



HOKKAIDO UNIVERSITY

Title	Studies on the molecular basis of pathogenicity of classical swine fever virus
Author(s)	田村, 友和
Degree Grantor	北海道大学
Degree Name	博士(獣医学)
Dissertation Number	甲第11966号
Issue Date	2015-09-25
DOI	https://doi.org/10.14943/doctoral.k11966
Doc URL	https://hdl.handle.net/2115/60007
Type	doctoral thesis
File Information	Tomokazu_Tamura.pdf



**Studies on the molecular basis of pathogenicity
of classical swine fever virus**

豚コレラウイルスの病原性の分子基盤に関する研究

Tomokazu Tamura

TABLE OF CONTENTS

Contents	i
Abbreviations	ii
Notes	iv
Preface	1
Chapter I	
Selection of classical swine fever virus with enhanced pathogenicity reveals synergistic virulence determinants in E2 and NS4B	
Introduction	8
Materials and Methods	10
Results	19
Discussion	38
Summary	45
Chapter II	
N^{pro} of classical swine fever virus contributes to pathogenicity in pigs by preventing type I interferon induction at local replication sites	
Introduction	47
Materials and Methods	50
Results	56
Discussion	72
Summary	75
Chapter III	
Intracellular membrane association of the N-terminal domain of classical swine fever virus NS4B determines viral genome replication and virulence	
Introduction	77
Materials and Methods	79
Results	86
Discussion	104
Summary	109
Conclusion	110
Acknowledgements	112
References	114
Summary in Japanese (和文要旨)	129

Abbreviations

AG:	adrenal glands
BDV:	border disease virus
BSL:	biosafety level
BVDV:	bovine viral diarrhea virus
CPK cell:	cloned porcine kidney cell
CSF:	classical swine fever
CSFV:	classical swine fever virus
DAPI:	4',6-Diamino-2-phenylindole, dihydrochloride
DNase:	deoxyribonuclease
EDTA:	ethylenediaminetetraacetic acid
EMCV:	encephalomyocarditis virus
EMEM:	Eagle's minimum essential medium
END:	exaltation of Newcastle disease virus
ER:	endoplasmic reticulum
FITC:	fluorescein isothiocyanate
GAPDH:	glyceraldehyde 3-phosphate dehydrogenase
GFP:	Green Fluorescent Protein
GPK cell:	guinea pig kidney cell
HCV:	hepatitis C virus
IFN:	interferon
IFN- α/β :	type I interferon
IL:	interleukin
IRES:	internal ribosome entry site
IRF:	interferon regulatory factor
mAb:	monoclonal antibody
MLN:	mesenteric lymph node

MOI:	multiplicity of infection
NDV:	Newcastle disease virus
NTPase:	nucleoside triphosphate phosphohydrolase
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
pDC:	plasmacytoid dendritic cell
pi:	post-inoculation
PLT:	platelet
pt:	post-transfection
qPCR:	quantitative real-time polymerase chain reaction
RNase:	ribonuclease
RT:	reverse transcription
SDS-PAGE:	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SK-L cell:	swine kidney L cell
SK-6 cell:	swine kidney 6 cell
SPF:	specific pathogen free
TCID ₅₀ :	50% tissue culture infective dose
TIR:	Toll-interleukin-1 receptor-like
TLR	Toll-like receptor
TNF:	tumor necrosis factor
TPB:	tryptose phosphate broth
UTR:	untranslated region
WBC:	white blood cell

Notes

Contents of the present thesis were published in the following articles.

1. **Tamura T, Sakoda Y, Yoshino F, Nomura T, Yamamoto N, Sato Y, Okamatsu M, Ruggli N, Kida H.** 2012. Selection of classical swine fever virus with enhanced pathogenicity reveals synergistic virulence determinants in E2 and NS4B. *J. Virol.* **86**:8602-8613.
Copyright © American Society for Microbiology
2. **Tamura T, Nagashima N, Ruggli N, Summerfield A, Kida H, Sakoda Y.** 2014. N^{pro} of classical swine fever virus contributes to pathogenicity in pigs by preventing type I interferon induction at local replication sites. *Vet. Res.* **45**:47.
Copyright © Creative Commons Attribution License CC BY 2.0
3. **Tamura T, Ruggli N, Nagashima N, Okamatsu M, Igarashi M, Mine J, Hofmann MA, Liniger M, Summerfield A, Kida H, Sakoda Y.** 2015. Intracellular membrane association of the N-terminal domain of classical swine fever virus NS4B determines viral genome replication and virulence. *J. Gen. Virol.* doi: 10.1099/vir.0.000200.
Copyright © Society for General Microbiology

Preface

Classical swine fever (CSF) is an economically important and highly contagious disease of swine (family *Suidae*), which is caused by classical swine fever virus (CSFV) (1). Field isolates of CSFV differ in virulence, resulting in clinical disease with different degrees of severity. Besides host factors such as breed, age, immune competence, and nutritional status of the animal, chiefly the virulence of the CSFV isolate *per se* determines disease severity (2). The infection can run peracute, acute, subacute, chronic, late onset, or inapparent course (Table 1). Acute CSF is caused by the highly virulent virus and generally results in high morbidity and mortality, whereas infections with low-virulent virus can go unnoticed. Pigs with acute CSF develop severe clinical manifestations within 6 days of exposure to a highly virulent virus, high fever reached around 42°C, anorexia, depression, marked discharge associated with conjunctivitis, constipation followed by a severe, watery, yellowish gray diarrhea, vomiting, weak hind legs, and purplish discoloration of the skin. Concurrent with fever, a leukopenia and thrombocytopenia are observed. Most pigs that suffer from acute CSF have viremia but not an antibody until they die between 8 and 20 days after infection. In subacute CSF, pigs show less severe signs of disease and succumb within 30 days. Persistence of CSFV in the host can result in different clinical syndromes. Leukopenia is a consistent manifestation of persistent CSF, but in the terminal stages of disease, leukocytosis may develop. Chronic CSF is caused by moderately virulent virus. There are three phases of chronic CSF based on the clinical signs observed. In the first, or acute, phase of illness, anorexia, depression, elevated temperature, and leukopenia are present. After several weeks, the appetite and general appearance of the pigs improve markedly, and their temperatures decrease to normal or slightly above normal values. Leukopenia of the infected pigs usually persists. This general clinical

Table 1. General features of classical swine fever

Feature	Acute	Chronic	Late onset
Virulence of virus	High	Moderate	Low
Course of disease	Short incubation period, high fever, anorexia, depression, eye conjunctivitis, constipation, diarrhea, vomiting, weak hind legs, purplish discoloration of the skin	Short incubation periods, three phases: depression, fever, anorexia; clinical improvement; terminal, exacerbation of disease	Late onset of disease, gradually aggravating depression and anorexia, normal to slightly elevated body temperatures, conjunctivitis, dermatitis, locomotion disturbances
Viremia	High level	Temporary reduction or disappearance	Persistent high level
Leukocytopenia	Develop quickly	Develop quickly followed by leukocytosis	Develop late during infection
Immune response	Absent	Present	Absent
Death	10-20 days	1-3 months	2-11 months
Gross pathology	Multiple hemorrhages particularly in lymph nodes and kidney, infraction of spleen	“Button” ulcers of cecum and colon, infraction of spleen and rib lesions	Lymph node swelling, thymic atrophy

improvement characterizes the second phase of illness. In the third phase, pigs again become anorectic and depressed, with temperatures often elevate until shortly before death. Runt pigs can develop during the course of chronic CSF. Such pigs are severely retarded in growth, have skin lesions, and frequently stand with arched backs. Pigs with chronic CSF may survive for more than 100 days. A 'late onset' disease is recognized as a sequel of congenital CSFV infection. The syndrome is characterized by an initial, relatively long period during which pigs remained free of disease. Pigs develop mild anorexia and depression, conjunctivitis, dermatitis, diarrhea, and locomotive disturbances leading to posterior paresis few months later after primary exposure of virus. Body temperatures of the pigs are normal. Most pigs survive for more than 6 months, but all eventually die. Pigs infected with low-virulent virus show mild disease or subclinical infections, which can be accompanied by leukopenia or remain apparently healthy.

In peracute cases of CSF, pathologic lesions are often absent. In acute and subacute disease, the most common lesion is hemorrhage. The lymph nodes become swollen, edematous, and then hemorrhagic. Petechial or ecchymotic hemorrhages can often be seen on serosal and mucosal surfaces, particularly on the kidney, urinary bladder, epicardium, larynx, trachea, intestines, subcutaneous tissues, and spleen. Straw-colored fluid may be found in the peritoneal and thoracic cavities, and the pericardial sac. Severe tonsillitis, sometimes with necrotic foci, is common. Infarction is occasionally seen in spleen. The lungs often become congested and hemorrhagic. The lesions of chronic disease are less severe and complicated by secondary infections. In addition, necrotic foci or "button" ulcers are sometimes found in cecum, colon, epiglottis, and larynx. In growing pigs that have survived for more than a month, bone lesions can also occur at the costochondral junction of the ribs and the growth plates of the long bones. In congenitally infected piglets, common lesions

include cerebellar hypoplasia, tic atrophy, ascites, and deformities of the head and legs. Edema and petechial hemorrhages may be seen in the skin and internal organs.

CSFV belongs to the genus *Pestivirus* of the family *Flaviviridae*, together with bovine viral diarrhea virus (BVDV) and border disease virus (BDV) of sheep (3) (Fig. 1A). Classification of pestiviruses previously referred to the host species from which they were isolated as described above. So far, numerous studies have shown that pestiviruses are not highly host-specific. BVDV and BDV can cause disease in domestic and wildlife ruminants and swine. However, CSFV infection is restricted to swine in nature. Currently, four species are recognized as tentative species, namely Giraffe-1 pestivirus, Pronghorn antelope pestivirus, Atypical pestivirus, and Bungowannah virus (3). CSFV possesses a single-stranded positive-sense RNA genome of approximately 12.3 kb with one large open reading frame flanked by 5' and 3' untranslated regions (UTRs) and coding for a polyprotein of approximately 3900 amino acids (Fig. 1B). Co- and post-translational processing of the polyprotein by cellular and viral proteases yields the twelve cleavage products N^{pro}, C, E^{ns}, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B finally (1). Recently, alternative cleavage products of NS3 were identified (4). These molecules probably regulate viral RNA replication. The structural components of the CSFV virion include the capsid (C) protein and the glycoproteins E^{ns}, E1, and E2. The nonstructural proteins NS3 through NS5B together with the 5' and 3' UTRs are essential for pestivirus RNA replication, while N^{pro}, p7, NS2, and all structural proteins are dispensable (1, 5).

Several groups studied the molecular biology of pestiviruses extensively with the aim of identifying the viral determinants of virulence. In all studies concluded there, highly conserved molecular viral sequence patterns essential for viral replication were considered as potential virulence factors. By mutation of these elements, attenuated viruses were created that knock out or frustrate specific viral properties, leading to less

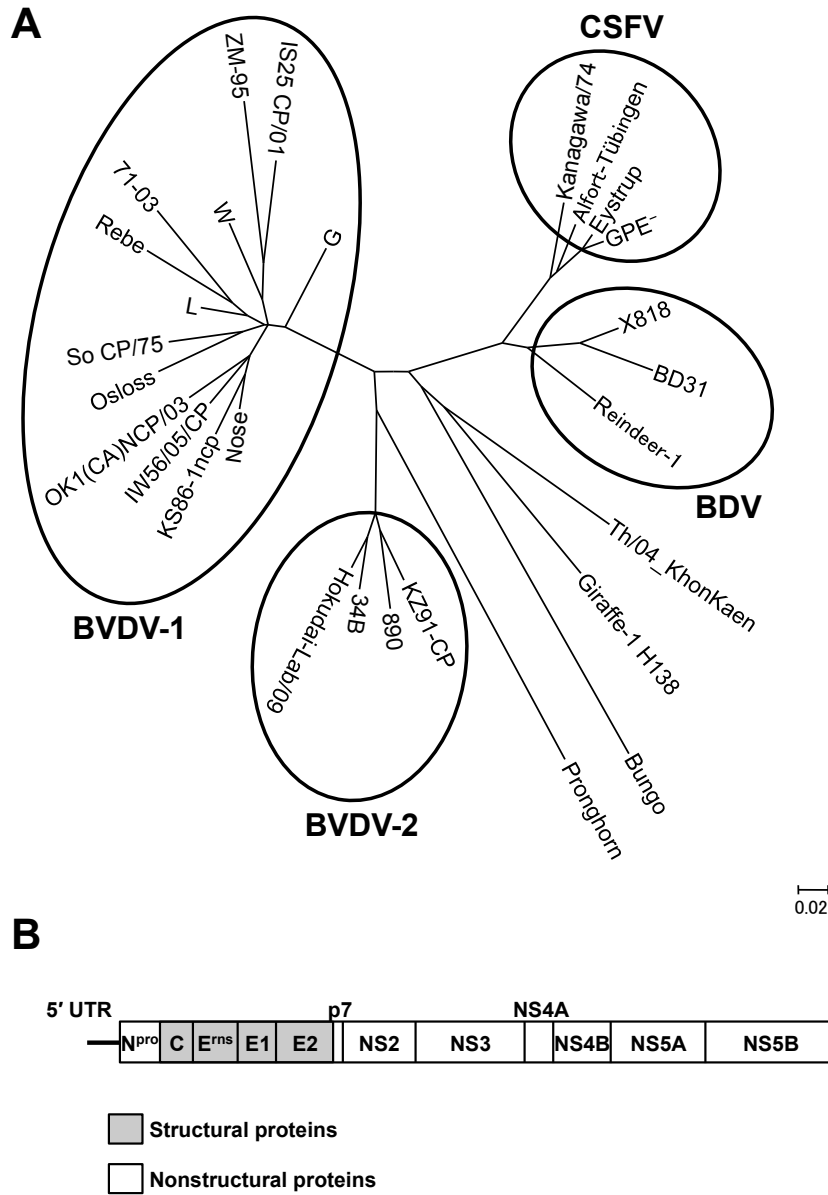


Fig. 1. Phylogeny and classification of pestiviruses and schematic representation of a pestivirus genome. (A) The genus *Pestivirus* belongs to the virus family *Flaviviridae*. In addition to the typical members of the four recognized species, CSFV, BVDV-1, BVDV-2, and BDV, a considerable number of novel pestiviruses have been isolated from different animal species. Giraffe-1 pestivirus (Giraffe-1 H138 strain), Pronghorn antelope pestivirus (Pronghorn strain), Bungowannah virus (Bungo strain), and Atypical pestivirus (Th/04_KhonKaen strain) are now considered as tentative species. (B) Four structural and eight nonstructural proteins are flanked by 5' and 3' UTRs. The structural proteins are indicated in gray boxes (the capsid protein C and three glycosylated envelope proteins E^{ms}, E1, and E2). The nonstructural proteins are shown in white boxes (the N-terminal protease N^{pro}, a viroporin p7, and the other nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B).

virulent phenotypes. The question of whether the essential elements differentiate various classes of virus virulence will remain conjectural. Thus, in the present thesis, the author investigated the viral determinants and functions responsible for pathogenicity of CSFV in pigs by “gain-of-function” approach. The present thesis consists of three chapters; in Chapter I, a live attenuated 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. In Chapter II, it was examined whether restoring the viral function to defend innate immune activation would contribute to its enhanced pathogenicity. In Chapter III, roles of a nonstructural protein in viral genome replication and pathogenesis were investigated compared between a live attenuated vaccine virus and a highly virulent virus.

Chapter I

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

Introduction

Live attenuated vaccine strains of CSF used worldwide were established from highly virulent strains by their attenuation through multiple passaging in cell culture or in nonnatural hosts such as guinea pigs and rabbits (6). Most countries have eradicated CSF successfully using vaccination together with stamping out of infected animals (6). In Japan that is currently free of CSF approved by the World Organisation for Animal Health OIE (7), a live attenuated vaccine based on the GPE⁻ strain was employed (6). The GPE⁻ vaccine strain was derived from the highly virulent CSFV strain ALD through multiple passages and biological cloning in primary cultures of swine testicle cells, bovine testicle cells, and guinea pig kidney (GPK) cells (8, 9). Pigs inoculated with the GPE⁻ vaccine strain were completely protected from challenge virus infection and replication (10, 11). Comparison of the viral genomes of the GPE⁻ vaccine strain and the parental ALD strain revealed 225 nucleotide substitutions and 46 amino acid differences (12), which did not provide any conclusive information on the molecular basis of the attenuation. Numerous reports suggest that the viral proteins C, E^{ms}, E1, E2, p7, and NS4B may be involved in determining the virulence of CSFV in pigs (13-26). So far, however, all conclusions on CSFV virulence determinants rely on mutations that result in down regulation of pathogenicity together with knock out of the viral functions.

In the study described here, the live attenuated GPE⁻ vaccine strain 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 eleven 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 11th passage had acquired a substitution in E2 (T830A) and two 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

Ethical statement

The animal experiments described in Chapter I were approved by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan with approval number 10-0106. All experiments were performed according to the guidelines of the committee.

Viruses and cells

The GPE⁻ vaccine strain was generated from the highly virulent ALD strain by multiple passages and biological cloning in swine testicle cells, bovine testicle cells, and the GPK cells (8, 9). The ALD/A76 strain was derived from the ALD strain by serial passages in swine testicle cells (27). The ALD/A76 and GPE⁻ viruses were kindly provided by the National Institute of Animal Health, Japan, and propagated in the swine kidney cell lines, swine kidney L (SK-L) cells (28) and cloned porcine kidney (CPK) cells (27). These cell lines were maintained in Eagle's minimum essential medium (EMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.295% tryptose phosphate broth (TPB) (Becton Dickinson, San Jose, CA, USA), 0.3 mg/ml L-glutamine, and 10% horse serum (Invitrogen, Carlsbad, CA, USA) at 37°C in the presence of 5% CO₂. The guinea pig 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 EMEM and slow stirring for 8 h at 4°C. After treatment, the cells were seeded in culture flasks in

EMEM supplemented with 0.295% TPB, 0.3 mg/ml L-glutamine, 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 transcription (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 polymerase chain reaction (PCR) with CSFV-specific primers and KOD FX polymerase (Toyobo, Osaka, Japan). The 5' ends of the viral cDNA were amplified using the 5' RACE System for Rapid Amplification of cDNA Ends, 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 MgCl₂, 250 mM NaCl, 1 mM DTT (Invitrogen), 2.5 mM MnCl₂, 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 System for Rapid Amplification of cDNA Ends (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, USA) and a 3500 Genetic Analyzer (Applied Biosystems). The sequencing data were analyzed using the GENETYX[®]-MAC version 13 software (Genetyx, Tokyo, Japan).

For preparation of a bead-bound clonal amplicon of the original inoculum of serial passages (GPE⁻ strain), emulsion PCRs 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 *SalI* and *BamHI*. The cDNA sequence was flanked by a modified T7 promoter sequence at the 5' end and a *SrfI* restriction site at the 3' end in analogy to a construct described previously (29). 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 (29) by using appropriate restriction enzymes. Details of the constructions can be obtained on request. The seven full-length cDNA clones with all possible combinations of the three amino acid substitutions T830A, V2475A, and A2563V were constructed in the pGPE⁻ backbone using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany) and KOD FX. The plasmids pGPE⁻/T830A, pGPE⁻/V2475A, and pGPE⁻/A2563V have a single amino acid substitution at position 830, 2475, and 2563 of pGPE⁻, respectively. The plasmids pGPE⁻/T830A; V2475A, pGPE⁻/T830A; A2563V, and pGPE⁻/V2475A;

A2563V carry double amino acid substitutions. The plasmid pGPE⁻/T830A; V2475A; A2563V harbors all triple amino acid substitutions.

The replicon cDNA clone pGPE⁻-N^{pro}-Luc-IRES-NS3 was constructed by replacing the genes C, E^{ms}, 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) from encephalomyocarditis virus (EMCV) obtained from the replicon cDNA clone pA187-N^{pro}-Luc-IRES-C-delE^{ms} (30). The cDNA cloning was performed using the In-Fusion[®] HD Cloning Kit according to the manufacturer's protocol (Clontech, Mountain View, CA, USA). The three 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 replicon cDNA clones 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 2475 and 2563 of pGPE⁻-N^{pro}-Luc-IRES-NS3, respectively. The replicon plasmid pGPE⁻-N^{pro}-Luc-IRES-NS3/2475A; A2563V carries double amino acid substitutions in NS4B.

Rescue of vGPE⁻ and mutant viruses

The cDNA-derived viruses were rescued as described previously (31). Briefly, full-length genomic pGPE⁻ and mutant cDNA clones were linearized with *SrfI* 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, USA), and electroporated immediately by using a Gene Pulser Xcell Electroporation System (Bio-Rad) set at 200 V and 500 μ F. After electroporation, the cells were plated in 60-mm dishes and incubated at 37°C and 5% CO₂. 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 (32, 33). 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 in the presence of 5% CO₂. 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% CO₂. After 4 days of incubation, viral NS3 was detected by immunoperoxidase staining using the mAb 46/1. The virus titers were calculated using the method of Reed and Muench formula (34) and expressed as 50% tissue culture infective dose (TCID₅₀) 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} TCID₅₀ 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 two new naïve pigs. This process was 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 was isolated from the tonsils of one pig only, since the other pig died accidentally before the experiment. The animal experiments described here were carried out in self-contained isolator units (Tokiwa Kagaku, Tokyo, Japan) in a biosafety level 3 (BSL-3) facility of the Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan.

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, five 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 ethylenediaminetetraacetic acid (EDTA) (Terumo, Tokyo, Japan) on days 0, 3, 5, 7, 9, 11, and 14 pi. Total white blood cell (WBC) and platelet

(PLT) 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 (MLN) 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 EMEM to obtain a 10% suspension for virus titration. The virus titers were expressed as TCID₅₀ 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% CO₂ 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, USA) 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 EMEM containing 0.295% TPB, 0.3 mg/ml L-glutamine, 1.0% Bacto-agar (Becton Dickinson), and 10% horse serum, and incubated at 37°C in the presence of 5% CO₂. After 72 h, the cells were fixed with ice-cold 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 fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1: 2,500) (MP Biomedicals, Morgan Irvine, CA, USA). 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 per focus was determined for 30 independent foci ($n = 30$).

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, add the fresh medium, and then incubated at 37°C in the presence of 5% CO₂. Total RNA was extracted from the cells at 2, 7, and 12 h pi using TRIzol Reagent and RT as described above. The cDNA of CSFV was analyzed with quantitative real-time polymerase chain reaction (qPCR) using the KAPA PROBE Fast qPCR Kit (Kapa Biosystem, Woburn, MA, USA), 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.* (35). 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 pi were recorded from three independent experiments, and each experiment was performed in duplicate.

Luciferase assay

Replicon RNA was transcribed *in vitro* from linearized plasmid DNA with *SrfI* as described above. In order to assess the efficiency of viral RNA replication by

luciferase expression, $10^{6.8}$ CPK cells were electroporated with 1 μ g of replicon RNA and 100 ng of pRL-TK (Int-) reporter plasmid (Promega, Madison, WI, USA) expressing *Renilla* luciferase. Electroporation was performed at 200 V and 500 μ F as described above. The cells were seeded in 6-well plates and incubated at 37°C in the presence of 5% CO₂. After 6 h, 12 h, and 24 h of transfection, cell extracts were prepared with 250 μ l of passive lysis buffer (Promega), 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 three independent experiments, and each experiment was performed in duplicate.

Statistical analysis

The Student's *t*-test was used to perform the statistical analysis. Differences were considered to be significant when $p < 0.05$.

Results

The virus titers in the tonsils increased by serial passages of the GPE⁻ vaccine strain in pigs

The live attenuated GPE⁻ vaccine strain was obtained after multiple passages of the highly virulent ALD strain in nonnatural host cells (8, 9). Under field conditions, the GPE⁻ vaccine virus is not transmitted from pig to pig (8). 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 two pigs by intramuscular injection of 1 ml cell culture supernatant containing $10^{7.6}$ TCID₅₀ of the GPE⁻ strain. On day 4 pi the tonsils were collected, homogenized, and re-inoculated intramuscularly into two 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 $10^{4.0}$ TCID₅₀/g (Table 2). The virus titers increased after the third and sixth passages to $10^{6.3}$ TCID₅₀/g and $10^{6.8}/10^{7.5}$ TCID₅₀/g, respectively, and remained stable for the following passages.

Amino acid changes acquired during the 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 eleven amino acid changes occurred during the serial 15 passages.

Table 2. Amino acid substitutions during serial passages of the GPE⁻ strain and virus recovery from the tonsils of infected pigs.

Virus	Amino acid at the indicated position [†] :											Virus recovery (log ₁₀ TCID ₅₀ /g)	
	E _{rms}	E2	p7					NS3	NS4B		NS5A		
			480	748	779	794	830		1085	1597			2447
GPE ⁻	K	N	S	S	T	V	V	V	T	V	A	A	NT [‡]
GPE ⁻ /P-1	§	.	.	.	.	.	.	.	.	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 [*]	.	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

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

[‡] NT: not tested.

[§] The periods indicate identity with the GPE⁻ sequence.

^{*} The two amino acids were found (quasispecies).

Three amino acid substitutions were retained until the end of the serial passages (Table 2). A valine to alanine substitution at position 2475 in NS4B (V2475A) occurred after the first passage already, and was maintained during all subsequent passages. After the fifth 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 2563 (A2563V), emerged after the sixth passage and also remained stable.

The virus passaged in pigs acquired the pathogenicity

In order to determine the effect on pathogenicity of the three mutations T830A, V2475A, and A2563V acquired by the GPE⁻ virus during adaptation in pigs, five 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 $10^{6.9}$ TCID₅₀ of infectious virus. As control, two groups of five pigs were inoculated with $10^{7.0}$ TCID₅₀ of the parental GPE⁻ virus and the ALD/A76 virus, respectively. In the pigs inoculated with the GPE⁻ strain, transient leukocytopenia and thrombocytopenia were observed without clinical signs (Fig. 2A, B and Table 3). Small amounts of viruses were recovered from blood samples of three out of the five pigs on days 3 and 5 pi, and from tonsils of one pig on day 14 pi. In contrast, two out of the five pigs inoculated with the GPE⁻/P-11 virus had diarrhea and dysstasia, and one pig died on day 11 pi. All five pigs had severe leukocytopenia and thrombocytopenia. Accordingly, virus was detectable in blood of all pigs on day 3 pi, and detected in one pig for a period of 14 days. Infectious virus was also isolated from tonsils, kidney, and/or MLN in all five pigs and from all three organs in two pigs (Table 3). With the

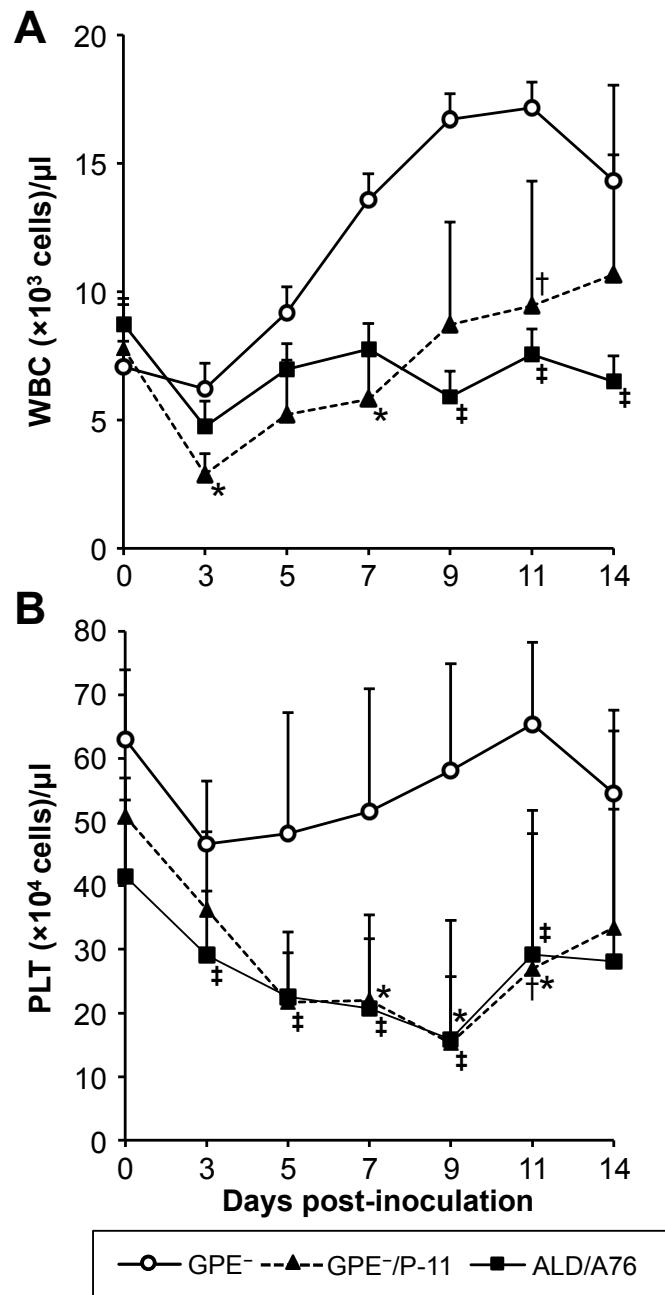


Fig. 2. WBC and PLT counts in pigs inoculated with the GPE⁻, GPE⁻/P-11, or ALD/A76 strains. Groups of five pigs were inoculated with the indicated viruses and blood was collected on days 0, 3, 5, 7, 9, 11, and 14 pi. WBC (A) and PLT (B) counts were determined for each timepoint and are shown as mean 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 Student's *t*-test. * indicates $p < 0.05$ between GPE⁻ and GPE⁻/P-11. ‡ 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.

Table 3. Clinical symptoms and virus recovery from pigs inoculated with the GPE⁻, GPE⁻/P-11, and ALD/A76 strains.

Inoculated virus	Clinical symptoms [†]	Virus recovery from:									
		Blood (log ₁₀ TCID ₅₀ /ml) [‡] on day pi:									
		3	5	7	9	11	14	Tonsils	Kidneys	MLN	Tissue (log ₁₀ TCID ₅₀ /g) [§]
GPE ⁻	-	+	-	-	-	-	-	-	-	-	-
	-	-	+	-	-	-	-	+	-	-	-
	-	-	-	-	-	-	-	-	-	-	-
	-	-	+	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-
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	+	-	-	-	≤2.8	-	+	+
	-	2.8	3.0	3.8	3.3	4.0	3.6	4.3	+	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	-

[†] -: not observed.

