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Effects of substitution of viral hemorrhagic septicemia virus genotype IVa glycoprotein with vesicular stomatitis virus (VSV) glycoprotein on cell line preference

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The glycoprotein of novirhabdoviruses is known to play a critical role in the determination of host specificity. Viral hemorrhagic septicemia viruses (VHSVs) in different genotypes have different glycoprotein sequences and show different preferences for specific cell lines. In this study, to know whether the glycoprotein is solely responsible for the host cell preference of VHSV, a recombinant VHSV expressing vesicular stomatitis virus (VSV) glycoprotein instead of VHSV IVa glycoprotein (rVHSV-VSV-G) was generated by reverse genetics and inoculated into several fish cell lines, then, cytopathic effect (CPE) and viral growth caused by rVHSV-VSV-G infection were compared with those caused by rVHSV-wild that was previously generated and has the same genomic sequence with wild-type VHSV except a few nucleotides. The plaque numbers of rVHSV-VSV-G were significantly higher in EPC, BF-2 and GF cells than those of rVHSV-wild. However, in HINAE cells (originated from olive flounder), rVHSV-VSV-G titer was significantly lower than rVHSV-wild titer, and both recombinant VHSVs were not grown well in CHSE-214 cells. Although statistical significances were detected in the titers between rVHSV-wild and rVHSV-VSV-G in several cell lines, the cell line-preference order of rVHSV-VSV-G was not different from that of rVHSV-wild. These results suggest that the replacement of VHSV glycoprotein may not completely change host cell preference, and other regions of VHSV might also involve in the determination of host cell preference.

Key words: Viral hemorrhagic septicemia virus IVa, Vesicular stomatitis virus, Glycoprotein substitution, Cell line preference

Rhabdoviruses are negative sense, single stranded RNA viruses and have a broad range of host species including vertebrates, invertebrates, and plants (Hogenhout et al., 2003; Rose and Whitt, 2001). The genome of all rhabdoviruses basically contains five genes encoding nucleoprotein (N), polymerase-associated phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase (L). Viral hemorrhagic septicemia virus (VHSV) is belonging to the genus *Novirhabdovirus* and possesses the non-virion (NV) gene between G and L genes of the genome. Based on the sequence of G gene, VHSV is divided into 4 genotypes, I (Ia-Ie), II, III, and IV (IVa-IVc) (Einer-Jensen et al., 2004; Snow et al., 2004). Epizootics caused by VHSV has been one of the major causes of mass mortality in cultured fish worldwide, and, among those genotypes, VHSV IVa has been responsible for severe damage in olive

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flounder (*Paralichthys olivaceus*) farms at low water temperature period in Korea (Kim et al., 2009).

The envelope of rhabdoviruses is spiked with the trimeric G proteins which bind to receptors on the host cell membrane. The attached virus enters into cell through endocytosis, and then the G protein fuses to endosomal membrane to release viral nucleocapsid into cytoplasm (Albertini et al., 2012; Langevin et al., 2002). Thus, rhabdoviral G protein is a critical factor for host cell specificity, and the viral tropism can be changed by substitution of G protein with other rhabdoviral G proteins or other viral envelope proteins. For instance, Foley et al. (2002) showed the alteration of rhabdoviral tropism by substitution of Rabies virus (RABV) glycoprotein with the human immunodeficiency virus type I (HIV-1) envelope protein, in which the recombinant RABV showed pH-independent cell entry and HIV-1 specific cell tropism.

Vesicular stomatitis virus (VSV) is a member of the genus *Vesiculovirus*, and infects a wide variety of animals including not only mammals but also insects (Kuzmin et al., 2009; Letchworth et al., 1999). This extremely broad host range has made the VSV glycoprotein be used to generate pseudotyped lentivirus that can infect a broad range of cell lines or organisms (Matsubara et al., 1996; Que et al., 1999; Boulo et al., 2000). Considering broad host range of VSV, it can be expected that the host cell preference of VHSV IVa may be changed by replacement of VHSV glycoprotein with VSV glycoprotein. In the present study, we successfully generated a recombinant VHSV that expressing VSV glycoprotein instead of VHSV glycoprotein (rVHSV-VSV-G) using the reverse genetics. To know whether the glycoprotein is solely responsible for the host cell preference of VHSV, the replication ability of rVHSV-VSV-G in various fish cell lines was analyzed, and compared with that of a recombinant wild-type VHSV (rVHSV-wild) that was previously produced (Kim et al., 2011).

