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Selection of classical swine fever virus with enhanced pathogenicity reveals synergistic virulence determinants in E2 and NS4B

Tomokazu Tamura[1], Yoshihiro Sakoda[1*], Fumi Yoshino[1], Takushi Nomura[1], Naoki Yamamoto[1], Yuka Sato[1], Masatoshi Okamatsu[1], Nicolas Ruggli[2], Hiroshi Kida[1, 3]

[1]Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

[2]Institute of Virology and Immunoprophylaxis, Sensemattstrasse 293, CH-3147 Mittelhäusern, Switzerland

[3]Research Center for Zoonosis Control, Hokkaido University, Sapporo 001-0020, Japan

[*]Corresponding author

Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, North 18, West 9, Kita-ku, Sapporo 060-0818, Japan

Phone: +81-11-706-5208; Fax: +81-11-706-5208

E-mail: sakoda@vetmed.hokudai.ac.jp

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Virulence determinants in E2 and NS4B of CSFV

Abstract

Classical swine fever virus (CSFV) is the causative agent of classical swine fever (CSF), a highly contagious disease of pigs. There are numerous CSFV strains that differ in virulence, resulting in clinical disease with different degrees of severity. Low virulent and moderate virulent isolates cause a mild and often chronic disease, while highly virulent isolates cause an acute and mostly lethal hemorrhagic fever. The live attenuated vaccine strain GPE$^-$ was produced by multiple passages of the virulent ALD strain in cells of swine, bovine, and guinea pig origin. With the aim of identifying the determinants responsible for the attenuation, the GPE$^-$ vaccine virus was re-adapted to pigs by serial passages of infected tonsil homogenates until prolonged viremia and typical signs of CSF were observed. The GPE$^-$/P-11 virus isolated from the tonsils after the 11th passage *in vivo* had acquired 3 amino acid substitutions in E2 (T830A) and NS4B (V2475A and A2563V) when compared with the virus before passages. Experimental infection of pigs with the mutants reconstructed by reverse genetics confirmed that these amino acid substitutions were responsible for the acquisition of pathogenicity. Studies *in vitro* indicated that the substitution in E2 influenced virus spreading, and that the changes in NS4B enhanced the viral RNA replication. In conclusion, the present study identified residues in E2 and NS4B of CSFV that can act synergistically to influence virus replication efficiency *in vitro* and pathogenicity in pigs.

Introduction

Classical swine fever (CSF) is an economically important, highly contagious disease of pigs caused by classical swine fever virus (CSFV). The virus belongs to the genus *Pestivirus* of the family *Flaviviridae*, together with bovine viral diarrhea virus (BVDV) and border disease virus (30). CSFV possesses a single-stranded positive-sense RNA genome of approximately 12.3 kb with one large open reading frame flanked by a 5' and 3' untranslated region (UTR) and coding for a polyprotein of approximately 4,000 amino acids (30). Co- and posttranslational processing of the polyprotein by cellular and viral proteases yields the 12 cleavage products N^{pro}, C, E^{rns}, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B (2, 3, 4, 6, 16, 17, 27, 45, 53, 59, 60). The structural components of the CSFV virion include the capsid (C) protein and the glycoproteins E^{rns}, E1, and E2. The non-structural proteins NS3 through NS5B are essential for pestivirus RNA replication, while N^{pro}, p7, NS2 and all structural proteins are dispensable (1, 30).

Most countries have eradicated CSFV successfully using stamping out and vaccination (57). The areas of the world in which the virus is still endemic represent a constant threat for disease-free countries. In Japan that is currently free of CSF, a live attenuated vaccine based on the GPE^- strain was employed. The GPE^- vaccine virus was derived from the virulent CSFV strain ALD through multiple passages and biological cloning in swine testicle cells, bovine testicle cells, and primary guinea pig kidney cells (48, 51). Pigs inoculated with the GPE^- vaccine were completely protected from challenge virus infection and replication (34, 49). Comparison of the genomes of the GPE^- vaccine virus and the parent ALD virus revealed 225

nucleotide substitutions and 46 amino acid differences (22), which did not provide any conclusive information on the molecular basis of the attenuation. Numerous reports suggest that the viral proteins N^{pro}, C, E^{rns}, E1, E2, p7, and NS4B may be involved in determining the virulence of CSFV in pigs (7, 8, 9, 10, 11, 12, 13, 32, 36, 37, 38, 39, 40, 43, 46, 54). So far however, all conclusions on CSFV virulence determinants rely on mutations that result in down regulation of pathogenicity.

In the present study, the live attenuated GPE^- vaccine virus was re-adapted to its natural host by multiple passages in pigs in order to rescue virus with increased replication efficiency, with the aim of identifying determinants of CSFV virulence. After 11 serial inoculations of tonsil homogenate from infected pigs, prolonged viremia with clinical signs typical of CSF were observed. The genome of the GPE^-/P-11 virus isolated after the 11^{th} passage had acquired a substitution in E2 (T830A) and 2 substitutions in NS4B (V2475A and A2563V). The relevance of these loci for virulence was confirmed by reverse genetics *in vivo* and by viral spreading and RNA replication assays *in vitro*.

Materials and Methods

Viruses and cells.

The GPE^- strain was generated from the virulent ALD strain by multiple passages and cloning in swine testicle cells, bovine testicle cells, and primary guinea pig kidney cells (48, 51).

The ALD/A76 strain was derived from ALD strain by serial passages in swine testicle cells (25). The ALD/A76 and GPE^- strains were kindly provided by the National Institute of Animal Health, Japan, and propagated in the swine kidney cell lines SK-L and CPK (25). These cell lines were maintained in Eagle's minimum essential medium (MEM) (Nissui, Tokyo, Japan) supplemented with 0.295% tryptose phosphate broth (TPB) (Becton Dickinson, San Jose, CA) and 10% horse serum (Invitrogen, Carlsbad, CA) at 37°C in the presence of 5% CO_2. The primary guinea pig kidney (GPK) cells were isolated from kidney tissue from specific-pathogen-free (SPF) guinea pigs (Japan SLC, Shizuoka, Japan) by treatment with 350 protease units/ml of dispase II (EIDIA, Tokyo, Japan) in MEM and slow stirring for 8 h at 4°C. After treatment, the cells were seeded in culture flasks in MEM supplemented with 0.295% TPB and 10% BVDV-free fetal calf serum (Mitsubishi Chemical, Tokyo, Japan) and incubated at 37°C.

Sequencing.

Viral RNA was extracted from infected SK-L cells with TRIzol Reagent (Invitrogen) and subsequently reverse-transcribed (RT) using Random Primer $(N)_9$ (Takara Bio, Shiga, Japan) and SuperScript III Reverse Transcriptase (Invitrogen). The viral cDNA, without the 5' and 3' ends, was amplified by PCR with CSFV-specific primers. The 5' ends of the viral cDNA were amplified using the 5' Rapid Amplification of cDNA ENDs (RACE) kit Version 2.0 (Invitrogen) and TaKaRa EX Taq polymerase (Takara Bio) according to the manufacturer's protocol. For

the amplification of the 3' ends, RNA was extracted from the supernatant of infected SK-L cells with TRIzol LS Reagent (Invitrogen), and polyadenylated by using 50 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 250 mM NaCl, 1 mM DTT (Invitrogen), 2.5 mM $MnCl_2$, 0.1% BSA, 1 mM ATP (Takara Bio), and Poly(A) Polymerase (Takara Bio). The polyadenylated RNA was purified on S-300 HR columns (GE Healthcare, Buckinghamshire, UK), and amplified using the 3'-RACE kit (Invitrogen) and TaKaRa Ex Taq polymerase. Nucleotide sequencing was carried out with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and a 3500 Genetic Analyzer (Applied Biosystems). The sequencing data were analyzed using the GENETYX®-MAC version 13 software (Genetyx, Tokyo, Japan).

