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A missense polymorphism in porcine interferon- γ cDNA affects antiviral activity of the protein variant

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Abstract

We determined the interferon- γ (IFN- γ) cDNA sequence from three porcine breeds, Duroc, Landrace/Duroc hybrid, and Landrace breeds. Five single nucleotide polymorphisms (SNPs) of porcine IFN- γ (PoIFN- γ) were identified, respectively, at positions 269 (A/G), 376 (C/T), 426 (T/C), and 465 (T/C) of the coding sequence in Landrace/Duroc hybrid, and at position 251 (A/G) in Landrace breed. Among them, A269G and A251G polymorphisms resulted in Q67R and K61R replacements in the mature protein. PoIFN- γ cDNAs of Duroc breed (PoIFN- γ -W) and Landrace/Duroc hybrid (PoIFN- γ -M), which, respectively, encoded Q67 and R67, were introduced into a prokaryotic expression vector pET32 to express recombinant PoIFN- γ -W (rPoIFN- γ -W) and rPoIFN- γ -M protein variants in *Escherichia coli*. The identity of both protein variants was further confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). We then compared bioactivities of these two recombinant proteins. Although both recombinant protein variants exhibited comparable activities in antiproliferation of PK-15 cells and in nitric oxide (NO) induction of porcine peripheral monocytes, antiviral activity of rPoIFN- γ -W protein was significantly higher ($P < 0.001$) than that of rPoIFN- γ -M protein in a plaque inhibition assay using pseudorabies virus (PRV). IC₅₀ values of rPoIFN- γ -W and rPoIFN- γ -M protein in anti-PRV assay were determined as 5.3 ± 1.3 and 9.3 ± 4.3 nM, respectively.

In conclusion, we have identified five novel SNPs in PoIFN- γ cDNA, including two missense polymorphisms that result in Q67R and K61R replacements. Our results further demonstrate that Q67R can markedly reduce antiviral activity of the PoIFN- γ protein. This is the first report that shows the functional SNP in the coding region of IFN- γ . In the future, it is imperative to determine whether Q67R replacement in IFN- γ may have disease association.

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1. Introduction

Interferon-gamma (IFN- γ) is a pleiotropic cytokine produced by T lymphocytes and natural killer (NK) cells. As the only member of type II IFN, IFN- γ not only possesses antiviral activity (Charley et al., 1988; Samuel, 1991), but also modulates immune responses via its immunoregulatory activities, including mediating tumor cell differentiation, stimulating

the expression of major histocompatibility complex (MHC) molecules, activation of macrophage/monocyte, antiproliferative effect on normal and tumor cells, and immunoglobulin isotype switch of B cells (Charley et al., 1988; Farrar and Schreiber, 1993; Fromm and Ehrlich, 2001; Weining et al., 1996; Williams et al., 1993).

Following isolation of human IFN- γ cDNA and gene in 1982 (Devos et al., 1982; Gray et al., 1982; Ullrich et al., 1982), cDNA of porcine IFN- γ (PoIFN- γ) was isolated in 1988 (Charley et al., 1988). Nevertheless, the cDNA sequence has not been formally published. Although sequence of PoIFN- γ gene was reported and aligned with human IFN- γ gene to deduce

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the exon and intron structure (Dijkmans et al., 1990), little is known about the polymorphism of PoIFN- γ gene or cDNA. Human population studies have revealed several polymorphic sites in both intron and exon regions of human IFN- γ gene (Iwasaki et al., 2001; Tiroch et al., 2005). Two polymorphisms located in intron 1 of human IFN- γ gene were further shown to affect the production of IFN- γ (Pravica et al., 1999, 2000), and which might influence either disease manifestation of IgA nephropathy (Skena et al., 2006), or the infection susceptibility to parvovirus B19 (Kerr et al., 2003) and severe acute respiratory syndrome coronavirus (SARS-CoV) (Chong et al., 2006). Nonetheless, it remains to be explored whether any polymorphic sites in the coding region of IFN- γ gene may affect its protein activity.

In this study, our specific aim was to explore functional polymorphism in the coding region of PoIFN- γ gene. By employing reverse transcription-polymerase chain reaction (RT-PCR), three cDNA clones of PoIFN- γ were isolated, respectively, from Duroc, Landrance/Duroc hybrid, and Landrance breeds. Nucleotide and encoded amino acid sequences of these three clones were aligned and compared with that of PoIFN- γ gene (Dijkmans et al., 1990) to identify any polymorphic sites. Two PoIFN- γ cDNA variants were cloned, respectively, into pET32 vector for expressing rPoIFN- γ proteins. By performing a pseudorabies virus (PRV)-based plaque inhibition assay, we evaluated the antiviral activity of both rPoIFN- γ protein variants. Other activities of IFN- γ , including antiproliferative effect on cells and stimulating nitric oxide (NO) production in porcine monocytes, were also assessed.