Materials and methods

Cells and viruses

Epitheliomia papulosum cyprini (EPC), bluegill fry caudal trunk (BF-2), hirame natural embryo (HINAE), grunt fin (GF), and chinook salmon embryo (CHSE-214) cell lines were grown in Leibovitz medium (L-15, Sigma) supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml, Gibco) and 10% fetal bovine serum (FBS, Sigma). The wild-type VHSV KJ2008 was isolated in 2008 from a moribund olive flounder in a natural outbreak of VHSD on a commercial farm in Korea.

Generation of rVHSV-VSV-G

To construct a recombinant VHSV cDNA vector harboring VSV-G gene instead of VHSV-G gene, the fragment encoding VSV-G gene ORF was PCR amplified using a plasmid DNA of pVSV-G (Retroviral Expression System, Clontech) as a template and the following primers; forward 5′-ACCGGTATGAAGT GCCTTTTGTACTTAGCCTTT-3′ (*Age*I site underlined) and reverse 5′-CCGCGGTTACTTTCCAAGT CGGTTCATCTCTATG-3′ (*Sac*II site underlined). The amplified PCR product was cloned into the pGEM T-easy vector (Promega) and sequenced. The cloned fragment was mutated to remove an *Age*I site by site-directed mutagenesis kit (SDM; Stratagene) with a primer pair (forward; 5′- GGCTCTATTCC TGTAGACACGGGTTATCACATGTATTGC-3′ and reverse; 5′-GCAATACATGTGATAACCCGTGTCT ACAGGAATAGAGCC-3′; mutagenesis site is underlined). The mutated vector was digested with *Age*I and *Sac*II, and the digested fragment encoding VSV-G gene was ligated to the previously constructed pVHSV-wild vector [14] that was predigested with the same enzymes, resulting in pVHSV-VSV-G.

EPC cells expressing T7 RNA polymerase (RNAP) were grown to about 80% confluence and transfected with a mixture of pVHSV-VSV-G (2 μg) and helper plasmids that expressing N (pCMV-N, 500 ng), P

(pCMV-P, 300 ng), and L (pCMV-L, 200 ng) proteins under the control of cytomegalovirus (CMV) promoter using FuGENE HD (Promega) according to manufacturer's instructions. Transfected cells were incubated for 12 h at 28°C, and shifted to 15°C. When total cytopathic effect (CPE) was observed, the cells were suspended by scratching the plates with a rubber policeman, and centrifuged at 4000 g for 10 min. The resulting supernatant (named P0) was used to inoculate fresh EPC cells monolayer in a T25 flask at 15°C. On 7-10 days post-inoculation, the supernatant (P1) was harvested, aliquoted, and stored at -80°C.

For verification of rVHSV-VSV-G generation, total RNA was extracted from the supernatant P1 using Trizol (Invitrogen) reagent, and treated with RNase-free DNase (Promega), and DNase was inactivated by adding stop solution. To synthesize first-strand cDNA, 1 μg of the total RNA was incubated with 0.5 μl of random primer (Enzynomics, Korea) at 80°C for 5 min and further incubated at 42°C for 60 min in reaction mixture containing 2 μl of each 2 mM dNTP mix (Enzynomics), 0.5 μl of MMLV reverse transcriptase (Promega) and 0.25 μl of RNase inhibitor (Promega) in a final reaction volume of 10 μl. RT-PCR was conducted using specific primer pairs between M gene (M-F; 5'-CCAGGTCGATAA GATCTGCATGG-3') and VSV G gene (VSV-G-R; 5'- TTATGGTGAAAGCAGGACCG-3') or between VSV-G gene (VSV-G-F; 5'- CCATGGACATCACC TTCTTCTCA-3') and NV gene (NV-R; 5'- TCATG GGGGAGATTCGGAGCCATTC-3'). The PCR products were analyzed on a 0.8% agarose gel.

Western blot

EPC cells in L-15 medium containing 2% FBS and antibiotics at 15°C were inoculated with the wild-type VHSV, rVHSV-wild or rVHSV-VSV-G. At 48 h post-inoculation, each group of infected cells was rinsed and suspended in 200 μl phosphate buffered saline (PBS), denatured by addition of 6 x sample buffer for 10 min at 95°C, and loaded on a 10% SDS-PAGE gel, then, transferred to a nitrocellulose membrane. The membrane was blocked with blocking solution (3% bovine serum albumin in TBS; 150 mM NaCl, 10 mM Tris-HCl, pH 7.5) for 2 h at room temperature (RT), washed with TTBS (0.05% Tween 20 in TBS, pH 7.5) and incubated with diluted VSV-G antibody (1:2000, Santa Cruz Biotechnology) for 2 h at RT. The membrane was washed three times with TTBS and incubated with alkaline phophatase conjugated goat anti-mouse IgG_1 (1:2000, Santa Cruz Biotechnology) for 2 h at RT. After washing off unbound secondary antibody, the specific antigen-bound antibody was visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoly phosphate (NBT-BCIP) substrate buffer (Sigma).