[‡] -: not isolated, NT: not tested, +: isolated in a 6-well plate and was lower detection limit of TCID₅₀ (10^{0.8} TCID₅₀/ml) in a 96-well plate.

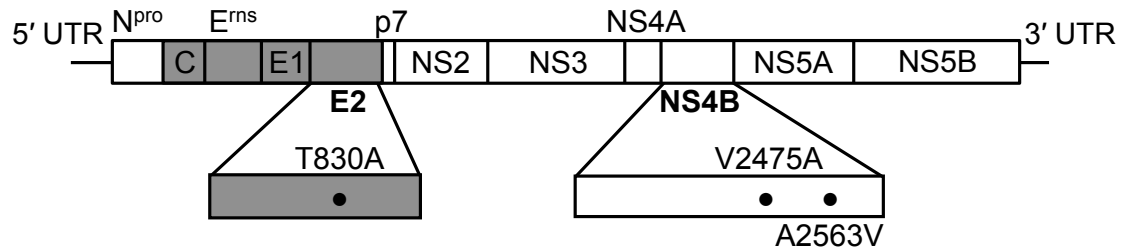
[§] -: not isolated, +: isolated in a 6-well plate and was lower detection limit of TCID₅₀ (10^{1.8} TCID₅₀/g) in a 96-well plate.

ALD/A76 strain, diarrhea, cyanosis, and/or weight loss were observed in four out of the five pigs, and all pigs had severe leukocytopenia and thrombocytopenia. Large amounts of virus were recovered in the blood and collected tissues 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.

All three amino acid substitutions observed after serial passages of the GPE⁻ vaccine strain are responsible for the acquisition of pathogenicity 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 2). 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 GPE⁻ backbone using reverse genetics (Fig. 3A and B). The pathogenicities of the reconstructed mutant viruses was determined in pigs and compared with the parental vGPE⁻ virus. For each recombinant virus, five pigs were infected by intramuscular injection of 10^{7.0} TCID₅₀ of virus. Clinical signs and virus content in blood and organs were monitored. Infection with the parental vGPE⁻ virus did not result in any clinical sign, with no or only very little amount of virus detection in blood and tonsils (Table 4-1). The virus with the single alanine to valine change in NS4B at position 2563 (vGPE⁻/A2563V) behaved similar to the parental vGPE⁻ virus. Similarly, the three viruses with a single amino acid change did not show any major leukocytopenia and thrombocytopenia (Fig. 4). Slightly better replication in pigs than the parental virus, also in the absence of any clinical signs, was observed with the virus carrying the single

A



B

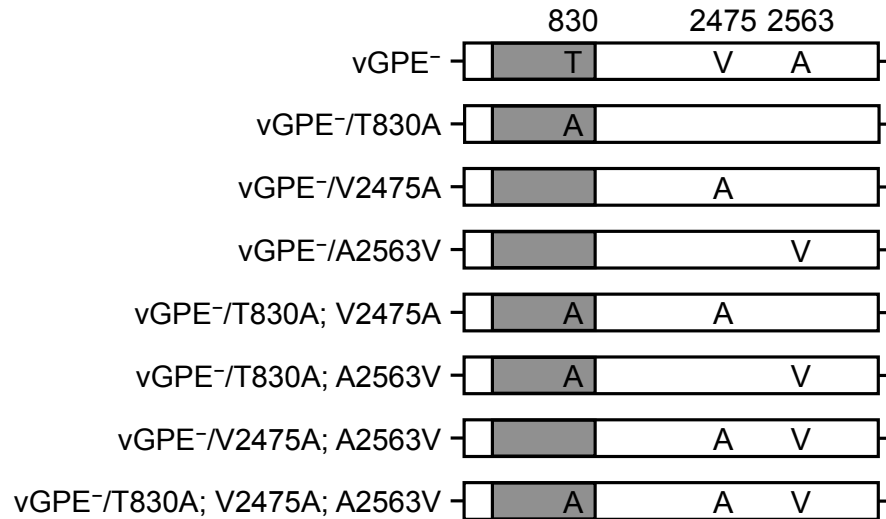


Fig. 3. Schematic representation of the cDNA clones of the GPE⁻/P-11 and mutant vGPE⁻ viruses. Three amino acid substitutions T830A, V2475A, and A2563V were found in the GPE⁻/P-11 virus (A). Seven recombinant viruses with all possible combinations of amino acid substitutions were generated by site-directed mutagenesis of the GPE⁻ backbone (B). The white and gray boxes indicate the nonstructural and structural proteins, respectively.

Table 4-1. Clinical symptoms and virus recovery from pigs inoculated with the vGPE⁻ and mutant viruses.

Inoculated virus	Clinical symptoms [†]	Virus recovery from:								
		Blood (log ₁₀ TCID ₅₀ /ml) [‡] on day pi:						Tissue (log ₁₀ TCID ₅₀ /g) [§]		
		3	5	7	9	11	14	Tonsils	Kidneys	MLN
vGPE ⁻	—	—	—	—	—	—	—	—	—	—
	—	+	—	—	—	—	—	+	—	—
	—	—	—	—	—	—	—	+	—	—
	—	—	+	—	—	—	—	+	—	—
	—	—	+	—	—	—	—	+	—	—
vGPE ⁻ /T830A	—	+	≤1.0	+	—	—	—	+	—	—
	—	+	2.6	≤2.1	+	—	—	3.8	+	—
	—	—	≤1.0	+	—	—	—	+	—	—
	—	+	≤1.5	—	—	—	—	+	—	—
	—	+	+	—	—	—	—	+	—	—
vGPE ⁻ /V2475A	—	—	+	—	—	—	—	+	—	—
	—	+	+	—	—	—	—	+	—	—
	—	—	+	+	—	—	—	4.0	—	—
	—	—	+	—	—	—	—	2.5	—	—
	—	—	+	—	—	—	—	+	—	—
vGPE ⁻ /A2563V	—	—	—	—	—	—	—	+	—	—
	—	—	—	—	—	—	—	+	—	—
	—	—	—	—	—	—	—	≤2.0	—	—
	—	+	+	—	—	—	—	—	—	—
	—	+	—	—	—	—	—	—	—	—

[†] —: not observed.

[‡] —: not isolated, +: isolated in a 6-well plate and was lower detection limit of TCID₅₀ (10^{0.8} TCID₅₀/ml) in a 96-well plate.

[§] —: not isolated, +: isolated in a 6-well plate and was lower detection limit of TCID₅₀ (10^{1.8} TCID₅₀/g) in a 96-well plate.

Table 4-2. Clinical symptoms and virus recovery from pigs inoculated with the vGPE⁻ and mutant viruses.

Inoculated virus	Clinical symptoms [†]	Virus recovery from:								
		Blood (log ₁₀ TCID ₅₀ /ml) [‡] on day pi:						Tissue (log ₁₀ TCID ₅₀ /g) [§]		
		3	5	7	9	11	14	Tonsils	Kidneys	MLN
vGPE ⁻ / T830A; V2475A	—	2.6	3.8	3.6	—	—	—	4.8	—	—
	—	+	≤1.0	—	—	—	—	3.3	—	—
	—	2.8	3.8	2.3	—	—	—	+	—	—
	—	2.6	3.3	≤1.5	+	—	—	2.1	—	—
	—	≤1.0	3.0	+	+	—	—	4.0	—	—
vGPE ⁻ / T830A; A2563V	—	≤1.0	≤1.6	≤1.0	—	—	—	4.3	—	—
	—	2.8	3.3	≤1.0	—	—	—	3.8	—	—
	—	+	2.8	3.0	—	—	—	2.8	—	—
	—	2.5	3.8	3.8	≤2.3	+	—	3.6	—	—
	—	≤2.1	2.3	≤1.8	—	—	—	3.9	—	—
vGPE ⁻ / V2475A; A2563V	—	+	≤1.0	—	—	—	—	≤2.3	—	—
	—	≤1.0	≤1.1	—	—	—	—	+	—	—
	—	≤1.0	≤1.1	—	—	—	—	3.3	—	—
	—	—	≤1.0	+	—	—	—	≤2.3	—	—
	—	+	+	—	—	—	—	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	+	—	3.3	—	+
	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	+	—	4.1	—	≤2.3

[†] —: not observed.

[‡] —: not isolated, +: isolated in a 6-well plate and was lower detection limit of TCID₅₀ (10^{0.8} TCID₅₀/ml) in a 96-well plate.

[§] —: not isolated, +: isolated in a 6-well plate and was lower detection limit of TCID₅₀ (10^{1.8} TCID₅₀/g) in a 96-well plate.

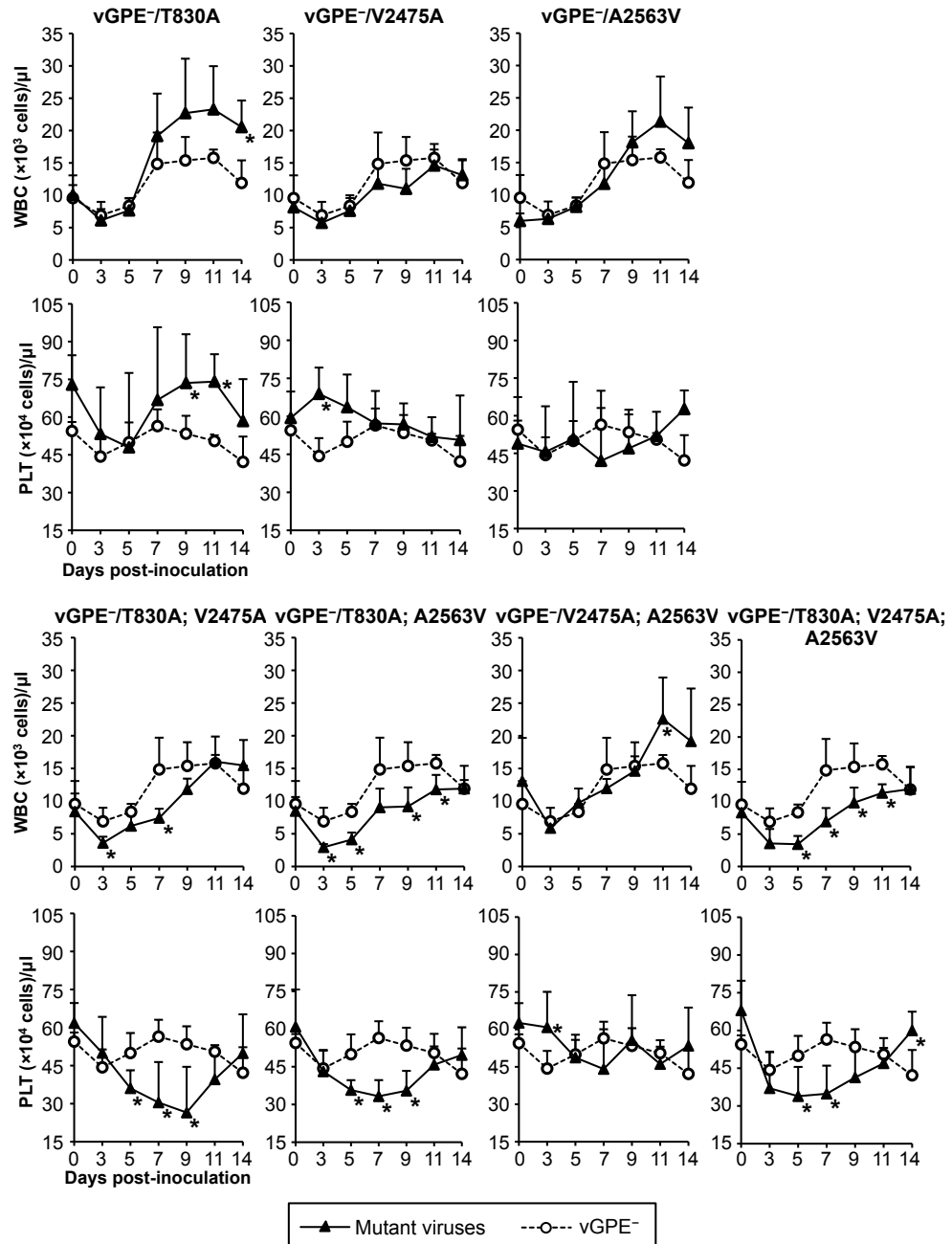


Fig. 4. WBC and PLT counts in pigs inoculated with the vGPE⁻ parent and mutant viruses. Groups of five pigs were inoculated with the indicated viruses and blood was collected on days 0, 3, 5, 7, 9, 11, and 14 pi. WBC and PLT counts were determined for each timepoint and are shown as mean value with error 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 Student's *t*-test. * indicates $p < 0.05$. In each graph the mean WBC and PLT counts of the pigs inoculated with the parental vGPE⁻ virus are shown for comparison purposes. The pigs were euthanized on day 14 pi.

mutation at position 2475 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 three amino acid substitutions (vGPE⁻/T830A; V2475A; A2563V) that resulted in cyanosis, diarrhea, and/or weight loss in four out of the five pigs, in severe leukocytopenia and thrombocytopenia in all five animals, and in prolonged virus shedding for up to 11 days after infection (Table 4-2 and Fig. 4). 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 two viruses (vGPE⁻/T830A; V2475A and vGPE⁻/T830A; A2563V), the highest virus 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 MLN of two out of five 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 (data not shown). Altogether, these results demonstrate that the three amino acid substitutions act in concert in the acquisition of pathogenicity after adaptation of the GPE⁻ vaccine strain in pigs.

All three amino acid substitutions found during adaptation in pigs act together to enhance virus replication in porcine cells and to abolish replication in nonhost cells

At 30°C, the GPE⁻ strain replicates efficiently in guinea pig cells in which it was adapted, as opposed to the parental ALD and other wild type strains that grow very poorly in these cells (8). Therefore, multistep growth curves of the recombinant

viruses carrying the different amino acid substitutions in the GPE⁻ backbone were evaluated in the porcine SK-L and in the guinea pig GPK cell cultures in comparison with the vGPE⁻ and ALD/A76 viruses (Fig. 5). 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 double amino acid substitutions (vGPE⁻/T830A; V2475A and vGPE⁻/V2475A; A2563V), and the virus carrying the triple mutations (vGPE⁻/T830A; V2475A; A2563V) did also replicate more efficiently than the parental vGPE⁻ virus in porcine 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 three adaptive mutations in the GPE⁻ backbone (vGPE⁻/T830A; V2475A; A2563V) did not replicate at all in the GPK cells, as opposed to the parental vGPE⁻ virus. These replication kinetics were confirmed with kinetics of parallel samples analyzed by qPCR (data not shown). Thus, these results show that the three amino acid substitutions found during adaptation in pigs act together to enhance virus replication in porcine cells and to abolish replication in GPK cells, which is in agreement with the effect of the three adaptive mutations on pathogenicity in pigs.

The single amino acid substitution T830A in E2 is responsible for the enhanced viral spreading in porcine cells

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 effects of these amino acid substitutions on viral spreading in porcine

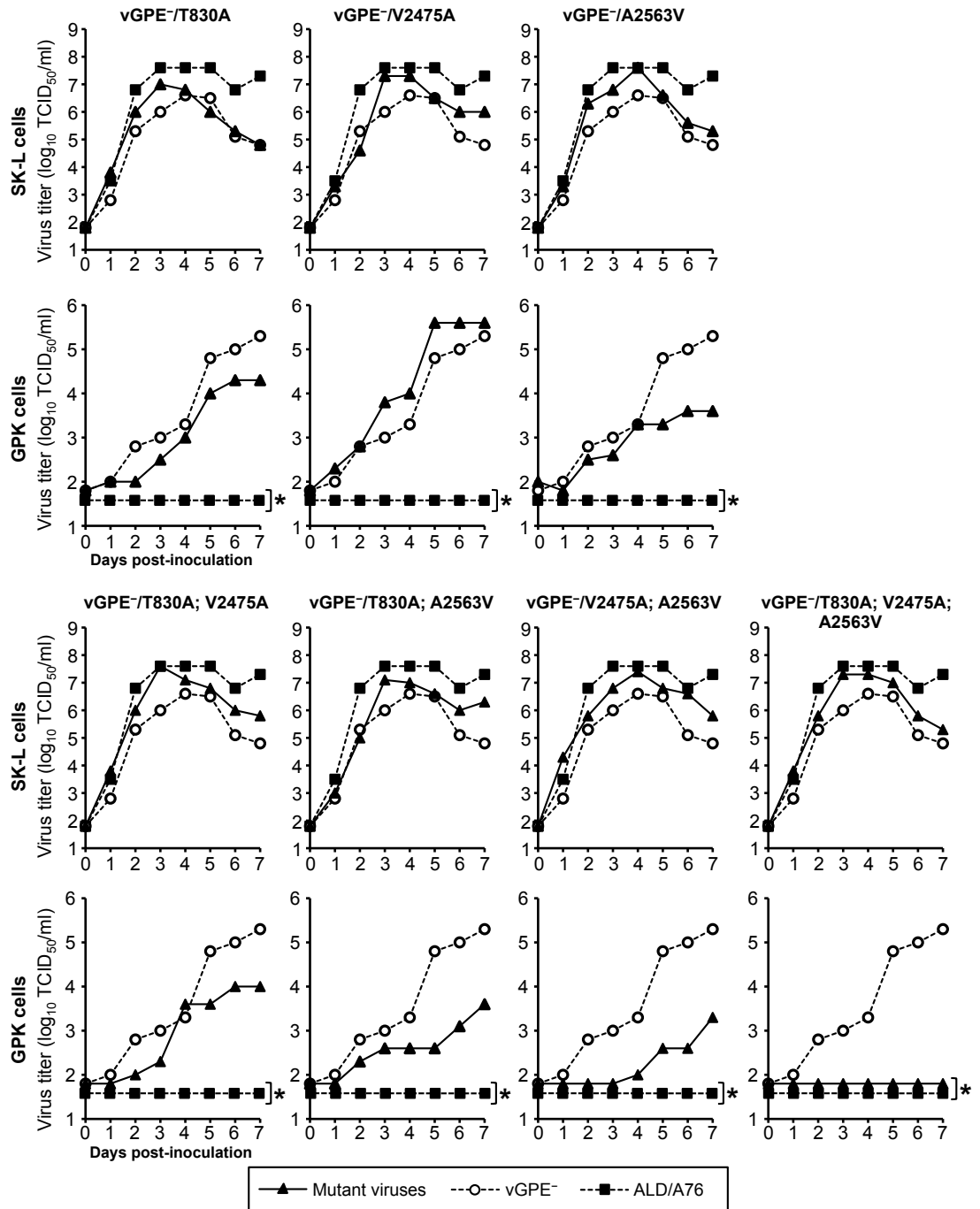


Fig. 5. Growth kinetics of the vGPE⁻ parent and mutant viruses and of the ALD/A76 strain in porcine cells and guinea pig cells. The porcine SK-L cells and the guinea pig GPK cells were inoculated at an MOI of 0.001 with the parent and mutant viruses as indicated. The SK-L and GPK cells were incubated at 40°C in the presence of 5% CO₂ and at 30°C, respectively. The supernatants were collected at the indicated times. The titers were determined in SK-L cells and expressed as TCID₅₀/ml. In each graph, the titers of the vGPE⁻ and ALD/A76 strains are included for comparison purposes. * indicates that the virus titer was below detection the limit of 10^{1.8} TCID₅₀/ml.

cells, monolayers of SK-L cells were infected with the parental and mutant 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 three adaptive mutations produced the largest foci, comparable in size with the foci obtained with the ALD/A76 strain (Fig. 6). The smallest foci were observed with the vGPE⁻ virus and with the mutants carrying one or two mutations in NS4B. All mutants that carry the T830A substitution alone or together with one 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 porcine cells.

Both amino acid substitutions V2475A and A2563V in NS4B contribute to enhanced viral RNA replication in porcine cells

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 pi (Fig. 7). At 12 h pi, the RNA replication efficiency of the two 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, a bi-cistronic reporter replicon was constructed with the GPE⁻ gene. This replicon consisted of the 5' and 3' UTRs flanked by a chimeric N^{pro}-firefly luciferase gene separated from the NS3 to NS5B genes of the replication complex by an EMCV IRES

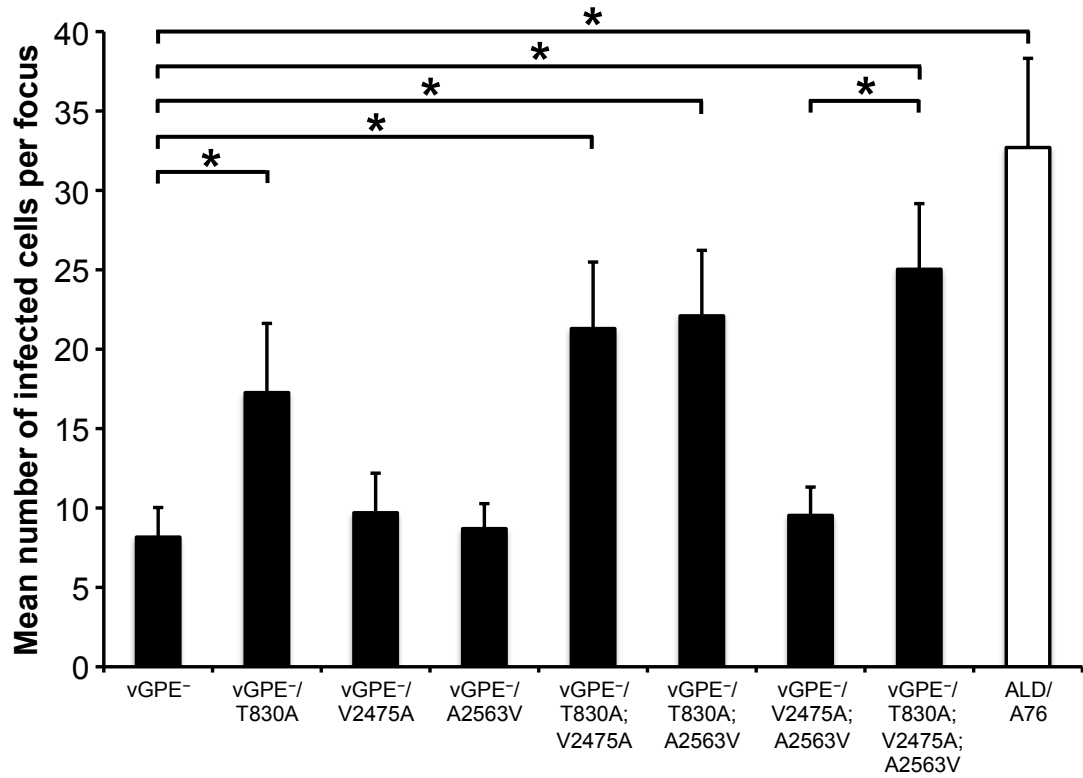


Fig. 6. Focus formation of vGPE⁻, mutant viruses, and ALD/A76 in SK-L cell cultures. The porcine SK-L cells were inoculated at an MOI of 0.0001 with the parent and mutant viruses as indicated. The cell monolayers were overlaid with EMEM containing 1.0% Bacto-agar and incubated at 37°C in the presence of 5% CO₂. 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 Student's *t*-test. * indicates $p < 0.05$.

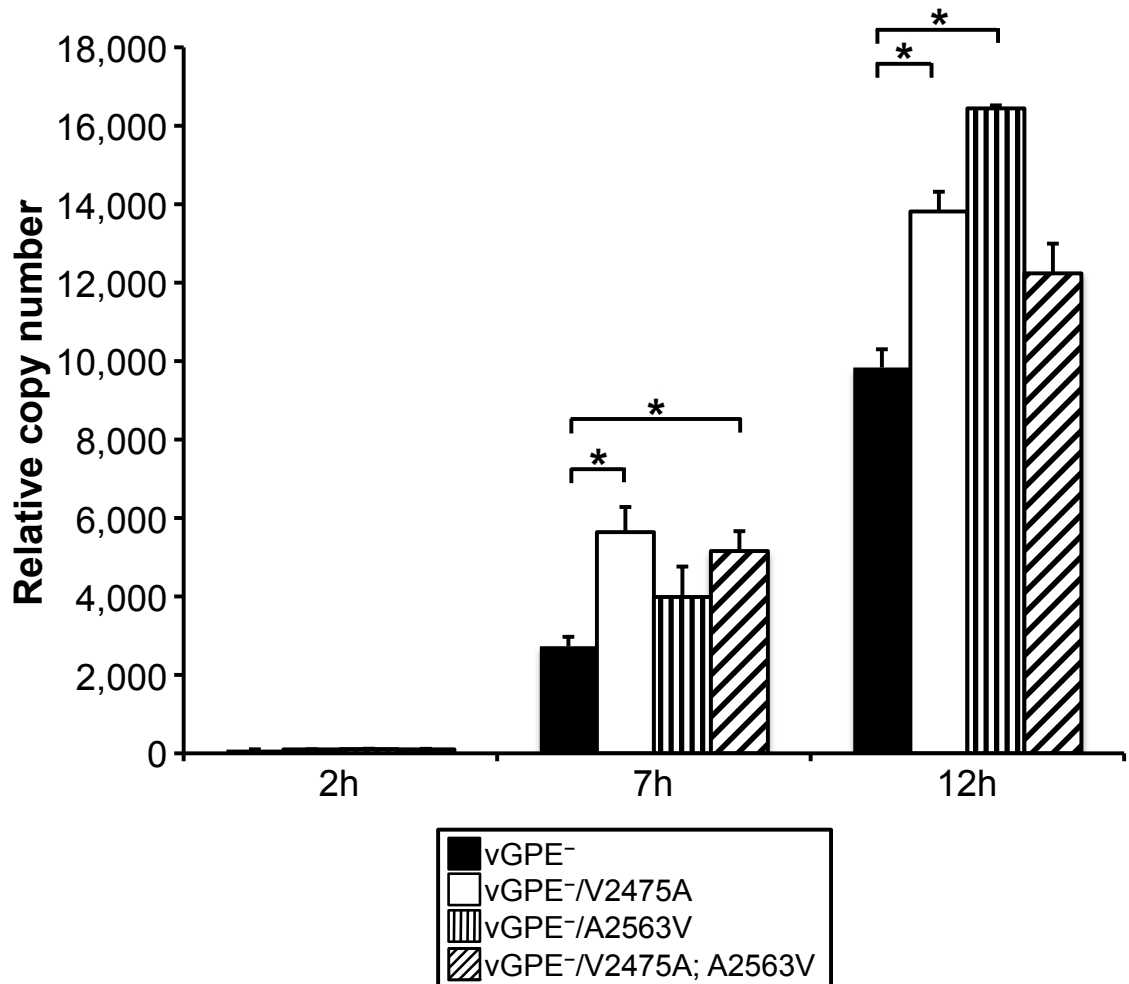


Fig. 7. Effect of amino acid substitutions in NS4B on the viral RNA replication efficiency. In order to assess the effect of the amino acid substitutions in NS4B on the viral RNA replication efficiency, confluent the porcine SK-L cell monolayers were inoculated at an MOI of 1.5 with the parent and mutant viruses carrying the indicated amino acid changes in NS4B. Parallel cultures of infected cells were incubated at 37°C in the presence of 5% CO₂ 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 pi are shown. Each bar represents the mean value of three independent experiments and the error bars show the standard deviation. Pairwise comparisons of the values were carried out using the Student's *t*-test. * indicates $p < 0.05$.

(Fig. 8A). The two 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 porcine CPK cell line. The replicon carrying the two adaptive mutations in NS4B resulted in ten-fold higher luciferase activity than the parental rGPE⁻-N^{pro}-Luc-IRES-NS3 replicon 24 h after transfection (Fig. 8B). At this timepoint, significantly higher luciferase activities when compared with the parental 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 viral RNA replication in porcine cells by increasing the activity of the viral replicase.

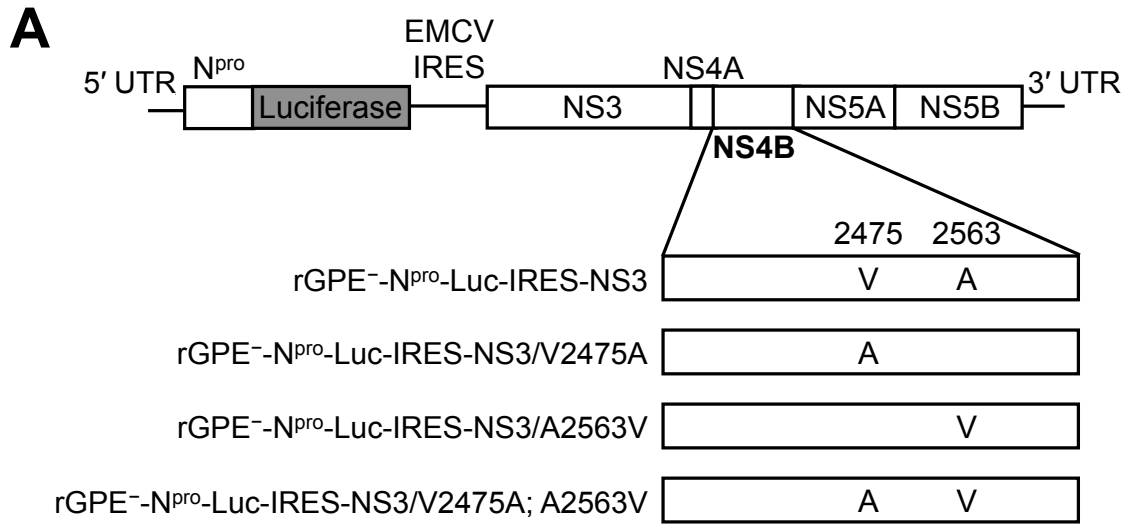


Fig. 8. Effect of amino acid substitutions in NS4B on RNA replication efficiency. (A) A GPE⁻-derived bi-cistronic replicon consisting of the 5' and 3' UTRs flanking a chimeric N^{pro}-firefly luciferase gene separated from the NS3 to NS5B genes by the IRES of EMCV was constructed. Single amino acid substitutions at position 2475 and 2563 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} of the porcine 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% CO₂, 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 three independent experiments with error bars showing the standard deviation. The values obtained with each construct were compared pairwise using the Student's *t*-test. * indicates *p* < 0.05.

