Nal), cyclohexylglycine (C or Chg), and pentafluorophenylalanine (P or Phe^{F5}) instead of residues GWI. These substitutions made the analog much more hydrophobic than native PG-4. Three other protegrin congener (PC) peptides were made: PC-303 (RWRLCYCRPRFCVCV-amide), PC-307 (RGWRACYCRPRFCACVGR-amide), and cyclic PC-307. These were analogs of PG-5, and their properties have been described elsewhere (13). The various peptides were lyophilized and dissolved in 0.01% acetic acid at 1 mg/mL (~0.5 mM) as a stock solution and stored at −135°C until use.

AHDP direct virus inactivation and cytotoxicity assays

To evaluate antiviral activity of the AHDPs, peptides were diluted in 50 μL of FBS-free MEM containing PRRSV (P7) or GFP-PRRSV at 10× the concentration of viral titers used for inoculation (MOI of 1 $TCID_{50}$/mL). Final concentrations of AHDPs were at 0, 5, 10, 20, and 40 μg/mL, and some peptides were used up to 240 μg/mL. After incubation at 37°C for 2 h, 10 μL of the mixture of virus and peptide were added to 90 μL medium and MARC-145 cells cultured in 96-well plates. The AHDPs were directly diluted in culture medium containing the virus at MOI of 0.1 $TCID_{50}$/cell and applied to the cells in 96-well plates (25). The plates were washed with fresh medium after infection for 2 h, and replenished with AHDP-containing medium. The infectivity of virus was examined at 24 or 48 h by immunostaining of PRRSV nucleocapsid protein N, or detection of GFP fluorescence in GFP-PRRSV-infected cells. Virus-infected cells were detected by two means (1) using monoclonal antibodies specific for PRRSV (2D6; VMRD, Inc., Pullman, WA), labeled with FITC-conjugated secondary antibodies, and counted using fluorescence microscopy, or (2) imaged using inverted fluorescent microscopy and fluorescence intensity was quantified with digital image software (AlphaEase FC, FluorChem™; Alpha Innotech Corp., San Leandro, CA). For evaluation of AHDP effects on viral infection in PAMs, viral supernatants were collected from PAMs infected with PRRSV for 18 h in the presence of AHDPs. Supernatants were serially diluted tenfold onto MARC-145 cell monolayers in 96-well plates. MARC-145 monolayers were fixed with buffered 4% formaldehyde at 48–72 h post-infection, and stained with crystal violet to identify viral plaques for calculation of $TCID_{50}$/mL (23,24).

Cytotoxicity of the AHDPs was quantified by determining the number of viable cells using a tetrazolium-based colorimetric (MTT) assay (Invitrogen Life Technologies, Carlsbad, CA). Cell viability was calculated using the equation: (mean OD of treated cells/mean OD of control cells) ×100 (17).

Results

Differential expression of AHDPs in PRRSV-infected lungs and PAMs

To determine if PRRSV influences the expression of AHDPs, we evaluated the expression of porcine defensins and cathelicidins in lungs and PAMs exposed to virus (Fig. 1). Lung samples were collected from either 14-d-old piglets or fetuses (108 and 111 d of gestation) from sows infected by

