Study on oligoadenylate synthetase as an innate immune factor against viral infection.

(ウイルス感染に対する自然免疫因子としてのオリゴアデニレート合成酵素に関する研究)

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Abbreviations

BHK: baby hamster kidney
DMEM: Dulbecco's modified Eagle medium
emOAS: emu OAS
GFP: green fluorescent protein
GWAS: genome-wide association study
HEK293FT: human embryonic kidney cells 293 FT
IFN: interferon
ISG: interferon-stimulated gene
MDA5: melanoma differentiation-associated gene 5
mOas: mouse Oas
JEV: Japanese encephalitis virus
OAS: oligoadenylate synthetase
osOAS: ostrich OAS
PCR: polymerase chain reaction
RIG-I: retinoic acid-inducible gene I
SEAP: secreted alkaline phosphatase
sfRNA: subgenomic flavivirus RNA
SNP: single nucleotide polymorphism
UTR: untranslated region
WNV: West Nile virus
YFV: yellow fever virus

CONTENTS

Preface

Innate immunity, the first line of defense against infection, is important for maintaining homeostasis in multicellular organisms. Pathogenic microorganisms threaten host homeostasis, and hosts have developed various mechanisms to counteract these pathogens. For viruses that invade cells during initial infection, interferons (IFNs) have been developed as innate immunity in vertebrate cells to induce proteins that effectively eliminate viruses[23, 32, 41]. IFNs are induced by various stimuli, including melanoma differentiation-associated gene 5 (MDA5), retinoic acid-inducible gene I (RIG-I), and viral RNA-sensing proteins that are expressed in virus-infected cells[17, 38, 61]. Genes induced by IFNs are referred to as interferon stimulated genes (ISGs), and several hundred genes have been currently identified[5, 23].

The oligoadenylate synthetase (OAS) gene cluster is a member of ISGs and plays a role in the elimination of foreign RNAs[19, 47, 65]. OASs recognize foreign double-stranded RNAs present in the cell and polymerize ATP to synthesize oligoadenylates. Oligoadenylates dimerize and activate RNase L, an RNA-cleaving protein to cleave intracellular single-stranded RNA. In this process, fragmented RNA is more easily recognized by RIG-I and MDA5, and induction of IFNs and ISGs is promoted. Therefore, OAS acts not only as a sensor for foreign RNA but also as an enhancer to eliminate RNAs entering from outside the cell and amplify IFN induction[47]. However, there remain many unresolved issues regarding the evolutionary phylogenetic and molecular biological aspects of the virus elimination function of the OAS.

OASs are possessed by many animal species and form various OAS families depending on the animal species[30, 59]. Although OASs have enzymatic activity in many animal species, OASs have not been well investigated for their flavivirus replication inhibition effects. Despite the five *OAS* genes possessed by humans and pigs, mice and rats possess 12 *OAS* genes. On the other hand, many bird species, such as chickens and ducks, have only one *OAS* gene, the OASL has both enzymatic activity and inhibitory effect on flavivirus replication[2, 40, 51]. In birds, only ostriches have been reported to have two *OAS* genes, with OAS1 having only enzymatic activity and OASL having only inhibition of flavivirus replication, indicating a division of functions. How the avian OAS family diversified and segregation of functions occurred remains unclear due to the small number of birds examined.

In mice possessing 12 *Oas* genes, *Oas1b* has been identified as a gene responsible for resistance to West Nile virus (WNV), Japanese encephalitis virus (JEV), and yellow fever virus (YFV), viruses of the genus *Olthoflavivirus*[16, 31, 36, 43]. These pathogens are major public health concerns since they are transmitted by arthropods and cause severe

symptoms in humans. It has long been reported that laboratory mouse strains are more susceptible to flaviviruses than wild-derived mouse strains[24, 42, 55]. This has been caused by translation of truncated proteins due to nonsense mutation of Oas1b in the laboratory mouse strains[31, 36]. On the other hand, other OASs in mice are known to have no inhibitory effect on flavivirus replication[8]. Interestingly, Oas1b of wild-derived mouse has no oligoadenylate synthetic activity and no binding capacity to double-stranded RNA[7, 8]. It has been reported that common OASs bind to double-stranded RNA, while Oas1b binds to stem-loop structures[7]. The inhibition of flavivirus replication is observed even when RNase L is knocked out, suggesting that the inhibitory mechanism is independent of the OAS/RNase L pathway, but the detailed mechanism is unknown[7, 25].

To clarify these issues, in chapter I, I focused on emu, a species closely related to the ostrich that has a unique OAS family in birds. In order to reveal the evolution process of OAS as an innate immune factor in birds and mammals, I sequenced the emu OAS (emOAS) and analyzed its function. In chapter II, I focused on the stem-loop structure in the untranslated region (UTR) of flavivirus genome RNA to clarify the mechanism of flavivirus replication inhibition of mouse Oas1b (mOas1b). I hypothesized that mOas1b inhibits viral replication by acting on the stem-loop in the UTR, and confirmed changes in the inhibitory effect of mOas1b on flavivirus replication by deleting sequences in the UTR of WNV replicons or expressing the sequence forming stem-loop in the cells.

Chapter I: Evolutionary diversity of oligoadenylate synthetase in emu (*Dromaius novaehollandiae*)

1. Introduction

OAS is a protein that acts as a defense mechanism against foreign RNA in cells. OAS is one of the ISGs and has been discovered as an enzyme that synthesizes oligoadenylates[15, 19].

OASs recognize double-stranded RNA of specific lengths and synthesizes oligoadenylates from intracellular ATP. Oligoadenylates dimerize RNase L into its active form, which recognizes and fragments RNA, resulting in decreased cellular protein expression. The fragmented RNA triggers RNA-sensing machinery such as MDA5 and RIG-I in the cell and enhances IFN expression. This activates the entire cellular antiviral machinery and promotes the expression of OAS as an ISG[27, 66]. In this mechanism, OASs play a dual role as an enhancer and a sensor in the antiviral machinery.