For bead-bound clonal amplicon of the original inoculum of serial passages (GPE^- strain), emulsion PCRs (emPCRs) were carried out with the GS Junior Titanium emPCR kit (Roche, Basel, Switzerland) according to the manufacturer's instructions, with 2 copies per bead. After bead recovery and enrichment, the beads were sequenced using the GS Junior Titanium Sequencing kit (Roche) and a GS Junior Bench Top System (Roche) according to the appropriate instrument run protocol. The resulting reads were sorted and assembled using the CLC Genomics Workbench software (CLC bio, Aarhus, Denmark).

Plasmid constructs.

The cDNA fragments of the GPE^- strain obtained by RT-PCR were cloned into the pBR322

vector (Takara Bio), between the restriction sites *Sal*I and *Bam*HI. The cDNA sequence was flanked by a modified T7 promoter sequence at the 5' end and a *Srf*I restriction site at the 3' end in analogy to a construct described previously (44). The cDNA was analyzed by nucleotide sequencing as described above. Subclones were assembled to the full-length clone termed pGPE$^-$ in the low copy number plasmid pACNR1180 (44) by using appropriate restriction enzymes. Details of the constructions can be obtained on request. The 7 full-length cDNA clones with all possible combinations of the 3 amino acid substitutions T830A, V2475A, and A2563V were constructed in the pGPE$^-$ backbone using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany). The plasmids pGPE$^-$/T830A, pGPE$^-$/V2475A, and pGPE$^-$/A2563V have a single amino acid substitution at position 830, 2,475, and 2,563 of pGPE$^-$, respectively. The plasmids pGPE$^-$/T830A; V2475A, pGPE$^-$/T830A; A2563V, and pGPE$^-$/V2475A; A2563V carry 2 amino acid substitutions. The plasmid pGPE$^-$/T830A; V2475A; A2563V harbors all 3 amino acid substitutions.

The replicon cDNA clone pGPE$^-$-N^{pro}-Luc-IRES-NS3 was constructed by replacing the genes C, E^{rns}, E1, E2, p7, and NS2 in the pGPE$^-$ backbone with a DNA cassette containing the firefly luciferase gene and the internal ribosome entry site (IRES) of encephalomyocarditis virus (EMCV) obtained from the replicon cDNA clone pA187-N^{pro}-Luc-IRES-C-delE^{rns} (52). The cDNA cloning was performed using the In-Fusion® HD Cloning Kit according to the manufacturers protocol (Clontech, Mountain View, CA). The 3 replicon cDNA clones carrying

all possible combinations of the amino acid substitutions in NS4B were constructed by site-directed mutagenesis of the pGPE$^-$-N^{pro}-Luc-IRES-NS3 backbone as described above. The plasmids pGPE$^-$-N^{pro}-Luc-IRES-NS3/V2475A and pGPE$^-$-N^{pro}-Luc-IRES-NS3/A2563V have a single amino acid substitution in NS4B, at positions 2,475 and 2,563 of pGPE$^-$-N^{pro}-Luc-IRES-NS3, respectively. The plasmid pGPE$^-$-N^{pro}-Luc-IRES-NS3/2475A; A2563V carries the 2 amino acid substitutions in NS4B.

Rescue of vGPE$^-$ and mutant viruses.

The cDNA-derived viruses were rescued as described previously (33). Briefly, full-length genomic pGPE$^-$ and mutant cDNA clones were linearized with *Srf*I and used as a template for run-off RNA transcription using the MEGAscript T7 kit (Ambion, Huntingdon, UK) according to the manufacturer's protocol. The *in vitro* transcribed RNA was purified on S-400 HR columns (GE Healthcare). For electroporation, SK-L cells were washed twice with ice-cold phosphate buffered saline (PBS). Approximately $10^{7.3}$ of cells were mixed with 1 μg of RNA, transferred to a 0.2 cm electroporation cuvette (Bio-Rad, Hercules, CA), and electroporated immediately by using a Gene Pulser Xcell Electroporation System (Bio-Rad) set at 200 V and 500 μF. After electroporation, the cells were plated in 60 mm dishes and incubated at 37°C and 5% CO_2. After 3 days, the supernatants were harvested and used to infect fresh SK-L cells. The viruses were termed according to the plasmid they were rescued from, replacing "p" with "v" in the

nomenclature. The viral antigens were detected by immunoperoxidase staining using the anti-NS3 monoclonal antibody (MAb) 46/1 as described previously (23, 47). The entire genomes of rescued viruses were verified by nucleotide sequencing to exclude any accidental mutation. The rescued viruses were stored at -80°C.

Virus titration.

The virus titers were determined in SK-L cells. For the titration of virus from SK-L or GPK cell supernatants, ten-fold dilutions of the supernatants were mixed with trypsinized SK-L cells, and the mixtures were seeded in 96-well plates and incubated for 4 days at 37°C and 5% CO_2. For the titration of virus from porcine blood and tissue samples, ten-fold dilutions of the samples were used to inoculate confluent monolayers of SK-L cells. After 1 h of incubation at 37°C, the inocula were replaced with fresh medium and the cells were incubated at 37°C and 5% CO_2. After 4 days of incubation, viral NS3 was detected by immunoperoxidase staining using MAb 46/1. The virus titers were calculated using the method of Reed and Muench formula (35) and expressed as 50% tissue culture infective dose ($TCID_{50}$) per ml or gram.

Serial passages of viruses in pigs.

The serial passage of the GPE^- strain in pigs was initiated by intramuscular injection of 1 ml of cell culture supernatant containing $10^{7.6}$ $TCID_{50}$ of GPE^- virus into two 4-week-old

crossbred Landrace × Duroc × Yorkshire SPF pigs (Yamanaka Chikusan, Hokkaido, Japan). The pigs were euthanized on day 4 post-inoculation (pi), and the tonsils were collected aseptically. The tonsils were homogenized using a pestle and mortar, and mixed to make a pool of 10% tissue suspension to be used for subsequent passaging. The tonsil suspensions were injected intramuscularly into 2 new naïve pigs. This process were repeated for a total of 15 times. The viruses isolated after each passage were identified by the name of the parental strain and the passage number. For instance, GPE$^-$/P-15 indicates that the GPE$^-$ virus was passaged 15 times in pigs. The GPE$^-$/P-3 virus could be isolated from the tonsils of 1 pig only, since the other pig died accidentally before the experiment.

All animal experiments were carried out in self-contained isolator units (Tokiwa Kagaku, Tokyo, Japan) in a BSL-3 facility of the Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan. The institutional animal care and use committee of the Graduate School of Veterinary Medicine authorized this animal experiment (approval number: 10-0106) and all experiments were performed according to the guidelines of this committee.

Experimental infection of pigs.

In order to assess the pathogenicity of the GPE$^-$, GPE$^-$/P-11, and ALD/A76 isolates, and of the recombinant vGPE$^-$ and mutants thereof, 5 pigs per group were inoculated by intramuscular injection of 1 ml of virus from cell culture supernatant. The pigs were monitored for clinical

signs over a period of 14 days and their blood collected in tubes containing EDTA (TERUMO, Tokyo, Japan) on days 0, 3, 5, 7, 9, 11, and 14 pi. Total white blood cell and platelet numbers were counted using a pocH-100iV Diff apparatus (Sysmex, Hyogo, Japan). The pigs that survived the infections were euthanized on day 14 pi, and tissue from tonsils, kidneys, and mesenteric lymph nodes were collected aseptically. The tissues were also collected for virus recovery from pigs that died of disease during the course of the experiments. The tissue samples were homogenized in MEM to obtain a 10% suspension for virus titration. The virus titers were expressed as $TCID_{50}$ per ml (blood) or gram (tissue).

Virus replication kinetics.

The replication kinetics of the parent and mutant viruses in SK-L or GPK cells were determined by inoculation of confluent cell monolayers at a multiplicity of infection (MOI) of 0.001 and collection of cell culture supernatants at different times pi. After inoculation, the SK-L cells were incubated at 40°C in the presence of 5% CO_2 and the GPK cells at 30°C. The supernatants were collected on days 0, 1, 2, 3, 4, 5, 6, and 7 pi, and the virus titers were determined in SK-L cells.

