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Differential host cell gene expression regulated by the porcine reproductive and respiratory syndrome virus GP4 and GP5 glycoproteins

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Abstract

The porcine reproductive and respiratory syndrome virus (PRRSV) GP4 and GP5 proteins are two membrane-associated viral glycoproteins that have been shown to induce neutralizing antibodies. In the present study, the host cell gene expression profiles altered by the GP4 and GP5 proteins were investigated by the use of DNA microarrays. Sublines of Marc-145 and HeLa cells were established by stable transfection with open reading frame (ORF)4 and ORF5 of PRRSV, respectively, and differential gene expressions were studied using microarray chips embedded with 1718 human-expressed sequence tags. The genes for protein degradation, protein synthesis and transport, and various other biochemical pathways were identified. No genes involved in the apoptosis pathway appeared to be regulated in GP5-expressing cells. The microarray data may provide insights into the specific cellular responses to the GP4 and GP5 proteins during PRRSV infection.

Keywords: PRRS; GP4 protein; GP5 protein; Microarray; Gene regulation

1. Introduction

For viruses to replicate, they must enter a host cell and utilize host cell biosynthetic machinery and energy supplies. Infected cells activate innate and adaptive immune responses, and host antiviral defense is switched on to eliminate invading viruses. Viruses may persist in infected cells when antiviral defenses are insufficient. The series of interactive processes cause the differential expression of cellular genes, and it has been of interest to understand how altered gene expression plays a role during virus infection. RT-PCR, RNase protection assays, and Northern and Western blot analyses are commonly used techniques to identify altered gene expression. RT-PCR in particular, has been used to study porcine reproductive and respiratory syndrome virus (PRRSV)-mediated altered gene expression for IFN-α, IFN-γ, IL-10, IL-12, and TNF-α (Johnsen et al., 2002; Feng et al., 2003; Suradhat and Thanawongnuwech, 2003; Thanawong-

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nuwech and Thacker, 2003). Such techniques, however, are often time-consuming and labor-intensive and lead to high degrees of experimental variation. For such reasons, little is known concerning the molecular changes in cells upon PRRSV infection.

The recent development of DNA microarray technology allows for the simultaneous assessment of mRNA transcription patterns for thousands of genes, and is commonly applied to determine patterns of differential gene expression (Schena et al., 1995). Using this technique, it is now possible to define changes in gene expression that evaluate host cell–virus interaction and to obtain specific insights into the molecular nature of viral pathogenesis (Browne et al., 2001; Johnston et al., 2001). Microarrays are particularly useful in studying whether cellular mRNAs, differentially regulated by each viral protein, play a crucial role for virus multiplication in the cell.

The PRRSV-2 (North American) genome contains nine open reading frames (ORFs). ORF1a and ORF1b code for two partly overlapping non-structural polyproteins that are predicted to be post-translationally processed to 13 cleavage products. These non-structural proteins are believed to participate in viral genome replication and subgenomic mRNA transcription. ORFs 2–7 code for six structural proteins: GP2–GP5, membrane (M), and nucleocapsid (N) proteins (Meulenberg et al., 1995; Snijder and Meulenberg, 1998). A small internal ORF is found within ORF2, which encodes the E protein (Wu et al., 2001).

The GP5 protein, consisting of 200 amino acids, is a major glycosylated structural component of the virion. It resembles a type I integral membrane protein with a putative endoplasmic reticulum (ER) translocational signal of 31 amino acids at its N-terminus. GP5 however lacks the typical C-terminal hydrophobic anchor sequence. Instead, a triple membrane spanning region is found in the middle of the protein between residues 65 and 130, leaving a large stretch of a 70 amino acid cytoplasmic tail at the C-terminus (Mardassi et al., 1995; Meulenberg et al., 1995). GP5 exists as a heterodimer with the M protein in the virion (Mardassi et al., 1996), and heterodimerization has been shown to be essential for virus infectivity in LDV (Faaberg et al., 1995) and EAV (Snijder et al., 2003). The GP5 protein is reported to cause apoptosis (Suárez et al., 1996) and is able to induce neutralizing monoclonal antibodies in mice (Weiland et al., 1999; Ostrowski et al., 2002).