B

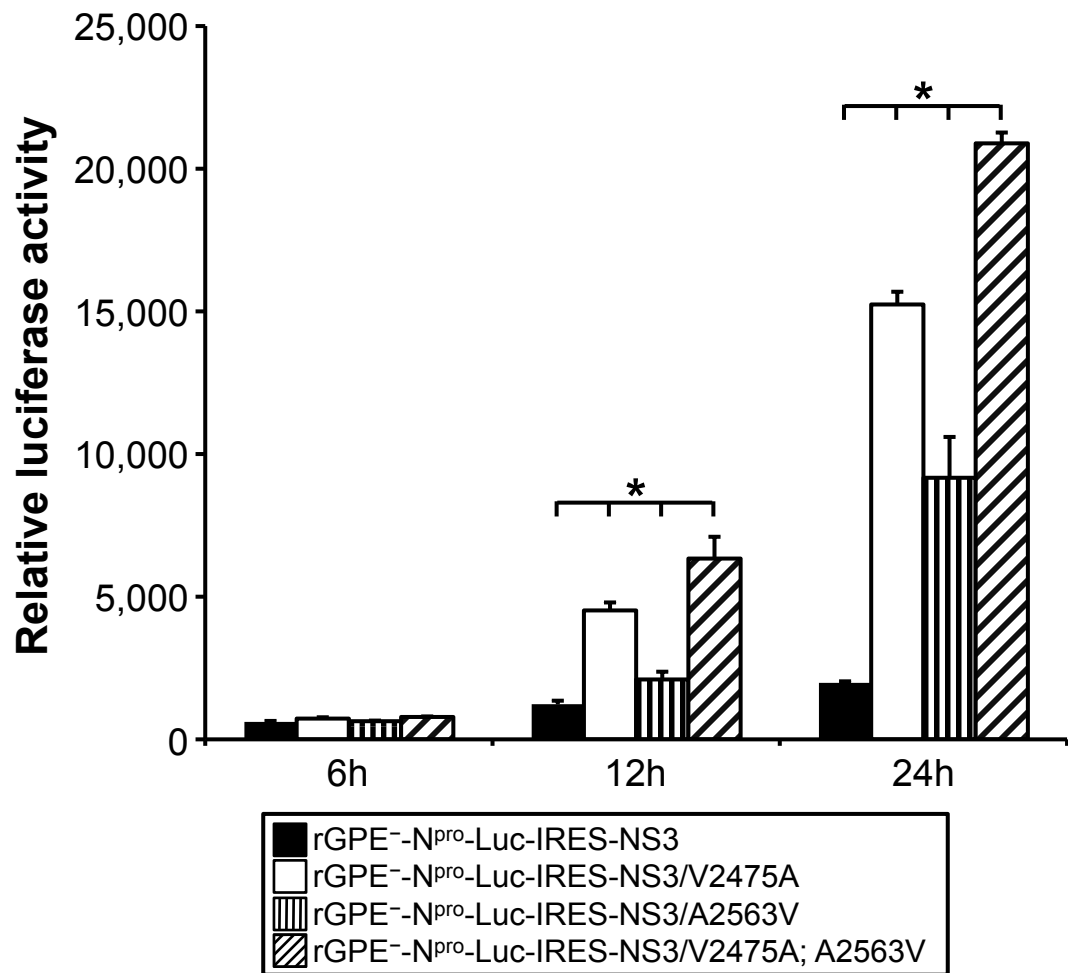


Fig. 8. (continued)

Discussion

The live attenuated vaccine strain GPE⁻ was widely used in Japan to control CSF (6). The GPE⁻ vaccine strain was generated from the highly virulent ALD strain through multiple passages and biological cloning in cells of swine, bovine, and guinea pig origin (8, 9). Field studies have proven that the GPE⁻ vaccine strain 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 (8, 10). 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, three 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 two genes had a synergistic effect on the pathogenicity *in vivo* and on virus spread and replication efficiency in cell culture. This is the first study 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 parental ALD virus and the GPE⁻ vaccine strain (12). Since the pathogenicity of the triple mutant vGPE⁻ virus is not as high as the pathogenicity of the original ALD strain, 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. Considering all of this, 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^{ns}, 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 (13-26).

The fact that the amino acid change V2475A was found in NS4B after one passage in pigs suggests that the alanine at position 2475 was already present in the quasispecies population of the original inoculum. This hypothesis was indeed confirmed by deep sequencing analysis (Table 5). 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 porcine 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^{ns} of pestiviruses mediate viral attachment and entry into host cells (36-40). 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 porcine cells. E2 is exposed on the outer surface of a viral particle and is the most

Table 5. Quasispecies composition of the original GPE⁻ virus at the codon sites at which adapted mutations were rescued.

Amino acid position and nucleotide sequence [†]	Rate (%)	Deduced amino acid [‡]
E2 830		
ACA	>99.9	T
NS4B 2475		
GUC	97.5	V
. C .	2.3	<u>A</u>
. G .	0.2	G
NS4B 2563		
GCA	99.0	A
. A .	0.8	E
. U .	0.2	<u>V</u>

[†] Periods indicate the same nucleotides as in the sequence above.

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

immunodominant protein inducing the major neutralizing antibodies in infected pigs (41). 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 (42, 43). Recent studies with the crystal structure of a pestivirus E2 suggest that a solvent exposed β -hairpin extrusion located in the linear epitope (amino acid of 829–837) of the N-terminal region is a candidate site for interacting with the BVDV cellular receptor CD46 (44, 45). Moreover, Risatti *et al.* (21) showed that in the Brescia strain, amino acid substitutions in this linear epitope, including the amino acid at position 830, attenuated virulence in pigs.

In NS4B, the amino acid substitutions at positions 2475 and 2563 have not yet been reported to be involved in virulence. On the basis of the predicted topology of CSFV NS4B, the both residues at position 2475 and 2563 are located in the trans-membrane domain. A recent study on the biosynthesis of the CSFV nonstructural proteins showed that NS4B is mostly found attached to NS5A (46). The role of trans-membrane 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 (47). Importantly, a study identified a putative Toll-interleukin-1 receptor-like (TIR) 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 2563 identified here, resulted in an attenuated phenotype along with enhanced activation of Toll-like receptor 7 (TLR-7)-induced genes (13). NS4B also harbors a nucleoside triphosphate phosphohydrolase (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 (48). The two

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 viral replicase of pestiviruses is not well understood. CSFV NS4B is relatively labile when compared to other nonstructural proteins (46). Several studies show that NS4B of the other members of *Flaviviridae* is an integral membrane protein of the endoplasmic reticulum (ER) 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 (49-53). Further investigation of the roles of NS4B in the pathogenesis of CSF will be discussed in Chapter III of the present thesis.

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 type I interferon (IFN- α/β) induction, a property attributed to the viral proteins N^{pro} and E^{ms}. N^{pro} interferes with the induction of IFN- α/β by promoting the proteasomal degradation of interferon regulatory factor 3 (IRF3) (54, 55). 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 (56). In the parental ALD strain, N^{pro} is functional in terms of inhibition of IFN- α/β induction (57). Interestingly, during the re-adaptation of the GPE⁻ vaccine strain 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 will be discussed in Chapter II. Previous data suggest that the IRF3 degrading function of N^{pro} may support the pathogenicity of low and moderately 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 Eystруп and resulted only in slight attenuation of the moderately virulent Alfort/187 virus (56). E^{ns} forms disulfide-linked homodimers and possesses ribonuclease (RNase) activity (25, 58). E^{ns} interacts with extracellular double-stranded RNA, thereby targeting a major viral interferon (IFN)-inducing signal (59). Knocking out the RNase activity in the CSFV Alfort-Tübingen backbone resulted in attenuation in pigs (19). Amino acid substitution at the glycosylation sites of E^{ns} of the Brescia strain did not affect RNase activity but attenuated the virus (25). The amino acids of E^{ns} 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^{ns} 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 nonnatural 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.

Summary

There are numerous CSFV strains that differ in virulence, resulting in clinical disease with different degrees of severity. Low virulent and moderately 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 highly 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 strain 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 eleventh passage *in vivo* had acquired three 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 are responsible for the acquisition of pathogenicity. Studies *in vitro* indicate that the substitution in E2 influences virus spreading, and that the changes in NS4B enhance 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.

Chapter II

**N^{pro} of classical swine fever virus contributes to pathogenicity
in pigs by preventing type I interferon induction at local
replication sites**

Introduction

Virus infection triggers innate immune activation. Pattern recognition receptors such as endosomal TLR, and retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5 in cytoplasm recognize double-stranded DNA or RNA produced in virally infected cells (60-63), and respond by activating a signaling pathway that culminates in IFN- α/β induction and establish an antiviral state (64). IFN- α/β production depends on a family of transcription factors, IRFs (65). IRF3, one of IRFs, is ubiquitously expressed in the cytoplasm, and acts as an important element in the early phase of the IFN- α/β induction (66). As a result of the signaling initiated by the cytoplasmic pattern recognition receptors, IRF3 is phosphorylated by the kinases IKK ϵ and TANK-binding kinase 1, dimerizes, and translocates to the nucleus, where it eventually up-regulates transcription of IFN- α/β mRNA (67, 68).

So far, numerous studies revealed that viruses can evade or subvert key elements of the host viral response. In the case of pestiviruses including CSFV, it is well documented that pestiviruses subvert innate immune defenses by preventing IFN- α/β induction. The viral protein responsible for this function is N^{pro}, encoded at the N terminus of the single open reading frame of the positive-sense RNA genome. N^{pro}, a nonstructural protein unique to pestiviruses encoding 168 amino acids, autocatalytically generates its own carboxy terminus through its cysteine protease activity in a Q22-H49-C69 (1). N^{pro} *per se* is dispensable for pestivirus replication (69). As accessory functions, N^{pro} interacts with and mediates the degradation of IRF3, blocking IFN- α/β induction in most target cells (70, 71). In plasmacytoid dendritic cells (pDCs), N^{pro} interacts with IRF7 without inducing IRF7 degradation, dampening

IRF7-dependent IFN- α induction by yet unknown mechanisms (72). The C-terminal half of N^{pro} harbors a zinc-binding TRASH domain consisting of C₁₁₂-X₂₁-C₁₃₄-X₃-C₁₃₈ that is required for the interaction with these factors (72-74). Recently, the colleagues of this author revealed that the N-terminal domain of N^{pro} is involved in its stability, in interaction of N^{pro} with IRF3, and subsequent degradation of IRF3 that leads to down-regulation of IFN- α/β production (75). By means of N^{pro}, pestiviruses interfere with IFN- α/β induction, which can be measured with strong viral IFN- α/β inducers such as Newcastle disease virus (NDV) (56, 76). This is termed ‘exaltation of Newcastle disease virus’ (END), referring to the observation that NDV replicates more efficiently in cells infected with pestivirus than in absence of pestivirus infection (76). During the generation of the live attenuated vaccine strain GPE⁻ from the parental ALD virus, the N^{pro}-mediated suppression of IFN- α/β induction was lost, resulting in a virus that did not exhibit the END phenomenon (9, 57). This biotype was designated END-negative (END⁻) CSFV. In the study described in Chapter I of the present thesis, the live attenuated CSF vaccine strain GPE⁻ was re-adapted to its natural host by multiple serial passages in pigs in order to select for increased replication efficiency and pathogenicity, with the aim of identifying determinants of CSFV virulence. The GPE⁻ virus became pathogenic after eleven serial passages in pigs by intramuscular injection of tonsil homogenates. Three amino acid substitutions, T830A in E2 and V2475A and A2563V in NS4B, were found to be responsible for the acquisition of pathogenicity. Interestingly, during the serial passages of the GPE⁻ vaccine strain in pigs, the IRF3-degrading function of N^{pro} was not recovered.

In a previous study, mutations designed to knock out N^{pro}-mediated IRF3 degradation did not alter the pathogenicity of the highly virulent CSFV strain Eystруп

and caused only slight attenuation of the moderately virulent Alfort/187 virus (56). Here, it was examined whether restoring the N^{pro} function of an END⁻ virus would enhance its pathogenicity. To this end, viruses with a single amino acid substitution restoring N^{pro}-mediated IRF3 degradation in the GPE⁻ vaccine strain backbone and in a GPE⁻-derived moderately virulent mutant were generated. The pathogenicity and innate immune responses to infection with the parental and mutant viruses were investigated by experimental infection of pigs.

Materials and Methods

Ethical statement

The animal experiments described in Chapter II were approved by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University with approval numbers 10-0106 and 12-0013. All experiments were performed according to the guidelines of the committee.

Cells

The swine kidney cell line SK-L (28) was propagated in EMEM supplemented with 0.295% TPB, 10 mM N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (Sigma-Aldrich, St. Louis, MO, USA), 0.3 mg/ml L-glutamine, and 10% horse serum (Life Technologies, Carlsbad, CA, USA). The porcine SK6-MxLuc cell line carrying a Mx/Luc reporter gene (77) was propagated in EMEM supplemented with 0.3 mg/ml L-glutamine, and 7% horse serum. The human embryonic kidney cell line 293T was maintained in Dulbecco's Modified Eagle's Medium (Life Technologies), 0.3 mg/ml L-glutamine, and 10% fetal calf serum (Cambrex, Grand Island, NY, USA). All cells were incubated at 37°C in the presence of 5% CO₂.

Viruses

The CSFV strains vGPE⁻, vGPE⁻/T830A; V2475A; A2563V, and vEy-37 were derived from the full-length cDNA clones pGPE⁻, pGPE⁻/T830A; V2475A; A2563V, and pEy-37 (78), respectively. The vEy-37 virus is identical to the highly virulent Eystrup strain. The vGPE⁻ and the triple mutants vGPE⁻/T830A; V2475A;

A2563V-derived viruses with the N136D mutation in N^{pro} (vGPE⁻/N136D and vGPE⁻/N136D; T830A; V2475A; A2563V) were rescued from mutant cDNA plasmids that were constructed using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA), KOD FX, KOD -Plus- Neo (Toyobo), and oligonucleotide primers containing the respective mutation, applying standard techniques described in Chapter I. All cDNA-derived viruses were rescued as described previously (31). In brief, plasmid constructs were linearized at the *SrfI* site located at the end of the viral genomic cDNA sequence, and RNA was obtained by run-off transcription using the MEGAscript T7 kit (Life Technologies). After deoxyribonuclease I (DNase I) treatment and purification on S-400 HR Sephadex columns, RNA was quantified using a spectrophotometer and used to electroporate SK-L cells. The complete genomes of the rescued viruses were verified by nucleotide sequencing to exclude any accidental mutation. The rescued viruses were stored at -80°C.

Sequencing

The full-length cDNA clones and *in vitro*-rescued viruses were completely sequenced as described in Chapter I. In brief, nucleotide sequencing of cDNA clones and PCR fragments from viral RNA was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and a 3500 Genetic Analyzer (Life Technologies). Sequencing data were analyzed using Genetyx[®]-Mac version 13 software.

Virus titration

Virus titers were determined by end-point dilution on SK-L cells in 96-well plates and immunoperoxidase staining using the anti-NS3 mAb 46/1 as described earlier (32, 33). The titers were calculated using the formula of Reed and Muench (34) and expressed in TCID₅₀ per ml or gram.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

SDS-PAGE and Western blotting were performed as described previously (79). The concentration of the SDS polyacrylamide gels was 7.5%. The anti-porcine IRF3 mAb 34/1 (70) was used as primary antibody. Signals were detected with the Immobilon Western Detection Reagents (Millipore, Bedford, MA, USA) and the LumiVision PRO 400EX system (Aisin Seiki, Aichi, Japan) and quantified using the LumiVision Analyzer 2.0 software (Aisin Seiki).

Virus replication kinetics

The replication kinetics of the parental and mutant viruses in SK-L cells were determined by inoculation of confluent cell monolayers at an MOI of 0.1 TCID₅₀ and collection of cell culture supernatants at different times pi. After inoculation, the SK-L cells were incubated at 37°C in the presence of 5% CO₂. The supernatants were collected at 0, 16, 24, 48, 72, 96, 120, 144, and 168 h pi. The virus titers were determined in duplicate using the SK-L cells.

IFN bioassay

The bioactivity of porcine IFN- α/β was assayed using the Mx/Luc reporter gene assay as previously described (77). Viruses present in culture supernatants and in porcine serum samples were inactivated using a UV crosslinker (ATTO, Tokyo, Japan). SK6-MxLuc cells were seeded in 24-well plates at a density of $10^{5.2}$ cells per well. After 20 h, 400 μ l of samples diluted appropriately to allow measurement in the linear range of the assay or a porcine IFN- β standard were added to the cells which were then incubated at 37°C in the presence of 5% CO₂. Recombinant porcine IFN- β produced in 293T cells was used as a standard. The cell extracts were prepared with 100 μ l of passive lysis buffer, and firefly luciferase activities were measured using a Luciferase Assay System (Promega) and a PowerScan4 microplate reader (DS Pharma Biomedical, Osaka, Japan). The data were analyzed using the Gen5 software (DS Pharma Biomedical). Results were recorded for three independent experiments, and each experiment was performed in duplicate. Virus inactivation was monitored on SK-L cells using immunohistochemistry with mAb 46/1.

Experimental infection of pigs

In order to assess the pathogenicity of the cDNA-derived viruses, groups of eight 4-week-old crossbred Landrace $\times$ Duroc $\times$ Yorkshire SPF pigs were injected intramuscularly with 1 ml of virus from cell culture supernatant. For each group, five pigs were kept for 14 days and three pigs for five days, and clinical signs monitored daily. With the five pigs kept for 14 days, blood was collected in tubes containing EDTA on days 0, 3, 5, 7, 9, 11, and 14 pi. Total leucocytes and platelets were counted using a pocH-100iV Diff apparatus. The pigs that survived the infection were euthanized on

day 14 pi, and tissues from brain, tonsils, spleen, kidneys, adrenal glands (AG), MLN, and colon were collected aseptically. The collected samples were homogenized in EMEM to obtain a 10% suspension for virus isolation. Virus isolation was performed by inoculation of samples into SK-L monolayer of 6-well plates. As for virus antigen-positive samples, virus titration was conducted and their titers were expressed as TCID₅₀ per ml (blood) or gram (tissue). With the three pigs kept for 5 days, whole blood was collected for serum preparation at 0, 12, 24, 36, 48, 72, 96, and 120 h pi. These three pigs were euthanized at 120 h pi, and tissues from tonsils, spleen, and MLN were collected for quantitative measurement of mRNA expression. All animal experiments were performed in self-contained isolator units (Tokiwa Kagaku) in a BSL-3 facility of the Graduate School of Veterinary Medicine, Hokkaido University.

qPCR

For quantification of IFN- α , IFN- γ , interleukin-1alpha (IL-1 α), IL-6, IL-10, and tumor necrosis factor-alpha (TNF- α) gene expression, total RNA of tissues from brains, tonsils, spleens, kidneys, and MLN was extracted using a RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Contaminant genomic DNA was removed by DNase I treatment using a RNase-Free DNase set (Qiagen). Total RNA was quantified and cDNA synthesized using the Random Primers (N)₉ with SuperScript III Reverse Transcriptase in 20 μ l reaction mixtures containing 1 μ g of total RNA. The cDNA was analyzed by qPCR using the KAPA PROBE Fast qPCR Kit and a Light Cycler[®] 480 System II. The primers and cycling conditions reported by Lee *et al.* (80) for IFN- α , Duvigneau *et al.* (81) for IFN- γ , IL-1 α , IL-6, and IL-10, and Allen *et*

al. (82) for TNF- α were used. The gene expression data were normalized by assessing mRNA abundance of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH gene expression was quantified by qPCR according to a previous report (81). Quantitative values were calculated using the mean of duplicated results.

Statistical analysis

The Student's *t*-test was used to perform the statistical analysis. Differences were considered to be significant when $p < 0.05$.

Results

The amino acid substitution N136D in N^{pro} of the GPE⁻-derived viruses restores N^{pro}-mediated IRF3 degradation

Previous studies demonstrated that C₁₁₂, C₁₃₄, D₁₃₆, and C₁₃₈ in N^{pro} of CSFV form a zinc-binding domain and are essential for the interaction of N^{pro} with IRF3 and inducing proteasomal degradation of IRF3 (56, 73, 74). The GPE⁻ vaccine strain encodes an asparagine instead of an aspartic acid at position 136 of the zinc-binding domain (12, 56). It was shown previously that this substitution is sufficient to abolish the IRF3 degrading function of N^{pro} completely (56). As expected, substitution of asparagine with aspartic acid at position 136 (N136D) in N^{pro} of the END⁻ vGPE⁻ virus or of the triple mutant virus vGPE⁻/T830A; V2475A; A2563V restored the capacity of the virus to bind and degrade IRF3. Infection of the porcine SK-L cells with the vGPE⁻/N136D and vGPE⁻/N136D; T830A; V2475A; A2563V viruses, resulted in degradation of IRF3 to undetectable levels after 96 h (Fig. 9). Accordingly, the GPE⁻-derived viruses carrying the mutation in N^{pro} regained the capacity to cause the END phenomenon in porcine cells (data not shown).

Restoring N^{pro}-mediated IRF3 degradation in the vGPE⁻ and GPE⁻-derived triple mutant virus results in suppression of IFN- α/β production and affects virus production in cell culture

In order to assess the effect of restoring N^{pro}-mediated IRF3 degradation on viral growth kinetics in porcine cells, multistep growth curves of viruses carrying either asparagine or aspartic acid at position 136 in N^{pro} were determined in the type I IFN-

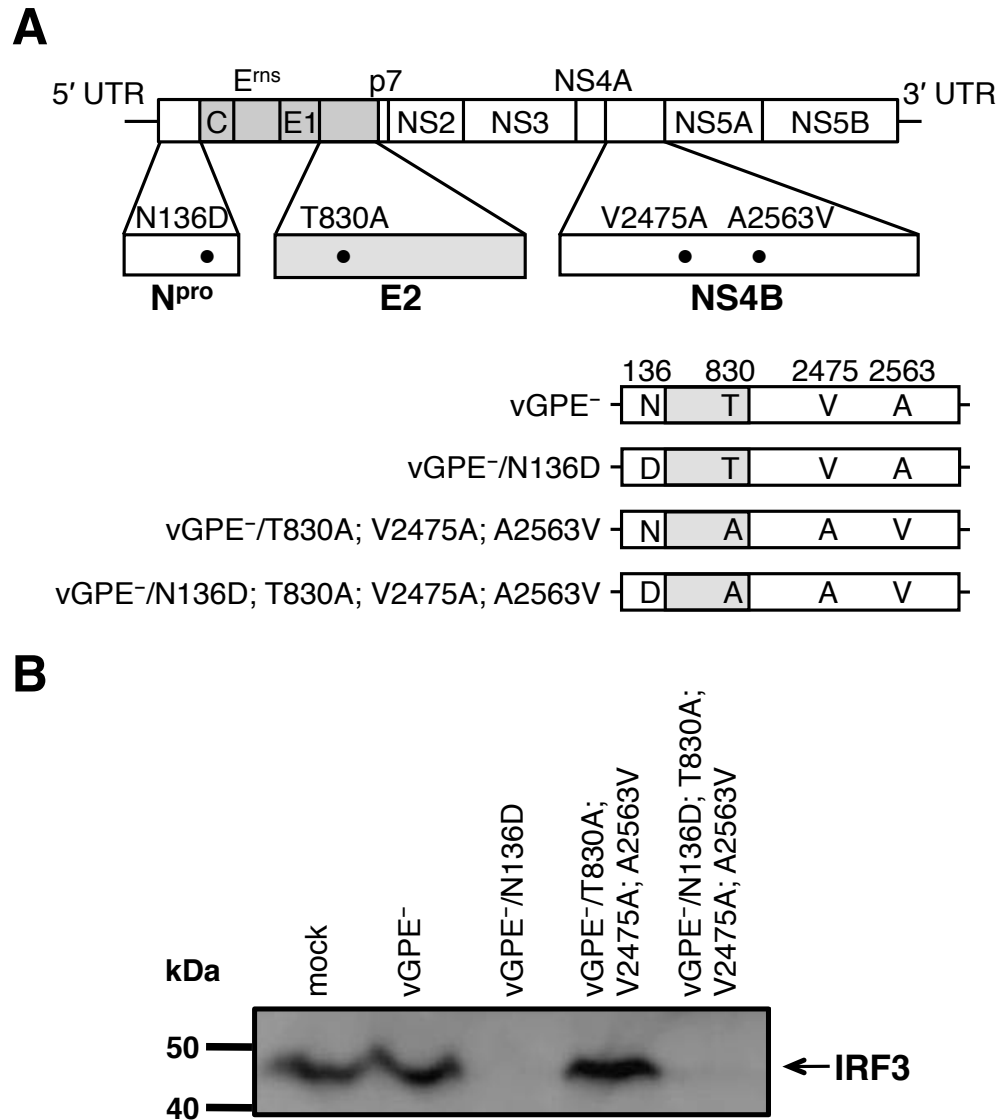


Fig. 9. Effect of restoring the zinc-binding domain in N^{pro} of GPE⁻-derived viruses on IRF3 degradation. (A) The amino acid substitution N136D was introduced in N^{pro} of vGPE⁻ and vGPE⁻/T830A; V2475A; A2563V by site-directed mutagenesis. (B) The porcine SK-L cells were inoculated at an MOI of 1 TCID₅₀ per cell with the parent and mutant viruses as indicated. The concentration of the SDS polyacrylamide gels was 7.5%. Proteins were detected at 96 h pi by Western blot analysis using the anti-porcine IRF3 mAb.

competent porcine SK-L cells. The viruses carrying the single N136D amino acid substitution in N^{pro} (vGPE⁻/N136D and vGPE⁻/N136D; T830A; V2475A; A2563V) showed an initial replication rate similar to that of the parental viruses and reached a peak titer at 72 h pi (Fig. 10A and B). After the peak titer was reached, the virus titers of the mutants that suppressed interferon induction tended to be higher than the titers of the corresponding END⁻ viruses. Complete suppression of IFN- α/β production conferred by the N136D substitution was confirmed by measuring IFN- α/β bioactivity in the supernatants of the porcine SK-L cells infected with the mutant and parental viruses. Taken together, these data suggest that the functional N^{pro} might affect virus production by dampening the innate immune response.

Restoring N^{pro}-mediated inhibition of IFN- α/β induction in the moderately virulent GPE⁻-derived triple mutant virus enhances pathogenicity in pigs

In order to determine the contribution of N^{pro}-mediated suppression of IFN- α/β induction to pathogenicity, five 4-week-old pigs were injected intramuscularly with 10^{7.0} TCID₅₀ of N136D mutant or parental GPE⁻-derived viruses. As a control, five pigs were inoculated with the highly virulent Eystrup strain. The four amino acid residues in N^{pro} (D₁₃₆), E2 (A₈₃₀), and NS4B (A₂₄₇₅ and V₂₅₆₃) are conserved in the highly virulent Eystrup strain, although a total of 62 amino acids differ between GPE⁻ and Eystrup strains. The pigs inoculated with the GPE⁻-derived virus carrying the functional N^{pro} (vGPE⁻/N136D) and with the vGPE⁻ parental virus had only a slight transient leukocytopenia and thrombocytopenia (Fig. 11A and B, top panels). No clinical symptoms were observed with any of these two viruses (Table 6-1). Very little or no virus was recovered from blood and tonsils of these pigs (Table 6-1). All five

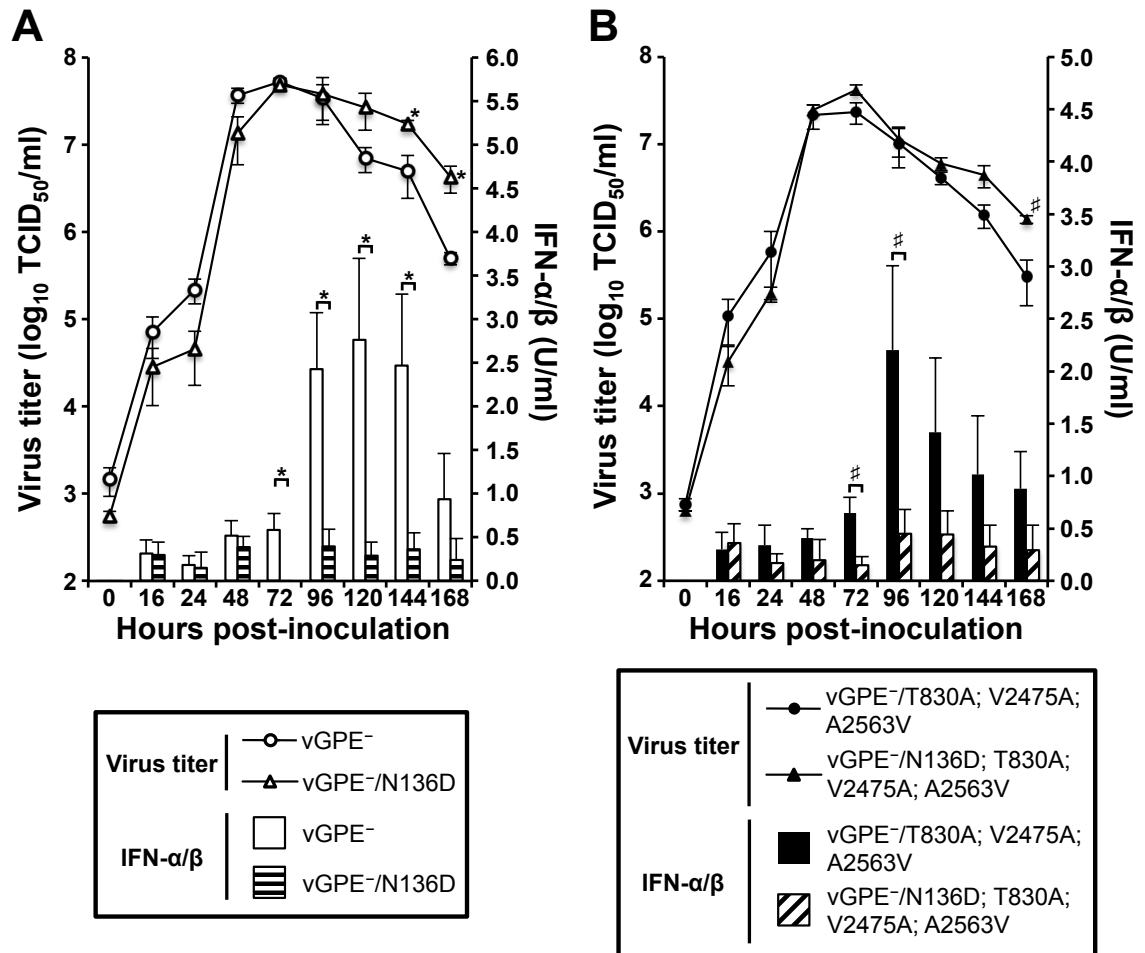


Fig. 10. Growth kinetics and IFN- α/β production after infection of porcine cells with the GPE⁻-derived viruses. The porcine SK-L cells were inoculated at an MOI of 0.1 TCID₅₀ per cell with the indicated viruses and incubated at 37°C in the presence of 5% CO₂. The supernatants were collected at 0, 16, 24, 48, 72, 96, 120, 144, and 168 h pi. Virus titers were determined in duplicate in SK-L cells and expressed as TCID₅₀/ml, and IFN- α/β bioactivity was measured in duplicate using the SK6-MxLuc reporter cells. These data were compared with vGPE⁻ and vGPE⁻/N136D (A), and with vGPE⁻/T830A; V2475A; A2563V and vGPE⁻/N136D; T830A; V2475A; A2563V (B). Each curve and bar presents the mean from three independent infections, with error bars showing the standard errors. The significance of the differences between was calculated using the Student's *t*-test. * indicates $p < 0.05$ between vGPE⁻ and vGPE⁻/N136D. # indicates $p < 0.05$ between vGPE⁻/T830A; V2475A; A2563V and vGPE⁻/N136D; T830A; V2475A; A2563V.