Focus formation assay.

SK-L cells were seeded in Lab-Tek II Chamber Slides (Thermo Fisher Scientific, Waltham,

MA) and infected with parent and mutant viruses at an MOI of 0.0001. After incubation at 37°C for 1 h, the cells were washed once with PBS and overlaid with MEM containing 0.295% TPB, 1.0% Bacto-agar (Becton Dickinson), and 10% horse serum, and incubated at 37°C in the presence of 5% CO_2. After 72 h, the cells were fixed with 100% acetone for 10 min and incubated for 1 h with MAb 46/1. The cells were then washed with PBS and incubated for 1 h with a FITC-conjugated goat anti-mouse IgG (MP Biomedicals, Morgan Irvine, CA). After incubation with the conjugate, the cells were washed again and the NS3-positive foci of infected cells were counted using a fluorescence microscope (Axiovert 200; Carl Zeiss, Oberkochen, Germany). The number of infected cells/focus was determined for 30 independent foci (n=30), and the statistical significance was calculated using the *t* test.

Viral RNA replication assay.

For RNA replication kinetics of the parental virus and mutant viruses carrying amino acid changes in NS4B, SK-L cell monolayers were inoculated at an MOI of 1.5. After 1 h, the cells were washed twice with PBS and incubated at 37°C in the presence of 5% CO_2. Total RNA was extracted from the cells at 2, 7, and 12 hours pi using TRIzol Reagent and reverse transcribed as described above. The cDNA of CSFV was analyzed with quantitative PCR (qPCR) using the KAPA PROBE Fast qPCR Kit (Kapa Biosystem, Woburn, MA) and a Light Cycler® 480 II (Roche) and the primers CSF-100F (sense), CSF-190R (antisense), and CSF-Probe 1 (probe) reported by

Hoffmann *et al.*, (18). Cycling consisted of denaturation at 95°C for 10 sec, followed by 42 amplification cycles (94°C for 15 sec, 57°C for 30 sec, and 68°C for 30sec). The results representing the relative copy number of viral RNA compared with the copy number at 2 h post-inoculation were recorded from 3 independent experiments, and each experiment was performed in duplicate. The statistical significance was determined with the *t* test.

Luciferase assay.

Replicon RNA was transcribed *in vitro* from linearized plasmid DNA with *Srf*I as described above. In order to assess the efficiency of viral genome replication by luciferase expression, $10^{6.8}$ CPK cells were electroporated with 1 μg of replicon RNA and 100 ng of pRL-TK (Int-) reporter plasmid (Promega, Madison, WI). Electroporation was performed at 200 V and 500 μF as described above. The cells were seeded in 6-well plates and incubated at 37°C in the presence of 5% CO_2. After 6 h, 12 h, and 24 h of transfection, cell extracts were prepared with 250 μl of passive lysis buffer, and firefly and *Renilla* luciferase activities were measured using the dual luciferase reporter assay system (Promega) and a Lumat LB9507 luminometer (Berthold, Freiburg, Germany). The firefly luciferase activity was normalized with the corresponding *Renilla* luciferase activity. The results were recorded from 3 independent experiments, and each experiment was performed in duplicate. The statistical significance was calculated using the *t* test.

Results

Serial passages of the GPE$^-$ strain in pigs.

The live attenuated GPE$^-$ vaccine virus was obtained after multiple passages of the virulent ALD virus in non-natural host cells (48, 51). Under field conditions, the GPE$^-$ vaccine virus is not transmitted from pig to pig (48). In order to assess whether the attenuated GPE$^-$ strain can re-adapt to pigs and recover pathogenicity when transmission from pig to pig is forced, the virus was serially passaged in pigs by injection of virus recovered from infected animals. The serial propagation of the virus was initiated in 2 pigs by intramuscular injection of 1 ml cell culture supernatant containing $10^{7.6}$ $TCID_{50}$ of the GPE$^-$ strain. On day 4 pi the tonsils were collected, homogenized, and re-inoculated intramuscularly into 2 naïve pigs. Passaging of tonsil homogenates from infected pigs was performed for a total of 15 times. After the first passage of tonsil homogenate, the GPE$^-$/P-1 titers in the tonsils of the infected pigs were $10^{3.8}$ and $10^{4.0}$ $TCID_{50}$/g (Table 1). The virus titers increased after the 3rd and 6th passages to $10^{6.3}$ $TCID_{50}$/g and $10^{6.8}$ /$10^{7.5}$ $TCID_{50}$/g, respectively, and remained stable for the following passages.

Amino acid changes acquired during serial passages in pigs.

In order to determine whether the serial passages of the GPE$^-$ virus in pigs resulted in selection of mutant viruses, the complete genome sequences of the viruses recovered after each

passage were analyzed by direct sequencing of DNA fragments amplified by RT-PCR. A total of 11 amino acid changes occurred during the 15 passages. Three amino acid substitutions were retained until the end of the serial passages (Table 1). A valine to alanine substitution at position 2,475 in NS4B (V2475A) occurred after the first passage already, and was maintained during all subsequent passages. After the 5th passage, a threonine to alanine change at position 830 in E2 (T830A) emerged and was also maintained for the 15 passages. In NS4B, a second substitution, alanine to valine at position at 2,563 (A2563V), emerged after the 6th passage and remained stable too.

Pathogenicity of the viruses passaged in pigs.

In order to determine the effect on pathogenicity of the 3 mutations T830A, V2475A, and A2563V acquired by the GPE$^-$ virus during adaptation in pigs, 5 pigs were inoculated intramuscularly with 1 ml of GPE$^-$/P-11 virus, a 10% tonsil suspension recovered from the pigs infected with the GPE$^-$/P-10 virus. The inoculum contained $10^{6.9}$ $TCID_{50}$ of infectious virus. As control, 2 groups of 5 pigs were inoculated with $10^{7.0}$ $TCID_{50}$ of GPE$^-$ and ALD/A76, respectively. In the pigs inoculated with the GPE$^-$ strain, transient leukocytopenia and thrombocytopenia were observed without clinical signs (Fig. 1A, B and Table 2). Small amounts of viruses were recovered from blood samples of 3 of the 5 pigs on days 3 and 5 pi, and from tonsils of 1 pig on day 14 pi. In contrast, 2 of the 5 pigs inoculated with the GPE$^-$/P-11

virus had diarrhea and dysstasia, and 1 pig died on day 11 pi. All 5 pigs had severe leukocytopenia and thrombocytopenia. Accordingly, virus was detectable in blood of all pigs on day 3 pi, and could be detected in 1 pig for a period of 14 days. Infectious virus could also be isolated from tonsils, kidney and/or mesenteric lymph nodes in all 5 pigs and from all 3 organs in 2 pigs (Table 2). With the ALD/A76 strain, diarrhea, cyanosis, and/or weight loss were observed in 4 of the 5 pigs, and all pigs had severe leukocytopenia and thrombocytopenia. High viruses titers were detected in the blood and tissue of all pigs. These data demonstrate that the GPE^- strain acquired pathogenicity by re-adaptation in pigs. However, the re-adapted virus did not reach the pathogenicity of the ALD/A76 strain.

Pathogenicity of the $vGPE^-$ and mutant viruses in pigs.