The GP4 protein is a minor structural protein consisting of 178 amino acids (Murtaugh et al., 1995), and as with GP5, is also able to induce neutralizing antibodies (Meulenberg et al., 1997; Weiland et al., 1999). The electrophoretic migration of the mature protein incorporated into virions is 31–35 kDa, suggesting that the GP4 protein is heavily glycosylated during transport through the ER–Golgi complex (van Nieuwstadt et al., 1996). Four potential *N*-glycosylation sites are found on the protein. The amino acid sequence shows that GP4 contains a putative N-terminal signal sequence at positions 1–22, and an additional hydrophobic sequence at positions 162–178 at the C-terminus. The hydrophobicity profile of GP4 suggests that it resembles a class I integral membrane protein. However, GP4 has a unique feature uncommonly seen in this class of proteins—the lack of a hydrophilic cytoplasmic tail on the carboxy terminus of the hydrophobic transmembrane region. This unique topology of GP4 is found to mimic a glycosylphosphatidylinositol (GPI) anchored protein (Ferguson and Williams, 1988). Indeed, GP4 has been shown to be a GPI-anchored protein in our laboratory (Bachand, 2003). The function of GPI anchors is poorly understood, but limited evidence suggests that they are involved in 'lipid rafts' (Varma and Mayor, 1998) or in cellular signal transduction (Jacobs et al., 2000).

The present study was designed to examine regulation of specific host cell gene expression by two PRRSV structural proteins. We established two independent cell sublines to stably express the GP4 and GP5 proteins of the North American type PRRSV, and investigated their effect on host cell gene expression using DNA microarray technology.

2. Materials and methods

2.1. Cells

Marc-145 cells (a subclone of MA 104 cells (Kim et al., 1993)) were grown in DMEM containing 8% fetal bovine serum (Invitrogen), 50 units/ml of penicillin, and 50 μg/ml of streptomycin. HeLa-Tet-off cells were purchased from Clontech. These cells constitutively express the chimeric tetracycline transactivator (Gossen and Bujard, 1992). HeLa-Tet-off cells and Marc-GP4 cells were maintained in Dulbecco's

modified Eagle's medium (DMEM) supplemented with 10% serum, 2 mM L-glutamine, 50 units/ml of penicillin, 50 μg/ml of streptomycin, and 100 μg/ml of G418 (Geneticin; Invitrogen). HeLa-GP5 cells were maintained in DMEM containing 10% serum, 2 mM L-glutamine, 50 units/ml of penicillin, 50 μg/ml of streptomycin, 100 μg/ml of G418, 1 μg/ml of doxycycline (Clontech), and 100 μg/ml of hygromycin B (Invitrogen). All cells were maintained at 37 °C with 5% CO_2 in a humidified incubator.

2.2. Cloning and DNA manipulation

DNA was manipulated according to standard procedures (Sambrook and Russell, 2001). The ORF4 gene of the North American type PRRSV strain ATCC VR2332 was PCR-amplified from the parental plasmid pGEM3zf-ORF4 (Wootton et al., 2000) and subcloned into the *Eco*RI and *Hin*dIII sites of the pCI-Neo mammalian expression vector (Promega) to generate pCI-Neo-ORF4. The GP5 expression plasmid pTRE-hyg-ORF5 was constructed by subcloning the VR2332 ORF5 gene from the parental plasmid pGEM3zf-ORF5 (Wootton et al., 2000) into pTRE-hyg (Clontech) using the *Bam*HI site such that the ORF5 gene was placed under control of the tetracycline-responsive element along with the hygromycin-resistance gene.

2.3. Generation of stably expressing cells

Marc-145 cells were grown to approximately 75% confluence in a 35 mm diameter dish and then transfected for 24 h with 1.5 μg pCI-Neo-ORF4 DNA using Lipofectin (Invitrogen) according to the directions of the manufacturer. After 24 h, the transfection solution was replaced with DMEM and incubated for 12 h to allow the cells to divide at least once. Cells were then trypsinized and seeded into fresh 35 mm diameter dishes at approximately 6×10^4 cells per dish. The pCI-Neo-ORF4 plasmid contains a gene conferring neomycin resistance and allows for the selection of cells that have integrated ORF4 into the cellular DNA. Freshly seeded cells were selected for neomycin resistance using 1 mg/ml of G418 (Invitrogen). Selection continued over the course of approximately 3 weeks, with G418 being replaced at least every 4 days. When the majority of cells died, resistant colonies of cells were picked using cell cloning cylinders and amplified for further characterization.