In humans, genome-wide association studies (GWAS) have shown that single nucleotide polymorphisms (SNPs) in OASs can alter the severity of infection and have a significant impact on virus elimination in the cell[1, 54].

The OAS family is a widely conserved protein family which has been reported in many vertebrates and sponges and is a widely conserved protein[37, 60]. There are several reports on OASs in birds, such as chickens, ducks, geese, and ostriches[2, 40, 48, 51]. Chickens, ducks, and geese have one *OAS* gene, 2'-5' oligoadenylate synthetase-like protein (*OASL*), which has both enzymatic activity and inhibitory effect on flavivirus replication. In contrast, ostriches have two *OAS* genes, *OAS1* and *OASL*, which have enzymatic activity and inhibitory effects on flavivirus replication, respectively[40, 51].

In addition, mOas1b has been reported to inhibit viral genome replication, specifically in viruses of the genus *Flavivirus*[20, 30]. This activity has been suggested to be independent of the RNase L pathway, because replication inhibition is still observed when genes constituting the OAS/RNase L pathway are knocked out, but the details of their functions have not been clarified. Mouse has 12 *OAS* family genes but only Oas1b has flavivirus replication inhibitory activity and only two OASs have enzymatic activity. The other nine OASs have neither enzymatic activity nor flaviviral replication inhibition, and their roles remain unknown[8].

Extant birds are classified into two groups: Palaeognathae and Neognathae. Neognathae consists of Galloanserae (chickens, ducks, and geese) and Neoaves. Recent

studies using numerous genes support that ostrich was the first to diverge among the palaeognaths and that there is a gap between the ostrich and other palaeognaths. The emu is a flightless bird classified as ratite, consisting of kiwi and rheas. The emu is closely related to the ostrich among palaeognaths in terms of molecular phylogeny[13, 53]. Therefore, in this study, I cloned the *OAS* genes of the emu, which is evolutionarily close to the ostrich, and investigated structures and functions of emu OASs to elucidate their roles.

2. Materials and Methods

2.1. RNA extraction

Emu spleen samples were kindly provided by Dr. K. Wada of the Tokyo University of Agriculture. Samples were collected after the emus were slaughtered for human consumption. Approval to collect the samples was obtained from the owner. Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) by crushing 200 mg of emu spleen, and the concentration of RNA was determined using SmartSpec™ (BioRad, Hercules, CA, USA). The extracted RNA was stored in a freezer at -80 °C until used.

2.2. Cloning

Reverse transcription reactions were performed using 1 μg of RNA to generate cDNA using ReverTra Ace® (Toyobo Co., Ltd. Osaka, Japan). cDNA of *OAS*s was cloned using the 5'/3'-RACE Kit, 2nd generation (Roche Diagnostics GmbH, Mannheim, Germany) by following the manufacturer's protocol. The primer sequences for PCR were based on the sequences predicted by whole genome sequencing (XM_026117387.1, XP_009671383.1). Table 1 lists the primers used in this study. The emu *OAS* PCR products were cloned into a pGEM-T-Easy vector and transfected into DH5α, and the plasmids were extracted and sequenced. For functional analysis, PCR was performed using FLAG-tagged primers and the PCR products were inserted into the pCAG-IRES-EGFP vector. Genetic recombination experiments were approved by Hokkaido University (approval number: 2020-034). After determining the OAS sequences, conserved motif analysis of emu OASs was performed using MOTIF Search (https://www.genome.jp/tools/motif/).

2.3. Phylogenetic tree

Protein sequence alignment with other birds was performed using MEGA X software[21] and the reported protein sequences were: *Gallus gallus* (chOASL, BAB19016.1), *Anser cygnoides* (goOASL, ANW12075), *Anas platyrhynchos* (duOASL, ANW12076), and *Struthio camelus* australis (osOASL, XP_009671383, and osOAS1, XP_009667960). The amino acid sequences were aligned by neighbor-joining algorism which create a tree based on the balanced minimum evolution criterion. The tree is drawn to scale with the branch lengths (next to the branches) in the same units as the evolutionary distances used to construct the phylogenetic tree.

2.4. Enzymatic activity assay

Enzymatic activity was measured as described previously[50, 51]. The HEK293FT cell line was obtained from Dr. N. Sasaki of Kitasato University. HEK293FT cells were grown in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Atlas Biological, Fort Collins, CO, USA) and 1% Penicillin-Streptomycin-L-Glutamine Solution (Fujifilm Wako Pure Chemical, Osaka, Japan) (final concentration: 100 unit/ml penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine). Enzymatic activity was determined by transfection of HEK293FT cells with a plasmid encoding OASs and lysis of the cells with Flag-lysis buffer after 72 h. Then, 2.5 μL of the lysate and 7.5 μL of reaction buffer [20 mM Tris-HCl at pH 7.4, 20 mM magnesium acetate, 2.5 mM dithiothreitol, 5 mM ATP, 50 μg/mL poly:IC, and 5 μCi of [α-^{32}P] ATP (3000 Ci/mmol)] in a final volume of 10 μL were mixed and reacted at 37 °C for 24 h. Subsequently, the mixtures were incubated at 95 °C for 5 min to terminate the reaction, and electrophoresis was performed on a 20% urea acrylamide gel for 1 h. After electrophoresis, the gels were soaked in the protection buffer (3% glycerol [w/v], 40% MeOH, 10% HOA) for 2 h, and then dried for 1 h using a Model 583 Gel Dryer (BioRad). The dried gels were exposed to a BAS 2000 imaging plate (FUJIFILM, Tokyo, Japan) for 30 min and quantified using BAS2000 Image Analyzer (FUJIFILM).