The pig-adapted viruses isolated from the tonsils are likely to possess some degree of heterogeneity, as suggested by the genome quasispecies distribution observed in the RNA preparations from passages 12 to 15 (Table 1). Therefore, in order to determine which amino acid changes are responsible for the increased pathogenicity acquired during passaging, recombinant viruses were generated by site-directed mutagenesis in the $vGPE^-$ backbone using reverse genetics (Fig. 2A and B). The pathogenicity of the reconstructed mutant viruses was determined in pigs and compared with the parent $vGPE^-$ virus. For each recombinant virus, 5 pigs were infected by intramuscular injection of $10^{7.0}$ $TCID_{50}$ of virus. Clinical signs and virus

content in blood and organs were monitored. Infection with the parent vGPE$^-$ did not result in any clinical sign, with no or only very little amount of virus detection in blood and tonsils (Table 3). The virus with the single alanine to valine change in NS4B at position 2,563 (vGPE$^-$/A2563V) behaved similar to the parent vGPE$^-$ virus. Also, the 3 viruses with a single amino acid change did not show any major leukocytopenia and thrombocytopenia (Fig. 3). Slightly better replication in pigs than the parent virus, in the absence of any clinical signs too, was observed with the virus carrying the single mutation at position 2,475 in NS4B (vGPE$^-$/V2475A). None of the other mutant viruses induced any obvious clinical signs either, despite higher titers in the tonsils, except for the virus with the 3 amino acid substitutions (vGPE$^-$/T830A; V2475A; A2563V) that resulted in cyanosis, diarrhea, and/or weight loss in 4 of the 5 pigs, in severe leukocytopenia and thrombocytopenia in all 5 animals, and in prolonged virus shedding for up to 11 days after infection (Table 3 and Fig. 3). Prolonged virus shedding was also observed up to day 9 pi in pigs infected with viruses carrying the T830A substitution alone and in combination with the V2475A or the A2563V mutation in NS4B. In pigs inoculated with these latter 2 viruses (vGPE$^-$/T830A; V2475A and vGPE$^-$/T830A; A2563V), the highest titers in blood and tonsils were observed, with severe leukocytopenia and thrombocytopenia, similar to the infection with the triple mutant virus. In addition, after infection with the triple mutant virus, infectious virus was also detected in the mesenteric lymph nodes of 2 out of 5 pigs, as opposed to infection with any of the other mutants. No

additional amino acid change was detected in viruses recovered from tonsils of pigs inoculated with the triple mutant virus on day 14 pi . Altogether, these results demonstrate that the 3 amino acid substitutions act in concert in the acquisition of pathogenicity after adaptation of the GPE$^-$ virus in pigs.

Comparison of *in vitro* growth kinetics of the mutant viruses.

At 30°C, the GPE$^-$ strain replicates efficiently in guinea pig cells in which it was adapted, as opposed to the ALD and other wild type strains that grow very poorly in these cells (48). Therefore, multistep growth curves of the recombinant viruses carrying the different amino acid substitutions in the vGPE$^-$ backbone were evaluated in SK-L and in the primary guinea pig (GPK) cell cultures in comparison with the vGPE$^-$ and ALD/A76 viruses (Fig. 4). Viruses that carry single amino acid change in NS4B (A2563V) replicated better than the GPE$^-$ virus in the porcine cell lines at 40°C at all time points. Two mutant viruses carrying the double amino acid substitutions (vGPE$^-$/T830A; V2475A and vGPE$^-$/V2475A; A2563V), and the virus carrying the triple mutations (vGPE$^-$/T830A; V2475A; A2563V) did also replicated more efficiently than the vGPE$^-$ virus in swine cells. Except for the vGPE$^-$/V2475A virus, the mutant viruses with single and double mutations in the vGPE$^-$ backbone lost replication efficiency in the GPK cells when compared with the vGPE$^-$ virus. Both, the ALD/A76 virus and the virus with 3 adaptive mutations in the GPE$^-$ backbone (vGPE$^-$/T830A; V2475A; A2563V) did not replicate at all in the

GPK cells, as opposed to the parent vGPE$^-$ virus. These replication kinetics were confirmed with kinetics of parallel samples analyzed by qPCR (data not shown). Thus, these results show that the 3 amino acid substitutions found during adaptation in pigs act together to enhance virus replication in swine cells and to abolish replication in GPK cells, which is in agreement with the effect of the 3 adaptive mutations on pathogenicity in pigs.

Effect of the adaptive mutations on virus focus formation.

The adaptive mutation in E2 is likely to influence virus spreading while the mutations acquired in NS4B may determine the RNA replication efficiency. In order to determine the effect of these amino acid substitutions on viral spreading in swine cells, monolayers of SK-L cells were infected with the mutant and parent viruses and the focus formation was monitored under a semi-solid overlay using viral antigen detection by immunofluorescence. The number of infected cells per focus was counted for 30 randomly selected foci and the data was analyzed statistically. The recombinant GPE$^-$ virus carrying all 3 adaptive mutations produced the largest foci, comparable in size with the foci obtained with the ALD/A76 virus (Fig. 5). The smallest foci were observed with the vGPE$^-$ virus and with the mutants carrying 1 or 2 mutations in NS4B. All mutants that carried the T830A substitution alone or together with 1 of the substitutions in NS4B resulted in foci of intermediate size. These results indicate that the T830A substitution acquired in E2 during adaptation in pigs is a major determinant of virus

spreading in swine cells.

Effect of the mutations in NS4B on viral RNA replication.

Two independent approaches were used to determine the effect of the adaptive mutations in NS4B on viral RNA replication. With qPCR, the RNA replication efficiency of the vGPE$^-$/V2475A and vGPE$^-$/V2475A; A2563V viruses was significantly higher than that of the parental vGPE$^-$ virus at 7 h post-infection (Fig. 6). At 12 h post-infection, the RNA replication efficiency of the 2 viruses carrying a single mutation was significantly higher than that of the parent vGPE$^-$ virus.

As an alternative approach to measure the activity of the viral RNA replicase complex, a bicistronic reporter replicon was constructed with the vGPE$^-$ genome. This replicon consisted of the 5' and 3' UTR flanked by a chimeric N^{pro}-firefly luciferase gene separated from the NS3 to NS5B genes of the replication complex by an EMCV IRES (Fig. 7A). The 2 adaptive mutations in NS4B were introduced separately or in combination in the reporter replicon backbone. The relative luciferase activities obtained with the parent and mutant replicons were determined in the swine CPK cell line. The replicon carrying the 2 adaptive mutations in NS4B resulted in 10-fold higher luciferase activity than the parent rGPE$^-$-N^{pro}-Luc-IRES-NS3 replicon 24 h after transfection (Fig. 7B). At this time point, significantly higher luciferase activities when compared with the parent replicon were also observed with the replicons carrying a single amino

acid substitution. These data indicate that the amino acid substitutions acquired in NS4B of the GPE$^-$ strain during adaptation in pigs contribute to enhanced virus replication in swine cells by increasing the activity of the viral replication complex.

Discussion

The live attenuated vaccine strain GPE$^-$ was widely used in Japan to control CSF (57). The GPE$^-$ strain was generated from the virulent ALD strain through multiple passages and cloning in cells of swine, bovine, and guinea pig origin (48, 51). Field studies have proven that the GPE$^-$ vaccine virus is safe and efficacious. The virus is not able to spread, neither horizontally nor vertically, and reduces the prevalence of CSF by increasing herd immunity (34, 49). However, the molecular basis of the attenuation was unknown. Using an artificial virus transmission protocol in order to force re-adaptation to pigs of the live attenuated guinea pig-adapted GPE$^-$ virus, 3 amino acid residues that are critical for determining the pathogenic difference between the GPE$^-$ and the ALD strains were identified in E2 and NS4B. Importantly, mutation of these residues in the 2 genes had a synergistic effect on the pathogenicity *in vivo* and on virus spread and replication efficiency in cell culture. To the best of our knowledge, this is the first report showing that the pathogenicity of CSFV can be enhanced by the substitution of selected amino acids in the backbone of a low virulent virus.