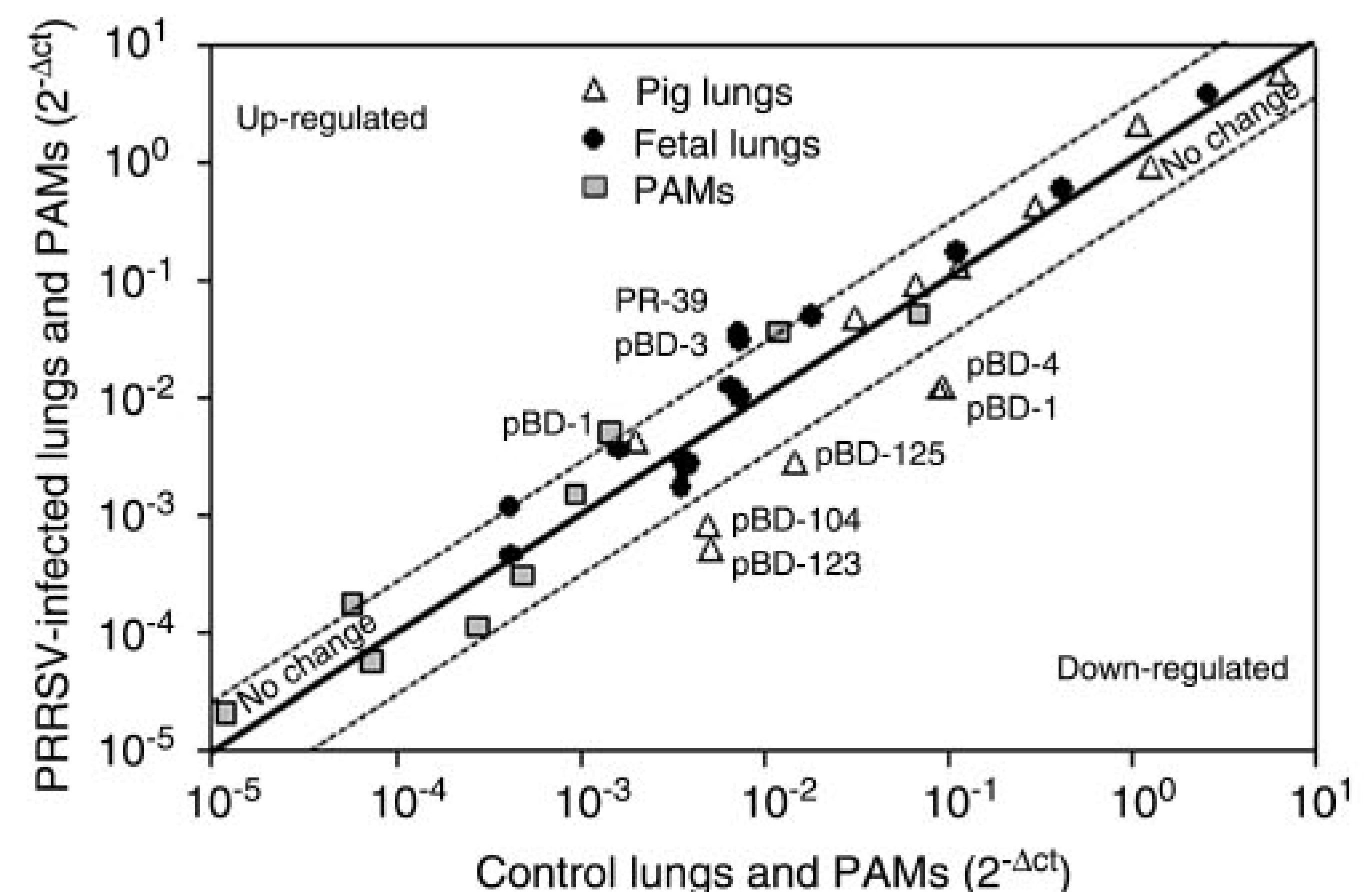


FIG. 1. Scatter plot of gene expression levels of porcine AHDPs between control and PRRSV-infected lungs and pulmonary alveolar macrophages (PAMs). Fetal and 14-d-old pig lungs from sows infected with PRRSV, and PAMs infected *in vitro* with PRRSV were evaluated for mRNA expression using a real-time RT-PCR array. Critical threshold (C_t) values of AHDP genes were normalized against C_t values of the housekeeping gene (GAPDH) amplified from same RNA samples to obtain $2^{-\Delta Ct}$ ($\log_{10}$). Values above the top-dashed diagonal line indicate upregulation by at least threefold, and values below the bottom-dashed diagonal line indicate downregulation by at least threefold. Data represent two independent experiments with similar results.

PRRSV at 85–90 d of gestation (23,24). PAMs were obtained by bronchoalveolar lavage from healthy pigs, and infected with PRRSV *in vitro* (36). Multiple β-defensins were expressed in lungs of 14-d-old pigs and fetuses. However, only pBD-1, pBD-4, pBD-104, pBD-123, and pBD-125 were downregulated (more than threefold) in PRRSV-infected pig lungs. Downregulation of pBD expression was not observed in lungs from PRRSV-infected fetuses or PRRSV-infected PAMs. pBD-1 in PRRSV-infected PAMs and pBD-3 in PRRSV-infected fetal lungs were modestly (slightly more than threefold) upregulated. Most porcine cathelicidins were expressed in lungs. However, only PR-39 was slightly upregulated (slightly more than threefold) in lungs of fetuses from PRRSV-infected sows. No porcine cathelicidins were detected in PAMs (data not shown).