2.5. Antiviral experiments

The inhibitory effect on flaviviral replication was measured using previous method[33]. The BHK-21 cells were obtained from the American Type Culture Collection. Briefly, BHK-21 cells were grown in DMEM (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Atlas biological) and 1% Penicillin-Streptomycin-L-Glutamine Solution (Fujifilm Wako Pure Chemical) (final concentration: 100 unit/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine). BHK-21 cells were seeded in 24-well plates at

a density of 1.0×10^5 cells/well. After 24 h, 10 μg of pIRES-EGFP (empty vector as a control), pemOAS1-EGFP, pemOASL-EGFP, posOAS1-EGFP, posOASL-EGFP, and pmOas1b-EGFP were transfected using Lipofectamine 2000® (Thermo Fisher Scientific) according to the manufacture's protocol. The posOAS1-EGFP, posOASL-EGFP, and pmOas1b-EGFP were constructed in the previous study, and mOas1b was cloned from MSM/Ms strain which is wild-derived strain[33, 51]. EGFP expression was observed using fluorescence microscope to estimate the transfection efficiency. WNV replicon RNA, harboring the secreted alkaline phosphatase (SEAP) reporter gene instead of viral structural genes, was propagated by mMESSAGE mMACHINE® Kit (Thermo Fisher Scientific), as previously reported[33]. Briefly, the WNV replicon DNA plasmid was linearized with *Not* I, and the single-stranded end was removed using mung bean nuclease. The 1 μg of linearized WNV replicon DNA was used as the template for transcription. WNV replicon RNA (500 ng) was lipofected into BHK-21 cells. The culture supernatants were collected after culturing the cells for 72 h post-lipofection and centrifuged under 12,000 xg for 30 s. The supernatants were collected, and stored at -80 °C until used. The amount of reporter protein in the culture supernatant was measured using Great EscAPe™ SEAP Chemiluminescence Kit 2.0 (Takara Bio Inc., Shiga, Japan) and an Infinite M200 PRO plate reader (TECAN Japan Co., Ltd., Kanagawa, Japan) according to the manufacturer's protocol.

2.6. Statistical analysis

The groups were compared with Dunnett's test and data were shown as means ± standard error. *P*-values less than 0.05 were considered to be significant.

3. Results

3.1. Cloning

The purity of the extracted RNA was evaluated by a ratio of OD260/280. The values of ratio of OD260/280 were ranged in 1.9±0.7. I attempted to clone the emu OASs to determine its amino acid sequence. Using the ostrich *OAS* (osOAS) sequence as a reference, I successfully determined the emu *OAS* sequence using the RACE method. The accession numbers for emu *OAS1* and *OASL* were LC788476 and LC788477, respectively.

The amino acid sequences of the ostrich and emu OASs were compared (Figs. 1A and 1B). Amino acid sequence homology was 80% for OAS1 and 78% for OASL. The

sequences of the two species were compared in terms of regions I-III, which have been reported to be essential for the function of the OAS: I and II sequences were completely identical, whereas the III sequence showed a little difference[2, 10, 51]. Domain structural analysis of these OASs showed that the OASL protein conserved three domains, the nucleotidyltransferase, OAS1_C, and two ubiquitin-like domains, UBL1 and UBL2, whereas osOAS1 possessed only the nucleotidyltransferase and OAS1_C domains.

3.2. Phylogenetic tree

The emu OAS1 and OASL sequences were compared with those of other bird species. emOAS1 was not present in other birds except for the ostrich. A phylogenetic tree was constructed using the neighborhood method (Fig. 1C). The OASL sequence of emu was similar to that of the ostrich and created a distinct group among the birds. Therefore, the emu and ostrich are the unique bird species among birds by possessing OAS1 with high homology.

3.3. Enzymatic activity

The enzymatic activities of OASs to synthesize 2'-5'-oligoadenylates from ATP were measured. OAS enzymatic activity was assessed using lysates of HEK293FT cells transfected with the pIRES-EGFP empty vector as a control, pmOas1b-EGFP, posOASL-EGFP, posOAS1-EGFP, pemOASL-EGFP, and pemOAS1-EGFP. As previously reported[8, 51], osOAS1 synthesized oligoadenylates, but osOASL and mOas1b did not have oligoadenylate synthesis activity (Fig. 2). The results showed that emOAS1 synthesized oligoadenylates via enzymatic activity, whereas no oligoadenylate synthesis was observed in emu OASL (Fig. 2).

3.4. Inhibitory activity on the WNV replicon replication

The replication inhibitory effects of OAS1 and OASL of the emu were compared with those of ostrich OASs and mOas1b. Inhibitory activity on the WNV replicon replication was measured using BHK-21 cells transfected with pIRES-EGFP empty vector as a control, pmOas1b-EGFP, posOASL-EGFP, posOAS1-EGFP, pemOASL-EGFP, and pemOAS1-EGFP (Fig. 3A). The results show that OASL of both emus and ostriches as well as mOas1b, inhibited the replication of the WNV replicon, whereas OAS1 of both emus and ostriches did not (Fig. 3B).

4. Discussion

In this study, I succeeded in sequencing the OASs of the emu, which was evolutionarily close to the ostrich. The amino acid sequence homology was 80% for OAS1 and 78% for OASL compared to those of the ostrich. This was almost consistent with the results of previously reported homology comparisons of other genes between the two species[12, 62]. The results of the phylogenetic tree analysis showed that the homologies of the OASLs could be compared and divided into Palaeognathae and Neognathae groups. Domain analysis revealed that OASL has three domains (nucleotidyltransferase, OAS1_C, and ubiquitin-like domain), similar to previously reported OASLs in other avian and mammalian species. These results suggest that the OASL may be important for their function in a wide range of animal species.