2. Materials and methods

2.1. Isolation and stimulation of porcine leukocytes

Anticoagulant blood containing 2.5 mM EDTA was collected from Duroc, Landrance/Duroc hybrid, and Landrance breeds of *Sus Scrofa*. Leukocytes from each breed were isolated by Histopaque (Sigma, St. Louis, MO, USA) and cultured at 37 °C in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Cells were stimulated with 40 μ g/ml of concanavalin A (Con A, Sigma) for 18 and 46 h, respectively.

2.2. Cloning of PoIFN- γ cDNA variants and construction of rPoIFN- γ expression plasmids

Messenger RNA (mRNA) was extracted from Con A-stimulated leukocytes by using a FastTrack 2.0 mRNA isolation kit (Invitrogen, Rockville, MD, USA). A total of 0.5 μ g of mRNAs were annealed with 0.5 μ g of oligo dT primer and reverse transcribed into cDNAs by AMV reverse transcriptase. PCR was performed in a 50 μ l reaction volume containing PCR buffer, dNTPs (0.2 mM each), cDNA template (10 ng), sense primer (20 pmole), antisense primer (20 pmole), and *Taq* DNA polymerase (1 U). After a denaturation step at 94 °C for 3 min, 35 PCR cycles were performed (denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1.5 min, extension at 72 °C for 1.5 min), followed by a final extension at 72 °C for 10 min. Based

on the sequence of PoIFN- γ gene (Dijkmans et al., 1990), sense primer (5'-CAGAAGCTAACTCTCTCCG-3') and antisense primer (5'-GTGAAATAGTCACAGGATAC-3') were synthesized for the amplification of coding region. PCR-amplified PoIFN- γ cDNA fragment was resolved by gel electrophoresis, cloned into pCR2.1 vector using a TA cloning kit (Invitrogen) and sequenced. DNA fragment encoding the mature PoIFN- γ protein plus *NcoI/XhoI* restriction sites was further amplified by PCR, and subcloned into pET32a expression vector. The resultant pETpIFN- γ -series plasmids were transformed into *Escherichia coli* strain BL21(DE3).

2.3. Sequence accession numbers

Sequences of three PoIFN- γ cDNA clones isolated in this study were deposited in GenBank under accession numbers AY188090 (PoIFN- γ -W), AY188089 (PoIFN- γ -M) and DQ839398 (PoIFN- γ -L).

2.4. Sequence alignment

Alignment of IFN- γ cDNA sequences was performed by using DNASTar 5.0 software with clustal W program (DNASTar, Madison, WI, USA). Alignment of amino acid sequences of IFN- γ among various animals was performed by using BioEdit software (Ibis Therapeutics, Carlsbad, CA, USA).

2.5. Expression and purification of rPoIFN- γ protein variants

E. coli strain carrying each pETpIFN- γ variant was grown at 37 °C in Luria broth medium with ampicillin (50 μ g/ml). The recombinant protein expression was induced by 0.5 mM isopropyl β -D-thiogalactopyranoside (IPTG) when the OD₅₉₅ reached 0.8. Aliquot of the bacterial culture was harvested at different time points to determine the timing for optimal induction. Upon optimal induction, bacterial pellet was collected by centrifugation and resuspended in 1/25 volume (original culture) of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Cells were lysed with a sonicator and rPoIFN- γ protein variants were purified by affinity chromatography on a nickel-chelate column (Invitrogen).

2.6. SDS-PAGE and Western blot analysis

Bacterial pellets or purified recombinant proteins were analyzed by 13% SDS-PAGE. Following electrophoresis, proteins were transferred onto nitrocellulose (NC) membranes. Each membrane was incubated with blocking solution (5% non-fat dry milk and 1% normal serum in PBS) for 30 min before it was incubated with rabbit anti-PoIFN- γ polyclonal antibody (1:2000 dilution in blocking solution) (R&D systems, Minneapolis, MN, USA) for 1 h at room temperature. After washing three times with phosphate-buffered saline containing 0.05% tween 20 (PBST), the membrane was further incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody

(1:3000 dilution). Finally, after PBST washings, NBT/BCIP was added for color detection.

2.7. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis

MALDI-TOF MS analysis was performed as previously described (Lin et al., 2006). Briefly, gel plugs containing rPoIFN- γ protein spots were excised from Coomassie blue stained gels to perform in-gel trypsin digestion. Using a Bruker ultraflex time-of-flight mass spectrometer equipped with a nitrogen laser and a 384 sample Scout source (Bruker Daltonics, Bremen, Germany), MALDI mass spectra were acquired by the reflectron mode. Peptide masses obtained were searched against a comprehensive nonredundant protein sequence database (NCBIInr) using the Mascot program (Perkins et al., 1999) for protein identification.