Cytopathic effect

EPC, BF-2, HINAE, GF, and CHSE-214 cells in 24-well plates (3×10^5 cells/well) were grown to 90% confluence at 15°C, then, inoculated with rVHSV-wild or rVHSV-VSV-G at MOI 0.1, and observed CPE under a microscope.

Plaque assay

EPC, BF-2, HINAE, GF, and CHSE-214 cells in 24-well plates (3×10^5 cells/well) were grown to 90% confluence at 15°C, then, inoculated with either rVHSV-wild or rVHSV-VSV-G at MOI 0.1. The experiment was conducted in triplicate. To assess the number of infectious viral particles, plaque assay was performed (Burke and Mulcahy, 1980). Briefly, EPC cells monolayer was inoculated with each kind of viral stocks that were serially diluted. After 1 h of incubation at 15°C, the cells were overlaid with plaquing medium (0.7% agarose in L-15 containing 10% FBS and antibiotics). After 7 d of incubation to allow plaque formation, the cells were fixed by 10% formalin and stained with 3% crystal violet for 30 min at RT. After rinsing of the cells with distilled water, the plaque-forming units (PFU) were counted.

Statistical analysis

Statistical analysis was performed using SPSS for Windows (Chicago, IL, USA). Data on each recombinant virus growth (plaque numbers) were analyzed by Student's t-test. A value of $P<0.05$ was considered as the statistical significance.

Results

Generation of recombinant VHSV harboring VSV-G gene

To rescue a recombinant VHSV expressing VSV-G gene instead of VHSV-G gene, we constructed a genomic mutant cDNA vector in which VHSV-G gene ORF was replaced with VSV-G gene ORF (Fig. 1). The recombinant VHSV (rVHSV-VSV-G) was rescued by transfection of EPC cells expressing T7 RNAP with the constructed mutant cDNA clone and supporting vectors. EPC cells transfected with the vectors showed evident CPE. The production of rVHSV-VSV-G in the stock supernatant was confirmed by RT-PCR, western blot and plaque assay. In RT-PCR analysis, the bands corresponding to the M-VSV-G and VSV-G-NV regions were detected (Fig. 2a), but M-VHSV-G and VHSV-G-NV regions were not amplified. In western blot analysis using a monoclonal antibody against VSV-G, the band corresponding to VSV-G protein (approximately 67 kDa) was detected only in rVHSV-VSV-G-inoculated cells (Fig. 2b). In plaque assay to verify the presence of infectious recombinant virus, 3×10^6 PFU/ml for rVHSV-VSV-G was counted in EPC cells inoculated with the stock supernatant.

Cytopathic effect and viral titers measured by plaque assay

To know whether replacement of VHSV IVa glycoprotein with VSV-G glycoprotein can influence on the viral growth in various fish cell lines, rVHSV-wild and rVHSV-VSV-G were inoculated into a variety of cell lines at 15°C, and titers of the newly produced viruses were analyzed by plaque assay. In EPC cells, the titers of rVHSV-VSV-G at 24, 48, and 72 h post-inoculation were significantly higher than titers of rVHSV-wild (Fig. 3a). In BF-2 and GF cells, plaque numbers of rVHSV-VSV-G were significantly higher than those of rVHSV-wild (Fig. 3b,d). The CPE induction in EPC, BF-2, and GF cells by rVHSV-VSV-G was faster than by rVHSV-wild (Fig. 3a,b,d). In HINAE cells, rVHSV-VSV-G induced weaker CPE and significantly lower viral titers than rVHSV-wild (Fig. 3c). In CHSE-214 cells, only a few plaques were observed by inoculation with rVHSV-wild and rVHSV-VSV-G (figures not shown).