The antiflavivirus replication activity of OASs was suggested by *Oas1b* as the gene responsible for the susceptibility to flavivirus infection in mice[31, 36]. However, Oas1b does not have the enzymatic activity to synthesize oligoadenylates from ATP. This antiflavivirus replication pathway was observed even when RNase L was knocked out, suggesting that there is a flavivirus replication inhibition pathway independent of the oligoadenylate synthetic activity. The inhibition of flavivirus replication has also been reported in other avian species. BHK-21 cells are one of the most commonly used cells for viral infection experiments due to the lack of interferon induction[22, 44]. The proteins that constitute the OAS/RNase L pathway are expressed at low levels in BHK-21 cells since they are a member of ISGs. Therefore, BHK-21 cells allow to measure the inhibitory activity of introduced OAS on flavivirus replication with minimizing the effects of the intrinsic OAS/RNase L pathway. In this experiment, the amount of reporter protein expressed by the WNV replicon was reduced by OASL, but not by OAS1 in both emus and ostriches. Alignment of the amino acid sequence of OAS1 showed that emu OAS1 had an additional 48 amino acids at the C-terminus compared to ostriches. This additional region may not affect the antiflaviviral activity. To elucidate the details of flavivirus replication inhibition by OASs, it is necessary to analyze common sequences and function-related domains by examining the sequences of OASs with flavivirus-specific viral replication inhibition in other animal species. OASL has been reported to lack enzymatic activity in many mammalian species, indicating that it differs from chicken, geese, and ducks[14, 48]. The OASL of ostriches and emus showed no oligoadenylate synthetic activity, suggesting that they have similar mammalian characteristics.

OASs synthesizes 2’5’-oligoadenylates from ATP. Enzyme activity is activated by recognition of double-stranded RNA. In the present experiment, stimulation with poly:IC

resulted in the synthesis of oligoadenylates in OAS1, but not in OASL. This mechanism is believed to be the basic function of this enzyme, which is responsible for cellular viral defense. Sequence comparisons with the ostrich OAS1 revealed that the regions of the sequences that are thought to be important for enzymatic activity were conserved[10]. These regions were highly conserved in other avian species, which support their functional importance.

The division of the function of the OASs was found to develop in emus as well as in the ostrich, although the significance of this division remains unclear. The OAS/RNase L pathway randomly cleaves and depletes intracellular single-stranded RNA[3]. Thus, it inhibits homeostatic protein synthesis in the cell. However, some ISGs evade degradation by RNase L to maintain their intracellular antiviral mechanisms. In mice, one of the ISGs that escape degradation by RNase L is mOas1b, which exhibits inhibitory effects on flaviviral replication without enzymatic activity. This protein also inhibits the oligoadenylate synthesis activity of mouse Oas1a which contributes to the OAS/RNase L pathway[7]. In mice, it has been suggested that division of functions and duplication of *OASs* allows rapid virus elimination with slightly suppressed OAS/RNase L by mOas1b during flavivirural infection, whereas the OAS/RNase L pathway by Oas1a is used for virus elimination during other viral infections. Therefore, the division of functions may contribute to viral defense through an optimal mechanism depending on the virus species. The division of functions may be advantageous to host organisms and the fact that emus and ostriches have two *OAS* genes, which play an allotted role in antiflavivirus replication and enzymatic activity, may be advantageous in viral defense compared to other avian species that have only one *OAS* gene. The division of OASs functions into multiple *OAS* genes has also been observed in mammals (Table 2). In particular, mouse *Oas1* paralogs have been well investigated for antiflaviviral replication and enzymatic activities[8].

In mice, only Oas1b has antiflaviviral replication, Oas1a and Oas1g have enzymatic activity, and the other Oas1 paralogs do not have either activity. Although other mammals, such as humans, swine, and rats have not been examined for these activities, they have multiple *OAS* genes. Emus and ostriches were evolutionarily different from other birds in terms of the OAS diversity. The enzymatically active OAS1 may be an ancestral OAS in invertebrates[59]. Most of birds such as the chickens, ducks, and geese acquired antiflavivirus activity in the same molecule, whereas the ratitae such as the ostriches and emus and mammals such as the miceacquired the same activity as the other OAS molecules, OASL or Oas1b. However, these evolutionary dynamics need to be clarified in future study.

The results of this study suggest that emus may be more resistant to flaviviruses than

other bird species. Experimental infections of WNV to birds has been conducted, and differences in susceptibility of bird species, including many wild birds, have already been reported[18]. Fatalities caused by WNV have been reported in a young ostrich, but not in adults[56]. However, there have been few reports of viral infections in emus, and the contribution of OAS to viral defense in emu remains unclear. Experimental viral infections are needed to deepen our knowledge of viral defense in emus. Hopefully, this study will open novel perspectives on defense against viruses in birds.

5. Summary

OAS is one of the proteins that act as a defense mechanism against foreign RNA in cells. Many birds are reported to have only one gene of the OAS family, *OASL*, which has both enzymatic activity and inhibitory effect on flavivirus replication. However, the ostrich has two OAS genes, *OAS1* and *OASL*, showing different functions, enzymatic and antiflaviviral activities, respectively.

In this study, emOASs were cloned to investigate sequence and function of them to elucidate the diversity of OASs in birds. The cloning results showed that emu had OAS1 and OASL, which were more closely related to the ostrich than to other birds. Amino acid sequence homology was 80% for OAS1 and 78% for OASL compared with ostrich. Functional investigation showed that emu OAS1 and OASL had enzymatic and antiflaviviral activities, respectively, similar to those of the ostrich.

The separation of OAS functions into multiple *OAS* genes as seen in mammals may be advantageous to the organism in the defense against the viral infection through an appropriate mechanism depending on the virus species. Emus and ostriches are birds evolutionally different from most of birds and may be more closely related to mammals with respect to the OAS diversity.

6. Tables and figures

Table 1. Primers for 5'/3' RACE and cloning of emu *OAS* cDNA.

Name	Direction	Primer sequence
OAS1-5'RACE	Reverse	CCTCTGCTTCTTGCACTCCA
OAS1-3'RACE	Forward	CTGTCAGCACCTCAACCTGCA
OASL-5'RACE	Reverse	TACCAGTGCTTGACCAGGC
OASL-3'RACE	Forward	GCCTGGTCAAGCACTGGTA
OAS1-*Xba* I	Forward	TGCTCTAGAGCAGCACGGGCGCTGTCACAG
OASL-*Xba* I	Forward	TGCTCTAGAGTATGGATGGGCTGGAGA
Emu OAS1 FLAG-*Xba* I	Reverse	TTATTATCTAGATCACTTGTCGTCATCGTCTTTGTAGTCGAG GACAGTGCAGAGGTC
Emu OASL FLAG-*Xba* I	Reverse	TTATTATCTAGATCACTTGTCGTCATCGTCTTTGTAGTCG TTTATTTCCGGCATGATA

Table 2. Diversity of OASs in birds and mammals.