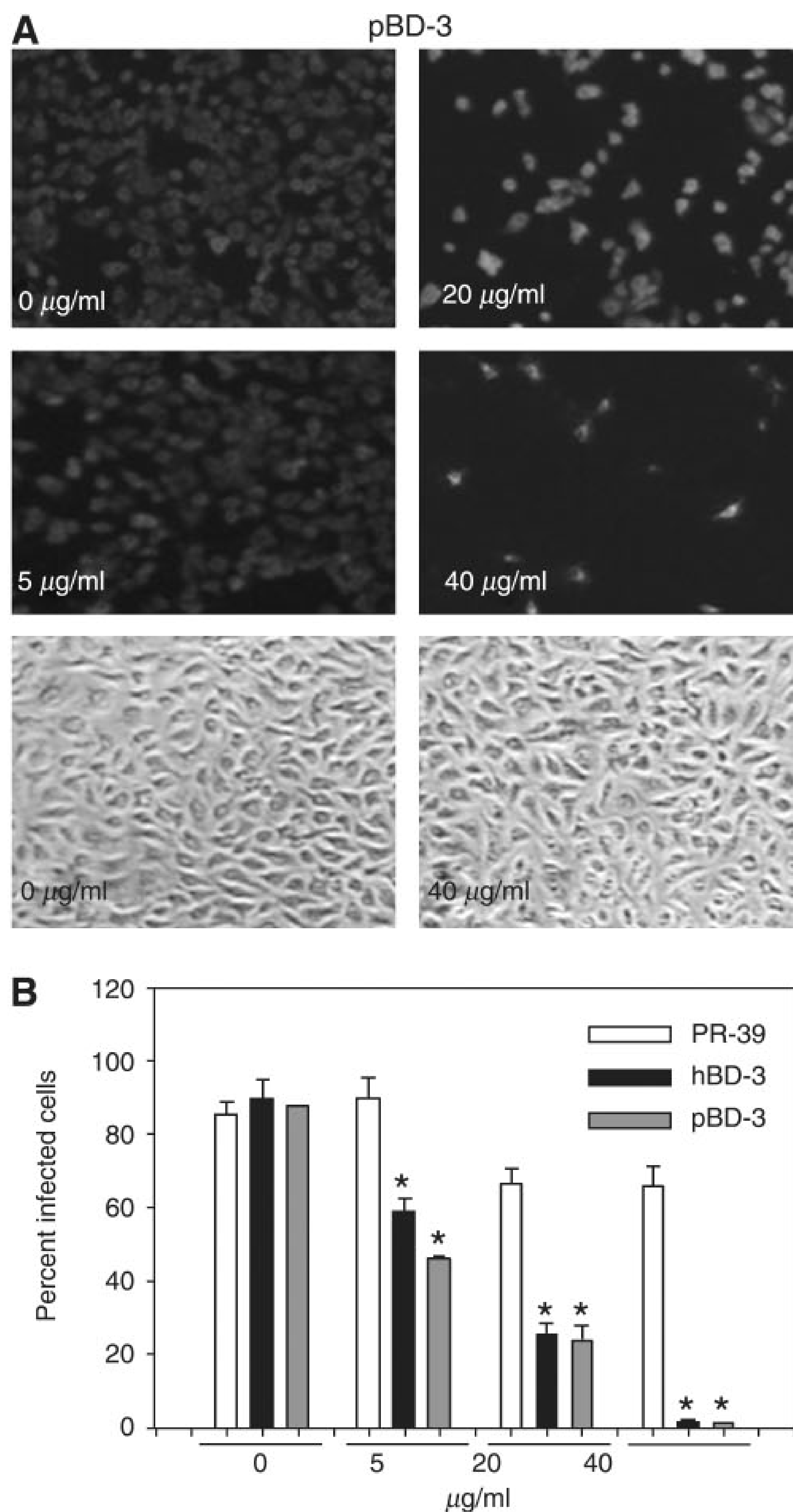


FIG. 2. pBD-3 and hBD-3 inhibit PRRSV infectivity. (**A**) PRRSV was incubated for 2 h with pBD-3 before infection of MARC-145 cells. The top four panels show immunofluorescence of cells detected with a mAb to PRRSV. The bottom two panels are brightfield micrographs. (**B**) Quantitative results of PRRSV-positive cells. PRRSV was incubated with pBD-3, hBD-3 and PR-39 before infection with MARC-145 cells, and PRRSV-infected cells were detected with mAbs specific for PRRSV, labeled with FITC-conjugated secondary antibodies and counted using fluorescence microscopy (*significantly different from controls, $p < 0.05$; n = 3).

Suppression of PRRSV infectivity in MARC-145 cells

Porcine AHDPs, including pBD-1, pBD-2, pBD-3, PR-39, PG-1, PG-4, and PG-5, were evaluated for anti-PRRSV activity. Two primate AHDPs, hBD-3 and RC-2, which have been shown to be active in suppression of multiple viruses, were also evaluated. As shown in Fig. 2A, pBD-3 suppressed PRRSV infectivity in MARC-145 cells when the virus was detected by immunofluorescence using a monoclonal antibody to PRRSV nucleocapsid protein N. As little as 5 μg/mL of hBD-3 and pBD-3 decreased PRRSV infectivity (Fig. 2B).