2.8. Plaque inhibition assay for antiviral activity

PK-15 cells (porcine kidney cells) purchased from ATCC (Manassas, VA, USA) were seeded on 24-well plates (1×10^5 well $^{-1}$) and grown in RPMI 1640 with 10% FBS. Upon adhesion, cells were treated either with each rPoIFN- γ protein variant expressed in this study or commercial rPoIFN- γ protein (R&D systems) for overnight. After three times of washes with PBS, cells were incubated with PRV (3.4×10^2 PFU/well) for 2 h at 37 °C. After removing unabsorbed virus, cells were covered with 1 ml of 1% methylcellulose in RPMI 1640. Three days later, cells were fixed by methanol for 10 min, stained with crystal violet, and plaque numbers were counted. Percentage of plaque formation inhibition was calculated as follows: [(mean number of plaques in the control) – (mean number of plaques in the treated sample)] \times 100/(mean number of plaques in the control). IC50 was calculated as the concentration of rPoIFN- γ that reached 50% of the inhibition.

2.9. Antiproliferation assay

PK-15 cells (1×10^5 well $^{-1}$) were cultured in 24-well plates with RPMI 1640 plus 10% FBS. Cells were grown in medium (control) or treated with various concentrations of each rPoIFN- γ protein variant for 72 h, washed with PBS and incubated at 37 °C for 3 h with *p*-nitrophenyl phosphate (pNpp) solution to measure acid phosphatase activity. The pNpp solution was prepared as described (Connolly et al., 1986). The reaction was stopped with 0.1 N NaOH (final concentration). The color development was determined at 405 nm using μ Quant spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA).

2.10. NO secretion assay

Adherent cells from porcine peripheral leukocytes (enriched monocytes) were cultured in 96-well plates (2×10^5 cells/well) and treated with various stimuli (in RPMI 1640/10% FBS) for 48 h. The supernatant of each stimulated culture was collected. By measuring nitrite and nitrate contents in the medium, NO

production was determined with a nitric oxide colorimetric assay kit (BioVision, Mountain View, CA, USA).

2.11. Statistical analysis

All statistical analyses were performed using a GraphPad Prism version 4 software (GraphPad Software, San Diego, CA). Differences between groups were analyzed by two-way ANOVA.

3. Results

3.1. Cloning and sequencing of PoIFN- γ cDNA variants

Using primers specific for PoIFN- γ , we obtained PoIFN- γ cDNA by RT-PCR. The PoIFN- γ cDNA fragment (501 bp) was cloned into pCR 2.1 vector and sequenced. Three cDNA clones, designated as PoIFN- γ -W (pIFN- γ -W), PoIFN- γ -M (pIFN- γ -M) and PoIFN- γ -L (pIFN- γ -L), were isolated, respectively, from Duroc, Landrace/Duroc hybrid, and Landrace breeds. These cDNA sequences were deposited to GenBank under accession numbers AY188090 (PoIFN- γ -W), AY188089 (PoIFN- γ -M), and DQ839398 (PoIFN- γ -L). The sequence of PoIFN- γ -W cDNA completely matches to the predicted coding sequence (CDS) of a previously reported PoIFN- γ gene (Dijkmans et al., 1990). On the other hand, PoIFN- γ -M clone has four nucleotide variations or single nucleotide polymorphisms (SNPs), at nucleotide positions 269 (A/G), 376 (C/T), 426 (T/C), and 465 (T/C) of CDS (Fig. 1). Among these four SNPs, only the A269G polymorphism would change the coded amino acid sequence (Gln67Arg or Q67R). PoIFN- γ -L clone has one SNP at position 251 (A/G), resulting in one amino acid replacement (Lys61Arg or K61R). Amino acid sequences of PoIFN- γ -W, -M and -L variants were compared with that of other mammalian homologs, including bovine (M29867) (Cerretti et al., 1986), ovine (DQ410715) (<http://www.ncbi.nlm.nih.gov/Genbank/>), equine (D28520) (Curran et al., 1994), bottle-nosed dolphin (AB022044) (<http://www.ncbi.nlm.nih.gov/Genbank/>), human (BC070256) (Devos et al., 1982) and murine (K00083) (Gray and Goeddel, 1983) IFN- γ . As shown in Fig. 2, all PoIFN- γ variants share higher amino acid identity to bovine (76.6% identity), ovine (77.8% identity), equine (70.1% identity) and bottle-nosed dolphin (tursiops) (80.2% identity) IFN- γ than to human (59.3% identity) and murine (39.1% identity) IFN- γ . Both K61 and Q67 are conserved in the mammalian IFN- γ (Fig. 2). Six α -helical regions are assigned according to the structure of human IFN- γ (Ealick et al., 1991).