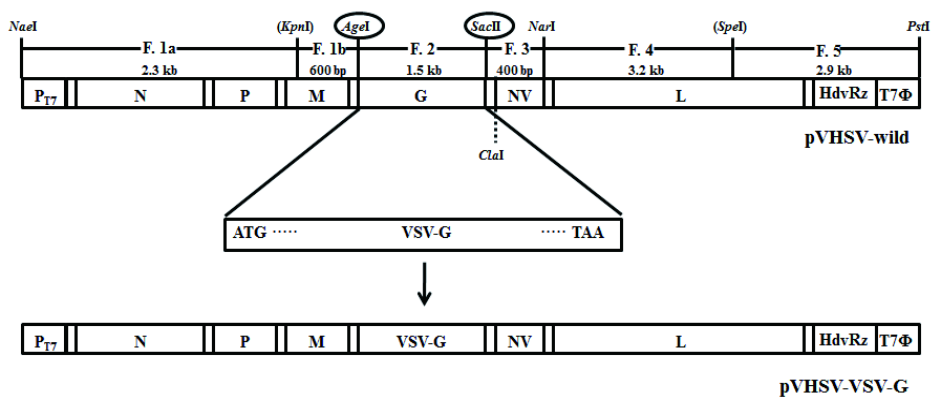

Fig. 1. Construction of the mutated cDNA vector harboring VSV-G gene (pVHSV-VSV-G). The VHSV-G gene ORF was replaced with the VSV-G gene ORF using *Age*I/*Sac*II restriction enzyme sites.

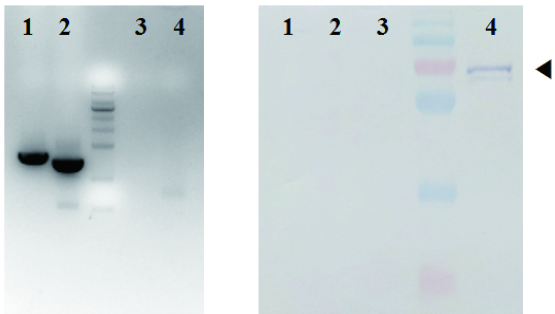

Fig. 2. Confirmation of rVHSV-VSV-G recovery by (a) RT-RCR and (b) western blot. (a) Bands were detected by primer pairs for amplification between M gene and VSV G gene (lane 1) or between VSV G gene and NV gene (lane 2). However, no bands were detected by primer pairs for amplification between M gene and VHSV-G gene (lane 3), or between VHSV-G gene and NV gene (lane 4). (b) Western blot analysis of VSV-G protein (approximately 67 kDa; indicated by an arrow head) using a monoclonal antibody against VSV-G. M, prestained protein ladder (Thermo); Lane 1, mock infected EPC cells; Lane 2, wild-type VHSV infected EPC cells; Lane 3, rVHSV-wild infected EPC cells; Lane 4, rVHSV-VSV-G infected EPC cells.

Discussion

Attachment of viruses to the cell surface receptors is the initial step for infection, so any alterations in the viral proteins that bind to the receptors can lead to change in cell tropism. VSV can replicate both mammals and insects, suggesting the receptor for VSV G glycoprotein is expressed in a wide range of cells (Davidson and Breakefield, 2003). Therefore, VSV G protein has been applied to generation of pseudotyped retrovirus and lentivirus to enhance transduction efficiency and cellular tropism (Farley et al., 2007; Yee et al., 1994). While several cell surface receptor candidates have been reported for VSV (Coil and Miller, 2004; Schloemer and Wagner, 1975; Schlegel and Wade, 1983; Schlegel et al., 1983; Yamada and Ohnishi, 1986), recently, Finkelshtein et al. (2013) demonstrated that members of the low-density lipoprotein receptor (LDLR) family that are expressed in all animal cells (Willnoe, 1999) act as the cell surface receptor for VSV. In VHSV, a glycoprotein, fibronectin, was reported as the major site for attachment of VHSV G protein to the plasma membrane (Bearzotti et al., 1999). In this study, rVHSV-VSV-G proliferated in EPC, HINAE, BF-2, and GF cell lines cultured at 15°C. These results suggested that binding to cell membrane receptor, pH-dependent fusion to endosomal membrane, and post-translational folding of VSV glycoprotein were well processed in those fish cell lines at 15°C.

Various fish cell lines are known to be susceptible to VHSV including EPC, BF-2, rainbow trout gonad (RTG-2), fathead minnow (FHM), and CHSE-214. However, the susceptibility of each cell line is different according to the VHSV genotypes and cell-line lineages (Olesen and Vestergard Jorgensen, 1992; Lorenzen et al., 2005; McAllister, 1997; Dixon, 1999; Sandlund et al., 2014), in which EPC cell line is more susceptible to genotype IV than BF2 cell line. In the present study, although the amplitude of titer difference was not high, the titers of rVHSV-VSV-G were statistically significantly higher than those of rVHSV-wild in EPC, BF-2, and GF cells, suggesting that the viral replication ability was enhanced to some extent by change with VSV glycoprotein. Interestingly, in HINAE cell line that is originated from olive flounder, the plaque numbers of rVHSV-VSV-G was significantly lower than those of rVHSV-wild, suggesting that the origin of virus isolation may be an important factor for the determination of VHSV preference for cell lines.