To generate cells expressing GP5, the Tet-off inducible gene expression system was chosen to prevent cell death that might occur due to the GP5 protein expression since GP5 was reported to induce apoptosis. The Tet-off inducible cell system was purchased from Clontech. HeLa-Tet-off cells were transfected with pTRE-hyg-ORF5 for 24 h using Lipofectin according to the manufacturer's instruction (Invitrogen). The transfection solution was removed and the cells were grown for additional 24 h in DMEM. For the transfected HeLa-Tet-off cells, the medium contained 1 μg/ml of doxycycline (Clontech). At 24 h of incubation, 300 μg/ml of hygromycin B was added, and cells were further incubated for 2 weeks until the majority of cells died. Hygromycin-resistant cell colonies were picked using cell-cloning cylinders and amplified in 24-well tissue culture plates.

2.4. Immunofluorescence

Cells were grown on microscope slide coverslips placed in 35 mm-diameter culture dishes in the maintaining medium. After 12 h, doxycycline was removed for 24 or 48 h to induce GP5 expression in GP5 expressing cells. Cells were washed twice in phosphate-buffered saline (PBS) and fixed immediately with cold methanol for 10 min. For immunofluorescence, cells on microscope coverslips were blocked using 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature. The cells were then incubated with a 1:50 dilution of porcine anti-PRRSV hyperimmune sera for 2 h. The cells were washed five times in PBS and incubated for 1 h at room temperature with a 1:100 dilution of fluorescein isothiocyanate (FITC)-labeled goat anti-swine secondary antibody (KPL). Cells were washed five times in PBS and the coverslips were mounted on microscope glass slides in the mounting buffer (60% glycerol and 0.1% sodium azide in PBS). Cell staining was visualized by a fluorescent microscope (model AX70, Olympus).

2.5. Protein expression and immunoprecipitation

Marc-GP4 and HeLa-GP5 cells were seeded in 100 mm-diameter cell culture dishes. HeLa-GP5 cells were grown in the presence of 1 μg/ml doxycycline. To

induce GP5 protein expression, doxycycline was removed and incubation was continued. At 48 h post-seeding or 48 h of induction, cells were starved for 30 min in methionine-deficient medium (Invitrogen) and labeled for 5 h with 100 μCi/ml of EasyTag EXPRESS protein labeling mix ([^{35}S]methionine and [^{35}S]cysteine, specific activity, 407 MBq/ml) (Perkin-Elmer). After labeling, cells were harvested, washed twice with cold PBS, and lysed with lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1% NP-40) containing 1 mM phenylmethyl-sulfonyl fluoride (PMSF). After incubation on ice for 20 min, cell lysates were centrifuged at 14,000 rpm for 30 min in a microcentrifuge (model 5415, Eppendorf), and supernatants were recovered. For immunoprecipitation, cell lysates equivalent to 1:15 of a 100 mm diameter dish were adjusted with RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris–HCl [pH 7.4], 10 mM EDTA, 0.1% SDS) to a final volume of 100 μl and incubated for 2 h at RT with 5 μl of swine anti-PRRSV hyperimmune serum. The immune complexes were adsorbed to 7 mg of protein-A Sepharose CL-4B beads (Amersham Biosciences) for 16 h at 4 °C. The beads were collected by centrifugation at 6000 rpm for 5 min, washed twice with RIPA buffer and once with wash buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl). The beads were resuspended in 20 μl of SDS–PAGE sample buffer (10 mM Tris–HCl [pH 6.8], 25% glycerol, 10% SDS, 0.12% (w/v) bromophenol blue) with 10% β-mercaptoethanol, boiled for 5 min, and analyzed by sodium dodecyl sulfate (SDS)–12% polyacrylamide gel electrophoresis (PAGE). Gels were dried on filter paper and radiographic images were obtained using PhosphorImager (Molecular Dynamics PhosphorImager SI, Amersham Biosciences).