Birds		Reference
Chicken	OASL*,**	52)
Goose	OASL*, **	51)
Duck	OASL*, **	51)
Ostrich	OASL*, OAS1**	51)
Emu	OASL*, OAS1**	(this study)
Mammals		
Human	OAS1**, OAS2**, OAS3**, OASL1	14)
Swine	OAS1a**, OAS1b**, OAS2, OASL	58, 64)
Rat	OAS1a, OAS1b, OAS1c, OAS1d, OAS1e, OAS1f, OAS1g, OAS1h, OAS1i, OAS2, OAS3, OASL	37)
Mouse	Oas1a**, Oas1b*, Oas1c, Oas1d, Oas1e, Oas1f, Oas1g**, Oas1h, Oas2, Oas3, OasL1, OasL2	8)

*, antiflaviviral activity. **, enzymatic activity. The underlined OASs are reported not to have either of the two activities. Other OASs not marked with asterisks or underlines are unknown for these two activities because of a lack of reports.

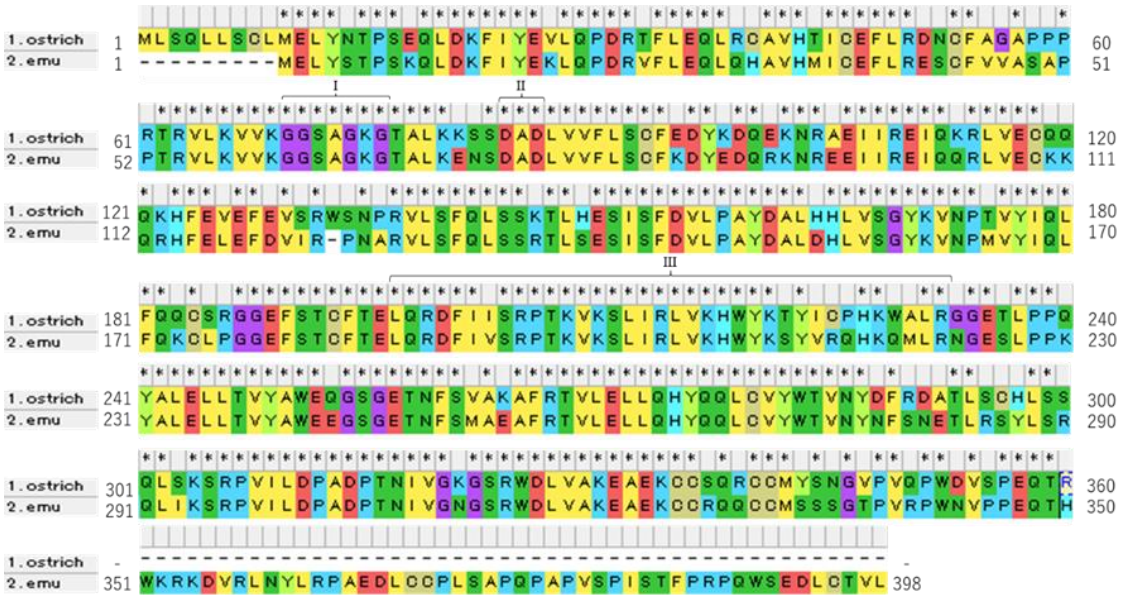

Fig. 1A. Amino acid sequences of emu OAS1. The amino acid sequence was aligned with those of ostrich. Homologous amino acid sequences are marked with * at the top. I-III indicate regions essential for the OAS enzymatic function.

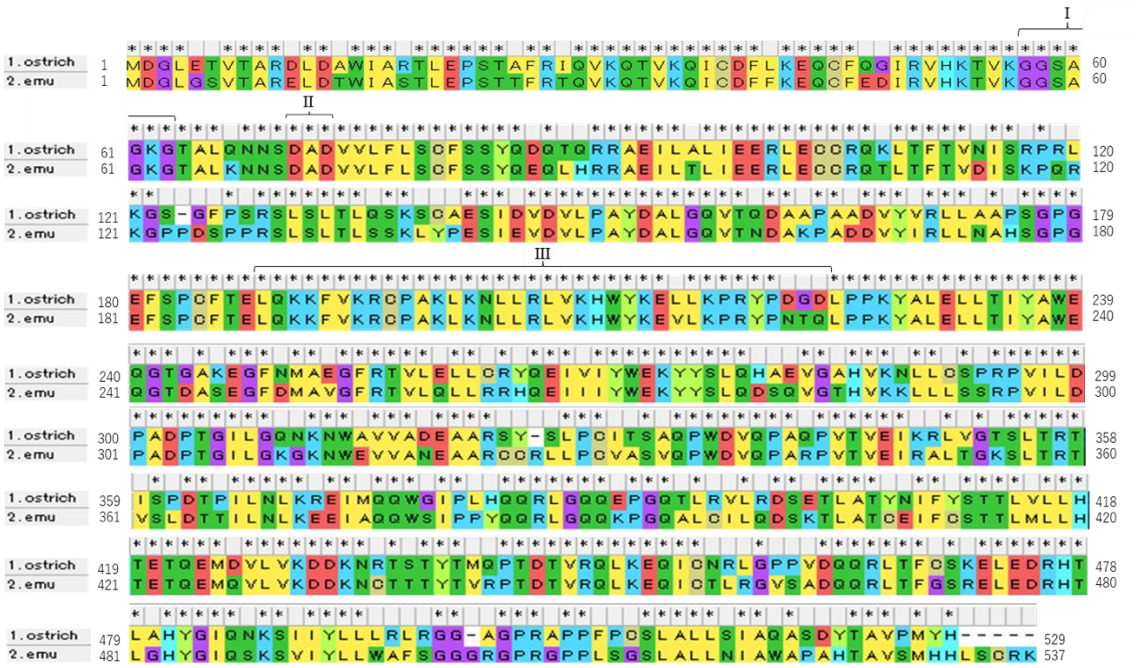

Fig. 1B. Amino acid sequences of emu OASL. The amino acid sequence was aligned with those of ostrich. Homologous amino acid sequences are marked with * at the top. I-III indicate regions essential for the OAS enzymatic function.