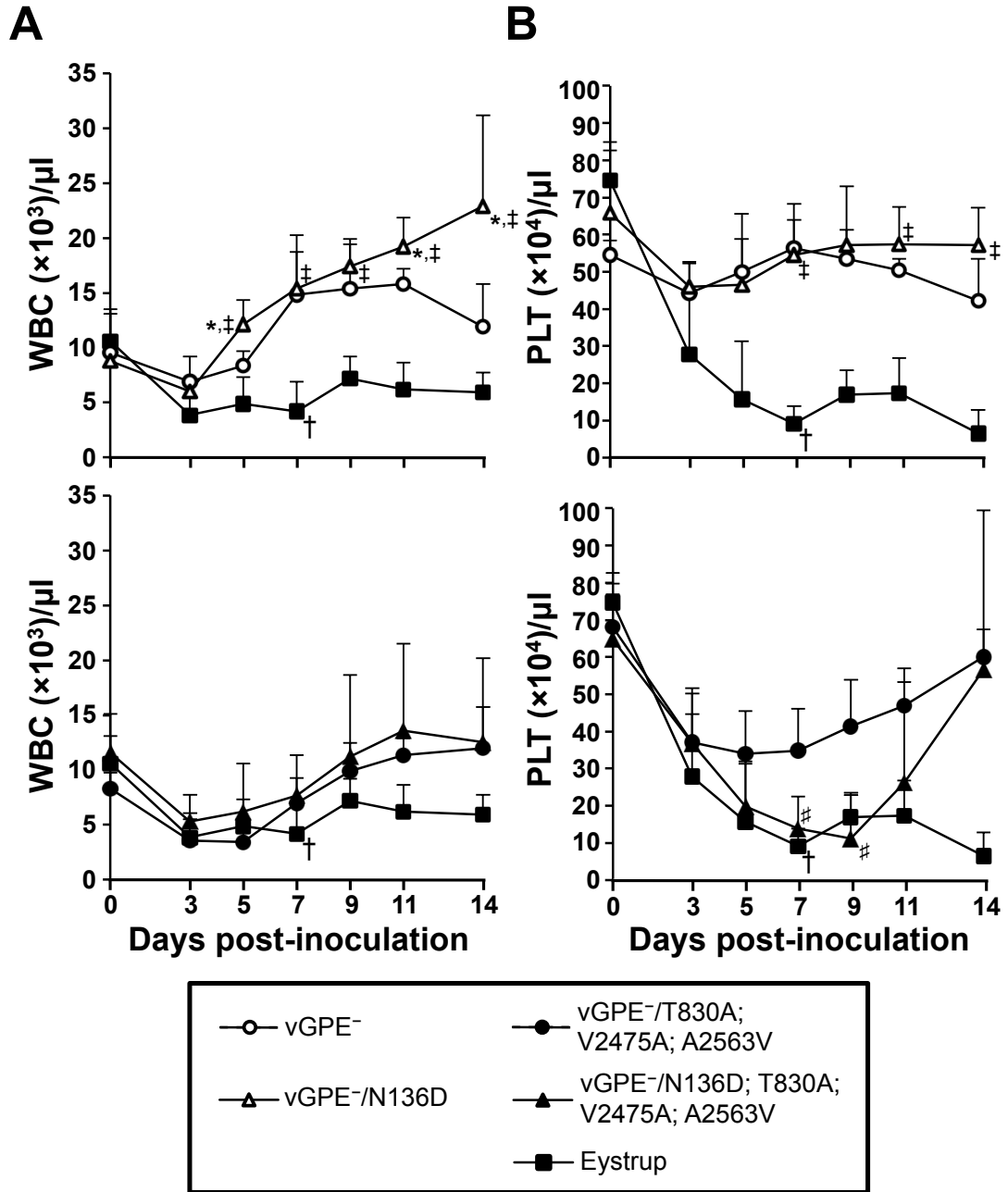


Fig. 11. WBC and PLT counts in pigs inoculated with the GPE⁻-derived viruses and with Eystруп strain. Groups of five pigs were inoculated with the indicated viruses and blood was collected on days 0, 3, 5, 7, 9, 11, and 14 pi. WBC (A) and PLT (B) counts were determined for each timepoint and are shown as mean values with error bars representing the standard deviation. The statistical significance of the differences between the cell counts was calculated using the Student's *t*-test. * indicates *p* < 0.05 between vGPE⁻ and vGPE⁻/N136D. ‡ indicates *p* < 0.05 between vGPE⁻/N136D and Eystруп. # indicates *p* < 0.05 between vGPE⁻/T830A; V2475A; A2563V and vGPE⁻/N136D; T830A; V2475A; A2563V. Two pigs inoculated with Eystруп strain died on day 7 pi (indicated by †). All remaining pigs were euthanized on day 14 pi.

Table 6-1. Clinical symptoms and virus recovery from pigs inoculated with the vGPE⁻ and mutant viruses.

Virus recovery from:														
Inoculated virus	Clinical symptoms [†]	Blood (log ₁₀ TCID ₅₀ /ml) [‡] on day pi:						Tissue (log ₁₀ TCID ₅₀ /g) [§]						
		3	5	7	9	11	14	Brain	Tonsils	Spleen	Kidneys	AG	MLN	Colon
vGPE ⁻	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	+	-	-	-	-	-	-	+	-	-	-	-	-
	-	-	-	-	-	-	-	-	+	-	-	-	-	-
	-	-	+	-	-	-	-	-	+	-	-	-	-	-
	-	-	+	-	-	-	-	-	+	-	-	-	-	-
vGPE ⁻ /N136D	-	+	-	-	-	-	-	-	+	-	-	-	-	-
	-	≤1.6	-	-	-	-	-	-	≤2.3	-	-	-	-	-
	-	+	-	-	-	-	-	-	≤2.1	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	+	-	-	-	-	-

[†] -: not observed.

[‡] -: not isolated, +: isolated in a 6-well plate and was lower detection limit of TCID₅₀ (10^{0.8} TCID₅₀/ml) in a 96-well plate.

[§] -: not isolated, +: isolated in a 6-well plate and was lower detection limit of TCID₅₀ (10^{1.8} TCID₅₀/g) in a 96-well plate.

Table 6-2. Clinical symptoms and virus recovery from pigs inoculated with the vGPE⁻ and mutant viruses.

Inoculated virus		Clinical symptoms [†]	Virus recovery from:															
			Blood (log ₁₀ TCID ₅₀ /ml) [‡]								Tissue (log ₁₀ TCID ₅₀ /g) [§]							
			on day pi:								Brain	Tonsils	Spleen	Kidneys AG	MLN	Colon		
3	5	7	9	11	14	3	5	7	9	11	14	Brain	Tonsils	Spleen	Kidneys AG	MLN	Colon	
vGPE ⁻ /T830A; V2475A; A2563V		≤1.0	≤2.1	≤1.0	≤1.0	—	—	—	—	—	—	—	—	4.0	—	—	—	—
	—	≤1.0	2.6	3.6	≤2.6	+	—	—	—	—	—	—	—	3.3	—	—	+	—
	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	+	—	—	—	—	—	—	—	4.1	—	—	≤2.3	—
vGPE ⁻ /N136D; T830A; V2475A; A2563V	Weight loss, coarse fur, cyanosis, astasia	2.6	4.8	5.3	5.6	5.1	4.6	4.3	4.3	7.0	6.3	5.6	5.8	6.0	5.8	5.8	5.8	5.8
	Weight loss, coarse fur	3.3	4.0	4.3	4.6	4.3	≤1.3	—	—	≤3.8	≤2.3	—	+	3.0	≤2.3	—	—	—
	Astasia, arthritis	3.3	4.0	3.6	+	—	—	—	—	3.0	≤2.1	—	—	—	≤2.0	—	—	—
	Weight loss, arthritis	2.8	3.6	2.9	+	—	—	—	—	4.1	—	3.8	—	—	≤2.0	—	—	—
	Weight loss, coarse fur	2.3	4.3	4.0	3.8	+	—	—	—	4.0	≤2.0	≤3.1	≤2.3	≤2.5	—	—	—	—

[†] –: not observed.

[‡] –: not isolated, +: isolated in a 6-well plate and was lower detection limit of TCID₅₀ (10^{0.8} TCID₅₀/ml) in a 96-well plate.

[§] –: not isolated, +: isolated in a 6-well plate and was lower detection limit of TCID₅₀ (10^{1.8} TCID₅₀/g) in a 96-well plate.

Table 6-3. Clinical symptoms and virus recovery from pigs inoculated with the vGPE⁻ and mutant viruses.

Inoculated virus	Clinical symptoms [†]	Virus recovery from:													
		Blood (log ₁₀ TCID ₅₀ /ml) [‡] on day pi:							Tissue (log ₁₀ TCID ₅₀ /g) [§]						
		3	5	7	9	11	14	Brain	Tonsils	Spleen	Kidneys	AG	MLN	Colon	
Eystrup	Fever, cyanosis, weight loss	5.5	5.0	5.8	6.0	5.8	4.6	6.0	6.8	7.6	7.8	6.0	8.0	7.0	
	Low-sprouted, anorexia, diarrhea, weight loss, cyanosis, death	4.0	6.0	6.6	NT	NT	NT	6.1	7.0	6.6	5.3	6.0	7.0	5.5	
	Fever, low-sprouted, anorexia, diarrhea, cyanosis, death	5.3	6.1	6.1	NT	NT	NT	5.0	6.6	6.3	5.6	5.3	6.8	5.8	
	Fever, anorexia, diarrhea, weight loss, cyanosis	5.3	6.6	6.7	6.0	6.0	5.1	6.0	7.3	6.8	7.6	6.6	7.5	4.6	
	Fever, anorexia, diarrhea, weight loss, cyanosis	4.8	6.0	6.0	6.0	6.8	6.6	6.3	7.6	7.8	7.5	6.8	8.0	7.8	

[†] —: not observed.

[‡] —: not isolated, NT: not tested, +: isolated in a 6-well plate and was lower detection limit of TCID₅₀ (10^{0.8} TCID₅₀/ml) in a 96-well plate.

[§] —: not isolated, +: isolated in a 6-well plate and was lower detection limit of TCID₅₀ (10^{1.8} TCID₅₀/g) in a 96-well plate.

pigs inoculated with the virus carrying the N136D mutation restoring functional N^{pro} along with the three mutations T830A, V2475A, and A2563V (vGPE⁻/N136D; T830A; V2475A; A2563V) had severe thrombocytopenia and showed clinical symptoms of CSF such as weight loss, cyanosis, and ataxia (Fig. 11B bottom panel and Table 6-2). Two of these pigs had arthritis. Leukocytopenia caused by vGPE⁻/N136D; T830A; V2475A; A2563V infection was similar to that caused by vGPE⁻/T830A; V2475A; A2563V infection (Fig. 11A bottom panel). Importantly, virus was detectable in the blood of all pigs as early as day 3 pi and remained detectable for a longer time period than in pigs infected with the triple mutant lacking the N136D substitution (Table 6-2). High titers of virus were detected in all collected tissues of one pig infected with the vGPE⁻/N136D; T830A; V2475A; A2563V virus. Infectious virus was detected in at least three collected tissues of the remaining four pigs. With the tonsils and MLN, virus was recovered from all infected pigs. In contrast, in pigs infected with the triple mutant virus carrying non-functional N^{pro} (vGPE⁻/T830A; V2475A; A2563V), virus was recovered only from tonsils and from MLN in two out of five animals (Table 6-2). With the highly virulent Eystrup strain, infection resulted in severe leukocytopenia, thrombocytopenia, and clinical signs in all five pigs, and two of five pigs died on day 7 pi (Table 6-3). High titers of virus were detected in all blood and tissue samples from Eystrup-infected pigs. These data demonstrate that N^{pro}-mediated suppression of IFN- α/β induction can prolong virus replication in tissue and enhance pathogenicity of a moderately virulent CSFV but does not render an avirulent virus pathogenic. Nevertheless, restoration of the N^{pro} function in the moderately virulent virus did not enhance pathogenicity to the level of the highly virulent Eystrup strain.

Local N^{pro}-mediated suppression of IFN- α / β induction and pro-inflammatory cytokine modulation by the moderately virulent GPE⁻-derived triple mutant virus carrying functional N^{pro} enhances systemic IFN- α / β responses

Next, it was examined whether restoring the N^{pro} function with the N136D substitution in the GPE⁻ parental and triple mutant viruses had any effect on the innate immune responses. To this end, three 4-week-old pigs per group were injected intramuscularly with the same dose of the parental or mutant viruses or with Eystруп strain as in the previous experiment. mRNA expression of the cytokines IFN- α , IFN- γ , IL-1 α , IL-6, IL-10, and TNF- α were measured in the brains, tonsils, spleens, kidneys, and MLN at 120 h pi. The IFN- α mRNA expression in the tonsils of pigs inoculated with the mutant vGPE⁻/N136D or vGPE⁻/N136D; T830A; V2475A; A2563V viruses was significantly reduced compared with IFN- α mRNA in pigs inoculated with the parental viruses, which is consistent with functional N^{pro} (Fig. 12). Conversely, IL-6 and TNF- α mRNA expression in the tonsils tended to be increased by infection with viruses carrying the N136D mutation in N^{pro} compared to infection with the parental viruses (Fig. 12). IL-6 mRNA expression in the kidneys was significantly increased by infection with viruses carrying the N136D mutation in N^{pro} compared to infection with the parental viruses (Fig. 13). With the other cytokines and tissue samples, no significant differences with respect to the N^{pro} mutant viruses were observed. In the serum, no IFN- α / β bioactivity was detected after infection with vGPE⁻ and vGPE⁻/N136D viruses at any timepoint pi (Fig. 14). Interestingly, significantly higher of IFN- α / β bioactivity was observed in sera from pigs inoculated with the vGPE⁻/N136D; T830A; V2475A; A2463V virus than in pigs inoculated with the parental vGPE⁻/T830A; V2475A; A2563V virus, despite N^{pro}-mediated suppression of

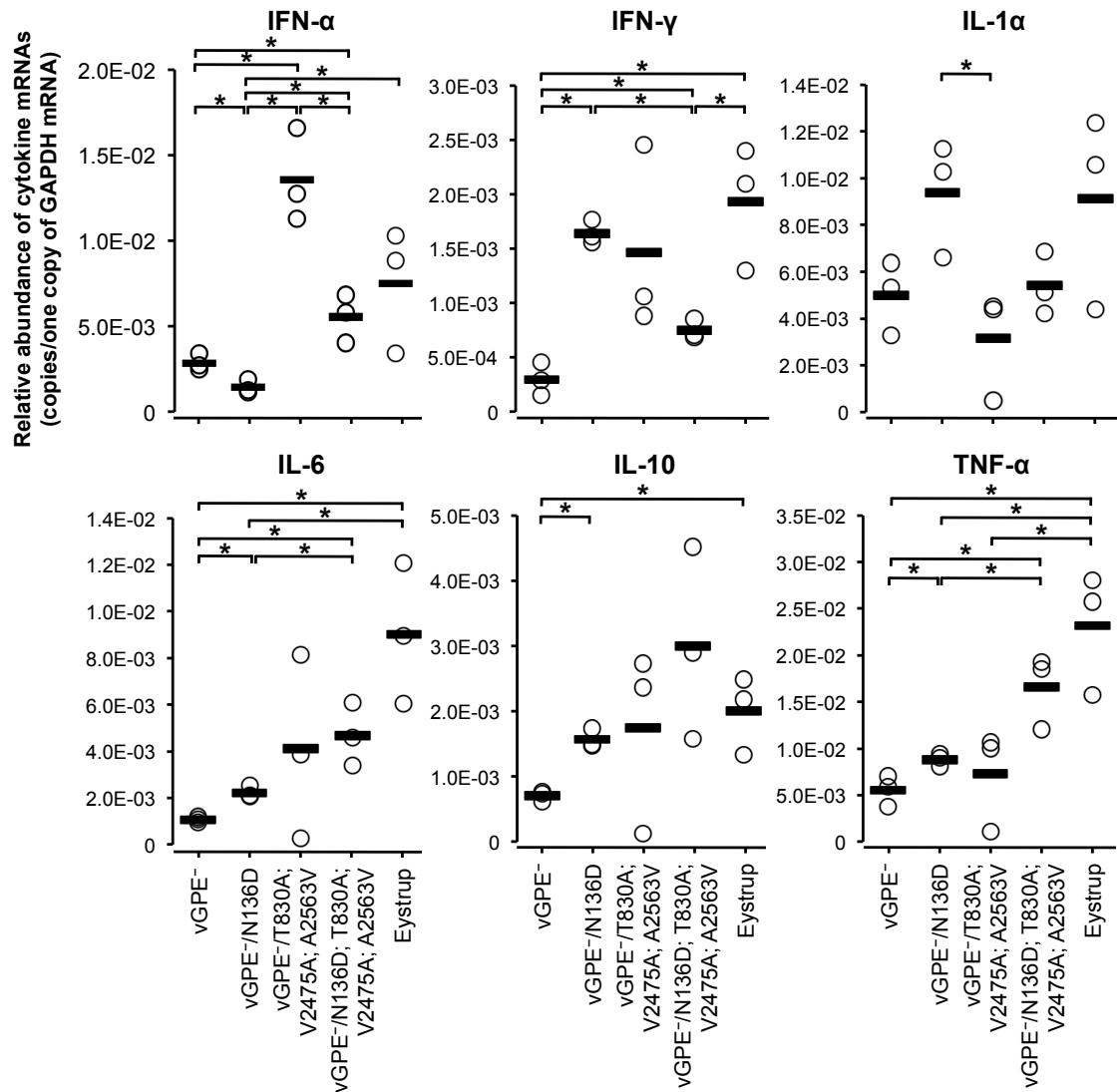


Fig. 12. Cytokine mRNA expression in tonsils of pigs inoculated with the GPE⁻-derived viruses and with Eystруп strain. Tonsils were collected from three pigs per group at 120 h pi, and the mRNA levels of IFN- α , IFN- γ , IL-1 α , IL-6, IL-10, and TNF- α were analyzed by RT and qPCR. The mRNA levels are shown as mean value after normalization with GAPDH mRNA content. The mean values with error bars represent the standard error. The statistical significance of the differences between the cytokine mRNA levels was calculated using the Student's *t*-test. * indicates $p < 0.05$.

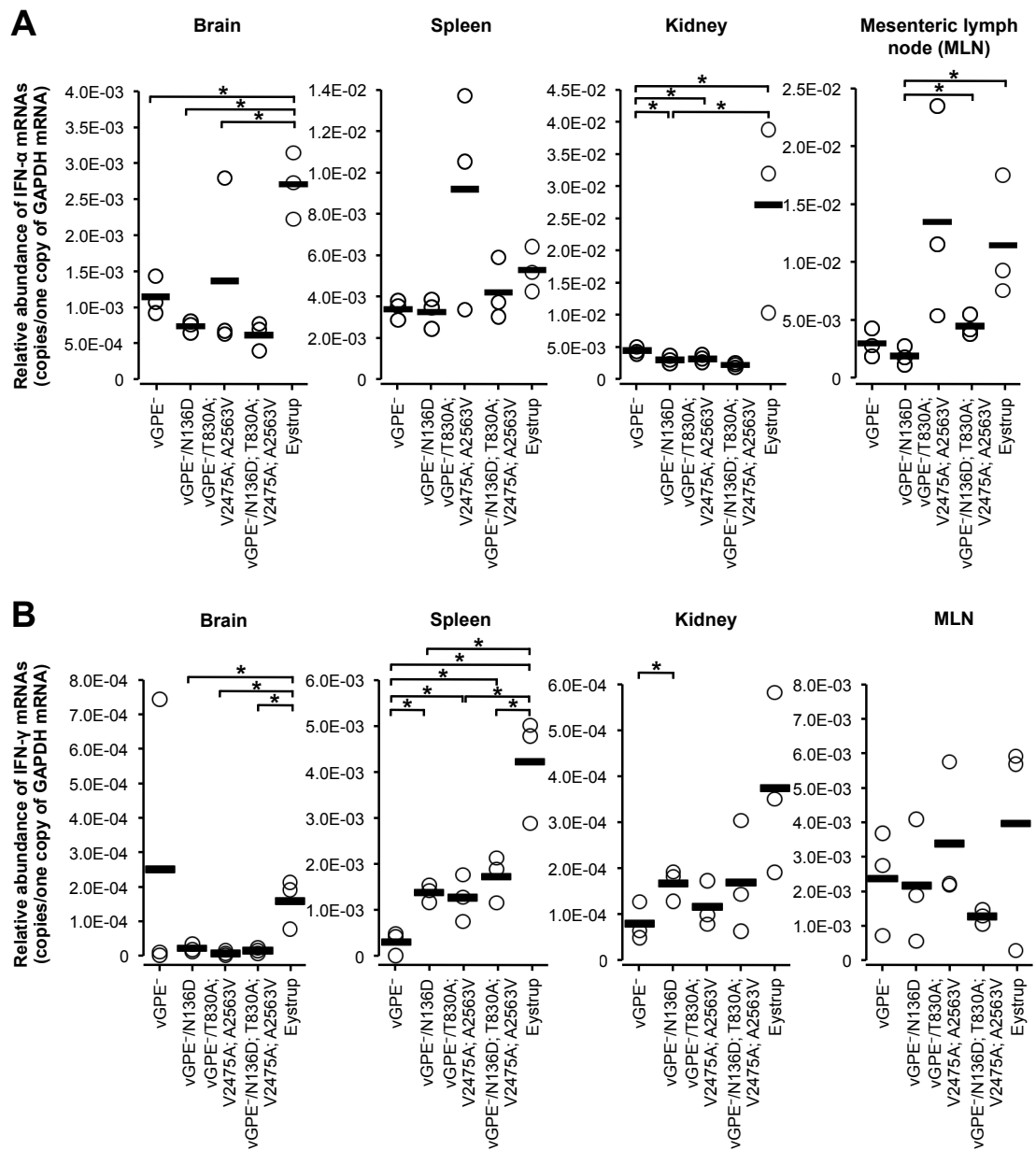


Fig. 13. Cytokine mRNA expression in the systemic tissue, brains, spleens, kidneys, and MLN, of pigs inoculated with the GPE⁻-derived viruses and with Eystруп virus. Tissues from brains, spleens, kidneys, and MLN were collected from three pigs per group at 120 h pi and the mRNA levels of IFN- α (A), IFN- γ (B), IL-1 α (C), IL-6 (D), IL-10 (E), and TNF- α (F) were analyzed by RT and qPCR. The mRNA levels are shown as mean values after standardization using the mRNA abundance of GAPDH. The mean values with error bars represent the standard error. Statistically significant differences between mRNA levels of cytokines were detected using the Student's *t*-test. * indicates $p < 0.05$.

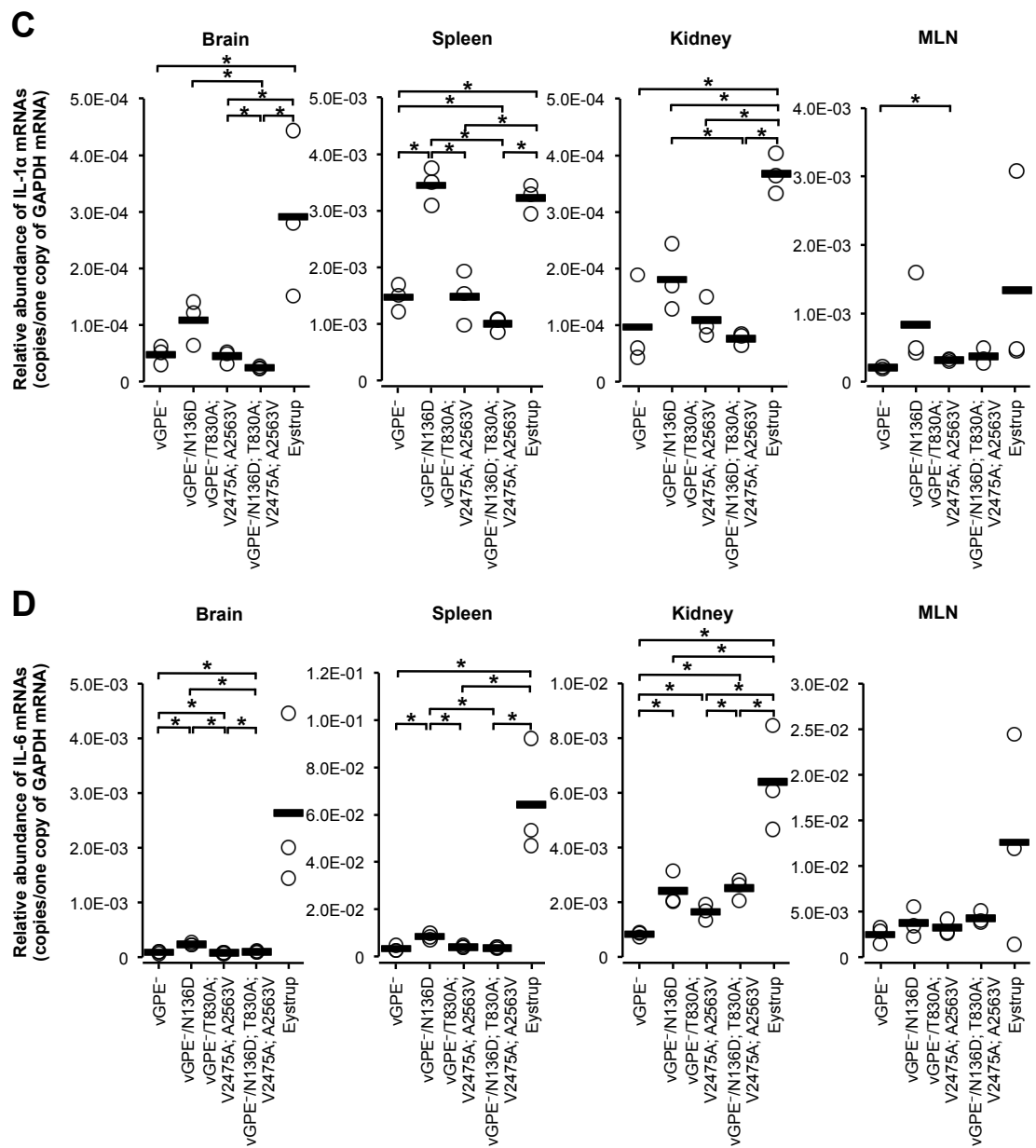


Fig. 13. (continued)

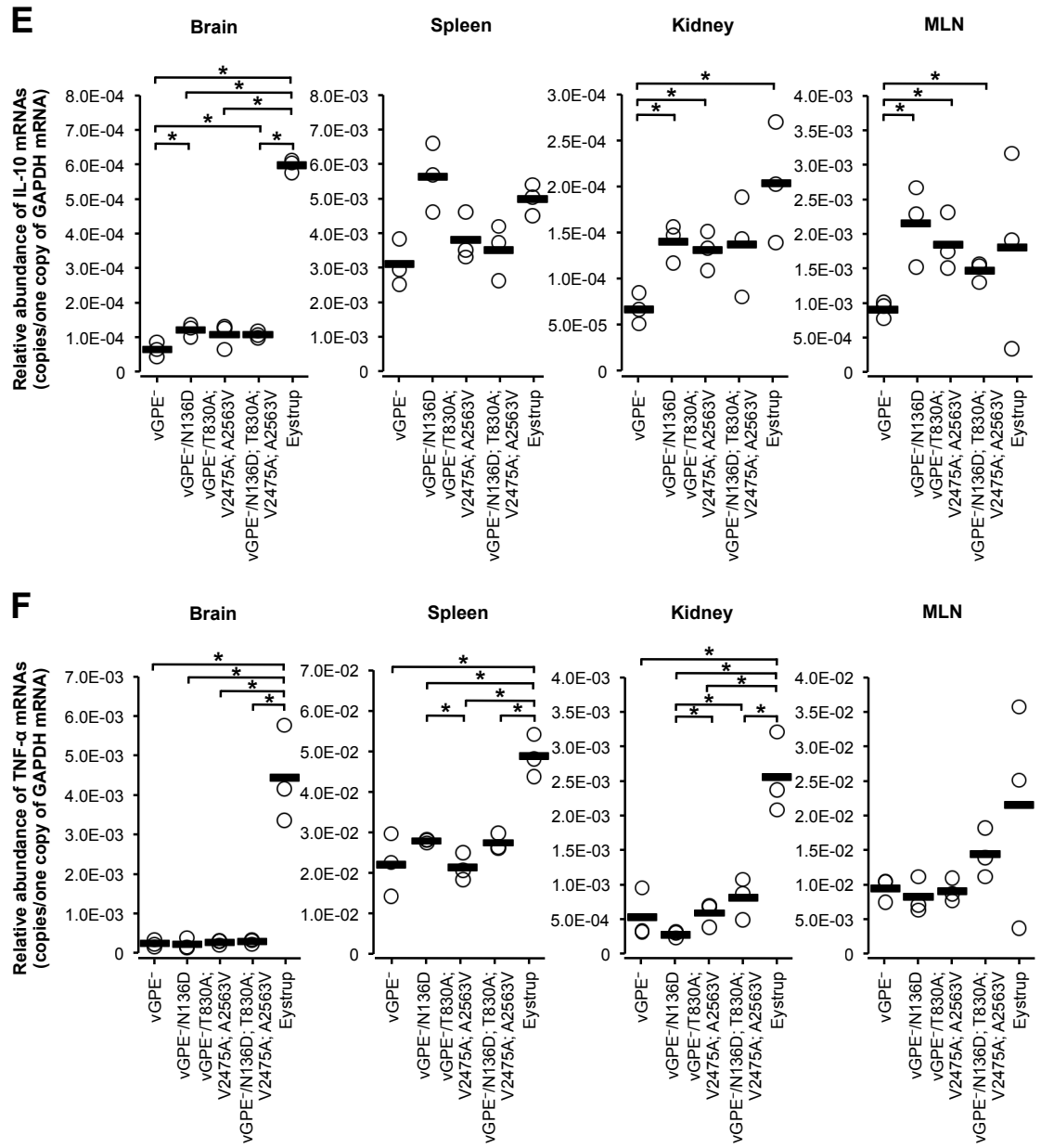


Fig. 13. (continued)

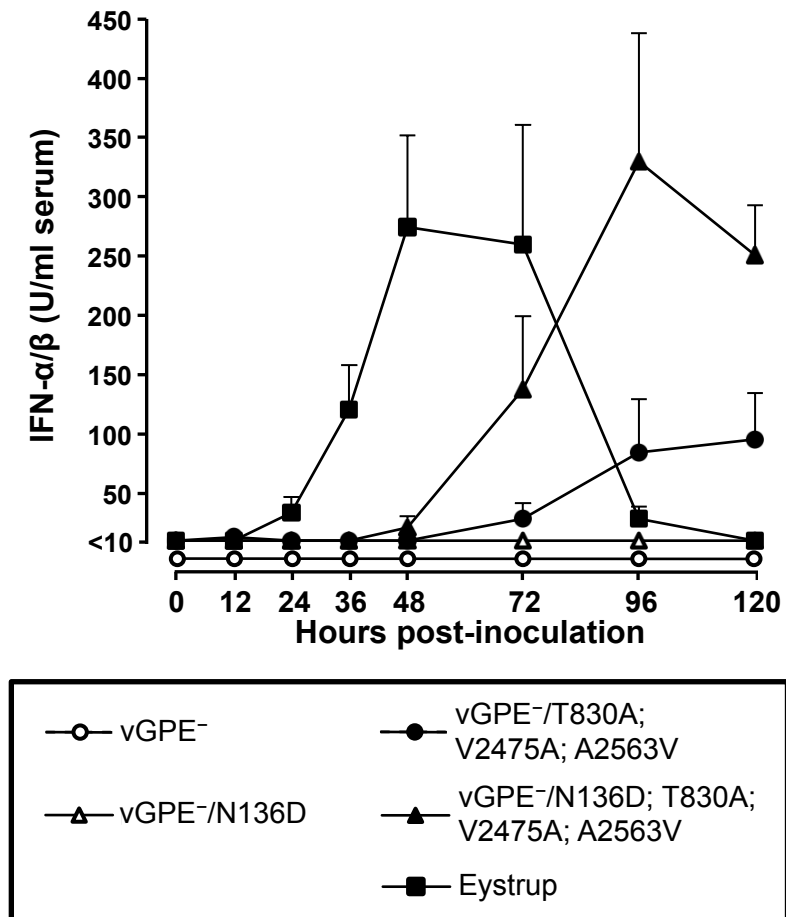


Fig. 14. IFN- α/β bioactivity in serum of pigs inoculated with the vGPE⁻-derived viruses and with Eystруп strain. Groups of three pigs were inoculated with the indicated viruses and blood was collected at 0, 12, 24, 36, 48, 72, 96, and 120 h pi. Serum IFN- α/β bioactivity was measured in duplicate using SK6-MxLuc cells. The error bars represent the standard deviation.