2.6. Microarray analysis

The DNA microarray used in this study was comprised of 1718 human expression sequence tag (EST) clones printed at the Microarray Centre (Toronto, Ont., Canada). The genes were arrayed in duplicate on one slide. Detailed information on the layout of the microarray is found at the Microarray Centre (http://www.microarrays.ca/support/glists.html). At 48 h post-induction, total cellular RNAs were extracted from each line of established cells, Marc-GP4 and HeLa-GP5, and from their corresponding parental cells, Marc-145 and HeLa-Tet-off, respectively. Complementary DNAs were synthesized by reverse transcription using 500 units of SuperScript II (Invitrogen) in a total reaction volume of 50 μl. Briefly, 10 μg of total RNA was primed with the AncT primer (Sigma Genosys; 5′T(20)-V-N 3′) and reverse transcription was carried out in the presence of 0.5 mM each of dATP, dCTP, and dGTP (Invitrogen), 0.15 mM dTTP, amino-allyl 0.15 mM dUTP, 10 mM DTT in 10× first-strand synthesis buffer (Invitrogen; 250 mM Tris–HCl [pH 8.3], 375 mM KCl, 15 mM $MgCl_2$). The mixture was heated at 65 °C for 5 min followed by 42 °C for 5 min. SuperScript II was added, and the reverse transcription reaction was further incubated at 42 °C for 2 h. The reaction was stopped by heating to 95 °C for 5 min, and the RNA template was degraded by the addition 10 μl of 1N NaOH followed by incubation at 65 °C for 5 min. The mixture was neutralized by the addition 10 μl of 1 M HCl and 5 μl of 1 M Tris–HCl (pH 7.5). The cDNA was purified using Microcon columns (Millipore) and labeled with Alexa dyes (Molecular Probes) at room temperature for 1 h in darkness. For each microarray, control cDNA from parental cell lines was labeled with Alexa 546 (Cy3 equivalent), whereas cDNA from Marc-GP4 or HeLa-GP5 cells was labeled with Alexa 647 (Cy5 equivalent). The fluorescent labeled cDNAs were purified again with the Qiaquick PCR purification kit (Qiagen) and precipitated by adding one volume of isopropanol and incubating on ice for 40 min. After rinsing with 70% ethanol, the labeled cDNA was resuspended in 2.5 μl of DNase-free, RNase-free water (Invitrogen). For hybridization, 40 μl of calf thymus DNA (10 mg/ml) was added to 80 μl of DIG Easy hybridization buffer (Roche) and the solution was heated at 65 °C for 2 min. Two 2.5 μl samples of labeled cDNA were combined with 30 μl of the hybridization solution as prepared above. This solution was then incubated at 65 °C for 3 min and pipetted onto the chip. The array chip was then covered with a 24 mm × 30 mm coverslip and incubated at 37 °C for 16 h. The slides were washed three times in 1 × SSC containing 0.1% SDS for 15 min at 50 °C, rinsed twice in 0.1 × SSC for 5 min each at room temperature, and dried. Array chips were scanned on a GenPix 4000A scanner (Axon Instruments Inc., Union City, CA). The normalization of raw

data and the analysis of the data sets were performed using GeneTraffic microarray data analysis software (Iobion Informatics, La Jolla, CA). The $\log_2 R/G$ (where R and G represent Cy5 and Cy3, respectively) normalized ratio was selected as the value, which was further calculated to a fold change in regulation.