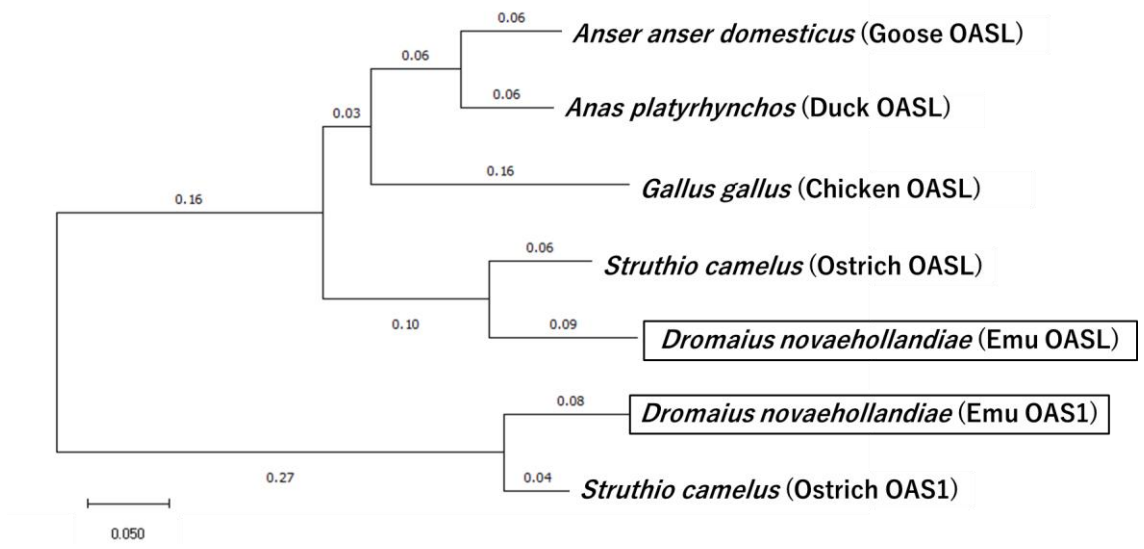

Fig. 1C. A phylogenetic tree of avian OASs with reported nucleotide sequences. The emu OASs of the sequences obtained in this study were put in squares.

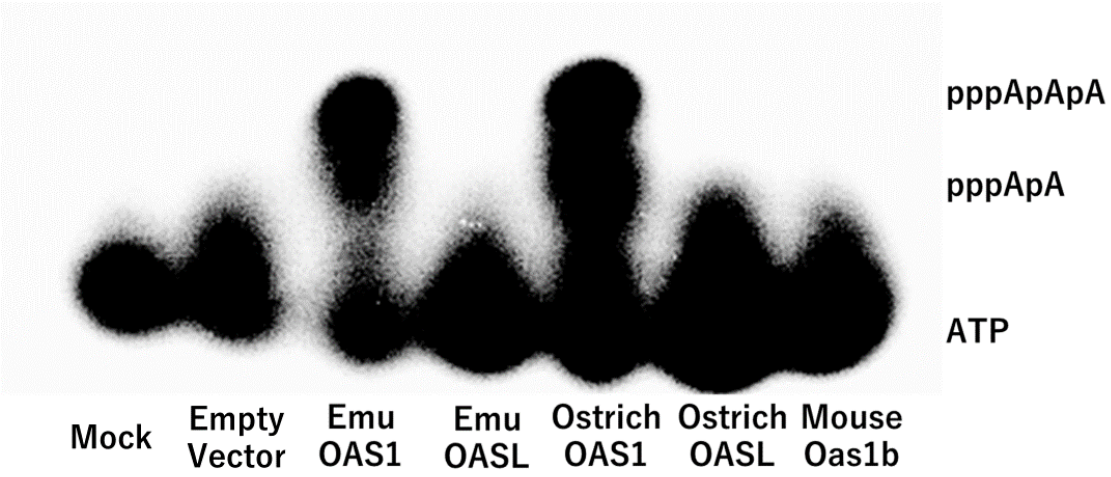

Fig. 2. Oligoadenylate synthetase activity was determined for emu, ostrich, and mouse OASs. The products after the enzymatic reaction were electrophoresed and detected as oligomerized ATP with ^{32}P.

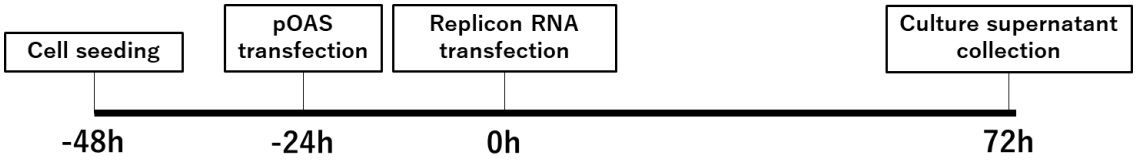

Fig. 3A. Timeline of the antiflaviviral activity experiment. The inhibitory effect of emu OAS on West Nile virus replication was measured. BHK-21 cells were seeded, then sequentially transfected with pOAS plasmid and WNV replicon RNA. The supernatant of the culture medium was collected after 72 h.