A total of 46 amino acid differences were found between the parent ALD virus and the GPE$^-$

vaccine strain (22). Since the pathogenicity of the triple mutant GPE$^-$ virus is not as high as the pathogenicity of the original ALD virus, additional changes are likely to determine the virulence of the ALD strain. Importantly, several virulent strains such as CSFV Brescia, Eystrup and Alfort/Tübingen carry the same amino acid residues as those acquired by the GPE$^-$ virus in E2 and NS4B during passaging in pigs. However, these amino acids are also present in the CSFV C-strain, a vaccine strain generated by adaptation in rabbits, indicating that other mutations are responsible for the attenuation of the C-strain. All this considered, one can conclude that the specific residues identified in this study to be critical for determining virulence in pigs do not represent general virulence determinants of CSFV, suggesting that virulence of CSFV is a complex multigenic trait involving different genes and functions. This is reflected in numerous reports in which mutations of different functional domains in the structural proteins C, E^{rns}, E1, E2, and in the nonstructural proteins p7 and NS4B were shown to reduce the pathogenicity in pigs, indicating that these genes may be involved in determining CSFV virulence (7, 8, 9, 10, 11, 12, 13, 32, 36, 37, 38, 39, 40, 46, 54).

The fact that the amino acid change V2475A was found in NS4B after 1 passage in pigs suggests that the alanine at position 2,475 was already present in the quasispecies population of the original inoculum. This hypothesis was indeed confirmed by deep sequencing analysis (Table 4). Additional direct sequencing data further indicate that the changes acquired during the passaging in pigs occurred from a shift of the quasispecies distribution during host-driven

selection *in vivo*.

In the present study, the T830A substitution in E2 acquired during passaging in pigs reduced the virus replication in guinea pig cells while enhancing the spreading and replication in pig cells, when compared with the parent vGPE$^-$ virus. These results suggest that the residue at position 830 in E2 is involved in host tropism and plays a role in cell to cell spreading, probably at the level of viral attachment and entry, as the glycoproteins E2 and E^{rns} of pestiviruses mediate viral attachment and entry into host cells (19, 41, 56). Unfortunately, there is only limited information available on the receptors and pathways involved in CSFV binding and entry. Further investigations are necessary to clarify the impact of the alanine at position 830 of E2 on virus infection of swine cells. E2 is exposed on the outer surface of the virus and is the most immunodominant protein inducing the major neutralizing antibodies in infected pigs (58). The amino acid at position 830 is located in the N-terminal region of E2 and this region is the minimal domain required for binding to pig antibodies generated during CSFV infection (29, 61). Recent studies support the importance of this domain for the pathogenicity of CSFV. In the Brescia strain, amino acid substitutions in this domain, including the amino acid at position 830, attenuated virulence in pigs (37).

In NS4B, the amino acid substitutions at positions 2,475 and 2,563 have not yet been reported to be involved in virulence. On the basis of the predicted topology of CSFV NS4B, the residues at position 2,475 and 2,563 are located in the N-terminal and C-terminal

transmembrane domain, respectively. A recent study on the biosynthesis of the CSFV nonstructural proteins showed that NS4B is mostly found attached to NS5A (28). The role of transmembrane domains of CSFV NS4B in the virus life cycle is not well understood, while these domains of hepatitis C virus (HCV) are reported to play a role in viral replication (15). Importantly, a study identified a putative Toll/interleukin-1 receptor-like domain in the C-terminal region of CSFV NS4B. Mutations in this domain of NS4B in the highly virulent CSFV Brescia backbone, immediately downstream of the residue 2,563 identified here, resulted in an attenuated phenotype along with enhanced activation of TLR-7-induced genes (7). NS4B harbors also an NTPase motif. The NTPase activity is an absolute requirement for CSFV replication, but there is no evidence yet that modulating the NTPase activity may affect virulence (10). The 2 amino acids of NS4B identified in the present study are not located within the NTPase motif, but are clearly determining the replication efficiency of the virus *in vivo* and *in vitro.* These substitutions may enhance the efficiency of viral RNA replication by influencing the stability of the replicase complex and/or the interaction of the replicase complex with host factors in swine. However, the function of NS4B in the replication complex of pestiviruses is not well understood. CSFV NS4B is relatively labile when compared to other non-structural proteins (28). Several studies show that NS4B of flaviviruses and HCV is an integral membrane protein of the endoplasmic reticulum and is involved in viral genome replication, interacting closely with other viral nonstructural proteins during the cycle of

replication, and in virus assembly and release (5, 14, 20, 24, 31).

With the adaptive mutations in E2 and NS4B combined in the GPE$^-$ backbone, the pathogenicity of the mutant viruses was clearly enhanced but did not equal the pathogenicity of the ALD/A76 strain, indicating that additional amino acid substitutions elsewhere in the genome may play a role in the pathogenicity of CSFV. Pestiviruses suppress innate immune defenses by preventing IFN-α/β induction; a property attributed to the viral proteins N^{pro} and E^{rns}. N^{pro} interferes with the induction of IFN-α/β by the promoting the proteasomal degradation of interferon regulatory factor 3 (IRF3) (26, 42). During the generation of the live attenuated GPE$^-$ vaccine strain, N^{pro} has acquired a mutation that abolishes its capacity to bind and degrade IRF3 and thus to interfere with the induction of IFN-α/β (43). In the parental ALD strain, N^{pro} is functional in terms of inhibition of IFN-α/β induction (42, 55). Interestingly, during the re-adaptation of the GPE$^-$ vaccine in pigs, the IRF3-degrading function of N^{pro} was not recovered. Whether restoring the N^{pro} function in the triple mutant GPE$^-$ virus would further enhance its pathogenicity is under current investigation. Previous data suggest that the IRF3 degrading function of N^{pro} may support the pathogenicity of low and moderate virulent strains but has only little influence on the pathogenicity of high virulent viruses: Mutations to knock out the N^{pro}-mediated IRF3 degradation did not alter the pathogenicity of the highly virulent CSFV strain Eystrup and resulted only in slight attenuation of the moderately virulent vA187-1 virus (43). E^{rns} forms disulfide-linked homodimers and possesses ribonuclease

(RNase) activity (45, 50). E^{rns} interacts with extracellular dsRNA, thereby targeting a major viral IFN-inducing signal (21). Knocking out the RNase activity in the CSFV Alfort/Tübingen backbone resulted in attenuation in pigs (32). Amino acid substitution at the glycosylation sites of E^{rns} of the Brescia strain did not affect RNase activity but attenuated the virus (46). The amino acids of E^{rns} reported to determine virulence of CSFV are found in virulent strains and in the GPE^- strain and as well. Therefore, it is not clear whether E^{rns} may also be involved in determining the pathogenicity of the ALD strain.

In summary, this is the first study showing that amino acid substitutions in E2 and NS4B can enhance the pathogenicity of CSFV in pigs by an additive effect enhancing viral spread and genome replication. The acquisition of pathogenicity is related to the selection of a pig-adapted mutant virus using a vaccine strain that had been depathogenized through adaptation in a non-natural host. Nevertheless, by demonstrating gain of virulence and increased virus replication after site-directed mutagenesis, these data show that a structural and a nonstructural protein of CSFV can carry virulence determinants that can function in a synergistic manner to determine the pathogenicity of the virus in pigs. Improved understanding of the molecular basis of CSFV pathogenicity contributes to understanding the molecular mechanisms involved in CSF pathogenesis, which is important for the implementation of efficient disease control and vaccine strategies.

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Figure legends

FIG. 1. White blood cell and platelet counts in pigs inoculated with the GPE$^-$, GPE$^-$/P-11, or ALD/A76 strains. Groups of 5 pigs were inoculated with the indicated viruses and blood was collected on days 0, 3, 5, 7, 9, 11, and 14 pi. The white blood cell (WBC) (A) and platelet (PLT) (B) counts were determined for each time point and are shown as mean value with error bars representing the standard deviation. The statistical significance of the differences between the cell counts from the pigs inoculated with GPE$^-$ virus and the pigs inoculated with the other viruses was calculated with the *t* test. The "a" indicates $p<0.05$ between GPE$^-$ and GPE$^-$/P-11. The "b" indicates $p<0.05$ between GPE$^-$ and ALD/A76. One pig died on day 11 pi (†). All remaining pigs were euthanized on day 14 pi.