3. Results

3.1. Establishment of cells expressing the PRRSV GP4 or GP5 proteins

To examine the effects of the PRRSV GP4 or GP5 protein on cell function, cells were first established to express the GP4 or GP5 proteins. Marc-145 cells were chosen to establish a cell line stably expressing the GP4 protein since they are cells susceptible to infection by PRRSV. For GP5 expression, we used the tetracycline-dependent inducible gene expression system (Tet-on/Tet-off) to avoid a possible cell death that may result from GP5 expression since the PRRSV GP5 protein was reported to induce cytotoxicity in African green monkey kidney cells (Suárez et al., 1996). HeLa-Tet-off cells, that were previously transformed with the regulatory plasmid pTet-off and therefore expressing the tetracycline transactivator (tTA), were chosen for additional transformation with the ORF5 gene. The presence of tetracycline (Tet) or its derivative doxycycline (Dox) in the culture medium prevents binding of tTA to the promoter, while the removal of Tet or Dox induces gene transcription placed under control of the promoter. Marc-145 and HeLa-Tet-off cells were transfected with pCI-Neo-ORF4 and pTRE-ORF5-hyg, respectively, and to confer resistance of transformed cells, neomycin (G418) or hygromycin were added. Transfected HeLa-Tet-off cells were maintained in the presence of doxycycline to prevent the GP5 expression during selection. Antibiotic-resistant cell clones were selected and individually confirmed for presence and transcription of the ORF4 or ORF5 gene by PCR and RT-PCR, and designated Marc-GP4 and HeLa-GP5, respectively.

Marc-GP4 and HeLa-GP5 cells were further examined for their respected protein expression by immunofluorescence (Fig. 1B and C). The cytoplasmic fluorescence was evident in these cells when incubated with PRRSV-specific hyperimmune sera, whereas no fluorescence was detected in parental Marc-145 and HeLa-Tet-off cells (data not shown), indicating that Marc-GP4 and HeLa-GP5 cells

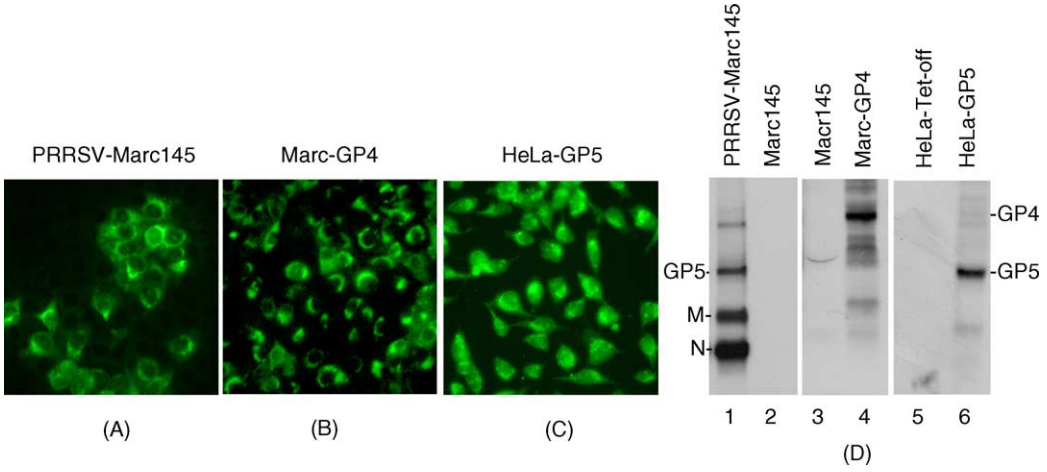

Fig. 1. Establishment of cells stably expressing the PRRSV GP4 or GP5 protein. Immunofluorescent cell staining was conducted in Marc-GP4 and HeLa-GP5 cells to confirm the expression of GP4 and GP5 proteins. Cells were grown on microscope coverslips, fixed with methanol, and incubated with anti-PRRSV-2 (North American) pig serum followed by FITC-conjugated goat anti-swine antibody. The fluorescence was visualized by a fluorescent microscope at 40× magnification. (A) Marc-145 cells infected with PRRSV-2 at 24 h post-infection; (B) Marc-GP4 cells at 48 h post-seeding; (C) HeLa-GP5 cells at 48 h post-induction in the absence of doxycline; (D) immunoprecipitation of GP4 and GP5 using anti-PRRSV-2 pig serum; lane 1: PRRSV-infected Marc-145 cells; lanes 2 and 3: un-infected Marc-145 cells; lane 4: Marc-GP4 cells; lane 5: HeLa Tet-off cells; lane 6: HeLa-GP5 cells.