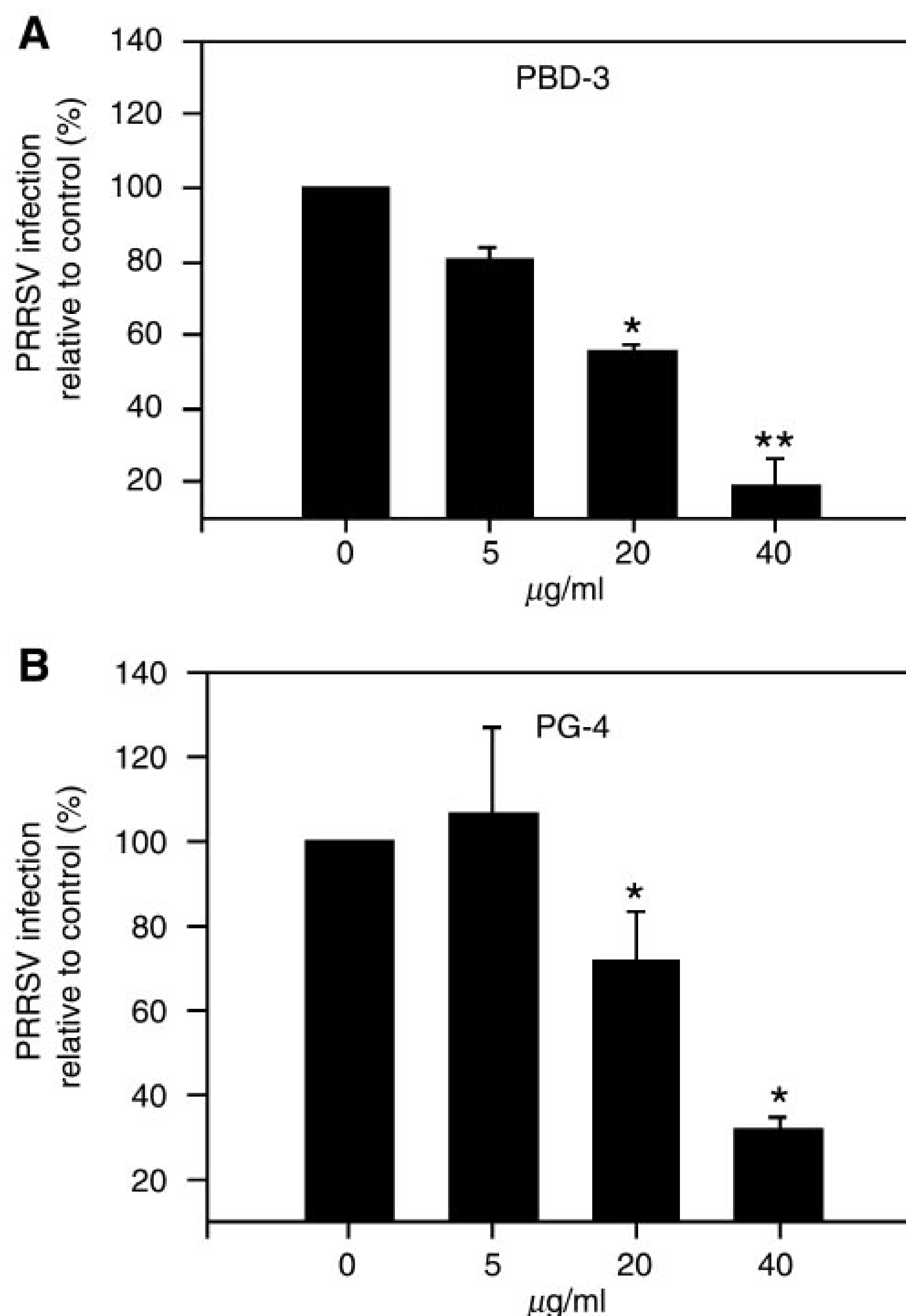


FIG. 3. pBD-3 and PG-4 inhibit PRRSV infectivity. PRRSV was incubated for 2 h with pBD-3 or PG-4 before infection of MARC-145 cells, and cells were cultured in the presence of pBD-3 or PG-4 for 48 h. PRRSV-positive cells were detected by measuring immunofluorescence intensity of 3–5 random areas and standardized relative to controls using a digital imaging system (significantly different from controls, *$p < 0.05$, **$p < 0.01$; n = 3).

Conversely, the cathelicidin PR-39 did not suppress PRRSV infectivity.

Incubation with either pBD-3 or PG-4 at 20 μg/mL or higher suppressed 50–80% of viral infectivity when measured by immunofluorescence intensity using an antibody to PRRSV nucleocapsid protein N (Fig. 3A and B). Peptides at concentrations lower than 10 μg/mL did not inactivate PRRSV, and complete elimination of PRRSV-positive cells could not be attained, even at 40 μg/mL (Fig. 3B). Other porcine AHDPs, including pBD-1, pBD-2, PR-39, PG-1, and PG-5 showed no significant inactivation at concentrations lower than 40 μg/mL (data not shown). Of the primate AHDPs evaluated, hBD-3 showed activity similar to pBD-3 (Fig. 2B); however, RC-2 was not active against PRRSV at concentrations lower than 40 μg/mL (data not shown). In most cases, either peptide pre-incubation for 2 h with virus in FBS-free medium prior to addition to cells, or simultaneous addition to cells in cell culture medium (unpublished data), yielded similar results with regard to inactivation of viral infectivity (25). In addition to the above activity assays, which used a wild-type PRRSV isolate (P7), a cell-culture-adapted PRRSV (P136) and a DNA infectious clone GFP-PRRSV (5) also were tested for sensitivity to the AHDPs. The efficacy of the AHDPs for suppression of PRRSV infectivity was consistent among these three PRRSV strains; however, the GFP-PRRSV infectious clone provided a real-time means of examining the effects of AHDP because of GFP's fusion to the viral Nsp2 protein.

Anti-PRRSV activity of protegrin-derived peptides

The ability of PG-4 to inactivate PRRSV, without significant cytotoxicity at 40 μg/mL (Fig. 4), prompted us to study this AHDP further. The alignment of the five isoforms of porcine protegrin mature peptides indicates that PG-4 differs the most from the other PGs (Fig. 5A). Whereas PG-4 has a hydrophobic β turn at residues 10–12 (GWI), the other PGs have arginine-rich polar turns with residues of (R)RRF or (R)PRF. In addition, PG-4 has a Phe (F) at residue 14, and the other PGs have Val (V) or Ile (I) at this position. Because cyclic PGs may exert a more favorable therapeutic ratio (30), we synthesized cyclic forms of PG-4 (Fig. 3B) and two protegrin congeners (PCs): PC-303 and PC-307. We also synthesized PG-4F-V, a β-hairpin analog of PG-4, in which Phe was replaced with Val, and cyclic PG-4/Nal/Chg/Phe-F5 (PG-4NCP), in which residues 10–12 (GWI) were replaced, in turn, by 1-naphthylalanine, cyclohexylglycine, and pentafluorophenylalanine, to make this analog more hydrophobic than either cyclic or native PG-4. The viability of cells treated with PG-4-derived peptides was similar, or perhaps even slightly greater, than that of linear PG-4 (Fig. 4).