3.2. Expression of rPoIFN- γ -W and rPoIFN- γ -M protein variants in *E. coli*

The open reading frame (ORF) of PoIFN- γ gene encodes a polypeptide of 167 amino acid residues, containing a signal peptide (23 residues) coding sequence followed by the mature protein (144 residues) coding sequence (Dijkmans et al., 1990). The mature protein coding sequence of PoIFN- γ -W and PoIFN- γ -M cDNA was subcloned, respectively, into pET32

PoIFN- γ -M			
PoIFN- γ -L			
PoIFN- γ -W	atgagttatacaacttatttcttagcttttcagctttgctgactttgtgttttctggc	60	S20
	M S Y T T Y F L A F Q L C V T L C F S G		
PoIFN- γ -M			
PoIFN- γ -L			
PoIFN- γ -W	tcttactgcccaggcgcccttttttaagaaataacgatcctaaggactattttaaagca	120	17
	S Y C Q A P F F K E I T I L K D Y F N A		
	↑ +1		
PoIFN- γ -M			
PoIFN- γ -L			
PoIFN- γ -W	agtacctcagatgtacctaattggtggacctcttttcttagaaattttgaagaattggaaa	180	37
	S T S D V P N G G P L F L E I L K N W K		
PoIFN- γ -M			
PoIFN- γ -L			
PoIFN- γ -W	gaggagagtgacaaaaaataattcagagccaaattgtctccttctacttcaaattcttt	240	57
	E E S D K K I I Q S Q I V S F Y F K F F		
PoIFN- γ -M			
PoIFN- γ -L			
PoIFN- γ -W	gaaatcttcaagataaccaggccattc aaaggagcatggatgtgatcaagcaagacatg	300	77
	E I F K D N Q A I Q R S M D V I K Q D M		
	R (PoIFN- γ -L) R (PoIFN- γ -M)		
PoIFN- γ -M			
PoIFN- γ -L			
PoIFN- γ -W	tttcagagggttcctaaatggttagctctgggaaactgaatgacttcgaaaagctgattaaa	360	97
	F Q R F L N G S S G K L N D F E K L I K		
PoIFN- γ -M			
PoIFN- γ -L			
PoIFN- γ -W	attccggtagataatctgacgatccagcgcaagccatcagtgaaactcatcaaagtgatg	420	117
	I P V D N L Q I Q R K A I S E L I K V M		
PoIFN- γ -M			
PoIFN- γ -L			
PoIFN- γ -W	aatgatctgtcaccaagatctaacctaagaaagcggaagagagaagtcagactatgttccaa	480	137
	N D L S P R S N L R K R K R S Q T M F Q		
PoIFN- γ -M			
PoIFN- γ -L			
PoIFN- γ -W	ggccagagagcatcaaaaataa	501	143
	G Q R A S K		

Fig. 1. Sequence alignment of porcine IFN- γ (PoIFN- γ) cDNA variants isolated from Duroc (PoIFN- γ -W), Landrace/Duroc hybrid (PoIFN- γ -M) and Landrace (PoIFN- γ -L) breeds. Four single nucleotide polymorphisms (SNPs) were identified in the coding sequence (CDS) of PoIFN- γ -M, including one missense polymorphism (nucleotide 269) that changed the coded amino acid (residue 67) from glutamine (Q) to arginine (R). Another SNP (nucleotide 251) identified in PoIFN- γ -L resulted in an amino acid replacement (residue 61) from lysine (K) to arginine (R). The signal peptide comprises residues S1 to S23. The signal peptide cleavage site is indicated with an arrow (\uparrow).

expression vector to generate bacterial expression constructs pETpIFN- γ -W and pETpIFN- γ -M. Upon induction with IPTG, *E. coli* strains carrying pETpIFN- γ -W and pETpIFN- γ -M unambiguously overexpressed a recombinant protein around 33.7 kDa, which was the expected molecular mass of the mature PoIFN- γ (15.8 kDa) fused with N-terminal tag (17.9 kDa). Five hours after IPTG induction, the recombinant protein reached a maximum amount and could be detected specifically by anti-PoIFN- γ polyclonal antibody. Both recombinant protein variants were further purified by nickel-chelate affinity chromatography (Fig. 3).

Purified recombinant protein variants were treated with trypsin and subjected to MALDI-TOF MS analysis. Mass spectra of trypsin-digested rPoIFN- γ -W and rPoIFN- γ -M protein variants matched to that of PoIFN- γ and thioredoxin fusion tag. Table 1 shows peptide mass and corresponding amino acid sequence of PoIFN- γ following trypsin cleavage of both protein variants. The 1655.867 Da peptide mass corresponding to the amino acid sequence FFEIFKDNQAIQR (residues 56–68) was detected exclusively in the spectrum of rPoIFN- γ -W, which carries Q67 (Fig. 4A, Table 1). On the other hand, the peptide mass 1527.828 Da detected in rPoIFN- γ -M (with R67) spectrum matched to FFEIFKDNQAIQR (residues 56–67) (Fig. 4B, Table 1).