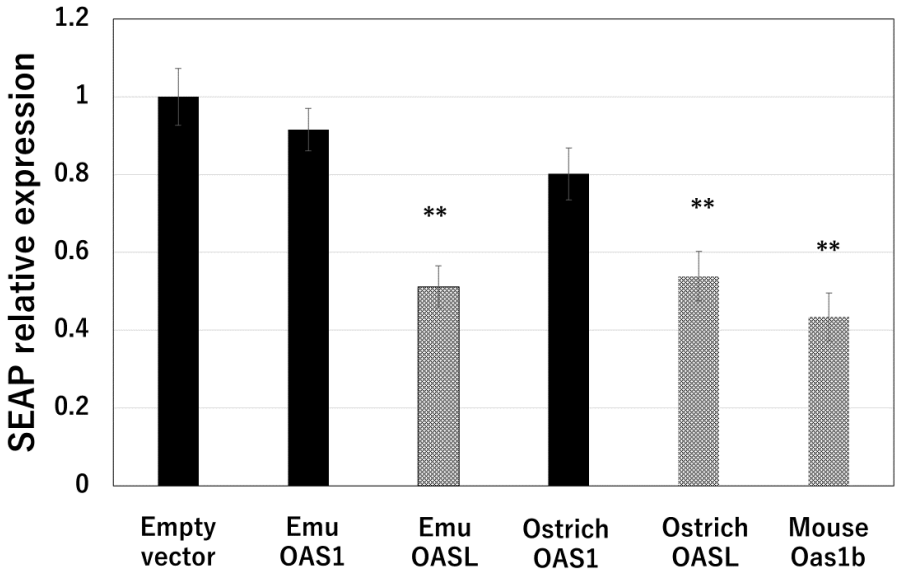

Fig. 3B. The inhibitory effect of emu OASs on WNV replication was measured. SEAP, a reporter protein in the culture supernatant was measured. Error bars indicate standard errors. ** indicates $p<0.01$ compared with empty vector.

Chapter II: Mouse oligoadenylate synthetase 1b inhibits West Nile virus replication by acting through the stem-loop 2 in the 3'-untranslated region.

1. Introduction

OAS is known to play a role in intracellular viral defense and conserved in many animal species. Oas is activated by the recognition of double-stranded RNA that enters the cell from the outside and polymerizes ATP to synthesize oligoadenylates[19]. Oligoadenylates dimerize RNase L to the active form, resulting in cleavage of single-stranded RNA at random[26]. Fragmented RNA is recognized by RNA sensing proteins such as MDA5 and RIG-I and induces IFN production. Because OAS is one of the ISGs, the OAS/RNase L pathway is further amplified. Thus, OAS plays a role as both sensor and enhancer of viral defense in cells.

Wild mice are resistant to WNV infection. Although wild-derived mouse strains such as MSM/Ms are also resistant, most laboratory mouse strains are susceptible to WNV. The gene responsible for the different susceptibility has been identified as *Oas1b* using positional cloning[31, 36]. The mouse Oas family consists of 12 members, *Oas1a* to *Oas1h*, *Oas2*, *Oas3*, *OasL1*, and *OasL2*, but only *Oas1b* is responsible for the susceptibility to WNV[8, 11, 37]. Sequence comparison of Oas1b has revealed that truncated Oas1b is translated due to a nonsense mutation in the fourth exon in most laboratory mice. Functional analysis of Oas1b has shown that it cannot bind double-stranded RNA and has no oligoadenylate synthetic activity[7, 16]. Furthermore, it has been shown that RNase L-knockout mice are resistant to the WNV infection, suggesting that the resistance by Oas1b is achieved with a different mechanism from the Oas/RNase L pathway, although the mechanism has not yet been clarified[30].

The inhibitory effect of *OAS* on flavivirus replication has been observed in other species than mice. Previous studies have shown that *OASL* in chicken, goose, and duck have replication inhibition similar to *Oas1b* in mice[2, 51, 52]. Previous studies and chapter I showed that ostriches and emus have *OASL*, which exhibits replication inhibitory effects independent of oligoadenylate synthetic activity[51]. However, the mechanisms have not been clarified yet in those OAS as well.

The UTR of flaviviruses is conserved among multiple viral species and has been reported to be involved in viral replication and virulence. The 5'-UTR and 3'-UTR ends are essential for forming circular structures during viral genomic RNA synthesis[57]. The 3'-UTR contains sequences that constitute multiple stem-loops (SLs). These SLs create a region that is resistant to RNA degradation by exoribonuclease 1 and accumulate in the

cell as RNA fragments[2, 4]. The accumulated RNA is called subgenomic flavivirus RNA (sfRNA) and is known to affect virulence; however the details have not been clarified[45].

OAS binds to double-stranded RNA, but also to single-stranded RNA that temporarily forms double-stranded structures to obtain enzymatic activity[57]. For example, it has been reported that the OAS/RNase L pathway is activated by single-stranded RNA viruses such as SARS-CoV-2 and Sindbis virus, which have sequences forming SL structures within the genomic RNA[9, 35, 49]. In a previous report, loss of the inhibitory effect of mOas1b on flavivirus replication was observed for a strain of tick-borne encephalitis virus (TBEV), lacking a part of the 3'-UTR[63]. These facts evoke a hypothesis that binding of mOas1b to SLs is the key of antiflavivirus activity of mOas1b. In this study, I investigated the role of SLs in the 3'-UTRs of WNV in the inhibition of WNV replication by mOas1b to clarify the viral factor that mOas1b act through.

2. Materials and Methods

2.1 Production of SL-deleted viral replicons

I deleted the SL2, SL3, and SL4 sequences of the WNV replicon, based on previous reports of deletion of the sequences constituting SLs in the 3'-UTR of flaviviruses[4, 45, 46]. Based on the WNV NY99 strain replicons, harboring the SEAP reporter gene instead of the viral structural genes[33], primers were designed for the sequences that are known to comprise the SL structures present in the 3'-UTR and inverted PCR reactions were performed (Fig. 4A). Table 3 lists the primers used in this study. The ends were then joined using In-Fusion® HD Cloning Kit (Takara Bio Inc.). WNV replicon RNAs were propagated using mMESSAGE mMACHINE® Kit (Thermo Fisher Scientific) as previously reported[33] These genetic recombinant experiments were approved by Hokkaido University (approval number: 2020-034).