IFN- α/β induction. The highest IFN- α/β bioactivity and cytokine expression levels in serum and tissue respectively were obtained with the highly virulent Eystруп strain (Fig. 12, Fig. 13, and Fig. 14). Taken together, these data indicate that the N^{pro}-mediated suppression of IFN- α/β induction impairs innate immune activation and modulates pro-inflammatory cytokines in local sites *in vivo* with the consequence of enhancing virus replication, systemic IFN- α/β responses, and disease. In addition, these data support the model of CSF pathogenesis in which local IFN- α/β inhibition by N^{pro} contributes to enhanced virus replication resulting in elevated systemic IFN- α production and disease.

Discussion

Here, in Chapter II, restoring the zinc-binding domain with the N136D substitution in N^{pro} of the GPE⁻-derived viruses was sufficient to recover the capacity of N^{pro} to suppress IFN- α/β production completely in cell culture and at the local replication sites *in vivo*. Moreover, pigs inoculated with the virus carrying functional N^{pro} in the triple mutant virus (vGPE⁻/N136D; T830A; V2475A; A2563V) showed a longer course of viremia than pigs infected with the parental END⁻ triple mutant virus. Several reports suggested a role for the IRF3-degrading function of N^{pro} in persistent infection in vascular endothelial cells (83), and in mononuclear cells and lymphocytes of lymph node cells, splenocytes, and gut-associated lymphoid tissue cells (84, 85). Meyers *et al.* (86) suggested that N^{pro} and E^{ms} of BVDV are involved in establishing persistence after trans-placental infection *in vivo*. All this strongly suggests that IRF3 degradation mediated by N^{pro} contributes to the establishment of persistent infections *in vivo*.

N^{pro}-mediated suppression of IFN- α/β induction affected viral replication *in vivo*. Virus recovery from collected organs of pigs inoculated with the N^{pro} mutant and parental viruses were significant different between vGPE⁻/N136D; T830A; V2475A; A2563V and vGPE⁻/T830A; V2475A; A2563V infection on day 14 pi. In pigs, viremia was detected earlier after infection with viruses carrying the N^{pro} mutation. These data indicate that functional N^{pro} suppressed IFN- α/β induction and enhanced virus replication in tissues at the local replication sites, allowing the virus to spread more efficiently. CSFV pathogenicity was enhanced by restoring N^{pro} function in the moderately virulent virus backbone, whereas functional N^{pro} in the avirulent virus backbone did not confer any pathogenic phenotype. These results indicate that

functional N^{pro} is not a virulence determinant on its own but acts as a co-factor of virulence by enhancing virus replication and spread through local inhibition of innate immune activation.

The presence of functional N^{pro} altered the production of IFN- α / β and pro-inflammatory cytokines in infected pigs. Functional N^{pro} did significantly reduce the local IFN- α mRNA expression in the infected tissues while it enhanced the quantities of IFN- α / β in the circulation. The sera from the animals infected with the vGPE⁻/N136D; T830A; V2475A; A2563V virus had significantly higher IFN- α / β bioactivity than the sera from the vGPE⁻/T830A; V2475A; A2563V-infected pigs. The difference between local IFN- α / β inhibition and systemic IFN- α / β levels may be explained with the following hypothesis. The pDCs are the major producer of IFN- α / β during CSFV infection (87) and rely essentially on IRF7 for IFN- α induction (88). In contrast, the non-pDC such as macrophages, conventional dendritic cells, endothelial cells, and epithelial cells which are the major replication sites targeted by the virus induce IFN- α / β mainly through activation of IRF3 (89). While in pDCs IRF7 is expressed constitutively, non-pDCs signal through IRF7 only after IRF7 induction by the IFN-dependent autocrine–paracrine amplification loop (90). IRF7 is the most potent transcription factor for IFN- α production (91), and wild-type CSFV induces IFN- α synthesis more efficiently in pDCs compared with CSFV devoid of functional N^{pro} (70). Previous data demonstrated that functional N^{pro} inhibits the IRF3 pathway completely while it does only slightly reduce the activity of IRF7-dependent pathways (70, 72). Thus, in the different tissues representing the major replication sites of the virus, the GPE⁻-derived viruses with restored N^{pro} function suppress IFN- α / β production by blocking the IRF3 pathway, supporting local replication. The GPE⁻-derived virus

that carries a functional N^{pro} and the triple mutations conferring virulence, as well as the highly virulent Eystrup strain can then spread and replicate to high titers and infect pDC resulting in high systemic IFN- α levels.

mRNA expression of the cytokines IL-6 and TNF- α was increased by infection with CSFV. However, in serum, all of these pro-inflammatory cytokines was under detectable levels or not related to the virus infection (data not shown). This conclusion might be due to host factors, *e.g.*, age, breeds, and also virus virulence itself. Relationship between virus virulence and systemic immune responses should be clarified in further studies. Infection with the highly virulent virus resulted in a marked increase of these cytokine mRNAs compared to infection with a virus of moderate or low virulence. Previous immunopathogenesis studies of CSF and other viral hemorrhagic fevers suggested that augmented secretion of pro-inflammatory cytokines such as IL-1, IL-6, and TNF- α by viral infection causes intravascular coagulation, thereby inducing thrombocytopenia (92, 93). It is conceivable that the thrombocytopenia observed in pigs inoculated with vGPE⁻/N136D; T830A; V2475A; A2563V and Eystrup viruses is related to the increased IL-6 and TNF- α mRNA induction as a consequence of efficient replication.

In summary, the present study in this Chapter II demonstrates that the viral protein N^{pro} influences immune response *in vivo* and is required for enhanced pathogenicity. The new insights described here provide improved understanding the molecular mechanisms involved in CSF pathogenesis, including persistent infections.

Summary

The viral nonstructural protein N^{pro} of CSFV interferes with IFN- α/β induction by promoting the degradation of IRF3. During the establishment of the live attenuated CSF vaccine strain GPE⁻, N^{pro} acquired a mutation that abolished its capacity to bind and degrade IRF3, rendering it unable to prevent IFN- α/β induction. In Chapter I, it was shown that the GPE⁻ vaccine strain became pathogenic after forced serial passages in pigs, which was attributed to the amino acid substitutions T830A in the viral proteins E2 and V2475A and A2563V in NS4B. Interestingly, during the re-adaptation of the GPE⁻ vaccine strain in pigs, the IRF3-degrading function of N^{pro} was not recovered. Therefore, it was examined whether restoring the ability of N^{pro} to block IFN- α/β induction of both the avirulent and moderately virulent GPE⁻-derived viruses would enhance pathogenicity in pigs. Viruses carrying the N136D substitution in N^{pro} regained the ability to degrade IRF3 and suppress IFN- α/β induction *in vitro*. In pigs, functional N^{pro} significantly reduced the local IFN- α mRNA expression in lymphoid organs while it increased quantities of IFN- α/β in the circulation, and enhanced pathogenicity of the moderately virulent virus. In conclusion, the present study demonstrates that functional N^{pro} influences the innate immune response at local sites of virus replication in pigs and contributes to pathogenicity of CSFV in synergy with viral replication.

Chapter III

Intracellular membrane association of the N-terminal domain of classical swine fever virus NS4B determines viral genome replication and virulence

Introduction

A typical feature of positive-strand RNA viruses is the close association of RNA replication with intracellular membranes, which often induces membrane rearrangements (94). The membrane association of the viral nonstructural proteins forming the replication complex is mediated by specialized integral protein determinants. Examples are protein A of flock house virus that targets replication complexes to the outer mitochondrial membranes (95), nsP1 of Semliki Forest virus that binds to the inner surface of the plasma membrane (96), or NS4A of dengue virus, NS4B and NS5A of HCV, and nsp8 of coronavirus that all target RNA replication on the ER membrane (97-99). In the case of CSFV, nonstructural proteins NS3–NS5B are essential for pestivirus RNA replication (5). Therefore, these nonstructural proteins at least consist of the viral replication complex together with unknown host factors, the replicating viral RNA, and intracellular membranes.

The nonstructural protein NS4B is a 38-kDa intracellular membrane-associated protein of the viral replicase, which is analogous to HCV NS4B and other related members of the family *Flaviviridae* (51, 100, 101). However, NS4B is a poorly characterized protein and its roles in CSFV replication and pathogenesis are not well understood. A putative TIR domain was identified in the C-terminal region of NS4B (13). Mutations in this domain of NS4B in the highly virulent Brescia strain resulted in an attenuated phenotype along with enhanced activation of TLR-7-induced genes. In addition, NS4B harbors an NTPase motif (48). The NTPase activity of NS4B is essential for virus replication, but there is no evidence that modulating the NTPase activity may affect virulence. In BVDV, NS4B can act as a modulator of virus

cytopathogenicity (102). In Chapter I of the present thesis, it was demonstrated that two amino acid residues within the predicted trans-membrane domains of NS4B are involved in viral genome replication and that they contribute to the pathogenicity of CSFV in pigs. These two residues are a part of the seven amino acids that differ in NS4B between the highly virulent Eystrup strain and the GPE⁻ vaccine strain.

Here, in this Chapter III, it was examined whether the five additional amino acid differences in the N-terminal domain of NS4B would further determine viral RNA replication *in vitro* and the pathogenicity of CSFV in pigs. To this end, a chimeric virus and corresponding replicons carrying the complete NS4B gene of the highly virulent Eystrup strain in a modified GPE⁻ vaccine strain backbone were generated and analyzed for pathogenicity *in vivo* and for replication efficiency in cell culture, respectively. An *in silico* approach was used to predict the localizations of these amino acid residues in the N-terminal domain of NS4B. Intracellular membrane localization and the association of the N-terminal NS4B in these two viruses were examined.

Materials and Methods

Ethical statement

The animal experiments described in Chapter III were approved by the Animal Welfare Committee of the Canton of Berne, Switzerland with the license number BE94/12, and conducted in compliance with the Swiss animal protection law and with the national and international animal experimentation guidelines.

Viruses and cells

The CSFV strains, vGPE⁻/N136D; T830A; V2475A; A2563V, vGPE⁻/N136D; T830A; Eystруп NS4B, and vEy-37 which is identical to Eystруп, were derived from the full-length cDNA clones, *i.e.*, pGPE⁻/N136D; T830A; V2475A; A2563V, pGPE⁻/N136D; T830A; Eystруп NS4B, and pEy-37 (78), respectively. All of the cDNA-derived viruses were rescued as aforementioned (31). The complete genomes of the rescued viruses were verified by nucleotide sequencing to exclude any accidental mutations. The swine kidney 6 (SK-6) cells were propagated at 37°C in the presence of 5% CO₂ in EMEM supplemented with 0.3 mg/ml L-glutamine, and 7% horse serum.

Plasmid constructs

The cDNA clone of the mono-cistronic replicon carrying the *Gaussia* luciferase reporter gene, pGPE⁻2GL, was constructed from rPad2GL (103) by replacing the genome of the Paderborn strain with that of the GPE⁻ strain applying the In-Fusion[®] HD Cloning techniques as described in Chapter I. The cDNA clone of rPad2GL was a gift of Dr. TB. Rasmussen (DTU National Veterinary Institute, Kalvehave, Denmark).

The infectious cDNA clone (pGPE⁻/N136D; T830A; Eystrup NS4B) and the replicon cDNA clones (pGPE⁻-N^{pro}-Luc-IRES-NS3/Eystrup NS4B and pGPE⁻2GL/Eystrup NS4B) were constructed by replacing NS4B in the backbone with the corresponding gene of the highly virulent strain Eystrup as described above. The two cDNA clones of the replication-deficient replicons (pGPE⁻-N^{pro}-Luc-IRES-NS3/GAA and pGPE⁻2GL/GAA) encoding the amino acid sequence ‘GAA’ instead of ‘GDD’ in the polymerase active site of the nonstructural protein NS5B (104) were constructed from the replicon cDNA clones, pGPE⁻-N^{pro}-Luc-IRES-NS3 and pGPE⁻2GL using the QuikChange XL Site-Directed Mutagenesis Kit, KOD FX, KOD -Plus- Neo, and oligonucleotide primers containing the respective mutations based on the aforementioned standard techniques. The replicon cDNA clones of the disrupted amphipathic character (helix mutant: HMT) in the N-terminal domain of GPE⁻ NS4B (pGPE⁻-N^{pro}-Luc-IRES-NS3/NS4B HMT and pGPE⁻2GL/NS4B HMT) were accordingly generated from the parental clones (pGPE⁻-N^{pro}-Luc-IRES-NS3 and pGPE⁻2GL) by mutagenesis described above.

In order to construct plasmids expressing Green Fluorescent Protein (GFP), NS4B with a C-terminal GFP-tag, and the truncated versions thereof, the cDNA fragments of the respective regions of the GPE⁻ and Eystrup strains were obtained using RT and PCR, and the GFP fragments were cloned into the pCI Mammalian Expression Vector (Promega) with In-Fusion[®] HD Cloning and appropriate restriction enzymes. Details of the constructs are available on request.

Sequencing

The cDNA clones, *in vitro*-rescued viruses, and expression vectors were completely sequenced as described in Chapter I. In brief, nucleotide sequencing of the

cDNA clones, expression vectors, and PCR fragments from viral RNA was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit and a 3500 Genetic Analyzer. The sequencing data were analyzed using the Genetyx[®]-Network version 12 (Genetyx).

Antibodies and markers

The E2-specific mAb HC/TC26 (105) was kindly provided by Dr. I. Greiser-Wilke (University of Veterinary Medicine Hannover, Hannover, Germany). The mAb against viral NS3, 46/1 was generated previously (32). The antibodies against cellular organelles, GAPDH and calnexin were purchased from Cell Signaling Technology (Danvers, MA, USA) and Enzo Life Sciences (Farmingdale, NY, USA), respectively. The mAb against GFP was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). A secondary antibody, FITC-conjugated goat anti-mouse IgG, was purchased from MP Biomedicals, and F(ab')₂-Goat anti-Mouse IgG (H+L) secondary antibody, Alexa Fluor 488[®] conjugate was obtained from Life Technologies. Fluorescent probes for detecting the ER (ER-Tracker), the mitochondria (MitoTracker), and the lysosomes (LysoTracker) were purchased from Life Technologies. For counterstaining of the cell nuclei, 4',6-Diamino-2-phenylindole, dihydrochloride (DAPI) was obtained from Dojindo Molecular Laboratories (Kumamoto, Japan).

Virus titration

The virus titers were determined by end-point dilution with SK-6 cells and immunoperoxidase staining using anti-E2 mAb HC/TC 26 or anti-NS3 mAb 46/1 as described earlier (33, 106). The titers were calculated using the Reed and Muench formula and expressed as TCID₅₀ per ml (34).

Experimental infection of pigs

In order to assess the pathogenicity of the cDNA-derived viruses, groups of six 10-week-old to 13-week-old Large White SPF pigs obtained from the breeding unit of The Institute of Virology and Immunology (IVI, Middelhäusern, Switzerland) were inoculated intranasally with $10^{6.0}$ TCID₅₀ of the respective viruses. All pigs were kept in separate isolation units in the BSL-4 facility of the IVI. During the animal experiment, this facility was used as a BSL-3 Agriculture. The body temperature and clinical scores were monitored daily according to a defined scoring system as described previously (107). Whole blood was collected for serum preparation at 3 days before infection and on days 3, 4, 5, 6, 7, 10, and 12 pi. The pigs that survived the infection were euthanized on day 14 pi. The virus titers in the serum samples were expressed as the TCID₅₀/ml.

Luciferase assay

Luciferase assays using bi-cistronic replicons were conducted as previously described, with some modifications. In brief, $10^{6.0}$ SK-6 cells were electroporated with 1 µg of replicon RNA. Electroporation was performed at 200 V and 500 µF. At different times after transfection, cell extracts were prepared with 200 µl of passive lysis buffer, and the firefly luciferase activity was measured using a Luciferase Assay System and a Lumat LB9507 luminometer.

Luciferase assays with mono-cistronic replicons were performed as aforementioned, with some modifications (103). Electroporation was conducted under the conditions described above. At the time indicated, 50 µl of cell medium was harvested from the supernatants of cells transfected with replicons, which was then

frozen at -20°C . After thawing, 20 μl of the cell medium was analyzed to determine the *Gaussia* luciferase activity using *Renilla* luciferase substrate and a Lumat LB9507 luminometer.

The results representing the luciferase activities that were compared with the luciferase activity of the replicon devoid of polymerase activity at the initial timepoints were measured on the basis of three independent experiments.

Immunofluorescence assay

SK-6 cells were transfected with the expression vectors using Lipofectamine 3000 (Life Technologies) and Opti-MEM (Life Technologies), according to the manufacturer's protocol. At the time indicated, SK-6 cells grown on an eight-well chamber slide (Matsunami Glass, Osaka, Japan) were fixed and stained as aforementioned (108, 109). Anti-GFP mAb as the primary antibody was used at a concentration of 1 mg/ml. As the secondary antibodies, FITC-conjugated goat anti-mouse IgG (1: 2,500) or Alexa-Fluor-conjugated IgG (1: 1,000) were used. In order to counterstain the cell nuclei, the cells were incubated for 30 min with 2 μg DAPI/ml in PBS at room temperature. For labeling the cell organelles, the cells were grown on 35-mm glass-based dishes (Asahi Glass, Tokyo, Japan) and washed with PBS, before staining for 30 min with 100 mM ER-Tracker, MitoTracker, and LysoTracker in complete medium at 37°C with 5% CO_2 . The cells were then washed twice with PBS and pre-warmed PBS was added. The fluorescent cells were analyzed with a BZ-9000 (KEYENCE, Osaka, Japan) microscope and a BZ-II Analyzer (KEYENCE). The confocal fluorescent images were acquired using Zeiss LSM700 (Carl Zeiss) microscopes (upright and inverted) and ZEN 2012 (Carl Zeiss). Quantitative

co-localization analysis was performed as described elsewhere (110). The co-localization ratio of the GFP signals with the organelle marker that represents a co-localization coefficient was quantified using ZEN 2012 software.

Membrane flotation assay

SK-6 cells were grown on 10-cm dishes overnight and transfected with the GFP-tagged NS4B and truncated vectors or with the control GFP constructs according to the conditions described above. At 30 h post-transfection (pt), the cells were re-suspended in hypotonic buffer (10 mM Tris-HCl [pH 7.5], 10 mM KCl, 5 mM MgCl₂) containing a protease inhibitor (Complete Mini; Roche). The cells were then disrupted by 20 passages through a 25G needle with 1ml syringe, ensuring approximately 90% disruption. The samples were spun at $1,000 \times g$ for 5 min at 4°C to pellet the cellular debris and nuclei, thereby obtaining postnuclear supernatants. A discontinuous iodixanol (OptiPrep; Alere Technologies AS, Oslo, Norway) gradient (5%, 25%, and 30%) was layered on top of the samples, followed by centrifugation at $230,140 \times g$ for 24 h at 4°C in a P40ST Rotor (Hitachi Koki, Tokyo, Japan). In total, eight equal fractions were collected from the top to bottom. Each fraction was precipitated with methanol-chloroform and analyzed by SDS-PAGE followed by immunoblotting using anti-GFP, anti-GAPDH, and anti-calnexin as described previously (79). For the membrane dissociation experiments, the postnuclear supernatants were adjusted to 0.25 M sucrose and centrifuged at $100,000 \times g$ for 45 min at 4°C. The pellet containing the membranes and their associated proteins was re-suspended in either high salt buffer (1 M NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA), 100 mM sodium carbonate (pH 11.5), or 1% Triton X-100. After incubation

at 4°C for 30 min, the samples were subjected to membrane flotation followed by immunoblotting, as described above. Typically, the membrane-bound proteins were associated with fractions 1–4 whereas the soluble proteins were generally present in fractions 5–8, which were followed by the respective protein markers.

Structure predictions and sequence analyses

Multiple sequence alignment and amino acid conservation analyses were performed with the CLUSTALW2 program (111) using the default parameters. The protein secondary structure predictions were generated using DSC (112), HNN (113), MLRC (114), PHD (115), Jpred 3 (116) Porter (117), PSIPRED (118), and APSSP2 (119). The trans-membrane domains were predicted using TopPred II (120). HeliQuest (121) was used for α -helix projections.

Statistical analysis

The Student's *t*-test was used to perform the statistical analysis. Differences were considered to be significant when $p < 0.05$.

Results

The N-terminal domain of NS4B of the highly virulent CSFV strain Eystrup confers enhanced pathogenicity to a low virulent GPE⁻-derived virus

So far, the studies described in Chapter I and II of the present thesis demonstrate that the combination of the four amino acid residues in N^{pro} (D₁₃₆), E2 (A₈₃₀), and NS4B (A₂₄₇₅ and V₂₅₆₃) can confer pathogenicity to the CSFV GPE⁻ vaccine strain in pigs (Fig. 15). These four amino acid residues are conserved in the highly virulent Eystrup strain, although a total of 62 amino acids differ between GPE⁻ and Eystrup strains. The two amino acid residues in the central part of NS4B (A₂₄₇₅ and V₂₅₆₃) are part of a total of seven amino acid differences in NS4B. The remaining five amino acid differences (positions at 2377, 2391, 2398, 2399, and 2414) were observed in the N-terminal domain of NS4B. In order to assess whether the five amino acids differing from the GPE⁻ strain in the N-terminal domain of Eystrup NS4B contribute to pathogenicity in pigs, NS4B of the low virulent GPE⁻-derived virus (vGPE⁻/N136D; T830A; V2475A; A2563V) was replaced with NS4B from the highly virulent Eystrup strain. This chimeric virus was termed vGPE⁻/N136D; T830A; Eystrup NS4B. Groups of six 10-week-old to 13-week-old pigs were inoculated intranasally with 10^{6.0} TCID₅₀ of these two GPE⁻-derived viruses. As a control, six pigs were inoculated with the highly virulent Eystrup strain. Infection with the parental virus (vGPE⁻/N136D; T830A; V2475A; A2563V) did not result in any clinical symptoms in this trial (Fig. 16A, left panel). Nevertheless, the body temperature of these pigs was slightly elevated between days 3 and 7 after infection, and low-level viremia was detected during 2 days (Fig. 16B and C, left panels). In contrast, all of the six pigs inoculated with the vGPE⁻/N136D;

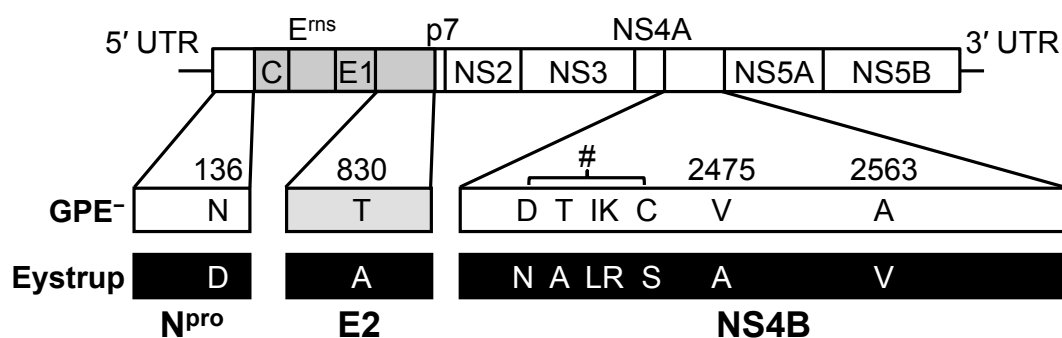


Fig. 15. Schematic representation of the viral genome and of the amino acids of the viral proteins relevant for this chapter III. The CSFV genome and the viral proteins N^{pro}, E2, and NS4B of the GPE⁻ vaccine strain and of the highly virulent Eystруп strain are depicted schematically. The amino acid differences related to virulence presented in Chapter I and II, and the five additional residues in the N-terminal half of NS4B (2377, 2391, 2398, 2399, and 2414) evaluated in the present study (#) are shown. Numbering starts with the methionine encoded by the AUG start codon.

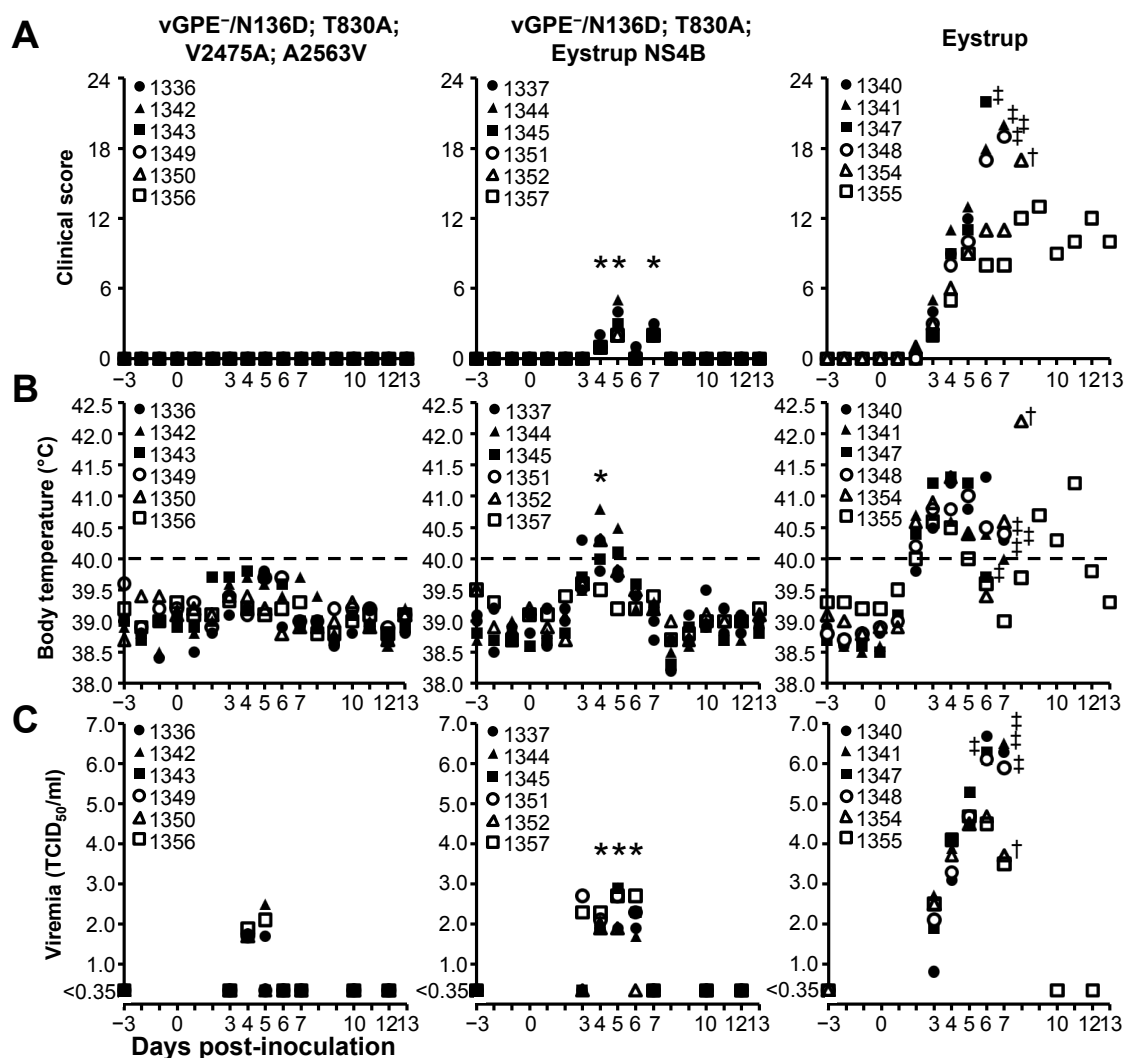


Fig. 16. Evaluation of the contribution of NS4B to CSFV pathogenicity. Three groups of six 10-week-old to 13-week-old SPF pigs were inoculated intranasally with $10^{6.0}$ TCID₅₀ of the parental vGPE⁻/N136D; T830A; V2475A; A2563V virus, the mutant vGPE⁻/N136D; T830A; Eyrstrup NS4B virus, and the highly virulent Eyrstrup strain, respectively. Clinical scores (A), body temperatures (B), and virus titer in the serum (C) were monitored. A body temperature of above 40°C was considered as fever. At terminal stages of the disease, the animals were euthanized according to defined endpoint criteria (‡) or died unexpectedly (†). The statistical significance of the differences was calculated using the Student's *t*-test. * indicates significance ($p < 0.05$) between the GPE⁻-derived mutant viruses (vGPE⁻/N136D; T830A; V2475A; A2563V and vGPE⁻/N136D; T830A; Eyrstrup NS4B).