FIG. 2. Schematic representation of the cDNA clones of the GPE$^-$/P-11 and mutant vGPE$^-$ viruses. Three amino acid substitutions T830A, V2475A, and A2563V were found in the GPE$^-$/P-11 virus (A). Seven recombinant viruses with all possible combinations of amino acid substitutions were generated by site-directed mutagenesis of the GPE$^-$ backbone (B). The white and gray boxes indicate the non-structural and structural proteins, respectively.

FIG. 3. White blood cell and platelet counts in pigs inoculated with the vGPE$^-$ parent and mutant viruses. Groups of 5 pigs were inoculated with the indicated viruses and blood was

collected on days 0, 3, 5, 7, 9, 11, and 14 pi. The WBC and PLT counts were determined for each time point and are shown as mean value with error bars representing the standard deviation. The statistical significance of the differences between the cell counts from the pigs inoculated with vGPE$^-$ virus and the pigs infected with the other viruses was calculated with the *t* test. The "*" indicates $p<0.05$. In each graph the mean WBC and PLT counts of the pigs inoculated with the vGPE$^-$ parent are shown for comparison purposes. The pigs were euthanized on day 14 pi.

FIG. 4. Growth kinetics of the vGPE$^-$ parent and mutant viruses and of the ALD/A76 strain in swine cells and guinea pig cells. Swine kidney cell lines (SK-L cells) and the primary guinea pig kidney (GPK) cells were inoculated at a multiplicity of infection (MOI) of 0.001 with the parent and mutant viruses as indicated. The SK-L and GPK cells were incubated at 40°C in the presence of 5% CO_2 and at 30°C, respectively. The supernatants were collected at the indicated times. The titers were determined in SK-L cells and expressed as 50% tissue culture infective dose ($TCID_{50}$) per ml. In each graph, the titers of the vGPE$^-$ and ALD/A76 strains are included for comparison purposes. The "*" indicates that the virus titer was below detection the limit of $10^{1.8}$ $TCID_{50}$/ml.

FIG. 5. Focus formation of vGPE$^-$, mutant viruses, and ALD/A76 in SK-L cell cultures. SK-L

cells were inoculated at an MOI of 0.001 with the parent and mutant viruses as indicated. The cell monolayers were overlaid with MEM containing 1.0% Bacto-agar and incubated at 37°C in the presence of 5% CO_2. After 72 h of incubation, the cells were fixed with acetone and viral NS3 was detected by immunofluorescence using MAb 46/1. The number of infected cells per focus was counted for 30 independent foci (n=30). Error bars represent the standard deviation and the significance of the differences was calculated with the *t* test. The "∗" indicates $p<0.05$.

FIG. 6. Effect of amino acid substitutions in NS4B on RNA replication efficiency. In order to assess the effect of the amino acid substitutions in NS4B on the viral RNA replication efficiency, confluent the SK-L cell monolayers were inoculated at an MOI of 1.5 with the parent and mutant viruses carrying the indicated amino acid changes in NS4B. Parallel cultures of infected cells were incubated at 37°C in the presence of 5% CO_2 for 2 h, 7 h and 12 h. Total RNA was extracted from the infected cells at the indicated times. The relative RNA copy numbers compared with the copy number at 2 h post-inoculation are shown. Each bar represents the mean value of 3 independent experiments and the error bars show the standard deviation. Pairwise comparisons of the values were carried out using the *t* test. The "∗" indicates $p<0.05$.

FIG. 7. Effect of amino acid substitutions in NS4B on the viral RNA replication efficiency. (A)

A GPE$^-$-derived bicistronic replicon consisting of the 5' and 3' UTR flanking a chimeric N^{pro}-firefly luciferase gene separated from the NS3 to NS5B genes by the IRES of EMCV was constructed. Single amino acid substitutions at position 2,475 and 2,563 were introduced by site-directed mutagenesis as indicated. (B) In order to analyze the effect of the amino acid substitutions on the viral RNA replication efficiency, $10^{6.8}$ CPK cells were transfected with 1 μg of each replicon RNA and with 100 ng of pRL-TK (Int-) reporter plasmid for constitutive *Renilla* luciferase expression. After 6 h, 12 h, and 24 h of incubation at 37°C in the presence of 5% CO_2, the firefly and *Renilla* luciferase activity expressed in relative light units was assayed in cell lysates. The firefly luciferase activity was normalized with the corresponding *Renilla* luciferase activity from the same lysate. Each bar represents the mean value of 3 independent experiments with error bars showing the standard deviation. The values obtained with each construct were compared pairwise using the *t* test. The "*" indicates $p<0.05$.