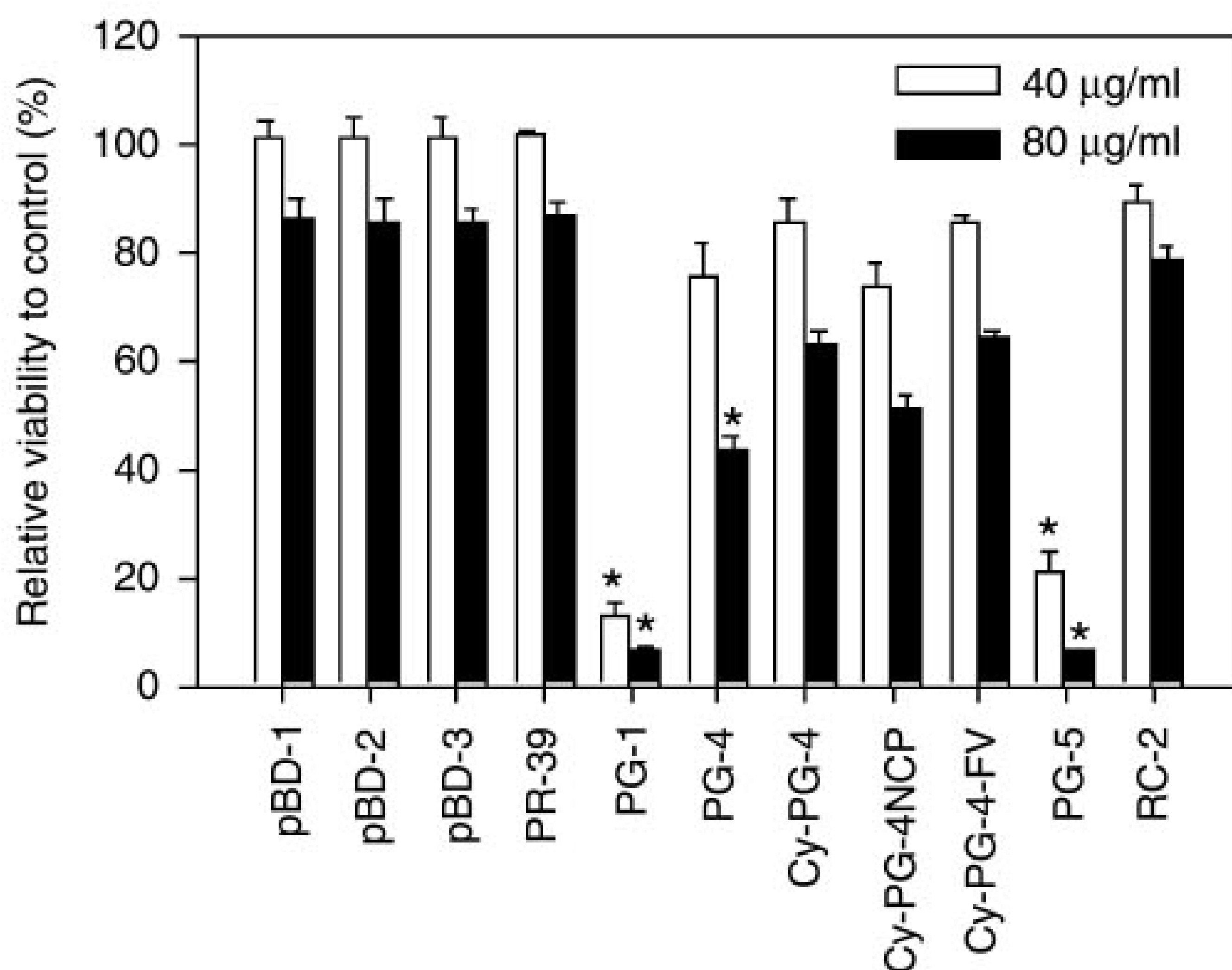


FIG. 4. Cytotoxicity of ADHPs on MARC-145 cells. Peptide cytotoxicity was determined by measuring cell viability using a tetrazolium-based colorimetric (MTT) assay (*significantly different from controls, $p < 0.001$; n = 3).