expressed the GP4 and GP5 proteins, respectively. Every cell expressed GP4 or GP5 indicating a homogenous population of cells. The GP4 and GP5 protein expressions were further confirmed by radioimmunoprecipitation (Fig. 1D). A specific band of 31 kDa protein was identified in Marc-GP4 cells (lane 4). This band was absent in the parental Marc-145 cells (lane 3) and was considered the GP4 protein. We were not, however, able to detect the same protein from PRRSV-infected cells, and this is probably due to the low abundance of GP4 in PRRSV-infected cells as it is a minor protein. The GP5 protein was readily produced in HeLa-GP5 cells (lane 6), and its migration was similar to that of the authentic GP5 protein seen in PRRSV-infected cells (lane 1).

3.2. Gene expression profiles in Marc-GP4 and HeLa-GP5 cells

To investigate the effects of the PRRSV GP4 and GP5 proteins on cellular gene expressions, a microarray DNA chip technology was employed. Total cellular RNAs were extracted at 2 days post-induction and were reverse transcribed. The cDNAs were labeled using fluorescent dyes and used for hybridization of the human 1.7k microarray chip. This chip contained probes designed to detect 1718 human transcripts. The slides were scanned and the obtained images were analyzed using GeneTraffic microarray data analysis software. The $\log_2$ Cy5/Cy3 normalized ratio 1.00, which corresponds to a two-fold change in regulation, was initially chosen as the cut-off value as used for standard and further calculated to a fold change. When a fold-change calculated by $\log_2$ Cy5/Cy3 was consistently 2.0 or greater in two independent experiments (four hybridizations), the value was considered significant.

Transcription of a total of 16 cellular mRNAs was altered by the GP4 protein expression in Marc-GP4 cells. Of these mRNAs, six genes were up-regulated and 10 genes were down-regulated (Table 1). The classifications of altered genes included those involved in cell adhesion, cell growth, replication, transcription, and protein degradation, and other

Table 1
Cellular genes regulated by the PRRSV ORF4 gene expressed in Marc-145 cells

Name of gene	Gene ID[a]	Accession no.[b]	Fold change[c]
Cell adhesion and structure			
Syndecan 4 (SDC4)	428077	AA002237	−2.1
Cell cycle regulators and cancer signals			
Growth factor independent 1B (GFI1B)	1707378	AI097490	−2.64
Replication, transcription, translation, degradation machinery, and protein modification			
Cathepsin L (CTSL)	308921	H58586	2.22
Zinc finger protein 36, C3H type-like 2 (ZFP36L2)	469768	AA028065	3.25
Angiotensinogen proteinase inhibitor (Clade A; AGT)	51693	H22747	−2.83
Myeloid cell nuclear differentiation antigen (MNDA)	145130	R77519	−2.89
Proliferating cell nuclear antigen (PCNA)	415202	W91932	−3.03
Synthesis, transport, and biochemical pathways			
Microsomal triglyceride transfer protein (MTP)	294724	W01491	3.73
Phosphorylase kinase α-2 (PHKA2)	308941	W25385	3.84
Farnesyltransferase β (FNTB)	3170737	BE221171	−2.64
Ornithine aminotransferase (OAT)	347414	W81245	−2.83
Metallothionein 1E (MT1E)	241879	H93255	−2.83
Metallothionein 2A (MT2A)	241629	H91613	−3.03
N-acylsphingosine amidohydrolase 1 (ASAH1)	4608918	BG430149	−4.29
Miscellaneous			
Peroxisomal membrane protein 3 (PXMP3)	665099	AA195577	2.67
Hypothetical protein FLJ 20378	489747	AA101987	2.97

[a] Gene identification number of the Ontario Genomics Institute Microarray Centre (http://www.microarrays.ca/support/glists.html).
[b] GenBank accession numbers.
[c] Fold changes calculated by the ratio of Cy5/Cy3. Negative figures indicate down-regulations.