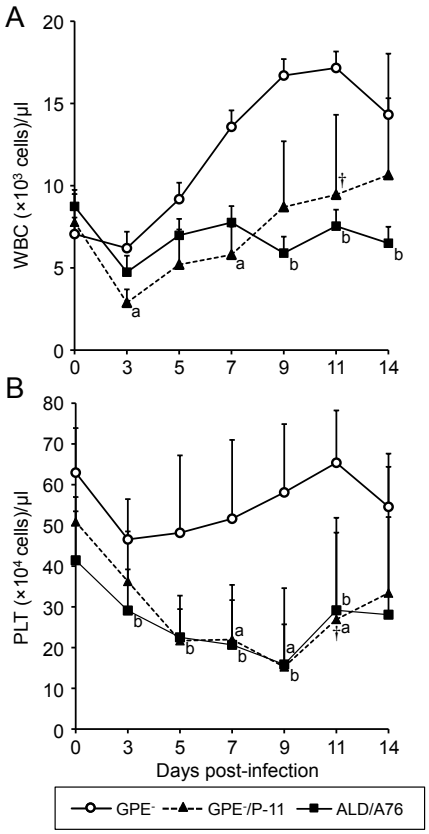

Figure 1. Tamura *et al.*

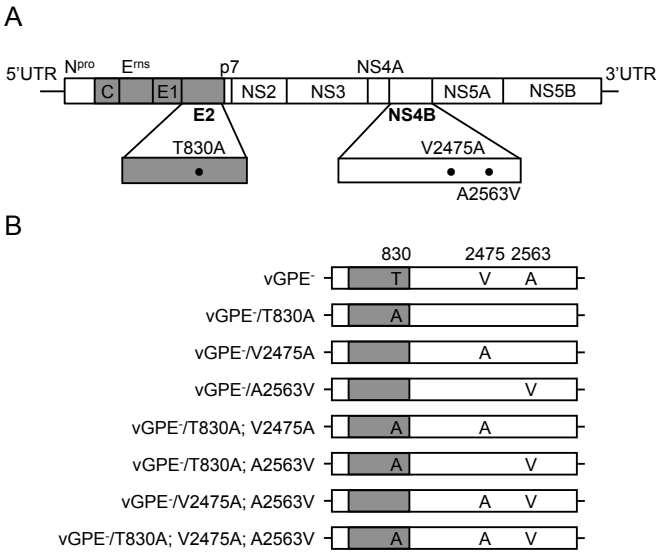

Figure 2. Tamura *et al.*

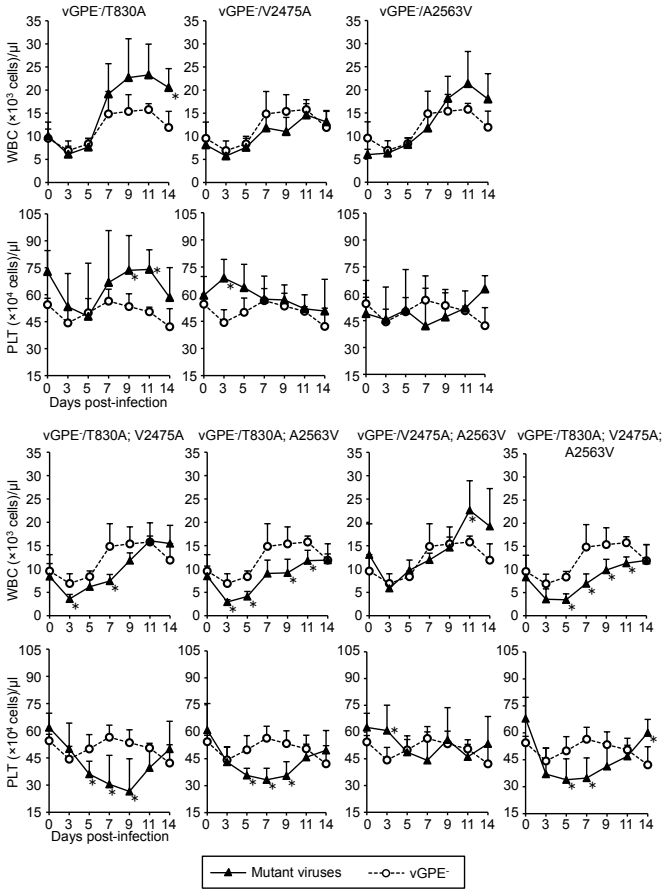

Figure 3. Tamura *et al.*

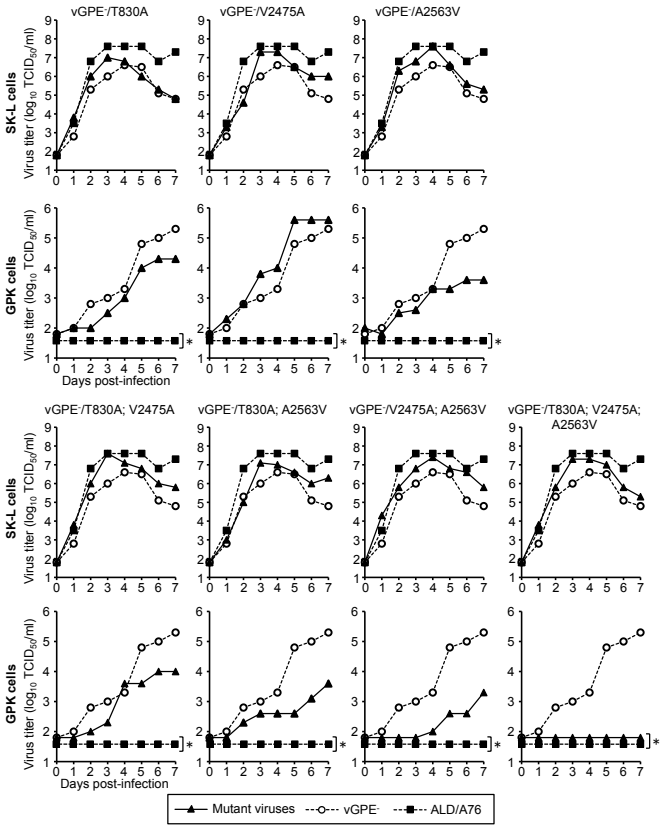

Figure 4. Tamura *et al.*

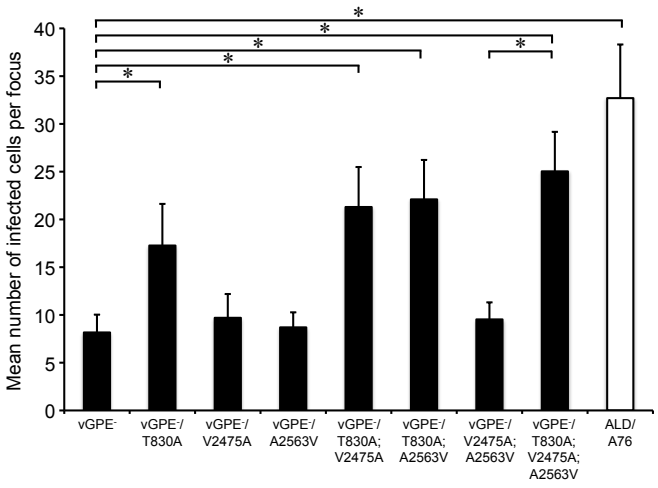

Figure 5. Tamura *et al.*

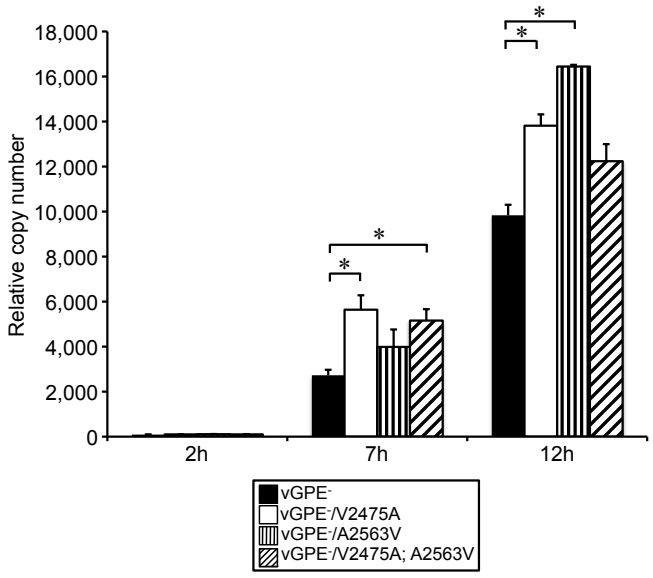

Figure 6. Tamura *et al.*

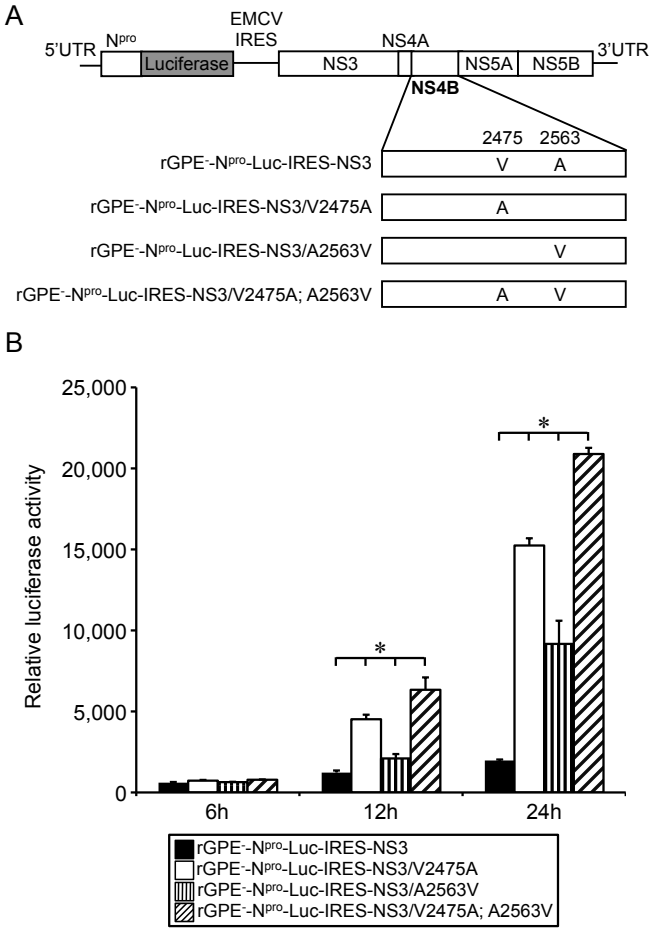

Figure 7. Tamura *et al.*

TABLE 1. Amino acid substitutions during serial passages of the GPE$^-$ strain and virus recovery from the tonsils of infected pigs.