3.3. Antiviral activity of rPoIFN- γ protein variants

The antiviral activity of both rPoIFN- γ -W and rPoIFN- γ -M protein variants was determined by a PRV-based plaque inhibition assay on PK-15 cells. As shown in Fig. 5, both protein variants were able to inhibit the plaque formation of PRV in a dose-dependent manner ($P < 0.001$). However, anti-

Table 1
MALDI-TOF MS peptide mass and corresponding amino acid sequence of the recombinant porcine IFN- γ following trypsin cleavage

Amino acid position of porcine IFN- γ	Mass ^a (Da)	Peptide sequence
13–34	2397.200	DYFNASTSDVPPNGGPLFLEILK
44–55	1472.790	IQSQIVSFYFK
56–61	830.412	FFEIFK
56–68	1655.867 ^b	FFEIFKDNQAIQR
56–67	1527.828 ^c	FFEIFKDNQAIQR
95–107	1549.961	LIKIPVDNLQIQR
98–107	1195.696	IPVDNLQIQR
109–123	1685.836	AISELIKVMNDLSPR
132–140	1082.477	SQTMFQQQR

^a Molecular mass larger than 500 Da.

^b Detected exclusively in the spectrum of rPoIFN- γ -W.

^c Detected exclusively in the spectrum of rPoIFN- γ -M.

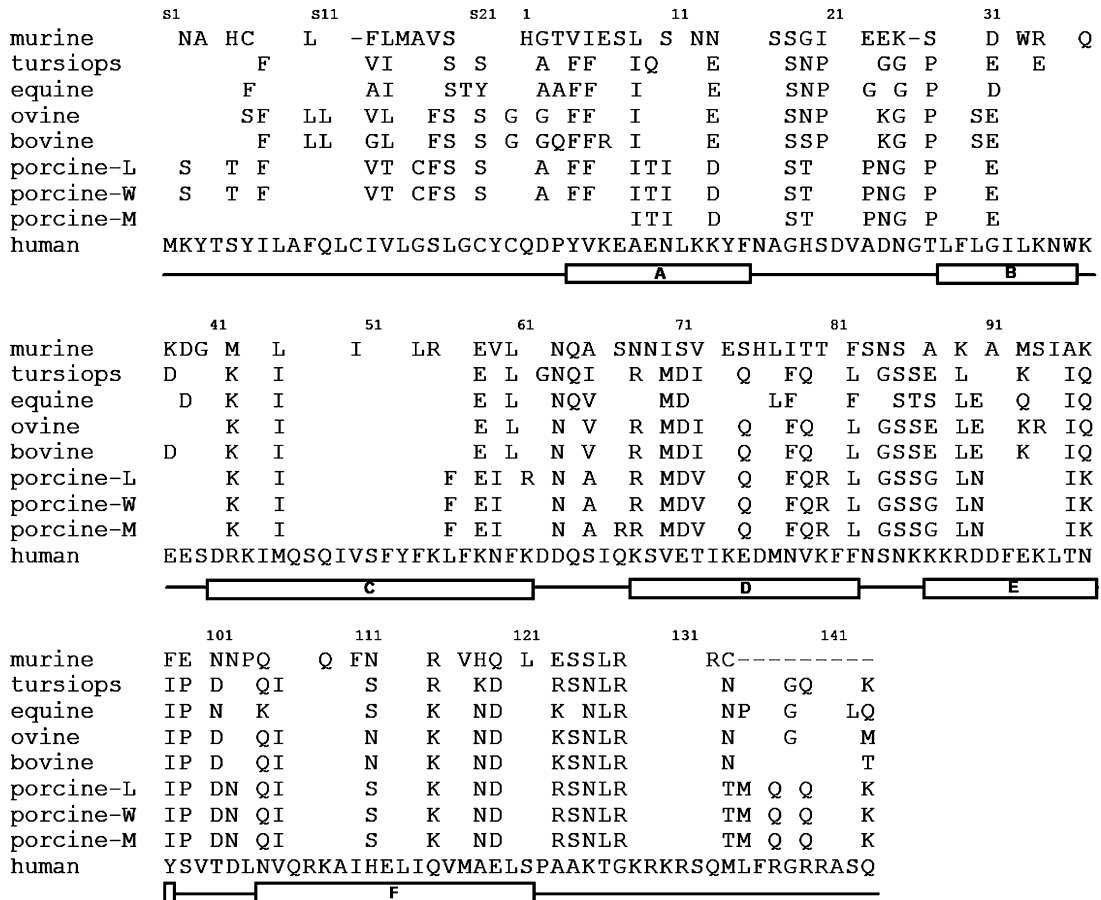


Fig. 2. Alignment of the deduced amino acid sequence of porcine IFN- γ with that of other mammals. Gaps in the alignment are indicated by dashes (-). Six α -helical regions that are alphabetically labeled are assigned according to the structure of human IFN- γ .