T830A; Eystrup NS4B virus developed overt clinical manifestations between days 4 and 7 pi, with five out of six pigs showing fever (Fig. 16A and B, middle panels). Viremia was detected during 4 days (Fig. 16C, middle panel). Infection with the highly virulent Eystrup strain resulted in severe clinical signs with high and prolonged fever and viremia in all six pigs. Four pigs were euthanized when they reached a clinical score above 18, between days 6 and 7 pi. One pig died unexpectedly on day 8 pi, and one pig recovered by day 14 pi. These data demonstrate that the N-terminal domain of NS4B contributes to pathogenicity of CSFV in pigs. Nevertheless, NS4B of the highly virulent Eystrup virus is not solely responsible for the high pathogenicity of this virus.

The N-terminal domain of NS4B of the highly virulent CSFV strain Eystrup contributes to enhanced RNA replication

Mono-cistronic and bi-cistronic replicons (Fig. 17A and B) were used to determine the effect on viral RNA replication of the amino acids of NS4B differing between the two strains. In SK-6 cells at 24 h post-electroporation, the bi-cistronic replicon carrying NS4B gene of the highly virulent Eystrup strain (rGPE⁻-N^{pro}-Luc-IRES-NS3/Eystrup NS4B) resulted in two-fold higher luciferase activity than the replicon carrying only the two amino acid substitutions in the predicted trans-membrane domains of NS4B (rGPE⁻-N^{pro}-Luc-IRES-NS3/V2475A; A2563V), and in five-fold higher activity of the firefly luciferase than the parental GPE⁻ virus-derived replicon (Fig. 17C). Consistent with the results obtained with the bi-cistronic replicons, Eystrup NS4B conferred significantly higher *Gaussia* luciferase production to the GPE⁻-derived mono-cistronic replicon compared to the replicon harboring only the two V2475A and

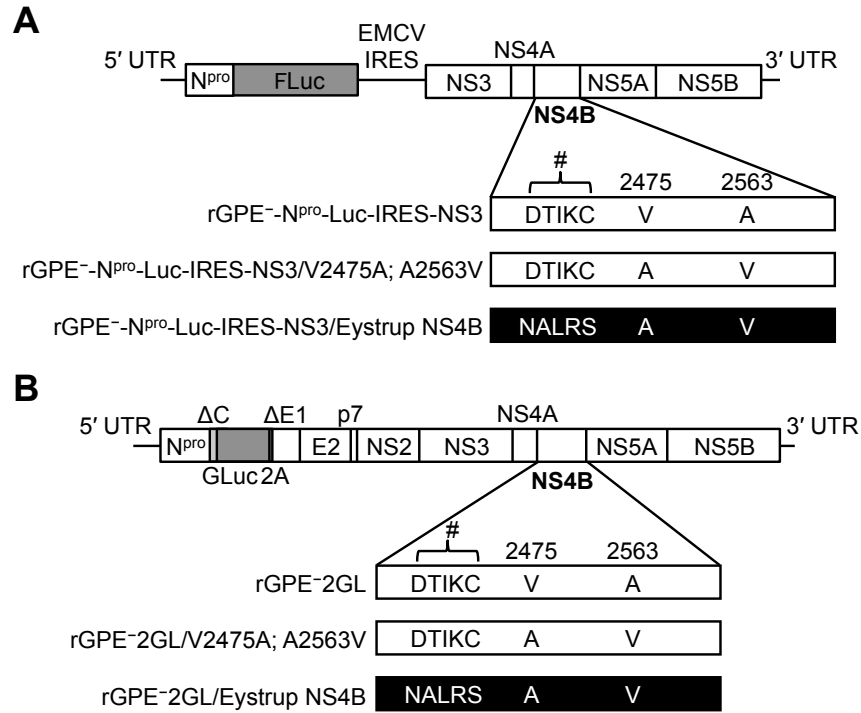
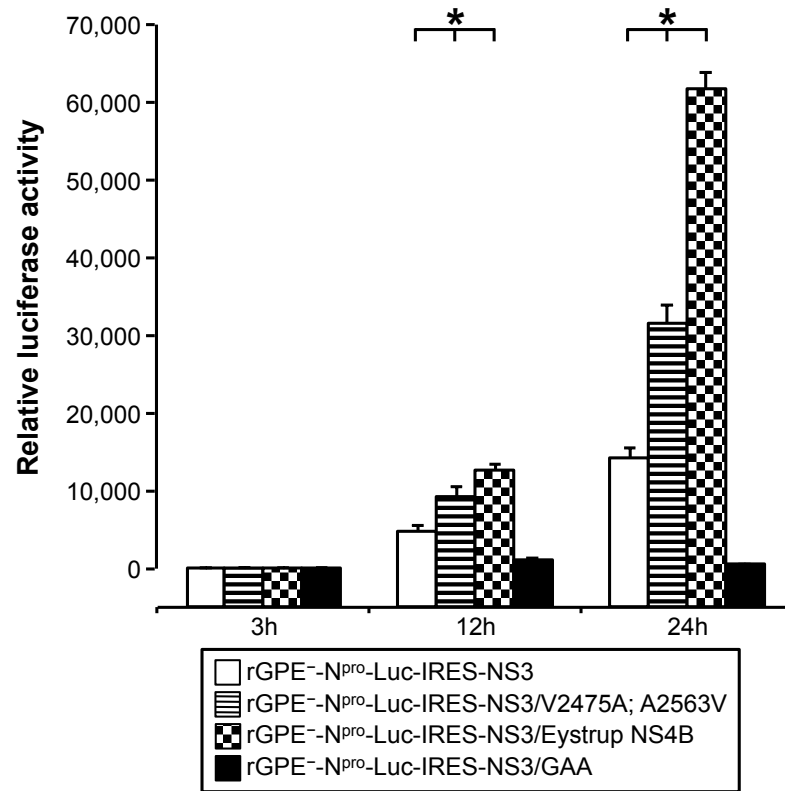
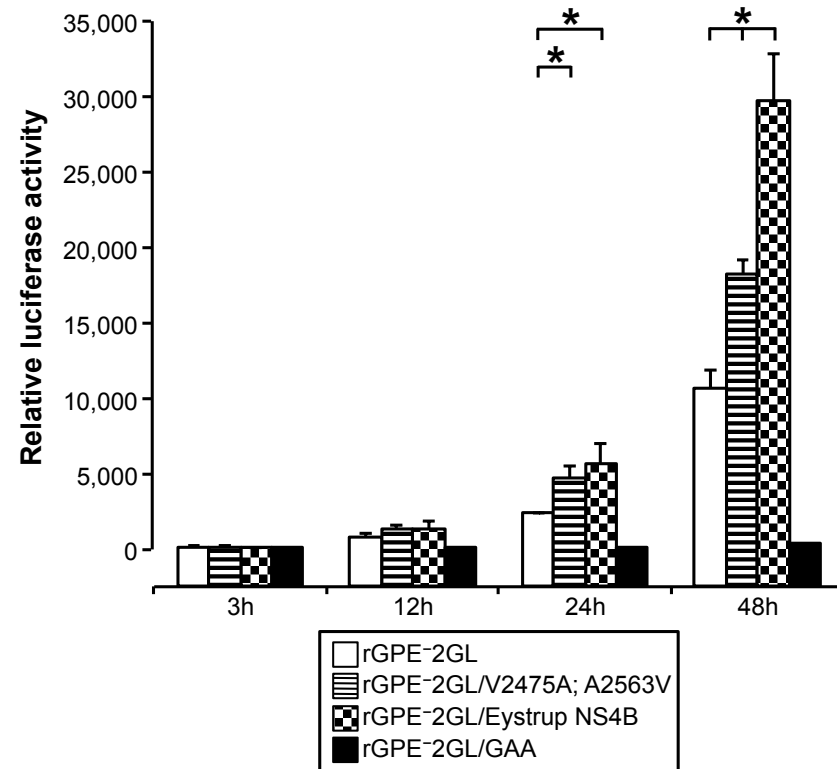


Fig. 17. Comparison of the effect of NS4B from low and highly virulent CSFV on the replication efficiency of GPE⁻-derived replicons. GPE⁻-derived bi-cistronic (A) and mono-cistronic (B) luciferase reporter replicons carrying the GPE⁻ NS4B or its NS4B with the two mutations V2475A and A2563V or the complete NS4B of the highly virulent Eystrup strain were utilized. # indicates the locations of the five amino acid differences (2377, 2391, 2398, 2399, and 2414) in the N-terminal domain of these strains. Corresponding bi-cistronic (rGPE⁻-N^{pro}-Luc-IRES-NS3/GAA) and mono-cistronic (rGPE⁻-2GL/GAA) replicons encoding an inactive NS5B polymerase were used as controls. After the indicated incubation periods, the firefly luciferase activity from cell lysates (C) and the *Gaussia* luciferase activity from cell culture supernatants (D) was measured. The mean values of the luciferase activity compared with the luciferase activity of the replicons devoid of NS5B polymerase activity at the initial timepoints were obtained from three independent experiments. The error bars represent the standard error. The statistical significance of the differences was calculated using the Student's *t*-test. Significance (*p* < 0.05) is indicated with an asterisk.

C**D****Fig. 17. (continued)**

A2563V substitutions in GPE⁻ backbone that was also more efficient at expressing the reporter protein than the replicon representing the parental GPE⁻ vaccine strain (Fig. 17B and D). These data indicate that the additional five amino acids that differ from GPE⁻ in the N-terminal domain of Eystруп NS4B contribute to enhanced RNA replication efficiency in cell culture.

The N-terminal domain of NS4B forms a predicted amphipathic α -helix and mediates intracellular membrane association

Secondary-structure prediction was carried out with the NS4B polyprotein sequence of the GPE⁻ and Eystруп strains by using several different software. The consensus structures are shown in Fig. 18A. CSFV NS4B comprises an N-terminal domain (amino acids [aa] of NS4B 1–81), a central part that harbors four predicted trans-membrane segments (TM) (aa 82–245), and a C-terminal domain (aa 246–347). The N-terminal domain contains two predicted α -helices from aa 5 to 30 (α 1) and from aa 40 to 81 (α 2), respectively. Interestingly, five out of the seven amino acid differences between NS4B of the GPE⁻ vaccine strain and of the Eystруп strain are located within the second α -helix, α 2. This α -helix (aa 40–81) is predicted to have an amphipathic structure (Fig. 18B). In order to elaborate on this, the plasmids were constructed for cytomegalovirus promoter-driven expression of the N-terminal α 1-helix (aa 1–39), of the helix α 2 (aa 40–81), of the two α -helices together (aa 1–81), and of the full-length NS4B protein, all tagged with the GFP at the C terminus (Fig. 19A). Then, the hydrophobic face of the helix was genetically disrupted by substitution of charged amino acids glutamates from the nonpolar amino acids (Fig. 19B). When these different GPE⁻-derived GFP-tagged NS4B variants were expressed in the porcine SK-6

A

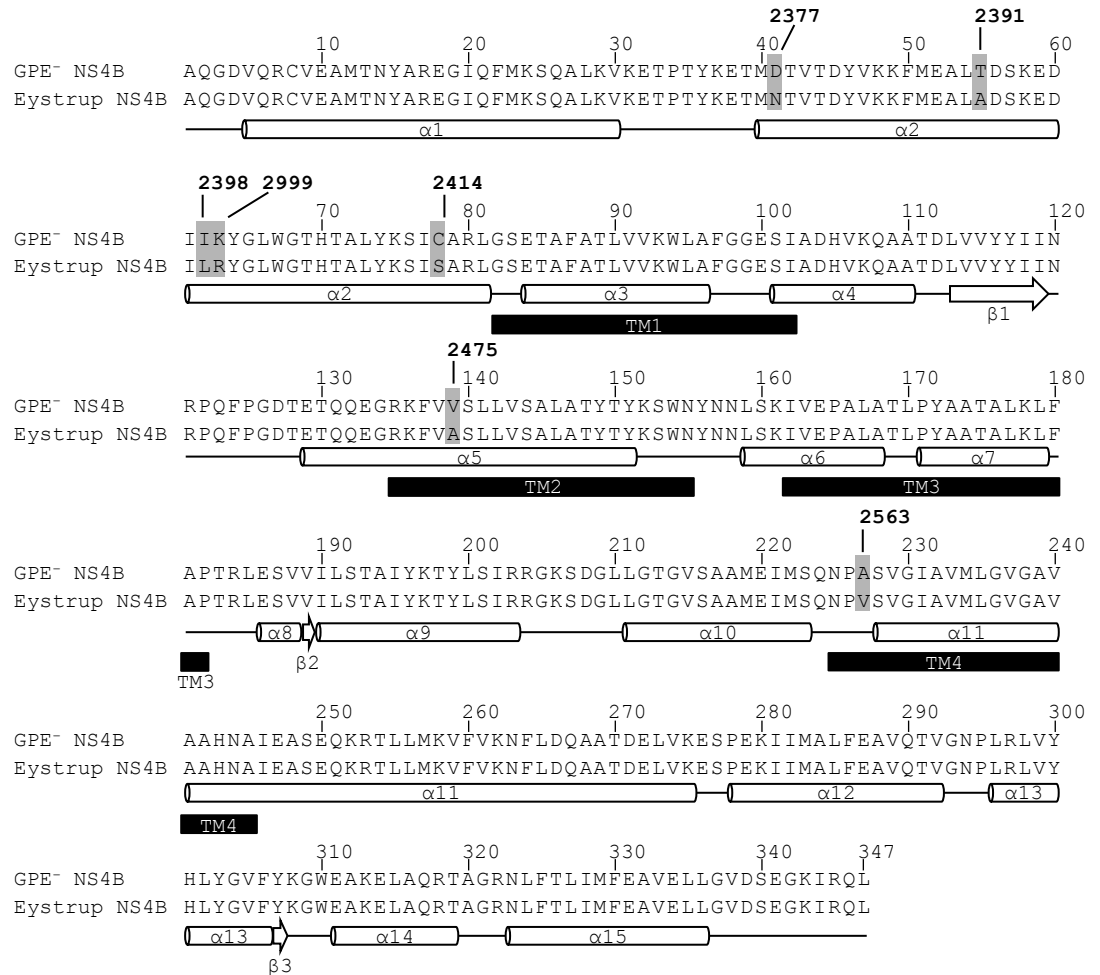


Fig. 18. Comparison of the NS4B amino acid sequences of the low virulent GPE⁻ and the highly virulent Eystруп strains. (A) Based on the secondary structure predictions and their consensus, the α-helices and β-strands are depicted by white columns and arrows, respectively. The trans-membrane domains (TM) are shown with black rectangles. Amino acid numbering starts with the beginning of NS4B. The amino acid differences are indicated based on their positions relative to the translation start site of the polyprotein, and they are highlighted in gray. (B) Amphipathic helix wheels in the N-terminal second α-helix (α2, amino acid [aa] of NS4B 40–81) of two strains are illustrated, with the amino acid differences indicated by an asterisk. White and black colors indicate hydrophobic and hydrophilic amino acids, respectively. The clustering of hydrophobic, nonpolar residues on one face of the helix suggests an amphipathic configuration. The broken lines separate the two faces of the helix.

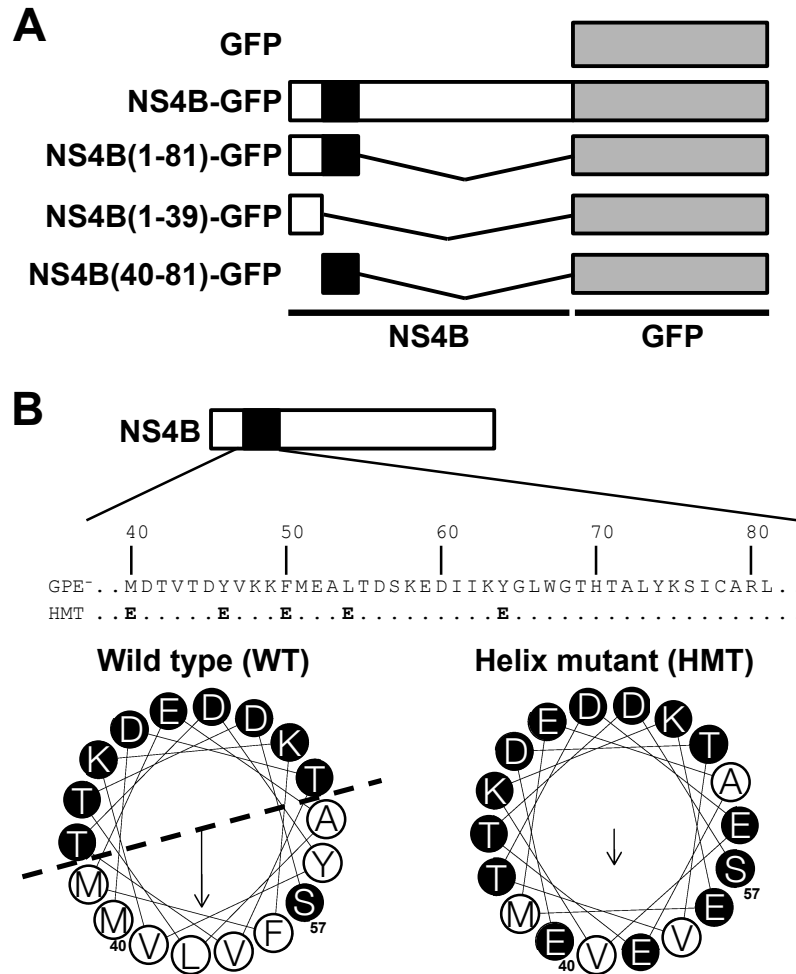


Fig. 19. Role of the predicted N-terminal amphipathic helix domain for protein localization in porcine cells. (A) Schematic representation of the NS4B-GFP chimeric proteins and of the truncated versions expressed from the pCI vector under the control of a cytomegalovirus promoter. The 2nd α -helix domain (aa 40–81) is shown in black. (B) Amino acid sequences and a helical wheel plot of N-terminal domain of NS4B amino acids showing the localization of the amino acid substitutions for disruption of its amphipathic character. (C) SK-6 cells were transfected with pCI constructs for expression of the indicated NS4B-GFP proteins from the GPE⁻ strain. GFP fluorescence (green) was analyzed by confocal microscopy after 30 h of culture. A plasmid expressing GFP served as control. Nuclei were stained with DAPI (blue). Bars = 10 μ m. (D) Membrane flotation analysis of SK-6 cells expressing the N-terminal domain (aa 40–81) of NS4B. SK-6 cells transfected with the pCI vectors, NS4B(40-81)-GFP and the amphipathic helix mutant [GPE⁻ NS4B(40-81HMT)-GFP], were disrupted mechanically at 36 h after transfection. Equal amounts of postnuclear supernatants were used for membrane flotation and Western blot analysis with an antibody to GFP, GAPDH, or calnexin. For the membrane extraction experiments, the postnuclear supernatants were treated with 100 mM Na₂CO₃ (pH 11.5), with 1 M NaCl, or with 1% Triton X-100 and subjected to membrane flotation followed by Western blot analysis.

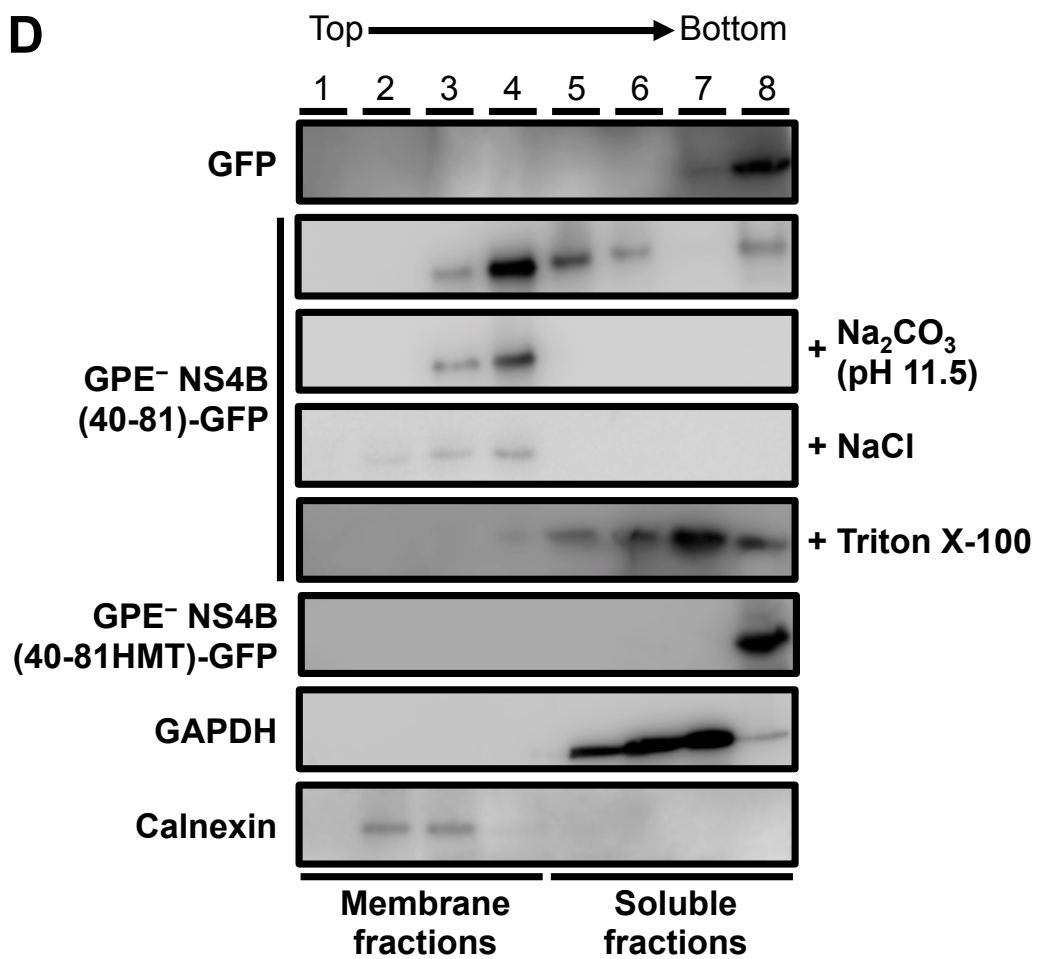
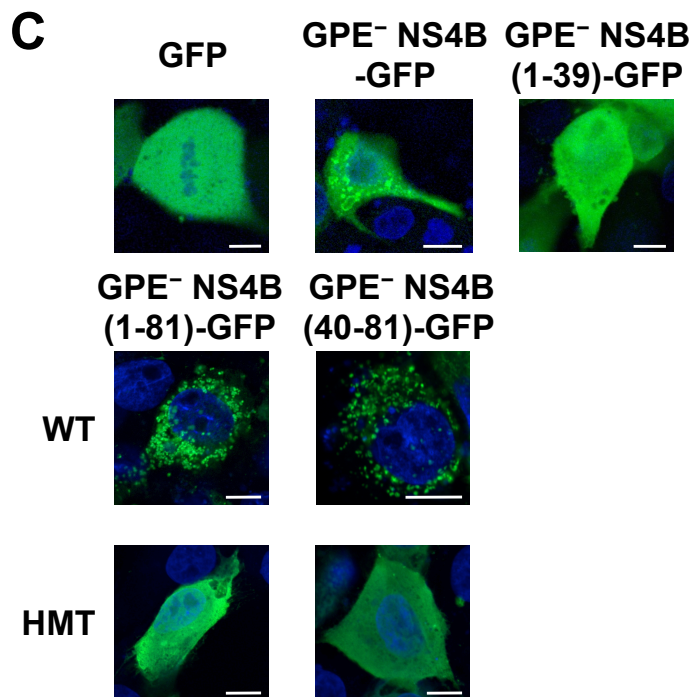


Fig. 19. (continued)

cells, the fluorescence signal of NS4B(40-81)-GFP, NS4B(1-81)-GFP, and NS4B-GFP was located essentially in the cytoplasm, with a tendency towards “dot-like” granular structures, which was clearly different from the pattern observed with NS4B(1-39)-GFP and with GFP alone, showing diffuse distribution in the cytoplasm and nucleus at the late stage of transfection. The fluorescence of the disrupted amphipathic helix mutant of NS4B(40-81)-GFP and NS4B(1-81)-GFP was diffuse distribution and did not observe the “dot-like” granular structures at all (Fig. 19C). These results indicate that the putative amphipathic α 2-helix (aa 40–81) is an essential segment for alternation of the fluorescent pattern.

A membrane flotation assay was used to further characterize the predicted membrane association of the α 2-helix of NS4B. A representative Western blotting from a membrane flotation experiment with extracts from SK-6 cells expressing NS4B(40-81)-GFP from the GPE⁻ strain is shown in Fig. 19D. Low-density membrane fractions are raised towards the top of the gradient, whereas soluble proteins remain with the denser fractions at the bottom. Accordingly, the GFP alone was retained at the bottom of the gradient (fractions 7 and 8) similar to the cytoplasmic soluble GAPDH protein. The GFP-tagged α 2-helix of NS4B came up in the low-density fractions 3 and 4 (from the top) of the flotation gradient. This was consistent with the accumulation of the membrane-associated calnexin in the gradient fractions 2 and 3 (Fig. 19D, bottom panel). In contrast, the disrupted amphipathic helix mutant of the GFP-tagged α 2-helix of GPE⁻ NS4B [GPE⁻ NS4B(40-81HMT)-GFP] detected at the soluble fraction. Next, to analyze the membrane association of this segment in more details, membrane flotation distribution was examined after treatments that discriminate between peripheral and integral

membrane association. To this end, the postnuclear supernatants from cells expressing NS4B(40-81)-GFP were centrifuged at $100,000 \times g$, and the pelleted membranes were subjected to different extraction methods. The GFP-tagged NS4B(40-81) remained in the membrane fraction under physiological conditions and after high-salt and alkaline extractions, while detergent extraction resulted in accumulation of NS4B(40-81)-GFP in the higher density soluble fractions. Taken together, the flotation assays confirmed strong intracellular membrane association of the N-terminal amphipathic α 2-helix of NS4B (aa 40–81).

Intracellular membrane association mediated by the N-terminal domain of NS4B is a determinant of CSFV RNA replication in cell culture

In order to clarify the roles of intracellular membrane association mediated by the N-terminal amphipathic domain of NS4B for RNA replication, the both cistronic replicons carrying the same mutations for disruption of the amphipathic α -helix were generated. The luciferase activity of the helix mutant (rGPE⁻-N^{pro}-Luc-IRES-NS3/NS4B HMT) was significantly lower compared with the luciferase activity of the parental GPE⁻ replicon (Fig. 20A). The activity of the mutant was similar to that of the replication-deficient replicon, indicating that the replication capability was completely abolished by the mutations. In the mono-cistronic replicon assay, the helix mutant behaved similarly as the replication-deficient replicon (Fig. 20B). Altogether, these data indicate that intracellular membrane association mediated by the N-terminal domain of NS4B is critical for RNA replication in cell culture.

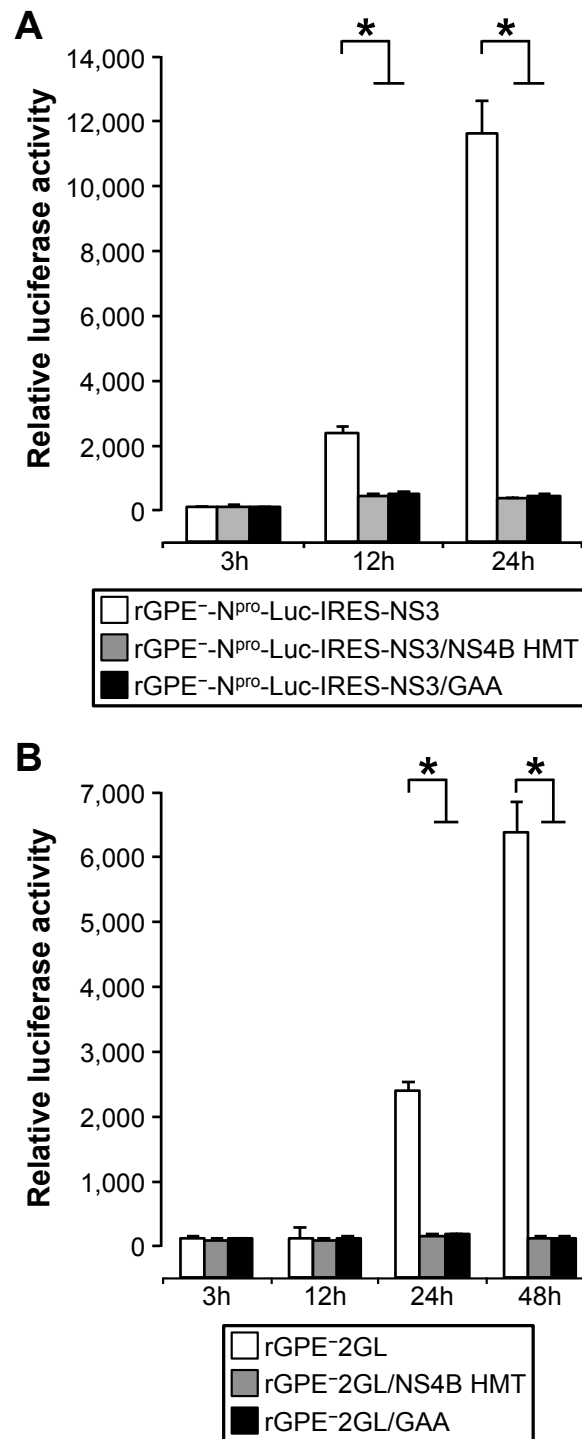


Fig. 20. Role of the predicted N-terminal amphipathic helix domain for RNA replication. GPE⁻-derived bi- and mono-cistronic luciferase reporter replicons carrying the mutations for disruption of the amphipathic character of the helix (rGPE⁻-N^{pro}-Luc-IRES-NS3/GAA and rGPE⁻2GL/GAA) were utilized. Respective firefly (A) and *Gaussia* (B) luciferase activities were analyzed as described above. Significance ($p < 0.05$) is indicated with an asterisk.