Table 2
Cellular genes regulated by the PRRSV ORF5 gene expressed in HeLa cells

Name of gene	Gene ID[a]	Accession no.[b]	Fold change[c]
Immune response and stress toxicity			
Beta-2-microglobulin (B2M)	131405	BG530085	2.62
Complement component 4 binding protein, α (C4BPA)	202665	H53489	−2.06
Cell adhesion and structure			
Keratin Type II cytoskeletal 1	364607	AA024415	2.1
ARP1 actin-related protein 1 homolog A, centractin α (ACTR1A)	267800	N34209	2.93
ARP1 actin-related protein 1 homolog B, centractin β (ACTR1B)	381596	AA059052	2.22
Signal transduction pathway			
S100 calcium binding protein A11 (calgizzarin, S100A11)	231145	BI834224	−2.1
S100 calcium binding protein A12 (calgranulin C, S100A12)	123640	R02722	−3.43
Cell cycle regulators and cancer signals			
Adenomatosis polyposis coli (APC)	49621	H29191	2.16
Cyclin D3 (CCND3)	240171	H89623	−3.14
Ras association (RalGDS/AF-6) domain family 2 (RASSF2)	488226	AA055910	−4.23
2,3-Bisphosphoglycerate mutase (BPGM)	206825	R98094	−6.68
Replication, transcription, translation, degradation machinery, and protein modification			
Transcription factor binding to IGHM enhancer 3 (TFE3)	264848	N29101	2.06
Cathepsin E (CTSE)	204519	H58586	2.33
ADP-ribosylation factor related protein 1 (ARFRP1)	21806	T65096	−2.01
Synthesis, transport, and biochemical pathways			
3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1 (HMGCS1)	39505	BG740145	2.25
2,4-Dehydrocholesterol reductase (DHCR24)	415303	W92108	2.26
Miscellaneous			
S164 protein (S164)	490982	AA136788	2.01
Hypothetical protein FLJ13657	2944520	AW592769	2.11
Diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein, DBI)	345809	W72686	2.13
Oviductal glycoprotein 1, 120 kD (oviductin, OVGP1)	247629	N58709	−2.97

[a] Gene identification number of the Ontario Genomics Institute Microarray Centre (http://www.microarrays.ca/support/glists.html).
[b] GenBank accession numbers.
[c] Fold changes calculated by the ratio of Cy5/Cy3. Negative figures indicate down-regulations.

unknown functional pathways. Seven genes associated with synthesis, transport, and biochemical pathway were also identified.

Microarray analysis of HeLa-GP5 cells showed an expression profile of 20 cellular genes altered by the GP5 protein expression in HeLa cells, with an increase in transcription of 12 genes and a decrease of eight genes (Table 2). The genes that were affected by the GP5 protein represented those involved in the immune response, cell adhesion and structure, signal transduction pathway, cell cycle and cancer signals, and other functions. Five additional genes involved in synthesis, replication, transcription, and protein degradation were found to be altered in GP5 expressing HeLa cells. Cellular genes involved in apopotis were expected to be regulated in HeLa-GP5, but none of these genes appeared to be regulated by GP5 expression, suggesting that the GP5 protein may be irrelevant to PRRSV apoptosis (see Section 4).

4. Discussion

In this study, we performed a global analysis of the transcriptional response of cells to expression of two PRRSV proteins. We established cell lines stably expressing the GP4 or GP5 protein, both protein genes under the control of human cytomegalovirus (hCMV) immediate early promoter, and experiments were designed to assess patterns of gene regulation in these

cells. In GP5-expressing HeLa cells, actin-related protein 1 (ARP1) homologs A and B were identified to be up-regulated. These genes encode a subunit of dynactin that binds to both microtubules and cytoplasmic dynein, and is involved in ER-to-Golgi transport (Lees-Miller et al., 1992). This may implicate an important function of GP5 in the transport of the viral components. The bisphosphoglycerate mutase (BPGM) gene was down-regulated by GP5 by more than six-folds. BPGM is an erythrocyte specific trifunctional enzyme regulating the level of 2,3-BPG in red blood cells. 2,3-BPG is the main allosteric effector of hemoglobin, shifting the equilibrium between the oxy and deoxy conformation of hemoglobins by stabilizing the unliganded form. Sickle cell anemia in humans is characterized by polymerization of deoxygenated hemoglobin mutants giving rise to deformed erythrocytes and vasoocclusive complications. 2,3-BPG has been shown to facilitate this polymerization in sickle cell anemia. In humans, deficiency of BPGM has been shown to be associated with anemia (Jacobasch and Rapoport, 1996). RASSF2 was also down-regulated by GP5. RASSF2 is a new member of the RASSF1 family and shares the properties of Ras effector/tumor suppressors (Vos et al., 2003). Similarly, cyclin D3 gene expression was found to be suppressed. D-type cyclins are the key regulators along with cyclin E for cell cycle progression from G1 to S phase. Complexes formed between cyclin D or cyclin E and their kinase partners are involved in phosphorylation of retinoblastoma protein, which ultimately leads to activation of E2F transcription factor and progression to S phase of the cell cycle. A recent study demonstrates a clear reduction of cyclin D3 and cell cycle arrest in G0/G1 phase in cells infected with mouse hepatitis coronavirus, a member of nidoviruses (Chen and Makino, 2004).