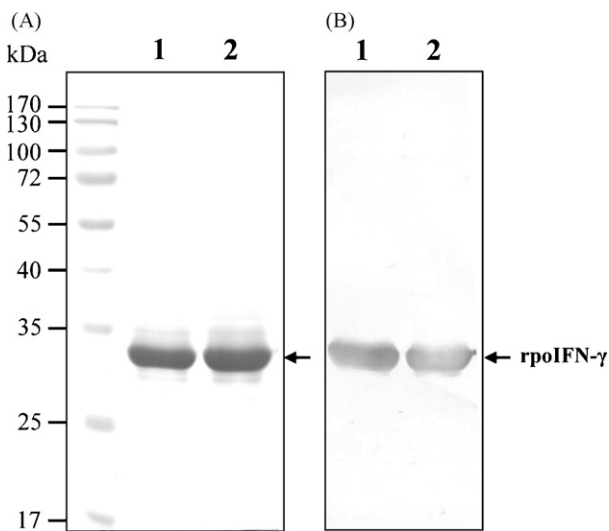


Fig. 3. SDS-PAGE (A) and Western blot (B) analysis of purified rPoIFN- γ -W and rPoIFN- γ -M protein variants. Purified rPoIFN- γ -W (lane 1) and rPoIFN- γ -M (lane 2) were resolved by 13% SDS-PAGE (A). The protein was transferred to nitrocellulose membrane, and detected by anti-PoIFN- γ antibody (B). Molecular weight of protein standards is indicated on the left.

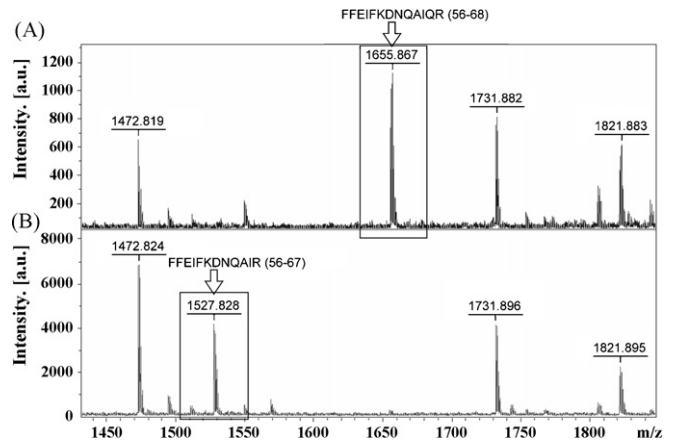


Fig. 4. MALDI-TOF mass spectra of trypsin-digested rPoIFN- γ -W (A) and rPoIFN- γ -M (B) protein variants. The figure displays a portion of the mass spectrum, with representative peptide mass to charge ratio (m/z) indicated above each peak. A peptide of mass 1655.867 Da specifies amino acid residues 56–68 (FFEIFKDNQAIQR) in rPoIFN- γ -W (A). A peptide of mass 1527.828 Da corresponds to residues 56–67 (FFEIFKDNQAIQR) in rPoIFN- γ -M (B).

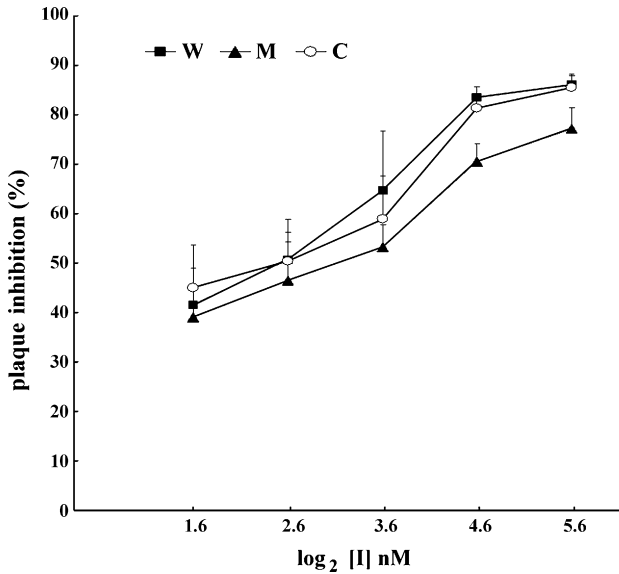


Fig. 5. Antiviral activity assay of rPoIFN- γ protein variants. PK-15 cells were incubated, respectively, with various concentrations of rPoIFN- γ -W (W), rPoIFN- γ -M (M) and commercial rPoIFN- γ (C) protein overnight before pseudorabies virus infection. Percent inhibition of plaque formation was expressed as the mean \pm S.D. ($n=6$).

ral activity of rPoIFN- γ -M protein was significantly lower than that of rPoIFN- γ -W and commercial rPoIFN- γ ($P<0.001$). Within a concentration range of 3.1–50 nM, rPoIFN- γ -M protein exhibited 4.2–13.1% less of antiviral activity compared with rPoIFN- γ -W and commercial rPoIFN- γ . IC₅₀ values for rPoIFN- γ -W, rPoIFN- γ -M and commercial rPoIFN- γ were 5.3 ± 1.3 , 9.3 ± 4.3 and 5.9 ± 1.7 nM. Tag protein expressed from pET32 vector did not show any antiviral activity at concentrations up to 50 nM (data not shown).