Furthermore, although statistical significances were detected in the titers between rVHSV-wild and rVHSV-VSV-G in EPC, BF-2, HINAE, and GF cells, the cell line-preference pattern of rVHSV-VSV-G was not different from that of rVHSV-wild. The order of rVHSV-VSV-G titers according to cell lines was identical with the order of rVHSV-wild titers including a few plaques of both rVHSV-wild and rVHSV-VSV-G in CHSE-214 cells. These results suggest that

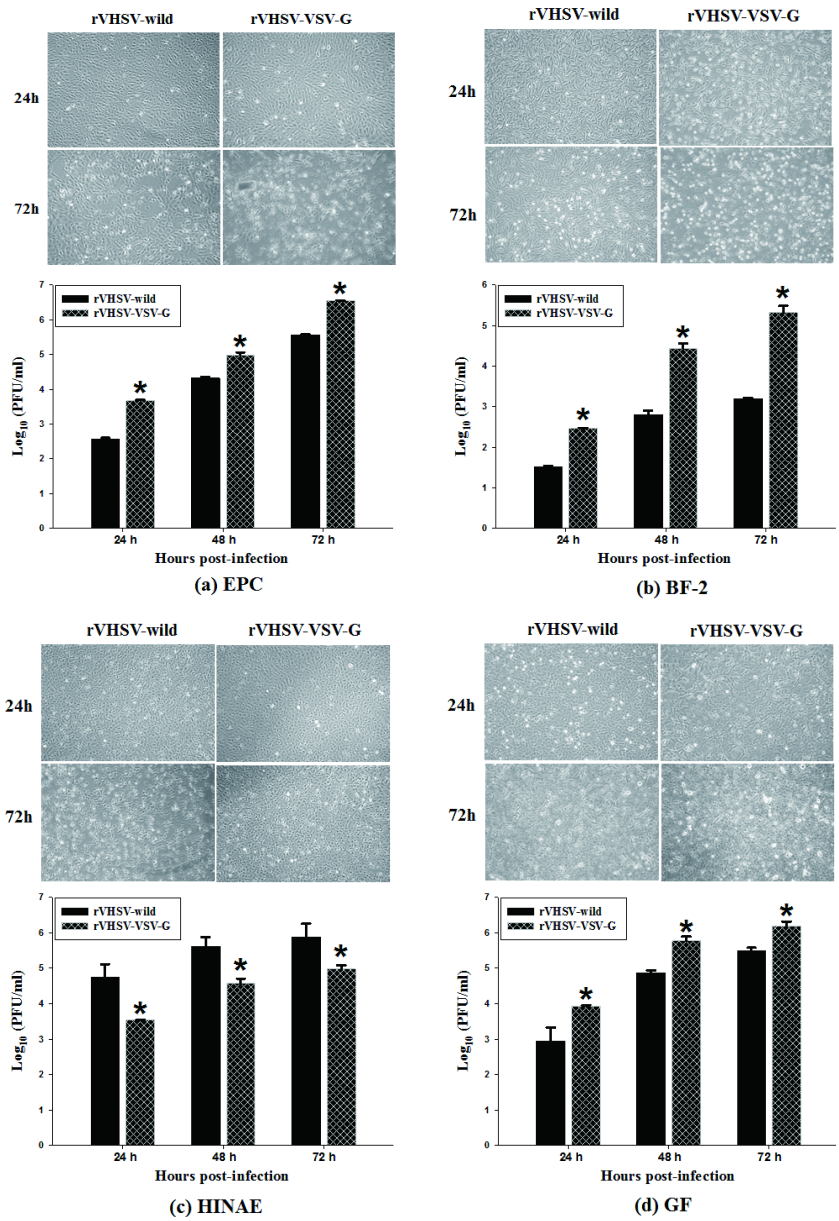

Fig. 3. Progression of cytopathic effect (CPE) and viral titers measured by plaque assay in EPC (a), BF-2 (b), HINAE (c), and GF (d) cell lines. The asterisk on the bar represents significantly different ($P<0.05$) in viral titers at the analyzed time.

not only glycoprotein but also other region(s) of VHSV might involve in the determination of cell preference. Further studies with other genotypes of VHSV are needed to elucidate the mechanism of host cell preference in VHSV genotypes.

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