The N-terminal domain of NS4B modulates subcellular localization

In order to determine the subcellular distribution of the N-terminal domain of NS4B, co-localization experiments were performed in SK-6 cells expressing the GFP-tagged fusion proteins. As shown in Fig. 21A, the GFP alone also showed diffuse distribution in the whole cells and did not co-localize with any specific subcellular organelles. The fluorescence pattern of the GFP-tagged N-terminal domain of NS4B, NS4B(1-81)-GFP, and NS4B(40-81)-GFP appeared to partially overlap with the ER. The co-localization efficient was the same level of the entire NS4B (Fig. 21B). The GFP with the N-terminal NS4B did not co-localize with the mitochondria and lysosomes (Fig. 21C). The same results were obtained by using the GFP-tagged N-terminal Eystруп NS4B vector (data not shown). These data indicate that the N-terminal amphipathic helix determines the subcellular localization of NS4B.

The Eystруп strain-specific residues in the predicated amphipathic α 2-helix of the N-terminal NS4B are involved in efficient intracellular membrane association

Next, the effect on intracellular localization and membrane association of the amino acids differing between the two viruses in the amphipathic α 2-helix (aa 40–81) was investigated. SK-6 cells were transfected with the plasmids expressing the GFP-tagged N-terminal 81 amino acids of NS4B of the GPE⁻ vaccine strain and of the highly virulent Eystруп strain, and the occurrence of “dot-like” fluorescence was monitored over time. The two constructs produced the chimeric GFP proteins in the cytoplasm as early as 6 h pt (data not shown). The fluorescence increased over time, and “dot-like” granular structures appeared in the cytoplasm at 12 h pt. At 12 h pt and later, the ratio of GFP-positive cells with the granular structures was significantly higher

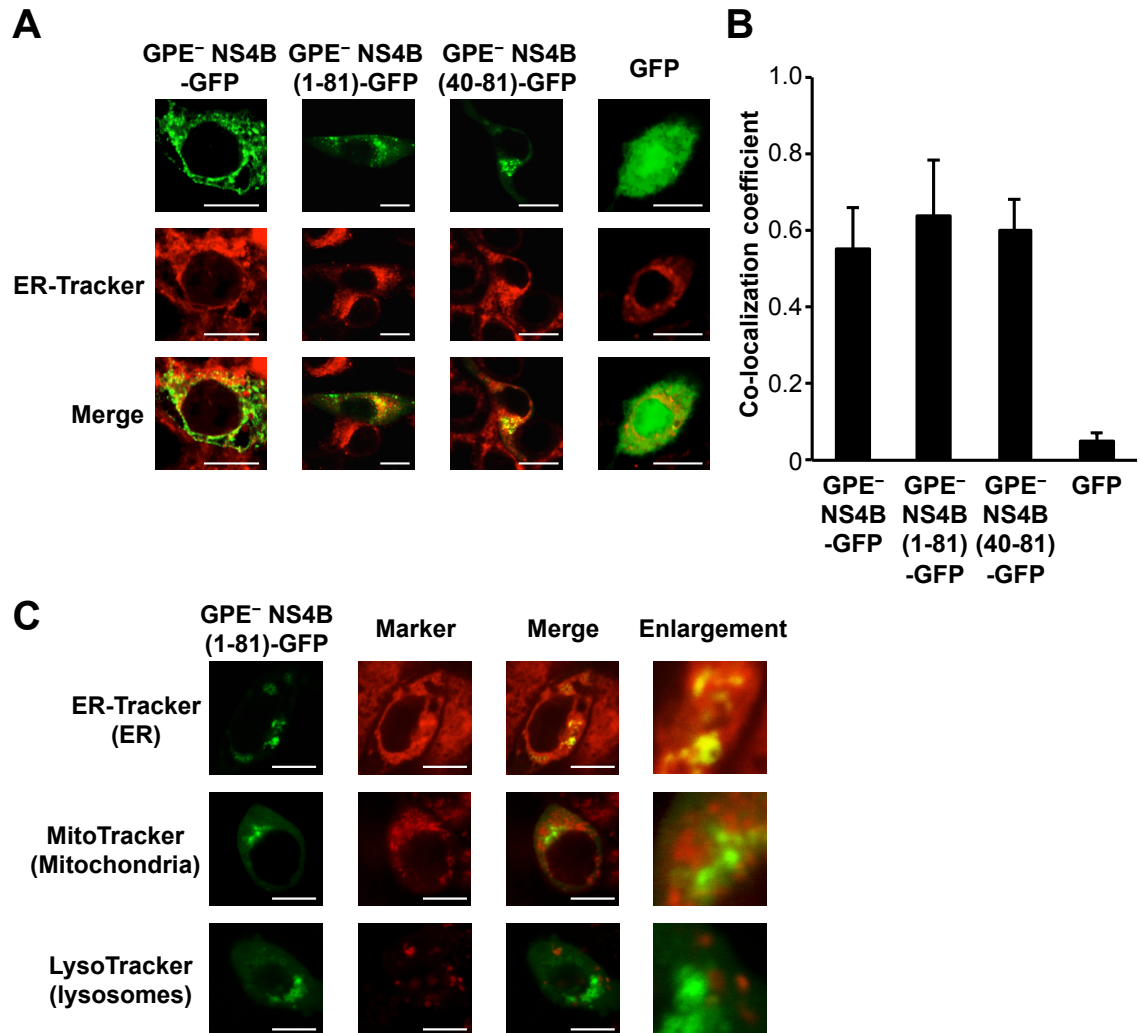


Fig. 21. Subcellular distribution of the N-terminal domain of NS4B in porcine cells. (A) SK-6 cells were transfected with pCI constructs expressing the NS4B-GFP chimeric proteins. At 36 h after transfection, the cells were processed for live-imaging fluorescence microscopy. The fusion proteins were detected by GFP fluorescence (Green). The ER-Tracker (red) was used as markers for the ER. (B) Co-localization coefficient with the ER was calculated. Mean and standard deviation of the values acquired from 15 fields of view are depicted. (C) At the same timepoint, the co-localization of the N-terminal domain (aa 1–81) of NS4B of the GPE⁻ strain with mitochondria and lysosomes were analyzed. The MitoTracker and LysoTracker were used as markers for the mitochondria and lysosomes, respectively. Yellow color indicates co-localization of the N-terminal NS4B domain (green) with the respective intracellular markers (red). Bars = 10 μ m.

for the Eystруп strain than for the GPE⁻ vaccine strain, with a two-fold difference observed at 30 h and 36 h pt (Fig. 22A). With the GFP-tagged α 2-helix of NS4B (aa 40–81) alone however, these differences were not observed (Fig. 22B).

In the membrane flotation assay, the flotation efficiency that is the ratio of the proteins in the membrane gradient fractions was also significantly different between the two strains with the GFP-tagged N-terminal 81 amino acids of NS4B, but not with the GFP-tagged α 2-helix of NS4B (aa 40–81) alone, which was consistent with the results above (Fig. 22C). Nearly 100% of the GFP-tagged NS4B(1-81) of the Eystруп strain was in the membrane fractions, compared to only 70% for the GPE⁻ vaccine strain. With the GFP-tagged NS4B(40-81), no difference was observed between the two strains. In conclusion, these data suggest that the entire N-terminal domain of NS4B comprising the α 1- and α 2-helices confer enhanced intracellular membrane association to NS4B of the Eystруп strain compared with NS4B of the GPE⁻ vaccine strain.

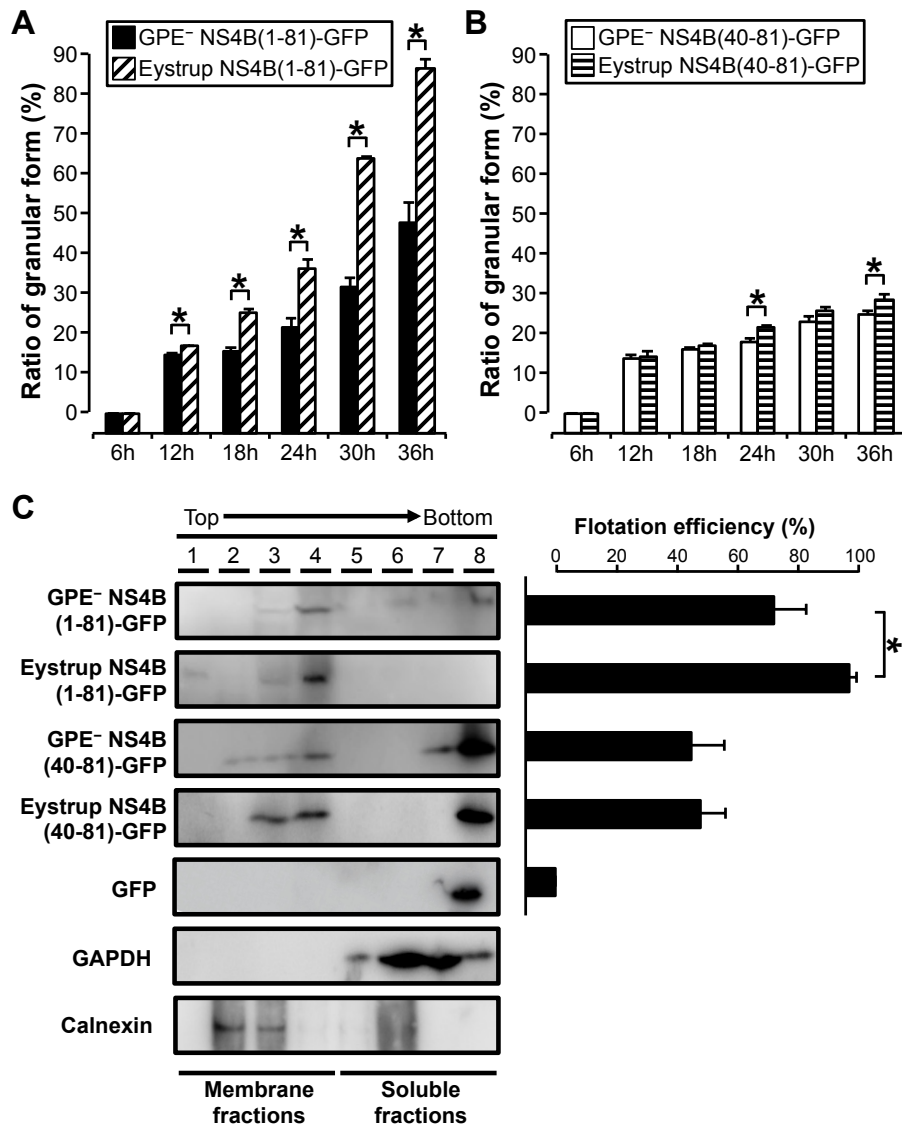


Fig. 22. Strain-specific differences in intracellular membrane association of the N-terminal domain of NS4B. SK-6 cells were transfected with pCI constructs for the expression of the GFP-tagged N-terminal domains encompassing aa 1–81 (A) or 40–81 (B) of NS4B from the GPE⁻ and Eystруп strains. At the indicated times after transfection, the cultures were monitored by fluorescence microscopy and the percentage of cells displaying “dot-like” granular structures was determined for a total number of at least 500 GFP-positive cells (ratio of granular form). (C) Membrane flotation analysis was performed with SK-6 cells transfected with the respective vectors as described above. The membrane and soluble fractions were analyzed by Western blotting after 30 h after transfection. The membrane flotation efficiency (%) was calculated from at least three independent experiments as the percentage of the total protein in the gradient that segregated in the membrane gradient fractions 1–4. All of the aforementioned experiments were independently repeated at least three times. Error bars represent the standard deviation. The significance of the differences was calculated with the Student’s *t*-test. Significance ($p < 0.05$) is indicated with an asterisk.

Discussion

In CSFV, the nonstructural protein NS4B is essential for viral replication (5). Alignment of NS4B from the GPE⁻ vaccine strain and from the highly virulent Eystrup strain –both genotype 1 viruses– revealed five amino acid differences in the N-terminal α 2-helix and two amino acid differences in the predicted second and fourth trans-membrane domains of NS4B respectively. In Chapter I, it was demonstrated that the two amino acid residues that differ between these two strains in the predicated trans-membrane domains of NS4B could modulate viral genome replication and influence the pathogenicity of the virus in pigs. Here, the GPE⁻-derived virus carrying the complete NS4B gene of the Eystrup strain was generated, in order to determine whether the additional five amino acid differences in the N-terminal domain of NS4B may contribute to the different pathogenicity in pigs. The GPE⁻-derived virus carrying the NS4B gene of the highly virulent Eystrup strain resulted in more severe clinical symptoms, earlier and prolonged viremia compared with the virus carrying only the two amino acid substitutions in the predicted trans-membrane domains. These data indicate that the N-terminal domain of NS4B may represent a virulence determinant *in vivo*. However, as expected, the NS4B gene from the Eystrup strain did not confer a full high virulent phenotype to the GPE⁻-derived virus, demonstrating that additional viral proteins and amino acid residues are involved in determining the virulence of the Eystrup strain. In the complete viral polyprotein, a total of 62 amino acids differ between these strains. Thus, it is difficult to determine how each amino acid difference contributes to viral pathogenicity. In addition, the specific residues identified in this study as critical for determining virulence in pigs are not general

virulence determinants in CSFV (Fig. 23). This suggests that the virulence of CSFV is more likely a multigenic trait determined by a complex interplay of several viral proteins or genes acting in concert as already postulated in Chapter I and in the previous study (122). Numerous studies suggest that the structural proteins C, E^{ms}, E1, E2, and the nonstructural proteins p7 and NS4B are involved in determining CSFV virulence, essentially by loss-of-function experiments (13-26). In a recent study, the amino acid residues in E2 were identified as CSFV virulence determinants *in vivo* by the gain-of-function experiments (123). Importantly, this is the first study to show that the N-terminal domain of CSFV NS4B can confer enhanced pathogenicity to a low virulent virus, which was related to an enhanced viral replication.

The five amino acid residues differing between the GPE⁻ and Eystrup strains in the N-terminal domain of NS4B clearly determine the RNA replication efficiency of replicons *in vitro*. In previous studies, two types of replicons were utilized for studying RNA replication: mono-cistronic (103) and bi-cistronic (5). The minimal viral elements required for RNA replication by pestiviruses including CSFV are NS3–NS5B, together with the 5' and 3' UTRs (5). In addition to these minimal elements, the bi-cistronic replicon carries an artificial IRES derived from the EMCV, a firefly luciferase reporter gene, and the viral auto-protease N^{pro} for optimal translation and processing of the reporter protein. However, the replicative properties of this replicon may be modified by the EMCV IRES. For these reasons, a mono-cistronic replicon was included. Comparable results of NS4B-dependent differences in RNA replication were obtained with the two types of replicons, strengthening the data.

The granular fluorescent signal derived from the N-terminal domain of NS4B was co-localized partially with the ER by confocal fluorescence microscopy (Fig. 21). In

the membrane flotation assay, the N-terminal domain of NS4B was also bound to the ER in part, suggesting that the ER could be a candidate site of the replication complex in infected cells. However, the major part of the N-terminal domain of NS4B was detected in different fractions than the ER marker calnexin. Thus, further studies will be needed to identify the exact subcellular localization of the replication complex including the natural NS4B in CSFV infected cells. It was described that BVDV NS4B alone can rearrange the host membrane (101) as well as HCV NS4B (49), suggesting that pestivirus NS4B might act as a trigger for building the replication complex. So far, however, pestivirus replication is considered not to remodel cytoplasmic host cell membranes (124) as opposed to HCV which induces organelle-like structures designated as the membranous web where the formation of the viral replication complex is triggered (98) and to other positive-strand RNA viruses (95-97, 99). Therefore, it is hypothesized that the role of pestivirus NS4B may be partially different from that in other members of the *Flaviviridae*. Further studies on the functions of the pestivirus NS4B will allow to clarify how the NS4B-dependent modulation of RNA replication may relate to differences in viral replication, morphogenesis, and virulence.

Intracellular membrane association mediated by the N-terminal amphipathic domain of NS4B is essential for RNA replication. This amphipathic structure is conserved among CSFV strains (Fig. 23). Interestingly, the amino acid differences in the N-terminal part of NS4B between the prototype vaccine virus and the highly virulent strain studied here are located on the predicated hydrophilic face of the amphipathic α 2-helix. These residues are not conserved with respect to virulence of CSFV, however, the amino acid differences among CSFV strains were found mostly on

the hydrophilic face of the amphipathic $\alpha 2$ -helix, suggesting that the functional differences observed may depend on subtle structure-related differences in association with host proteins and/or other viral nonstructural proteins, modulating replication complex formation, RNA replication, and eventually pathogenicity of the virus in pigs. For HCV, the N-terminal amphipathic $\alpha 2$ -helix of NS4B is critical for its polymerization, and thus for its accumulation in organelles (125, 126). A recent study with HCV demonstrated that hydrophilic amino acids in the N-terminal cytoplasmic NS4B are responsible for virus production (127).

In summary, this is the first study showing the roles of intracellular membrane association mediated by the N-terminal domain of CSFV NS4B in viral RNA replication and pathogenicity in pigs. Further studies are required in order to understand the functionality of the pestivirus replication complex at the molecular level, and how this affects pathogenicity in pigs.

Summary

The nonstructural protein NS4B is essential for viral replication. However, the roles of CSFV NS4B for viral genome replication and pathogenesis have not yet been elucidated. NS4B of the GPE⁻ vaccine strain and of the highly virulent Eystrup strain differ by a total of seven amino acid residues, two of which are located in the predicted trans-membrane domains of NS4B and were described previously to relate to virulence, and five residues clustering in its N-terminal part. Here, in Chapter III, the potential role of these five amino acids in modulating genome replication and determining pathogenicity in pigs was investigated. A chimeric low virulent GPE⁻-derived virus carrying the complete Eystrup NS4B showed enhanced pathogenicity in pigs. The *in vitro* replication efficiency of the NS4B chimeric GPE⁻ replicon was significantly higher than that of the replicon carrying only the two Eystrup-specific amino acids in NS4B. *In silico* and *in vitro* data suggest that the N-terminal part of NS4B forms an amphipathic α -helix structure. The N-terminal NS4B with these five amino acid residues is associated with intracellular membranes. Taken together, this is the first gain-of-function study showing that the N-terminal domain of NS4B can determine CSFV genome replication in cell culture and viral pathogenicity in pigs.

Conclusion

Numerous CSFV isolates with various degrees of virulence have been isolated worldwide, ranging from low virulent strains that do not result in any apparent clinical signs to highly virulent strains that cause a severe peracute hemorrhagic fever with lethal mortality. Understanding of the virulence determinants is important issues for effective CSF control and development of safe and effective marker vaccines. However, little is known on the viral molecular determinants of CSFV virulence. In the present thesis, the author identified the viral determinants and functions responsible for pathogenicity of CSFV in pigs by gain-of-function studies.

In Chapter I, it was examined whether the avirulent CSFV could acquire the pathogenicity by force re-adaptation to pigs of the live attenuated and nonhost adapted GPE⁻ vaccine strain. After the serial passages of the GPE⁻ vaccine strain in pigs, the virus acquired the pathogenicity. Three amino acid residues that are critical for determining the acquisition of pathogenicity were identified in E2 (T830A) and NS4B (V2475A and A2563V) by experimental infection of pigs with the GPE⁻ mutants constructed by reverse genetics. Studies *in vitro* revealed that E2 T830A enhanced virus spreading, and that both V2475A and A2563V in NS4B contributed to enhanced RNA replication efficiency synergistically.

In Chapter II, capabilities of the viral function to evade innate immune activation as virulence determinant were examined in the both avirulent and moderately virulent GPE⁻-derived viruses. Viruses carrying the N136D substitution in the nonstructural protein N^{pro} regained the ability to degrade IRF3 and suppress IFN- α/β induction *in vitro*. In pigs, restoring the function of N^{pro} resulted in significantly reduced the local

IFN- α mRNA expression in local sites of virus replication while it increased quantities of IFN- α/β in the circulation, and enhanced pathogenicity of the moderately virulent virus without any affect of an avirulent virus.

In Chapter III, the roles of the nonstructural protein NS4B, the essential components for viral RNA replication, in the pathogenesis of CSF was analyzed. By comparing the NS4B amino acid sequences of the GPE⁻ vaccine strain and of the highly virulent strain, the predicted amphipathic α -helix carrying five amino acid differences between these two viruses was identified in the N-terminal domain. The five residues from the highly virulent virus conferred enhanced viral RNA replication *in vitro* and pathogenicity *in vivo*. They also proved crucial for efficient intracellular membrane association, suggesting that membrane association mediated by the N-terminal domain of NS4B is critical for a functional viral replication complex.

These findings described here provide new insights into the molecular mechanisms determining CSFV virulence. The present study revealed that both structural and nonstructural proteins of CSFV carry virulence determinants that can act in concert to determine the pathogenicity of the virus in pigs. In addition, the specific residues identified in the present thesis as critical for determining virulence in pigs are not general virulence determinants in CSFV strains, suggesting that the virulence is more likely a multigenic trait determined by a complex interplay of several viral proteins or genes. Improved understanding of the molecular basis of CSFV pathogenicity contributes to reveal molecular mechanisms of CSF pathogenesis.

Acknowledgements

The author would like to acknowledge Prof. Y. Sakoda, Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University (Sapporo, Japan), for his guidance and encouragement during the course of this work.

The author is extremely grateful to Prof. H. Kida, Research Center for Zoonosis Control, Hokkaido University (Sapporo, Japan), and Dr. N. Ruggli, The Institute of Virology and Immunology IVI, Federal Food Safety and Veterinary Office FSVO and Vetsuisse-Faculty, University of Bern (Mittelhäusern, Switzerland), for their valuable advices, supports and encouragements throughout the present studies.

Great appreciation is extended to Prof. K. Ohashi, Laboratory of Infectious Diseases, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University; Prof. H. Kariwa, Laboratory of Public Health, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University; and Dr. M. Okamatsu, Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, for their critical review of the manuscript.

The author heartily thanks Y. Nomoto and Dr. A. Sawata, Kitasato Daiichi Sankyo Vaccine Co., Ltd. (Saitama, Japan), for technical help with the GPK cells; Dr. TB. Rasmussen; DTU National Veterinary Institute (Kalvehave, Demark), for providing the plasmid for the transcription of the mono-cistronic replicon rPad2GL; and the late Dr. Irene Greiser-Wilke, University of Veterinary Medicine Hannover (Hannover, Germany), for mAb HC/TC26.

The author also thanks Prof. A. Summerfield, Dr. KC. McCullough, Dr. I. Leifer, Dr. M. Liniger, M. Gerber, S. Python, B. Zumkehr, M. Mader, D. Brechbühl, J. Rappe, The Institute of Virology and Immunology IVI; Dr. H. Aoki, Nippon Veterinary and Life Science University (Musashino, Japan); Dr. N. Isoda, Dr. M. Igarashi, Dr. A. Ishii, and A. Ohnuma, Research Center for Zoonosis Control, Hokkaido University; and Dr. K. Matsuno, Dr. K. Yoshii, Dr. T. Yamasaki, Dr. Y. Okamatsu-Ogura, and Dr. S. Murata, Graduate School of Veterinary Medicine, Hokkaido University for their excellent contribution and support.

The author earnestly appreciates all colleagues of Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University for their invaluable help and friendships.

The author owes his deepest gratitude to his family for their encouragement, love, and devoted support to his education.

The Japan Society for the Promotion of Science (JSPS) Research Fellowship for Young Scientists, JSPS KAKENHI Grant Number 02501199, and the Program for Leading Graduate Schools (F01) from JSPS supported this work.

Finally, the author express his mourning for all of the animals sacrificed their precious lives for these studies.

References

1. **Lindenbach BD MC, Thiel HJ, Rice CM.** 2013. Flaviviridae, p 712-794. *In* Knipe DM, Howley PM (ed), Fields Virology, 6th ed, Vol 1. Lippincott Williams and Wilkins, Philadelphia, PA, USA.
2. **Kirkland P, Le Potier M, Vannier P, Finlaison D.** 2012. Pestiviruses, p 538-553. *In* Zimmerman JJ, Karriker LA, Ramirez A , Schwartz K, Stevenson GW (ed), Disease of Swine, 10th ed, John Wiley & Sons, Hoboken, NJ, USA.
3. **Simmonds P, Becher P, Collett MS, Gould EA, Heinz FX, Meyers G, Monath T, Pletnev A, Rice CM, Stiasny K, Thiel H-J, Weiner A, Bukh J.** 2011. Family Flaviviridae. p 1003-1020. *In* King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (ed), Virus Taxonomy Ninth Report of the International Committee on Taxonomy of Viruses, Elsevier Academic Press, San Diego, CA, USA.
4. **Lamp B, Riedel C, Wentz E, Tortorici MA, Rumenapf T.** 2013. Autocatalytic cleavage within classical swine fever virus NS3 leads to a functional separation of protease and helicase. *J. Virol.* **87**:11872-11883.
5. **Behrens SE, Grassmann CW, Thiel HJ, Meyers G, Tautz N.** 1998. Characterization of an autonomous subgenomic pestivirus RNA replicon. *J. Virol.* **72**:2364-2372.
6. **van Oirschot JT.** 2003. Vaccinology of classical swine fever: from lab to field. *Vet. Microbiol.* **96**:367-384.
7. **World Organisation for Animal Health.** 2015. Recognition of the Classical Swine Fever Status of Member Countries. Available from:
<http://www.oie.int/en/animal-health-in-the-world/official-disease-status/classical-swine-fever/list-of-csf-free-member-countries/>
8. **Sasahara J.** 1970. Hog Chorela: Diagnosis and Prophylaxis. *Natl. Inst. Anim. Health Q. (Tokyo)* **10**:57-81.

9. **Shimizu Y, Furuuchi S, Kumagai T, Sasahara J.** 1970. A mutant of hog cholera virus inducing interference in swine testicle cell cultures. *Am. J. Vet. Res.* **31**:1787-1794.
10. **Okaniwa A, Nakagawa M, Shimizu Y, Furuuchi S.** 1969. Lesions in swine inoculated with attenuated hog cholera viruses. *Natl. Inst. Anim. Health Q. (Tokyo)* **9**:92-103.
11. **Sasahara J, Kumagai T, Shimizu Y, Furuuchi S.** 1969. Field experiments of hog cholera live vaccine prepared in guinea-pig kidney cell culture. *Natl. Inst. Anim. Health Q. (Tokyo)* **9**:83-91.
12. **Ishikawa K, Nagai H, Katayama K, Tsutsui M, Tanabayashi K, Takeuchi K, Hishiyama M, Saitoh A, Takagi M, Gotoh K.** 1995. Comparison of the entire nucleotide and deduced amino acid sequences of the attenuated hog cholera vaccine strain GPE⁻ and the wild-type parental strain ALD. *Arch. Virol.* **140**:1385-1391.
13. **Fernandez-Sainz I, Gladue DP, Holinka LG, O'Donnell V, Gudmundsdottir I, Prarat MV, Patch JR, Golde WT, Lu Z, Zhu J, Carrillo C, Risatti GR, Borca MV.** 2010. Mutations in classical swine fever virus NS4B affect virulence in swine. *J. Virol.* **84**:1536-1549.
14. **Fernandez-Sainz I, Holinka LG, Gavrilov BK, Prarat MV, Gladue D, Lu Z, Jia W, Risatti GR, Borca MV.** 2009. Alteration of the N-linked glycosylation condition in E1 glycoprotein of Classical Swine Fever Virus strain Brescia alters virulence in swine. *Virology* **386**:210-216.
15. **Fernández-Sainz I, Holinka LG, Gladue D, O'Donnell V, Lu Z, Gavrilov BK, Risatti GR, Borca MV.** 2011. Substitution of specific cysteine residues in the E1 glycoprotein of classical swine fever virus strain Brescia affects formation of E1-E2 heterodimers and alters virulence in swine. *J. Virol.* **85**:7264-7272.
16. **Gladue DP, Holinka LG, Fernandez-Sainz IJ, Prarat MV, O'Donell V, Vepkhvadze N, Lu Z, Rogers K, Risatti GR, Borca MV.** 2010. Effects of the

interactions of classical swine fever virus Core protein with proteins of the SUMOylation pathway on virulence in swine. *Virology* **407**:129-136.

17. **Gladue DP, Holinka LG, Fernandez-Sainz IJ, Prarat MV, O'Donnell V, Vepkhvadze NG, Lu Z, Risatti GR, Borca MV.** 2011. Interaction between Core protein of classical swine fever virus with cellular IQGAP1 protein appears essential for virulence in swine. *Virology* **412**:68-74.
18. **Gladue DP, Holinka LG, Largo E, Fernandez Sainza I, Carrillo C, O'Donnell V, Baker-Branstetter R, Lu Z, Ambroggio X, Risatti GR, Nieva JL, Borca MV.** 2012. Classical Swine Fever Virus p7 protein is a viroporin involved in virulence in swine. *J. Virol.* **86**:6778-6791.
19. **Meyers G, Saalmüller A, Büttner M.** 1999. Mutations abrogating the RNase activity in glycoprotein E^{tns} of the pestivirus classical swine fever virus lead to virus attenuation. *J. Virol.* **73**:10224-10235.
20. **Risatti GR, Borca MV, Kutish GF, Lu Z, Holinka LG, French RA, Tulman ER, Rock DL.** 2005. The E2 glycoprotein of classical swine fever virus is a virulence determinant in swine. *J. Virol.* **79**:3787-3796.
21. **Risatti GR, Holinka LG, Carrillo C, Kutish GF, Lu Z, Tulman ER, Sainz IF, Borca MV.** 2006. Identification of a novel virulence determinant within the E2 structural glycoprotein of classical swine fever virus. *Virology* **355**:94-101.
22. **Risatti GR, Holinka LG, Fernandez Sainz I, Carrillo C, Kutish GF, Lu Z, Zhu J, Rock DL, Borca MV.** 2007. Mutations in the carboxyl terminal region of E2 glycoprotein of classical swine fever virus are responsible for viral attenuation in swine. *Virology* **364**:371-382.
23. **Risatti GR, Holinka LG, Fernandez Sainz I, Carrillo C, Lu Z, Borca MV.** 2007. N-linked glycosylation status of classical swine fever virus strain Brescia E2 glycoprotein influences virulence in swine. *J. Virol.* **81**:924-933.
24. **Risatti GR, Holinka LG, Lu Z, Kutish GF, Tulman ER, French RA, Sur JH, Rock DL, Borca MV.** 2005. Mutation of E1 glycoprotein of classical swine fever virus affects viral virulence in swine. *Virology* **343**:116-127.