Virus	E^{rns}	E2				p7	NS3	NS4B			NS5A	Virus recovery
	480[a]	748	779	794	830	1085	1597	2447	2475	2563	3136	(log_{10} $TCID_{50}$/g)
GPE$^-$	K	N	S	S	T	V	V	T	V	A	A	NT[d]
GPE$^-$/ P-1	.[b]	.	.	.	.	.	.	.	A	.	.	3.8/4.0
P-2	.	.	.	.	.	.	.	.	A	.	.	4.6/4.3
P-3	.	.	.	.	.	.	.	.	A	.	.	6.3
P-4	.	D	.	.	.	.	.	A	A	.	.	5.8/7.5
P-5	.	.	.	.	A	.	.	.	A	.	A/V	6.6/6.3
P-6	.	.	.	.	A	.	.	.	A	A/V	.	6.8/7.5
P-7	.	.	.	.	A	.	.	.	A	V	.	6.8/7.3
P-8	.	.	.	.	A	.	.	.	A	V	.	6.8/7.8
P-9	.	.	.	.	A	.	.	.	A	V	.	7.3/7.8
P-10	.	.	.	.	A	.	.	.	A	V	.	7.8/6.6
P-11	.	.	.	.	A	.	.	.	A	V	.	7.8/8.0
P-12	K/N[c]	.	S/P	.	A	.	.	.	A	V	.	7.0/7.0
P-13	.	.	S/P	.	A	.	.	.	A	V	.	7.1/7.3
P-14	.	.	S/P	.	A	.	.	.	A	V	.	7.6/7.3
P-15	.	.	.	S/F	A	V/A	V/I	.	A	V	.	7.8/6.8
ALD/A76	.	.	.	F	A	.	.	.	A	V	.	NT

[a] The methionine encoded by the AUG start codon is defined as position 1.

[b] The periods indicate identity with the GPE$^-$ sequence.

[c] The two amino acids were found (quasispecies).

[d] Not tested.

TABLE 2. Clinical signs and virus recovery from pigs inoculated with the GPE$^-$, GPE$^-$/P-11, and ALD/A76 strains.

Inoculated virus	Clinical signs[a]	Virus recovery								
		Blood (log_{10} $TCID_{50}$/ml)[b] on dpi						Tissue (log_{10} $TCID_{50}$/g)[c]		
		3	5	7	9	11	14	Tonsil	Kidney	MLN
GPE$^-$	-	<0.8	-	-	-	-	-	-	-	-
	-	-	<0.8	-	-	-	-	<1.8	-	-
	-	-	-	-	-	-	-	-	-	-
	-	-	<0.8	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-
GPE$^-$/P-11	-	≤1.6	NT	≤1.5	NT	-	-	4.0	-	-
	Diarrhea, Dysstasia, Dead	2.5	NT	4.0	NT	NT	NT	4.3	3.3	5.0
	-	2.0	2.3	<0.8	-	-	-	≤2.8	-	<1.8
	-	2.8	3.0	3.8	3.3	4.0	3.6	4.3	<1.8	3.8
	Diarrhea, Dysstasia	2.3	2.8	3.3	3.0	≤1.0	-	-	-	≤2.0
ALD/A76	Diarrhea, Weight loss	3.0	4.8	7.0	6.6	7.0	6.0	9.5	9.3	8.8
	-	3.6	4.5	6.0	5.8	6.8	6.0	8.3	7.3	7.5
	Weight loss	3.8	4.3	6.3	5.3	6.8	6.0	8.7	8.8	8.8
	Diarrhea, Cyanosis	3.6	5.0	6.6	7.1	6.8	6.8	8.8	9.1	9.3
	Diarrhea, Weight loss	3.1	4.0	6.0	6.0	6.3	6.6	8.0	7.1	7.5

[a] -: not observed.

[b] -: not isolated, NT: not tested.

[c] -: not isolated, MLN: mesenteric lymph node.

TABLE 3. Clinical signs and virus recovery from pigs inoculated with the vGPE$^-$ and mutant viruses.

Inoculated virus	Clinical signs[a]	Virus recovery								
		Blood (log_{10} $TCID_{50}$/ml)[b] on dpi						Tissue (log_{10} $TCID_{50}$/g)[c]		
		3	5	7	9	11	14	Tonsil	Kidney	MLN
vGPE$^-$	-	-	-	-	-	-	-	-	-	-
	-	<0.8	-	-	-	-	-	<1.8	-	-
	-	-	-	-	-	-	-	<1.8	-	-
	-	-	<0.8	-	-	-	-	<1.8	-	-
	-	-	<0.8	-	-	-	-	<1.8	-	-
vGPE$^-$/T830A	-	<0.8	≤1.0	<0.8	-	-	-	<1.8	-	-
	-	<0.8	2.6	≤2.1	<0.8	-	-	3.8	<1.8	-
	-	-	≤1.0	<0.8	-	-	-	<1.8	-	-
	-	<0.8	≤1.5	-	-	-	-	<1.8	-	-
	-	<0.8	<0.8	-	-	-	-	<1.8	-	-
vGPE$^-$/V2475A	-	-	<0.8	-	-	-	-	<1.8	-	-
	-	<0.8	<0.8	-	-	-	-	<1.8	-	-
	-	-	<0.8	<0.8	-	-	-	4.0	-	-
	-	-	<0.8	-	-	-	-	2.5	-	-
	-	-	<0.8	-	-	-	-	<1.8	-	-
vGPE$^-$/A2563V	-	-	-	-	-	-	-	<1.8	-	-
	-	-	-	-	-	-	-	<1.8	-	-
	-	-	-	-	-	-	-	≤2.0	-	-
	-	<0.8	<0.8	-	-	-	-	-	-	-
	-	<0.8	-	-	-	-	-	-	-	-
vGPE$^-$/T830A; V2475A	-	2.6	3.8	3.6	-	-	-	4.8	-	-
	-	<0.8	≤1.0	-	-	-	-	3.3	-	-
	-	2.8	3.8	2.3	-	-	-	<1.8	-	-
	-	2.6	3.3	≤1.5	<0.8	-	-	2.1	-	-
	-	≤1.0	3.0	<0.8	<0.8	-	-	4.0	-	-
vGPE$^-$/T830A; A2563V	-	≤1.0	≤1.6	≤1.0	-	-	-	4.3	-	-
	-	2.8	3.3	≤1.0	-	-	-	3.8	-	-
	-	<0.8	2.8	3.0	-	-	-	2.8	-	-
	-	2.5	3.8	3.8	≤2.3	<0.8	-	3.6	-	-
	-	≤2.1	2.3	≤1.8	-	-	-	3.9	-	-
vGPE$^-$/V2475A; A2563V	-	<0.8	≤1.0	-	-	-	-	≤2.3	-	-
	-	≤1.0	≤1.1	-	-	-	-	<1.8	-	-
	-	≤1.0	≤1.1	-	-	-	-	3.3	-	-
	-	-	≤1.0	<0.8	-	-	-	≤2.3	-	-
	-	<0.8	<0.8	-	-	-	-	3.5	-	-
vGPE$^-$/T830A; V2475A; A2563V	Cyanosis	≤1.0	≤2.1	≤1.0	≤1.0	-	-	4.0	-	-
	-	≤1.0	2.6	3.6	≤2.6	<0.8	-	3.3	-	<1.8
	Weight loss, Cyanosis	≤1.1	2.8	≤2.3	≤1.3	-	-	3.7	-	-
	Cyanosis	≤1.0	2.6	2.0	≤1.0	-	-	3.0	-	-
	Diarrhea, Cyanosis	≤2.1	2.3	2.6	3.1	<0.8	-	4.1	-	≤2.3

[a] -: not observed.

[b] -: not isolated, NT: not tested.

[c] -: not isolated, MLN: mesenteric lymph node.

TABLE 4. Quasispecies composition of the orignal GPE$^-$ virus at the codon sites at which adapted mutations were rescued.

Nucleotide sequence of the each amino acid position	Rate (%)	Deduced amino acid[b]
E2 830		
ACA	>99.9	T
NS4B 2475		
GUC	97.5	V
.[a] C .	2.3	A
. G .	0.2	G
NS4B 2563		
GCA	99.0	A
. A .	0.8	E
. U .	0.2	V

[a] Periods indicate same nucleotides as above.

[b] The amino acid found after adaptation in pigs are underlined.