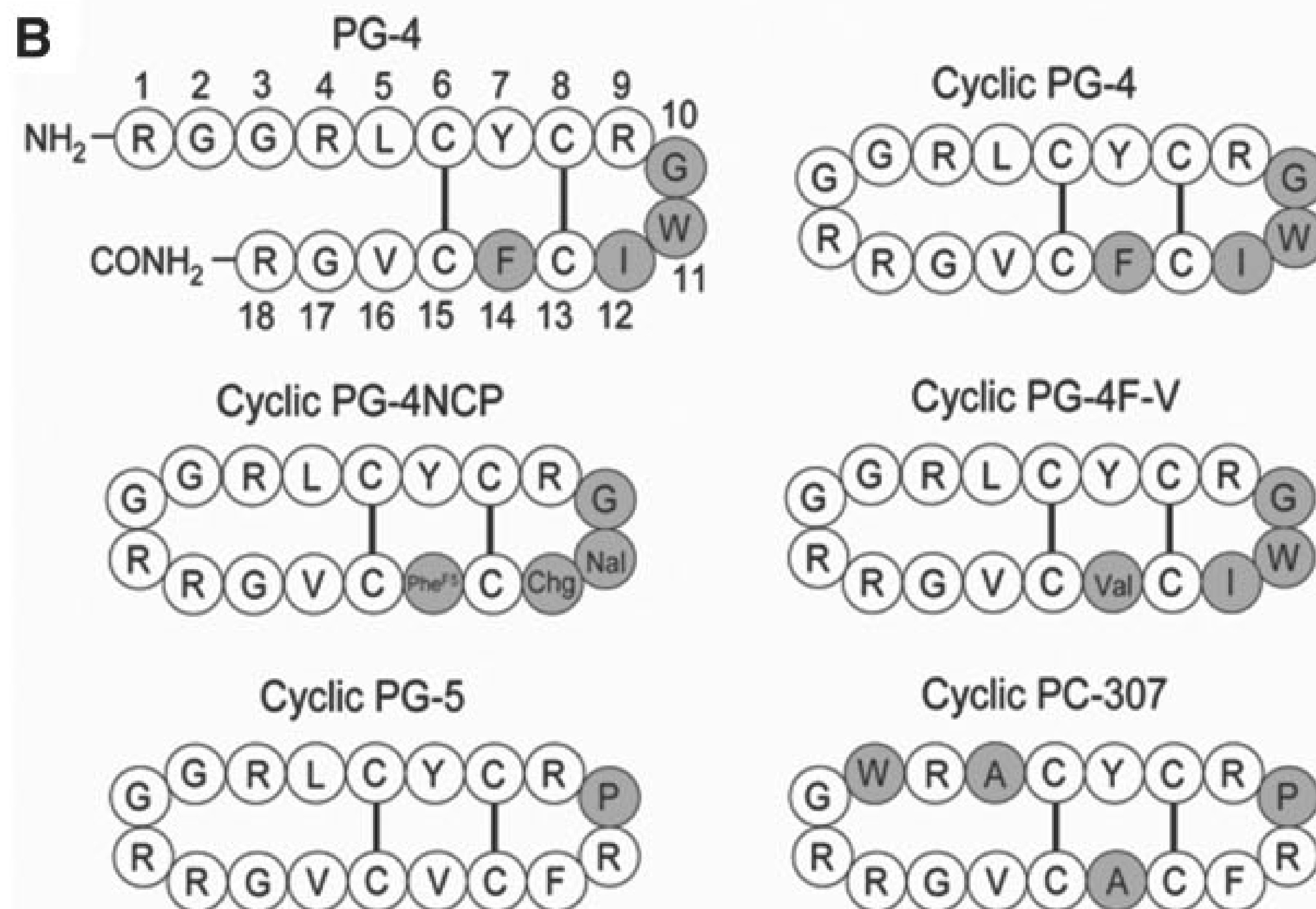
Antiviral activity of these protegrin-derived peptides was evaluated against both wild-type PRRSV and GFP-PRRSV. Examined at 48 or 72 h post-infection, the presence of linear PG-4, cyclic PG-4 (Cy-PG-4), and Cy-PG-4NCP, had similar anti-PRRSV activity at 5–20 μg/mL (Fig. 5C). The cyclization of PG-4 (Cy-PG-4 and Cy-PG-4NCP) showed some improvement in anti-PRRSV activity compared to PG-4 at 20 and 40 μg/mL. However, Cy-PG-4NCP was no better than Cy-PG-4. Clearly, cyclic PG-4 with substitution of Phe with Val (PG-4F-V) exhibited less anti-PRRSV activity than PG-4 and cyclic PG-4 at all concentrations. We also evaluated the activity of cyclic PG-5 and three other protegrin-like peptides: PG-303, PG-307, and cyclic PG-307 (Cy-PC-307). PC-303 (15 residues) was a truncated analog of PG-5 that contained a tryptophan residue (Fig. 5A). PC307, a more extensively modified protegrin congener, also contained a tryptophan (Fig. 5A). Both PG-303 and PG-307 have been shown to be more active against HIV-1 than PG-1 (unpublished data). However, PG-5 had little anti-PRRSV activity at <40 μg/mL; but cyclic PG-5 showed increased anti-PRRSV activity at an earlier time point in viral infection when tested with GFP-PRRSV (data not shown). In general, PG-303 and PG-307 showed no obvious activity in suppression of PRRSV infectivity.

Suppression of PRRSV infection/replication in PAMs

Because PAMs are an important site of PRRSV infection, we also evaluated virus inactivation of selected AHDPs directly on PAMs. Peptides were co-incubated with PAMs during virus infection for 18 h, and released virus in the PAM supernatants was titrated on MARC-145 cells. As shown in Table 2, at concentrations of 5 and 10 μg/mL, pBD-3 and PG-4 were the most active peptides suppressing PRRSV compared to controls. PG-1 and PG-5 also decreased PRRSV titers at 10 μg/mL. At 20 and 40 μg/mL, all porcine AHDPs showed some suppression of PRRSV infectivity (~1–3 $TCID_{50}$ units). Interestingly, replacing the six cysteines of pBD-3 with alanines (pBD-3ΔC), thus eliminating the three disulfide bonds, did not influence the ability of pBD-3 to inactivate PRRSV (Table 2).