3.4. Antiproliferation activity of rPoIFN- γ protein variants

After incubation for 3 days, both rPoIFN- γ -W, rPoIFN- γ -M protein variants showed a dose-dependent antiproliferative effect on PK-15 cells ($P<0.001$). Using tag protein expressed from pET32 as a mock control, we did not observe any antiproliferation activity at concentrations up to 50 nM (data not shown). IC₅₀ of rPoIFN- γ -W, rPoIFN- γ -M and commercial rPoIFN- γ protein was determined as 39.8 ± 4 , 32.5 ± 1.9 , and 27.1 ± 0.1 nM, respectively. Commercial rPoIFN- γ protein apparently exerted a better antiproliferation activity ($P<0.001$) than rPoIFN- γ -W and rPoIFN- γ -M. However, no significant difference in antiproliferation activity was found between rPoIFN- γ -W and rPoIFN- γ -M (Fig. 6).

3.5. NO induced in monocytes by rPoIFN- γ protein variants

IFN- γ can stimulate macrophages and monocytes to secrete NO (Kim and Son, 1996; Malu et al., 2003). We thereby measured NO induction in monocytes by each rPoIFN- γ protein variant. Untreated monocytes produced a low basal level of NO (Fig. 7). In contrast, NO secretion increased substantially in a

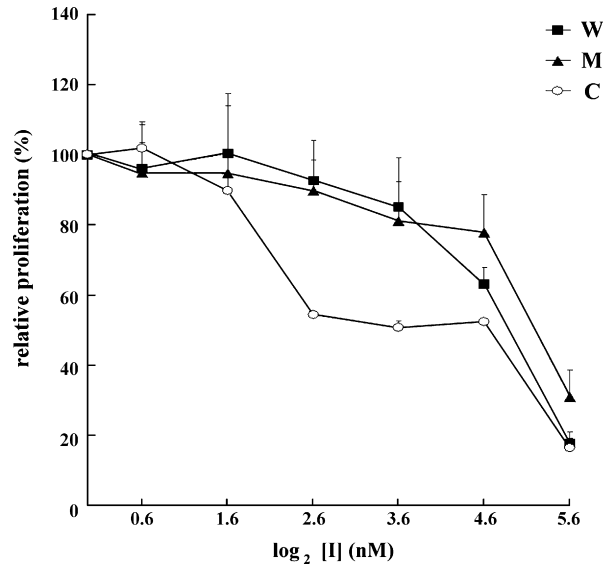


Fig. 6. Antiproliferation activity of rPoIFN- γ protein variants. PK-15 cells were grown in medium (control) or treated with various concentrations of rPoIFN- γ proteins (rPoIFN- γ -W (■), W; rPoIFN- γ -M (▲), M; commercial rPoIFN- γ (○), C) for 72 h before assaying for cell proliferation. Relative proliferation was determined as percent of control. Data were expressed as the mean \pm S.D. ($n=5$).

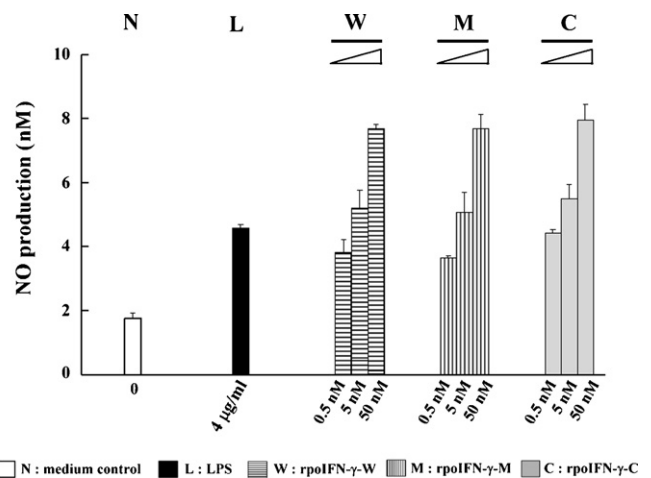


Fig. 7. The effect of rPoIFN- γ protein variants on NO production in porcine monocytes. Peripheral monocytes were treated with various concentrations of rPoIFN- γ (from 0.5 to 50 nM). NO production was expressed as the mean \pm S.D. ($n=3$).

dose-dependent manner when monocytes were treated with various concentrations of rPoIFN- γ (from 0.5 to 50 nM) ($P<0.001$). However, levels of NO synthesis stimulated by rPoIFN- γ -W, rPoIFN- γ -M, and commercial rPoIFN- γ were not significantly different.