25. **Sainz IF, Holinka LG, Lu Z, Risatti GR, Borca MV.** 2008. Removal of a N-linked glycosylation site of classical swine fever virus strain Brescia E^{ns} glycoprotein affects virulence in swine. *Virology* **370**:122-129.
26. **Tews BA, Schürmann EM, Meyers G.** 2009. Mutation of cysteine 171 of pestivirus E^{ns} RNase prevents homodimer formation and leads to attenuation of classical swine fever virus. *J. Virol.* **83**:4823-4834.
27. **Komaniwa H, Fukusho A, Shimizu Y.** 1981. Micro method for performing titration and neutralization test of hog cholera virus using established porcine kidney cell strain. *Natl. Inst. Anim. Health Q. (Tokyo)* **21**:153-158.
28. **Sakoda Y, Fukusho A.** 1998. Establishment and characterization of a porcine kidney cell line, FS-L3, which forms unique multicellular domes in serum-free culture. *In Vitro Cell. Dev. Biol. Anim.* **34**:53-57.
29. **Ruggli N, Tratschin JD, Mittelholzer C, Hofmann MA.** 1996. Nucleotide sequence of classical swine fever virus strain Alfort/187 and transcription of infectious RNA from stably cloned full-length cDNA. *J. Virol.* **70**:3478-3487.
30. **Suter R, Summerfield A, Thomann-Harwood LJ, McCullough KC, Tratschin JD, Ruggli N.** 2011. Immunogenic and replicative properties of classical swine fever virus replicon particles modified to induce IFN- α/β and carry foreign genes. *Vaccine* **29**:1491-1503.
31. **Moser C, Stettler P, Tratschin JD, Hofmann MA.** 1999. Cytopathogenic and noncytopathogenic RNA replicons of classical swine fever virus. *J. Virol.* **73**:7787-7794.
32. **Kameyama K, Sakoda Y, Tamai K, Igarashi H, Tajima M, Mochizuki T, Namba Y, Kida H.** 2006. Development of an immunochromatographic test kit for rapid detection of bovine viral diarrhea virus antigen. *J. Virol. Methods* **138**:140-146.
33. **Sakoda Y, Hikawa M, Tamura T, Fukusho A.** 1998. Establishment of a serum-free culture cell line, CPK-NS, which is useful for assays of classical swine fever virus. *J. Virol. Methods* **75**:59-68.

34. **Reed L, Muench H.** 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg. (Lond.)* **27**:493-497.
35. **Hoffmann B, Beer M, Schelp C, Schirrmeier H, Depner K.** 2005. Validation of a real-time RT-PCR assay for sensitive and specific detection of classical swine fever. *J. Virol. Methods* **130**:36-44.
36. **Chen J, He WR, Shen L, Dong H, Yu J, Wang X, Yu S, Li Y, Li S, Luo Y, Sun Y, Qiu HJ.** 2015. The laminin receptor is a cellular attachment receptor for classical swine fever virus. *J. Virol.* **89**:4894-4906.
37. **Drager C, Beer M, Blome S.** 2015. Porcine complement regulatory protein CD46 and heparan sulfates are the major factors for classical swine fever virus attachment *in vitro*. *Arch. Virol.* **160**:739-746.
38. **Maurer K, Krey T, Moennig V, Thiel HJ, Rumenapf T.** 2004. CD46 is a cellular receptor for bovine viral diarrhea virus. *J. Virol.* **78**:1792-1799.
39. **Ronecker S, Zimmer G, Herrler G, Greiser-Wilke I, Grummer B.** 2008. Formation of bovine viral diarrhea virus E1-E2 heterodimers is essential for virus entry and depends on charged residues in the transmembrane domains. *J. Gen. Virol.* **89**:2114-2121.
40. **Tscherne DM, Evans MJ, Macdonald MR, Rice CM.** 2008. Transdominant inhibition of bovine viral diarrhea virus entry. *J. Virol.* **82**:2427-2436.
41. **Weiland E, Stark R, Haas B, Rumenapf T, Meyers G, Thiel HJ.** 1990. Pestivirus glycoprotein which induces neutralizing antibodies forms part of a disulfide-linked heterodimer. *J. Virol.* **64**:3563-3569.
42. **Lin M, Lin F, Mallory M, Clavijo A.** 2000. Deletions of structural glycoprotein E2 of classical swine fever virus strain Alfort/187 resolve a linear epitope of monoclonal antibody WH303 and the minimal N-terminal domain essential for binding immunoglobulin G antibodies of a pig hyperimmune serum. *J. Virol.* **74**:11619-11625.
43. **Zhang F, Yu M, Weiland E, Morrissy C, Zhang N, Westbury H, Wang LF.** 2006. Characterization of epitopes for neutralizing monoclonal antibodies to

- classical swine fever virus E2 and E^{rms} using phage-displayed random peptide library. *Arch. Virol.* **151**:37-54.
44. **El Omari K, Iourin O, Harlos K, Grimes JM, Stuart DI.** 2013. Structure of a pestivirus envelope glycoprotein E2 clarifies its role in cell entry. *Cell Rep.* **3**:30-35.
 45. **Li Y, Wang J, Kanai R, Modis Y.** 2013. Crystal structure of glycoprotein E2 from bovine viral diarrhea virus. *Proc. Natl. Acad. Sci. U. S. A.* **110**:6805-6810.
 46. **Lamp B, Riedel C, Roman-Sosa G, Heimann M, Jacobi S, Becher P, Thiel HJ, Rümenapf T.** 2011. Biosynthesis of classical swine fever virus nonstructural proteins. *J. Virol.* **85**:3607-3620.
 47. **Han Q, Aligo J, Manna D, Belton K, Chintapalli SV, Hong Y, Patterson RL, van Rossum DB, Konan KV.** 2011. Conserved GXXXG- and S/T-like motifs in the transmembrane domains of NS4B protein are required for hepatitis C virus replication. *J. Virol.* **85**:6464-6479.
 48. **Gladue DP, Gavrillov BK, Holinka LG, Fernandez-Sainz IJ, Vepkhvadze NG, Rogers K, O'Donnell V, Risatti GR, Borca MV.** 2011. Identification of an NTPase motif in classical swine fever virus NS4B protein. *Virology* **411**:41-49.
 49. **Egger D, Wölk B, Gosert R, Bianchi L, Blum HE, Moradpour D, Bienz K.** 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J. Virol.* **76**:5974-5984.
 50. **Grant D, Tan GK, Qing M, Ng JK, Yip A, Zou G, Xie X, Yuan Z, Schreiber MJ, Schul W, Shi PY, Alonso S.** 2011. A single amino acid in nonstructural protein NS4B confers virulence to dengue virus in AG129 mice through enhancement of viral RNA synthesis. *J. Virol.* **85**:7775-7787.
 51. **Hügler T, Fehrmann F, Bieck E, Kohara M, Kräusslich HG, Rice CM, Blum HE, Moradpour D.** 2001. The hepatitis C virus nonstructural protein 4B is an integral endoplasmic reticulum membrane protein. *Virology* **284**:70-81.

52. **Kim M, Mackenzie JM, Westaway EG.** 2004. Comparisons of physical separation methods of Kunjin virus-induced membranes. *J. Virol. Methods* **120**:179-187.
53. **Lindström H, Lundin M, Häggström S, Persson MA.** 2006. Mutations of the Hepatitis C virus protein NS4B on either side of the ER membrane affect the efficiency of subgenomic replicons. *Virus Res.* **121**:169-178.
54. **Lackner T, Müller A, König M, Thiel HJ, Tautz N.** 2005. Persistence of bovine viral diarrhea virus is determined by a cellular cofactor of a viral autoprotease. *J. Virol.* **79**:9746-9755.
55. **Ruggli N, Bird BH, Liu L, Bauhofer O, Tratschin JD, Hofmann MA.** 2005. N^{pro} of classical swine fever virus is an antagonist of double-stranded RNA-mediated apoptosis and IFN- α/β induction. *Virology* **340**:265-276.
56. **Ruggli N, Summerfield A, Fiebach AR, Guzylack-Piriou L, Bauhofer O, Lamm CG, Waltersperger S, Matsuno K, Liu L, Gerber M, Choi KH, Hofmann MA, Sakoda Y, Tratschin JD.** 2009. Classical swine fever virus can remain virulent after specific elimination of the interferon regulatory factor 3-degrading function of N^{pro}. *J. Virol.* **83**:817-829.
57. **Toba M, Matumoto M.** 1969. Role of interferon in enhanced replication of Newcastle disease virus in swine cells infected with hog cholera virus. *Jpn. J. Microbiol.* **13**:303-305.
58. **Schneider R, Unger G, Stark R, Schneider-Scherzer E, Thiel HJ.** 1993. Identification of a structural glycoprotein of an RNA virus as a ribonuclease. *Science* **261**:1169-1171.
59. **Iqbal M, Poole E, Goodbourn S, McCauley JW.** 2004. Role for bovine viral diarrhea virus E^{ms} glycoprotein in the control of activation of beta interferon by double-stranded RNA. *J. Virol.* **78**:136-145.
60. **Ishii KJ, Coban C, Kato H, Takahashi K, Torii Y, Takeshita F, Ludwig H, Sutter G, Suzuki K, Hemmi H, Sato S, Yamamoto M, Uematsu S, Kawai T, Takeuchi O, Akira S.** 2006. A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. *Nat. Immunol.* **7**:40-48.

61. **Kawai T, Akira S.** 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* **11**:373-384.
62. **Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T.** 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* **5**:730-737.
63. **Yoneyama M, Kikuchi M, Matsumoto K, Imaizumi T, Miyagishi M, Taira K, Foy E, Loo YM, Gale M, Jr., Akira S, Yonehara S, Kato A, Fujita T.** 2005. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J. Immunol.* **175**:2851-2858.
64. **Levy DE, Garcia-Sastre A.** 2001. The virus battles: IFN induction of the antiviral state and mechanisms of viral evasion. *Cytokine. Growth Factor Rev.* **12**:143-156.
65. **Theofilopoulos AN, Baccala R, Beutler B, Kono DH.** 2005. Type I interferons (α/β) in immunity and autoimmunity. *Annu. Rev. Immunol.* **23**:307-336.
66. **Juang YT, Lowther W, Kellum M, Au WC, Lin R, Hiscott J, Pitha PM.** 1998. Primary activation of interferon A and interferon B gene transcription by interferon regulatory factor 3. *Proc. Natl. Acad. Sci. U. S. A.* **95**:9837-9842.
67. **Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, Coyle AJ, Liao SM, Maniatis T.** 2003. IKK ϵ and TBK1 are essential components of the IRF3 signaling pathway. *Nat. Immunol.* **4**:491-496.
68. **Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J.** 2003. Triggering the interferon antiviral response through an IKK-related pathway. *Science* **300**:1148-1151.
69. **Tratschin JD, Moser C, Ruggli N, Hofmann MA.** 1998. Classical swine fever virus leader proteinase N^{pro} is not required for viral replication in cell culture. *J. Virol.* **72**:7681-7684.
70. **Bauhofer O, Summerfield A, Sakoda Y, Tratschin JD, Hofmann MA, Ruggli N.** 2007. Classical swine fever virus N^{pro} interacts with interferon

- regulatory factor 3 and induces its proteasomal degradation. *J. Virol.* **81**:3087-3096.
71. **Hilton L, Moganeradj K, Zhang G, Chen YH, Randall RE, McCauley JW, Goodbourn S.** 2006. The NPro product of bovine viral diarrhea virus inhibits DNA binding by interferon regulatory factor 3 and targets it for proteasomal degradation. *J. Virol.* **80**:11723-11732.
 72. **Fiebach AR, Guzylack-Piriou L, Python S, Summerfield A, Ruggli N.** 2011. Classical swine fever virus N^{pro} limits type I interferon induction in plasmacytoid dendritic cells by interacting with interferon regulatory factor 7. *J. Virol.* **85**:8002-8011.
 73. **Gottipati K, Ruggli N, Gerber M, Tratschin JD, Benning M, Bellamy H, Choi KH.** 2013. The structure of classical swine fever virus N^{pro}: a novel cysteine Autoprotease and zinc-binding protein involved in subversion of type I interferon induction. *PLoS Pathog.* **9**:e1003704.
 74. **Szymanski MR, Fiebach AR, Tratschin JD, Gut M, Ramanujam VM, Gottipati K, Patel P, Ye M, Ruggli N, Choi KH.** 2009. Zinc binding in pestivirus N^{pro} is required for interferon regulatory factor 3 interaction and degradation. *J. Mol. Biol.* **391**:438-449.
 75. **Mine J, Tamura T, Mitsuhashi K, Okamatsu M, Parchariyanon S, Pinyochon W, Ruggli N, Tratschin JD, Kida H, Sakoda Y.** 2015. The N-terminal domain of N^{pro} of classical swine fever virus determines its stability and regulates type I interferon production. *J. Gen. Virol.* **96**:1746-1756.
 76. **Kumagai T, Shimizu T, Matsumoto M.** 1958. Detection of hog cholera virus by its effect on Newcastle disease virus in swine tissue culture. *Science* **128**:366.
 77. **Ocaña-Macchi M, Bel M, Guzylack-Piriou L, Ruggli N, Liniger M, McCullough KC, Sakoda Y, Isoda N, Matrosovich M, Summerfield A.** 2009. Hemagglutinin-dependent tropism of H5N1 avian influenza virus for human endothelial cells. *J. Virol.* **83**:12947-12955.

78. **Mayer D, Thayer TM, Hofmann MA, Tratschin JD.** 2003. Establishment and characterisation of two cDNA-derived strains of classical swine fever virus, one highly virulent and one avirulent. *Virus Res.* **98**:105-116.
79. **Kameyama K, Sakoda Y, Tamai K, Nagai M, Akashi H, Kida H.** 2006. Genetic recombination at different points in the N^{pro}-coding region of bovine viral diarrhea viruses and the potentials to change their antigenicities and pathogenicities. *Virus Res.* **116**:78-84.
80. **Lee SM, Schommer SK, Kleiboeker SB.** 2004. Porcine reproductive and respiratory syndrome virus field isolates differ in in vitro interferon phenotypes. *Vet. Immunol. Immunopathol.* **102**:217-231.
81. **Duvigneau JC, Hartl RT, Groiss S, Gemeiner M.** 2005. Quantitative simultaneous multiplex real-time PCR for the detection of porcine cytokines. *J. Immunol. Methods* **306**:16-27.
82. **Allen DG, Riviere JE, Monteiro-Riviere NA.** 2001. Cytokine induction as a measure of cutaneous toxicity in primary and immortalized porcine keratinocytes exposed to jet fuels, and their relationship to normal human epidermal keratinocytes. *Toxicol. Lett.* **119**:209-217.
83. **Bensaude E, Turner JL, Wakeley PR, Sweetman DA, Pardieu C, Drew TW, Wileman T, Powell PP.** 2004. Classical swine fever virus induces proinflammatory cytokines and tissue factor expression and inhibits apoptosis and interferon synthesis during the establishment of long-term infection of porcine vascular endothelial cells. *J. Gen. Virol.* **85**:1029-1037.
84. **Choi C, Chae C.** 2003. Localization of classical swine fever virus from chronically infected pigs by in situ hybridization and immunohistochemistry. *Vet. Pathol.* **40**:107-113.
85. **Sánchez-Cordón PJ, Romanini S, Salguero FJ, Ruiz-Villamor E, Carrasco L, Gómez-Villamandos JC.** 2003. A histopathologic, immunohistochemical, and ultrastructural study of the intestine in pigs inoculated with classical swine fever virus. *Vet. Pathol.* **40**:254-262.

86. **Meyers G, Ege A, Fetzer C, von Freyburg M, Elbers K, Carr V, Prentice H, Charleston B, Schürmann EM.** 2007. Bovine viral diarrhea virus: prevention of persistent fetal infection by a combination of two mutations affecting E^{rns} RNase and N^{pro} protease. *J. Virol.* **81**:3327-3338.
87. **Summerfield A, Alves M, Ruggli N, de Bruin MG, McCullough KC.** 2006. High IFN- α responses associated with depletion of lymphocytes and natural IFN-producing cells during classical swine fever. *J. Interferon. Cytokine Res.* **26**:248-255.
88. **Dai J, Megjugorac NJ, Amrute SB, Fitzgerald-Bocarsly P.** 2004. Regulation of IFN regulatory factor-7 and IFN- α production by enveloped virus and lipopolysaccharide in human plasmacytoid dendritic cells. *J. Immunol.* **173**:1535-1548.
89. **Marié I, Durbin JE, Levy DE.** 1998. Differential viral induction of distinct interferon- α genes by positive feedback through interferon regulatory factor-7. *EMBO J.* **17**:6660-6669.
90. **Honda K, Yanai H, Negishi H, Asagiri M, Sato M, Mizutani T, Shimada N, Ohba Y, Takaoka A, Yoshida N, Taniguchi T.** 2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* **434**:772-777.
91. **Sato M, Suemori H, Hata N, Asagiri M, Ogasawara K, Nakao K, Nakaya T, Katsuki M, Noguchi S, Tanaka N, Taniguchi T.** 2000. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN- α/β gene induction. *Immunity* **13**:539-548.
92. **Paessler S, Walker DH.** 2013. Pathogenesis of the viral hemorrhagic fevers. *Annu. Rev. Pathol.* **8**:411-440.
93. **Sánchez-Cordón PJ, Núñez A, Salguero FJ, Pedrera M, Fernández de Marco M, Gómez-Villamandos JC.** 2005. Lymphocyte apoptosis and thrombocytopenia in spleen during classical swine fever: role of macrophages and cytokines. *Vet. Pathol.* **42**:477-488.

94. **Romero-Brey I, Bartenschlager R.** 2014. Membranous replication factories induced by plus-strand RNA viruses. *Viruses* **6**:2826-2857.
95. **Kopek BG, Perkins G, Miller DJ, Ellisman MH, Ahlquist P.** 2007. Three-dimensional analysis of a viral RNA replication complex reveals a virus-induced mini-organelle. *PLoS Biol.* **5**:e220.
96. **Spuul P, Salonen A, Merits A, Jokitalo E, Kaariainen L, Ahola T.** 2007. Role of the amphipathic peptide of Semliki forest virus replicase protein nsP1 in membrane association and virus replication. *J. Virol.* **81**:872-883.
97. **Knoops K, Kikkert M, Worm SH, Zevenhoven-Dobbe JC, van der Meer Y, Koster AJ, Mommaas AM, Snijder EJ.** 2008. SARS-coronavirus replication is supported by a reticulovesicular network of modified endoplasmic reticulum. *PLoS Biol.* **6**:e226.
98. **Romero-Brey I, Merz A, Chiramel A, Lee JY, Chlanda P, Haselman U, Santarella-Mellwig R, Habermann A, Hoppe S, Kallis S, Walther P, Antony C, Krijnse-Locker J, Bartenschlager R.** 2012. Three-dimensional architecture and biogenesis of membrane structures associated with hepatitis C virus replication. *PLoS Pathog.* **8**:e1003056.
99. **Welsch S, Miller S, Romero-Brey I, Merz A, Bleck CK, Walther P, Fuller SD, Antony C, Krijnse-Locker J, Bartenschlager R.** 2009. Composition and three-dimensional architecture of the dengue virus replication and assembly sites. *Cell Host. Microbe* **5**:365-375.
100. **Miller S, Sparacio S, Bartenschlager R.** 2006. Subcellular localization and membrane topology of the Dengue virus type 2 Non-structural protein 4B. *J. Biol. Chem.* **281**:8854-8863.
101. **Weiskircher E, Aligo J, Ning G, Konan KV.** 2009. Bovine viral diarrhea virus NS4B protein is an integral membrane protein associated with Golgi markers and rearranged host membranes. *Virol. J.* **6**:185.
102. **Qu L, McMullan LK, Rice CM.** 2001. Isolation and characterization of noncytopathic pestivirus mutants reveals a role for nonstructural protein NS4B in viral cytopathogenicity. *J. Virol.* **75**:10651-10662.

103. **Risager PC, Fahnoe U, Gullberg M, Rasmussen TB, Belsham GJ.** 2013. Analysis of classical swine fever virus RNA replication determinants using replicons. *J. Gen. Virol.* **94**:1739-1748.
104. **Zhong W, Gutshall LL, Del Vecchio AM.** 1998. Identification and characterization of an RNA-dependent RNA polymerase activity within the nonstructural protein 5B region of bovine viral diarrhea virus. *J. Virol.* **72**:9365-9369.
105. **Greiser-Wilke I, Moennig V, Coulibaly CO, Dahle J, Leder L, Liess B.** 1990. Identification of conserved epitopes on a hog cholera virus protein. *Arch. Virol.* **111**:213-225.
106. **Mittelholzer C, Moser C, Tratschin JD, Hofmann MA.** 1997. Generation of cytopathogenic subgenomic RNA of classical swine fever virus in persistently infected porcine cell lines. *Virus Res.* **51**:125-137.
107. **Mittelholzer C, Moser C, Tratschin JD, Hofmann MA.** 2000. Analysis of classical swine fever virus replication kinetics allows differentiation of highly virulent from avirulent strains. *Vet. Microbiol.* **74**:293-308.
108. **Sharma R, Ghasparian A, Robinson JA, McCullough KC.** 2012. Synthetic virus-like particles target dendritic cell lipid rafts for rapid endocytosis primarily but not exclusively by macropinocytosis. *PLoS One* **7**:e43248.
109. **Yamasaki T, Suzuki A, Shimizu T, Watarai M, Hasebe R, Horiuchi M.** 2012. Characterization of intracellular localization of PrP^{Sc} in prion-infected cells using a mAb that recognizes the region consisting of aa 119-127 of mouse PrP. *J. Gen. Virol.* **93**:668-680.
110. **Dunn KW, Kamocka MM, McDonald JH.** 2011. A practical guide to evaluating colocalization in biological microscopy. *Am. J. Physiol. Cell Physiol.* **300**:C723-742.
111. **Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG.** 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* **23**:2947-2948.

112. **King RD, Sternberg MJ.** 1996. Identification and application of the concepts important for accurate and reliable protein secondary structure prediction. *Protein Sci.* **5**:2298-2310.
113. **Guermeur Y.** 1997. Thèse de doctorat l'. Université de Paris 6, Paris, Combinaison de classifieurs statistiques, application à la prédiction de la structure secondaire des protéines.
114. **Guermeur Y, Geourjon C, Gallinari P, Deleage G.** 1999. Improved performance in protein secondary structure prediction by inhomogeneous score combination. *Bioinformatics* **15**:413-421.
115. **Rost B.** 1996. PHD: predicting one-dimensional protein structure by profile-based neural networks. *Methods Enzymol.* **266**:525-539.
116. **Cole C, Barber JD, Barton GJ.** 2008. The Jpred 3 secondary structure prediction server. *Nucleic Acids Res.* **36**:W197-201.
117. **Pollastri G, McLysaght A.** 2005. Porter: a new, accurate server for protein secondary structure prediction. *Bioinformatics* **21**:1719-1720.
118. **Jones DT.** 1999. Protein secondary structure prediction based on position-specific scoring matrices. *J. Mol. Biol.* **292**:195-202.
119. **Raghava GPS.** 2002. APSSP2 : A combination method for protein secondary structure prediction based on neural network and example based learning. *CASP5 A*:132.
120. **Claros MG, von Heijne G.** 1994. TopPred II: an improved software for membrane protein structure predictions. *Comput. Appl. Biosci.* **10**:685-686.
121. **Gautier R, Douguet D, Antonny B, Drin G.** 2008. HELIQUEST: a web server to screen sequences with specific alpha-helical properties. *Bioinformatics* **24**:2101-2102.
122. **Leifer I, Ruggli N, Blome S.** 2013. Approaches to define the viral genetic basis of classical swine fever virus virulence. *Virology* **438**:51-55.
123. **Fahnoe U, Pedersen AG, Risager PC, Nielsen J, Belsham GJ, Hoper D, Beer M, Rasmussen TB.** 2014. Rescue of the highly virulent classical swine fever

virus strain "Koslov" from cloned cDNA and first insights into genome variations relevant for virulence. *Virology* **468-470**:379-387.

124. **Schmeiser S, Mast J, Thiel HJ, Konig M.** 2014. Morphogenesis of pestiviruses: new insights from ultrastructural studies of strain Giraffe-1. *J. Virol.* **88**:2717-2724.
125. **Gouttenoire J, Roingeard P, Penin F, Moradpour D.** 2010. Amphipathic α -helix AH2 is a major determinant for the oligomerization of hepatitis C virus nonstructural protein 4B. *J. Virol.* **84**:12529-12537.
126. **Yu GY, Lee KJ, Gao L, Lai MM.** 2006. Palmitoylation and polymerization of hepatitis C virus NS4B protein. *J. Virol.* **80**:6013-6023.
127. **Gouttenoire J, Montserret R, Paul D, Castillo R, Meister S, Bartenschlager R, Penin F, Moradpour D.** 2014. Aminoterminal Amphipathic α -Helix AH1 of Hepatitis C Virus Nonstructural Protein 4B Possesses a Dual Role in RNA Replication and Virus Production. *PLoS Pathog.* **10**:e1004501.

Summary in Japanese (和文要旨)

豚コレラは、豚に強い伝播力と高い致死率を特徴とするウイルス感染症で家畜伝染病に指定されている。豚コレラの原因ウイルスである豚コレラウイルスは、牛ウイルス性下痢ウイルスおよびボーダー病ウイルスと同じフラビウイルス科ペスチウイルス属に属する。ウイルスゲノムは全長約 12,300 塩基のプラス極性 1 本鎖 RNA であり、5' 末端より N^{pro}、C、E^{ms}、E1、E2、p7、NS2、NS3、NS4A、NS4B、NS5A および NS5B 蛋白をコードしている。豚コレラウイルスの病原性は株によって様々である。強毒株が感染した豚は、著しい白血球並びに血小板数の減少、発熱および播種性血管内凝固症候群に伴う多臓器不全により、多くの場合 1 週間以内に死亡する。中等毒株が豚に感染した場合は、慢性経過をたどり死亡あるいは耐過してひね豚となる。一方、実験室で作出された弱毒生ワクチン株を接種した豚は、臨床症状を殆ど示さず強固な免疫を獲得する。豚コレラの病態についてはよく知られているが、豚コレラウイルスの病原性の分子基盤は未だ不明である。そこで本研究では、日本で使用されていた弱毒生ワクチン GPE⁻株を用いて病原性発現に関与するウイルス蛋白を同定し、その機能を解析することで豚コレラウイルスの病原性発現機序の解明を試みた。

弱毒生ワクチン GPE⁻株は、野外強毒株 ALD を異種動物の培養細胞で継代することで樹立した。本稿第一章では、まず GPE⁻株を本来の宿主である豚で連続継代し、病原性を獲得するか調べた。GPE⁻株を豚で 11 代継代した結果、ウイルスは豚に対して病原性を獲得した。継代後のウイルスの全長の塩基配列を決定し、継代前後でのアミノ酸配列を比較したところ、ウイルスの表面糖蛋白 E2 に 1 箇所、非構造蛋白 NS4B に 2 箇所のアミノ酸の相違を認めた。次に GPE⁻株のリバースジェネティクスの系を確立し、それを用いてそれぞれのアミノ酸を置

換したウイルスを作出した。それらの変異ウイルスの豚への接種試験の結果から、認めた3箇所のアミノ酸置換全てが病原性発現に関与することがわかった。さらに、E2 蛋白のアミノ酸置換はウイルスの細胞間伝播の向上に、NS4B 蛋白のアミノ酸置換はウイルスゲノムの複製効率の向上に関与することを明らかにした。

豚コレラウイルスの野外株のほとんどは、宿主細胞に感染するとインターフェロン制御因子3を分解することでI型インターフェロンの産生を抑制する。しかし、弱毒生ワクチン GPE⁻株はその樹立の過程でそれを失っている。本稿第二章では、ウイルスによるI型インターフェロンの産生抑制能が病原性発現に関与するか調べた。まず、野外株とのウイルス遺伝子の比較およびリバーシジェネティクス法を用いて、GPE⁻株の非構造蛋白 N^{pro} 分子上のI型インターフェロン産生調節に関与するアミノ酸部位を同定した。次にその部位のアミノ酸を置換し、I型インターフェロンの産生抑制能を復帰させたウイルスの感染に対する豚の免疫応答と病原性を解析した。GPE⁻株のI型インターフェロン産生抑制能を復帰させたウイルスの豚に対する病原性は、GPE⁻株と比較して同程度だった。一方、豚継代ウイルスのI型インターフェロン産生抑制能を復帰させたウイルスの豚に対する病原性は、その親株と比較して高かった。以上から、N^{pro} 蛋白による宿主のI型インターフェロンの産生抑制は、先に同定したE2蛋白およびNS4B蛋白分子上のアミノ酸置換によるウイルスの増殖効率の向上に相乗作用することで、病原性発現に関与することが明らかになった。

ウイルスのゲノム複製と病原性発現に関してその分子機序を解明するため、機能が不明である非構造蛋白 NS4B に着目し研究を進めた。本稿第三章では、GPE⁻株の NS4B 蛋白遺伝子を野外強毒株のそれに置き換えたキメラウイルスを作出し、ウイルスのゲノム複製および病原性に及ぼす影響を調べた。その結果、

強毒株の NS4B 蛋白遺伝子を持つキメラウイルスは、親株と比較してウイルスゲノムの複製効率が向上し、豚に対する病原性が高かった。GPE⁻株と強毒株では NS4B 蛋白分子上に 7 つのアミノ酸相違を認め、うち 5 つは N 末端領域に、他の 2 つは膜貫通領域に位置した。後者の 2 つのアミノ酸部位は先の豚継代実験で同定したアミノ酸部位と同じだった。5 つのアミノ酸相違を認めた N 末端領域は両親媒性ヘリックス構造を有し、ウイルスゲノムの複製に重要であることを明らかにした。さらに、当該 N 末端領域が細胞内膜への親和性に関与し、アミノ酸相違によりそれが変化することを明らかにした。以上から、NS4B 蛋白の N 末端領域による細胞内膜への親和性は、ウイルスゲノムの複製および豚に対する病原性発現に関与することが明らかになった。

本研究により、豚コレラウイルスの病原性発現に関与するウイルス蛋白とその機能、さらには機能ドメインが明らかになった。また、それらウイルス蛋白の機能が相乗作用することで豚に対して病原性を発現することがわかった。以上、本研究の成績は豚コレラウイルスの病原性発現機序の一端を分子レベルで解明したものであると共に、豚コレラのみならず牛ウイルス性下痢症等のペスチウイルス感染症に有効で安全な生ワクチンおよび抗ウイルス薬の開発に役立つものと期待される。