Discussion

This study provides experimental support for new findings concerning the interaction of AHDPs and porcine arteriviral infections. Foremost, it provides the first characterization of porcine defensins and cathelicidins in animals and cells infected with PRRSV. Second, it shows that AHDPs can directly inactivate PRRSV. These findings suggest that antiviral therapies using AHDPs, such as viral suppressors



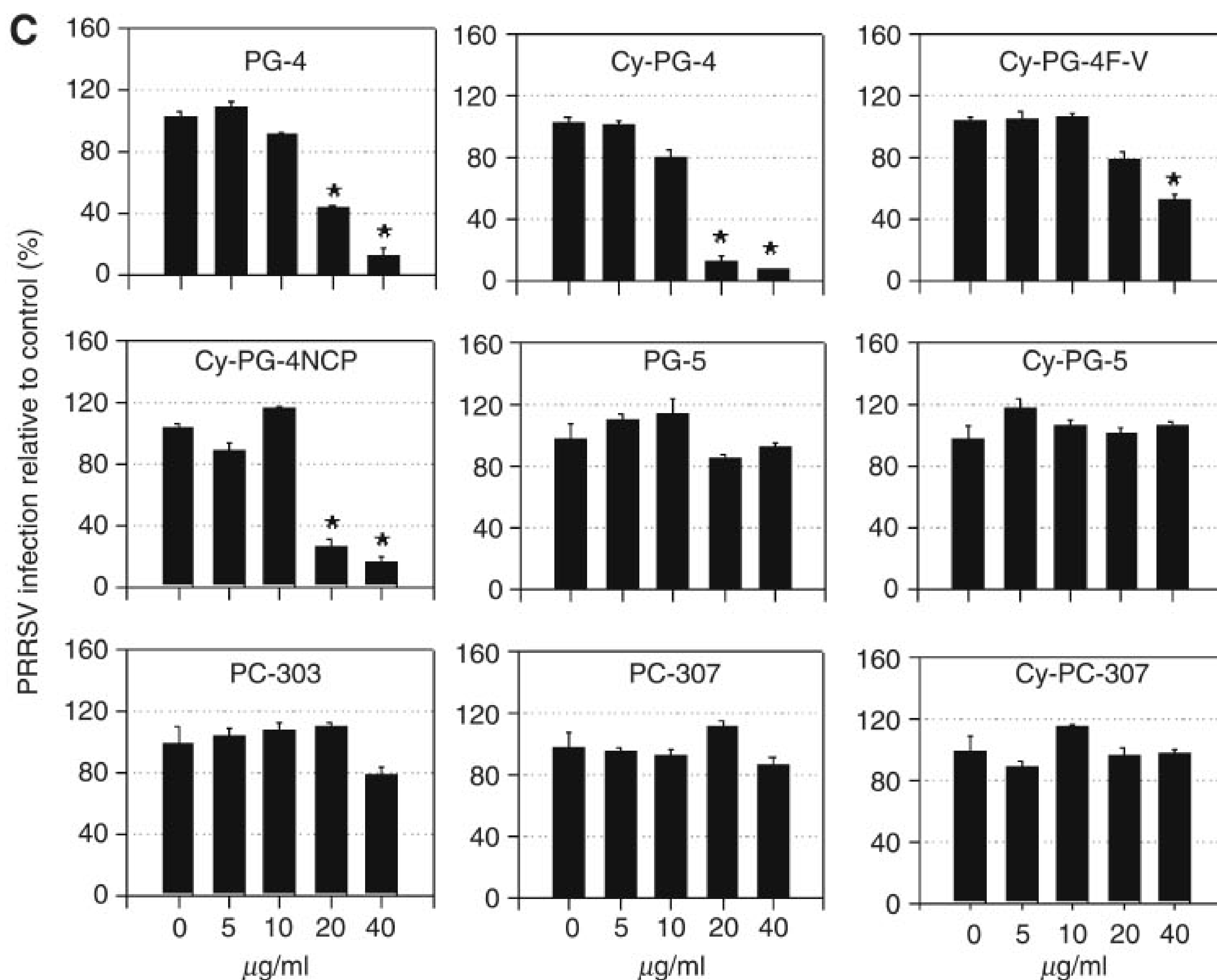


FIG. 5. Peptide sequences and PRRSV infectivity of porcine protegrins (PGs) and PG-4- and PG-5-derived peptides. (**A**) Peptide sequences (single letter code) of PG-1, PG-2, PG-3, PG-4, PG-5, and two protegrin congeners (PC-303 and PC-307). Unique residues relative to PG-1 are shaded. (**B**) Coin diagrams of PG-4 and cyclic peptides based on PG-4 and PG-5. The different residues of PG-4, including the aromatic beta turn (GWI) and Phe^{F5} (F), are shaded. The cyclic forms of PG-4-derived peptides have the one-letter codes replaced with three-letter codes at the replaced residues (Nal, 1-naphthylalanine; Chg, cyclohexylglycine; Phe^{F5}, pentafluorophenylalanine; cyclization was catalyzed at the positions mimicking Arg^1/Gly^2 and Gly^{10}/Trp^{11}, and synthetic peptides were oxidized to form intramolecular disulfide bonds [black bars]). (**C**) PRRSV was incubated with PG-4, PG-5, or their derived peptides for 2 h before infection of MARC-145 cells, and cells were cultured in the presence of the same AHDP for 48 h. PRRSV-positive cells were detected using immunofluorescence and quantified as indicated in Fig. 3.