4. Discussion

In this study, we identified five novel SNPs in PoIFN- γ cDNA clones, including four (A269G, C376T, T426C, and T465C) from Landrace/Duroc crossbreed and one (A251G) from Landrace breed (Fig. 1). A251G and A269G are located within exon 3 of PoIFN- γ gene at nucleotide positions 68 and 86

(Dijkmans et al., 1990). On the other hand, C376T, T426C and T465C are located within exon 4 of PoIFN- γ gene at nucleotide positions 10, 60 and 99 (Dijkmans et al., 1990). Four SNPs detected in PoIFN- γ -M (Landrace/Duroc) were absent in both PoIFN- γ -W (Duroc) and PoIFN- γ -L (Landrace), indicating that these SNPs may not be breed-specific. Allelic frequencies of these polymorphisms between Landrace and Duroc breeds, nevertheless, require further investigation. A269G polymorphism in PoIFN- γ -M would change the coded amino acid sequence (Q67R). A251G polymorphism in PoIFN- γ -L would also result in one amino acid replacement (K61R). Both K61 (within helix C) and Q67 (near helix D) are conserved in the mammalian IFN- γ (Fig. 2). However, we expect that the impact of K61R (PoIFN- γ -L), a similar amino acid replacement, would be minor. On the other hand, Q67R that increases positive charge of the protein variant (PoIFN- γ -M) could be a significant replacement. Consequently, in this study we expressed both PoIFN- γ -M (R67) and PoIFN- γ -W (Q67) variants to compare their biological activities. Interestingly, our results showed that Q67R could clearly inhibit antiviral activity of PoIFN- γ .

Antiviral activity of PoIFN- γ has been demonstrated in vitro on several swine viruses, including African swine fever (Esparza et al., 1988), transmissible gastroenteritis virus (Charley et al., 1988), influenza virus (Horisberger, 1992), porcine reproductive and respiratory syndrome virus (PRRSV) (Bautista and Molitor, 1999; Buddaert et al., 1998), and classical swine fever virus (CSFV) (Suradhat et al., 2001). All these viruses are RNA viruses that are highly sensitive to the antiviral mechanism of interferon. Instead of using RNA viruses, we evaluated the antiviral effect of PoIFN- γ on pseudorabies virus, a DNA virus. PRV, a swine alphaherpesvirus, is distantly related to the human herpes simplex virus type 1 (HSV-1). Unlike HSV-1 that is well known for its anti-interferon mechanism, PRV lacks homologs of some anti-IFN genes (e.g. γ 34.5 and Us11) of HSV-1 and the viral growth could be inhibited in IFN- β treated PK-15 cells (Brukman and Enquist, 2006). The result of this study showed that PoIFN- γ inhibited PRV in a dose-dependent manner, although the dosage of IFN- γ used for PRV inhibition assay was higher than that for other RNA viruses. Therefore, we have established a PRV-based plaque inhibition assay to determine antiviral activity of PoIFN- γ . Except for carrying an extra fusion peptide encoded by pET32 vector at N-terminus, the amino acid sequence of rPoIFN- γ -W was identical to that of commercial rPoIFN- γ protein (Vandenbroeck et al., 1991). The fact that antiviral activities of rPoIFN- γ -W and commercial rPoIFN- γ proteins were not significantly different indicated that the fusion peptide did not interfere with antiviral activity of IFN- γ . Results from plaque inhibition assay showed that rPoIFN- γ -W presented a better antiviral activity than rPoIFN- γ -M protein ($P < 0.001$). The missense polymorphism, Q67R, indeed has an impact on the biological activity of PoIFN- γ -M protein, and this is the first report that identified a functional SNP in the coding region of IFN- γ .

In terms of antiproliferative effect (Friesel et al., 1987; Hansson et al., 1988; Maekawa et al., 1988), our data showed that both rPoIFN- γ -M and rPoIFN- γ -W expressed comparably lower antiproliferation activity than commercial rPoIFN- γ

($P < 0.001$) on PK-15 cells. This could be resulted from the interference of fusion peptide carried in rPoIFN- γ -M and rPoIFN- γ -W proteins. In addition to growth inhibition, activation of macrophage/monocyte is another important immunoregulatory activity of IFN- γ . Measuring IFN- γ -induced NO secretion in macrophages is a common bioassay for evaluating the activity of murine IFN- γ (Kim and Son, 1996; Malu et al., 2003). Instead of using macrophages, we measured PoIFN- γ -induced NO secretion in porcine monocytes because peripheral monocytes were more easily obtained from pigs. Our result showed that, in response to PoIFN- γ stimulation, porcine peripheral monocytes produced NO in a dose-dependent manner. Nonetheless, induction levels of NO were not significantly different among monocytes treated with rPoIFN- γ -W, rPoIFN- γ -M, or commercial rPoIFN- γ . Taken altogether, Q67R and fusion peptide in the rPoIFN- γ protein can, respectively, reduce antiviral activity and antiproliferation activity of IFN- γ , with little effect on NO induction. Our data indicate that these three activities reside in independent functional domains of PoIFN- γ .

In conclusion, we have identified five novel SNPs in PoIFN- γ cDNA, including two missense polymorphisms that result in Q67R and K61R replacements. Interestingly, Q67R can markedly reduce antiviral activity of PoIFN- γ protein. This in and of itself could warrant a potential association with a yet to be determined disease mechanism, of which the reduced immune response may lead to high susceptibility of virus infection.

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