In cell expressing GP4, the majority of differentially expressed genes were involved in synthesis, transport, and biochemical pathways. This observation implicates that the GP4 protein may utilize or change host cell machinery to transport viral or cellular components to the cell surface. Zinc finger protein 36 (zfp36) and microsomal triglyceride transfer protein were readily up-regulated, while metallothineins, proliferating cell nuclear protein, and *N*-acylsphingosine amidohydrolase were down-regulated. Zinc finger protein 36-like 1 is a member of the tristetraprolin family of tandem CCCH finger proteins. Tristetraprolin can bind to AU-rich elements within the 3′-untranslated regions of the mRNAs encoding tumor necrosis factor (TNF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), leading to accelerated mRNA degradation (Stumpo et al., 2004). Tristetraprolin-knockout mice exhibit an inflammatory phenotype that is largely due to increased TNF secretion (Taylor et al., 1996). Microsomal triglyceride transfer protein is a protein complex required for the assembly of lipoprotein particles (Gordon et al., 1995). It is noteworthy that GP4 has recently been shown to be a lipid-anchored protein (Bachand, 2003). Metallothionein is a metal binding protein and has been shown to be regulated by a common virus infection (Ilback et al., 2004). Coxsackievirus B-type 3 virus infection altered the normal physiological trace element balance in the liver, kidney, spleen, and increased metallothionein in these organs. This may be a normal response in common infections that could adversely influence the pathogenesis when the host is concomitantly exposed to potentially toxic trace elements, even at levels in the physiological range. The function of *N*-acylsphingosine amidohydrolase is unclear.

We have observed consistent increases in the expression of cathepsin genes in both Marc-GP4 and HeLa-GP5 cells. Cathepsin is involved in protein degradation. Despite a specific role of cathepsin during virus replication remains to be determined, the up-regulated expression of the gene encoding protease may represent a cellular defense against expression of foreign proteins.

Interestingly, no pro-apoptotic genes were identified in HeLa-GP5 cells. This observation is contradictory to the previous report (Suárez et al., 1996) but is consistent with our recent finding that the GP5 expressing cells did not show any detectable level of cytotoxicity or cell death (Lee et al., 2004). Zhang et al. (1999) have shown that PRRSV infection induced the expression of IFN-inducible gene *Mx*1 and an ubiquitin-specific protease in porcine alveolar macrophages. These genes were not identifiable in the present study. This difference may be due to the use of different cell types since Marc-145 or HeLa cells were used in the present study to express the single GP4 or GP5 protein rather than using the whole virus to infect porcine macrophages in the previous report.

The DNA microarray has allowed us to identify the differential effects of PRRSV proteins on cellular genes. Confirmatory studies are further required as to the significance of the genes that have been identified in the present study. The method of choice to measure and confirm the differential mRNA expression mediated by the GP4 and GP5 proteins is real-time quantitative RT-PCR. It is also possible that altered mRNA profiles may not necessarily reflect altered production of corresponding proteins (Gygi et al., 1999). In this regard, additional techniques such as Western blot analysis or protein arrays may be needed to support our findings. Nevertheless, our data obtained from the microarray study will provide future insights into the understanding of host cell virus interactions and eventually of the pathogenic mechanisms of PRRSV and the host responses to PRRSV infection.

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