TABLE 2. AHDP DIRECT INACTIVATION OF PRRSV IN PAMs

	AHDP treatment			
	5 μg/mL	10 μg/mL	20 μg/mL	40 μg/mL
Control	7.4[1]	7.4	7.8	7.4
pBD-2	7.4	7.4	6.6	6.8
pBD-3	6.8	6.6	6.5	4.8[a]
pBD-3ΔC	7.3	6.7	6.8	5.3[a]
PR-39	7.2	7.5	7.4	5.8[a]
PG-1	7.4	6.6	5.8[a]	4.7[a]
PG-4	6.8	6.4	6.3	4.7[a]
PG-5	7.2	7.0	7.0	5.3[a]
RC-2	7.0	8.0	7.2	7.0

[a]Significantly different from controls, $p < 0.05$.
Pulmonary alveolar macrophages (PAMs) were incubated with PRRSV and AHDPs for 18 h, and virus released in PAM supernatants was titrated on MARC-145 cells.
Values are means of $\log_{10}$ $TCID_{50}$/mL.

based on direct antiviral activity or vaccine adjuvants based on immunomodulatory activity (12), may be developed if cost-benefit ratios become favorable.

Minimal regulation of innate immune genes, such as interferon-α, interleukin-1, and interleukin-6, appears to be a prominent feature in PRRSV infections (20). This was also true for most AHDP genes in lungs from neonatal pigs congenitally infected with PRRSV. However, pBD expression in 2-wk-old pig lungs was somewhat more responsive, as indicated by a decrease in expression of five pBD-1 genes, and a slight increase in pBD-1, pBD-3, and PR-39 gene expression. These findings suggest that pBD expression in lungs may be differentially responsive to PRRSV infection, ontogenically regulated, and that downregulation of multiple pBDs in pig lungs may be related to increased susceptibility to secondary infection in PRRSV-infected pigs (17). As innate antiviral effectors, the human β-defensins (hBDs) hBD-2 and hBD-3 were increased in airway epithelial cells infected by human rhinovirus (21) or HIV-1, but hBD-1 was not altered in HIV-infected epithelial cells (22). Conversely, recombinant hBD-2 and hBD-3 showed dose-dependent *in-vitro* inhibition and *in-vivo* suppression of HIV replication (22,29). Although we found that pBD-3 had similar antiviral activity against PRRSV as hBD-3, gene expression of pBD-3 was not altered in PRRSV-infected lungs and macrophages. Finally, expression of several porcine cathelicidins in lungs but not in PAMs is consistent with results reported in the literature indicating that porcine cathelicidins are expressed in neutrophils but not in macrophages (37).

Our findings showed that pBD-3 had direct inactivation activity on PRRSV in both MARC-145 cells and primary macrophages. These results are similar to findings for hBD-3 that showed direct inactivation of HIV (6). Two mechanisms of virus inactivation by hBD-3 are known to involve direct interaction with virus fusion to the cell membrane, or internalization of the cellular receptor (6,15). Although the mechanism by which pBD-3 inhibits PRRSV infection remains to be determined, our data show that the direct PRRSV antiviral activity of pBD-3 is independent of intramolecular disulfide bonds. This finding is consistent with other reports showing that intramolecular disulfide bonds are not required for antimicrobial activity (32,35).

Although several porcine cathelicidins may have indirect antiviral activity *in vivo* (28), we did not find significant direct anti-PRRSV activity related to the porcine cathelicidins PR-39, PG-1, and PG-5. Conversely, PG-4 repeatedly showed direct inactivation of PRRSV infectivity. Because PG-4 differs from other protegrin isoforms solely at the aromatic turn (residues 10, 11, and 12 [GWI]) and residue 14 (F), two analogs of PG-4, cyclic PG-4 and cyclic PG-4NCP, were used to further elucidate anti-PRRSV activity. Whereas cyclization of PG-4 enhanced anti-PRRSV activity, increasing its hydrophobicity (GWI to NCP) did not further improve anti-PRRSV activity. In contrast, substitution of phenylalanine at position 14 with valine (cyclic PG-4F-V) diminished most of the anti-PRRSV activity compared to the linear (PG-4) or the cyclic peptide (Cy-PG-4). This finding suggests that the aromatic side chain of Phe plays an important role in activity against PRRSV.

Animal AHDPs may suppress viral infectivity via interaction directly with virion glycoproteins or cellular receptors adopted by viruses, and indirectly through stimulation of other antiviral responses (12). The mechanisms by which pBD-3 and PG-4 suppress PRRSV infectivity are not known. However, because combinations of pBD-3 and PG-4 showed no significant synergistic anti-PRRSV activity (unpublished data), it is tempting to speculate that these two porcine AHDPs may interact with the same or related target(s) in a pathway of PRRSV infection.

Conclusion

In summary, we have found differential expression patterns of porcine AHDPs in lungs of PRRSV-infected pigs that indicate a potential weak induction mechanism for these innate immune effectors. Direct inactivation studies identified the porcine defensin pBD-3 and the porcine cathelicidin PG-4 as promising candidates for limiting PRRSV infectivity. Furthermore, using peptides derived from pBD-3 and protegrins suggest that critical motifs interact with PRRSV. Whereas the six cysteine residues were found to not be essential for the antiviral activity of pBD-3, the stereo-structure formed by the GWI and F residues appears critical for the interactions of PG-4 with PRRSV and merits further investigation.

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Author Disclosure Statement

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