2.2 Cloning of SL2

SL2 (10502-10564 nt in WNV NY99) was amplified by PCR from the WNV replicon plasmid by adding restriction enzyme (*Bam* HI and *Cla* I) sites using PrimeSTAR® Max DNA Polymerase (Takara Bio Inc.) (Table 3). The synthesized product was gel-purified and inserted into the pSIN-hU6 plasmid using restriction enzyme sites.

2.3 Intracellular transfection of ΔSL2, ΔSL3, and ΔSL4

The plasmids used to express functional mOas1a and mOas1b in the cells were named as pCAG-Oas1a-IRES-EGFP and pCAG-Oas1b-IRES-EGFP, respectively, which were constructed previously[8, 33]. *mOas1a* and *mOas1b* were cloned from C57BL/6J and MSM/Ms strain, respectively. MSM/Ms mice express the full-length Oas1b protein, which cause resistance for flavivirus[34]. The empty vector, pCAG-IRES-EGFP was used as a control.

BHK-21 cells were grown in DMEM (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Atlas biological) and 1% Penicillin-Streptomycin-L-Glutamine Solution (Fujifilm Wako Pure Chemical) (final concentration: 100 unit/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine). BHK-21 cells were seeded in 24 well plates at a concentration of 1.0×10^5 cells/well and transfected with 1.5 μg of pCAG-Oas1a-IRES-EGFP, pCAG-Oas1b-IRES-EGFP or pCAG-IRES-EGFP and subsequently with 0.5 μg of WNV replicon RNA by Lipofectamine 2000® (Thermo Fisher Scientific) according to the timeline shown in Fig. 4B. The efficiencies of pCAG-Oas1a-IRES-EGFP and pCAG-Oas1b-IRES-EGFP transfection were confirmed by GFP expression using a fluorescence microscope. At 72 h after transfection with WNV replicon RNA, culture supernatants were collected, centrifuged at 12,000 xg, and stored at -80 ℃ until used. The amount of reporter protein, SEAP in the culture supernatant was measured by Great EscAPe™ SEAP Chemiluminescence Kit 2.0 (Takara Bio Inc.) and an Infinite M200 PRO plate reader (TECAN Japan Co., Ltd.) according to the manufacturer's protocol.

In experiments co-expressing pSIN-SL2-hU6, it was introduced into cells according to the timeline shown in Fig. 5A at concentrations of 0.25, 0.5, 1.0, and 2.0 μg/well. The pSIN-hU6 plasmid (1.0 μg/well) was used as a control for pSIN-SL2-hU6.

2.4 Immunofluorescence

Immunofluorescence was performed on BHK-21 cells in 72 h post-transfection with WNVrep ΔSL2. The cells were fixed in 4% paraformaldehyde, treated with 0.1 % triton X-100, 1% BSA in PBS for 30 min at room temperature for permeabilization and blocking, and incubated with mouse anti-dsRNA antibody (clone rJ2, 1:100, Merck, Darmstadt, Germany) overnight at 4 °C, followed by Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor™ 546 (1:1000, Thermo Fisher Scientific) for 1h at room temperature in the dark. A fluorescence microscope BZ-X800 (Keyence, Osaka, Japan) was used to detect positive cells.

2.5 Statistical analysis

The groups were compared with Dunnett's test and data were shown as means ± standard error. *P*-values less than 0.05 were considered to be significant.

3. Results

Since the 3'-UTR of WNV is known to have a secondary structure, I attempted to confirm whether mOas1b exerted inhibitory activity against the WNV replicon lacking each SL. To construct viral replicons lacking each SL, I cloned and joined the sequences excluding the RNA sequences constituting each SL structure using inverse PCR on the previously constructed WNV replicon[33]. As a result, three types of WNV replicons were constructed by removing SL2, SL3 or SL4 (Fig. 4A). RNA was synthesized from the created WNV replicons and transfected into BHK-21 cells expressing mOas1a or mOas1b. The amounts of SEAP, a reporter protein synthesized by WNV replicon, in the culture supernatant at 72 h after transfection were measured (Fig. 4B). To confirm the influence of the WNV replicon lacking SL on replication, immunostaining by anti-dsRNA antibody was performed. The results showed that dsRNA was detected 72 h post-transfection, indicating that the viral RNA had replicated (Fig. 4C). As in previously reports[8], SEAP expression was not changed in the cells expressing mOas1a but was suppressed in the cells expressing mOas1b (Fig. 4D). ΔSL2 and ΔSL3 showed an entire decrease in SEAP expression compared to the original replicons. The same phenomenon was observed in a previous experiment in which the same WNV region was deleted[45]. However, the SEAP expression in the cells transfected with ΔSL2 reamained unchanged in the presence of Oas1b. In the cells transfected with ΔSL3 and ΔSL4, the expressions of SEAP in the supernatant were suppressed by mOas1b as in the original WNV replicons (Fig. 4D). These results suggested that mOas1b could not inhibit WNV replicon replication lacking SL2 in the 3'-UTR.

Since mOas1b did not inhibit replication of the ΔSL2-WNV replicon, the SL2 was predicted to be the site of action of mOas1b. To confirm this possibility, I generated a plasmid expressing RNA from the SL2 of the WNV and examined the effect of SL2 on the WNV replicon replication by co-transfection with plasmids expressing RNA from SL2 and those expressing the WNV replicon. First of all, the effect of SL2 on the WNV replicon replication in the absence of mOas1b was examined. The result showed that SEAP expression did not change regardless of the concentrations of pSIN-SL2-hU6 (Fig.

5B). When SL2 was expressed in mOas1b-expressing cells, SEAP levels increased with the concentration of pSIN-SL2-hU6 (Fig. 5B), suggesting that excess SL2 interrupts the inhibitory effect of mOas1b on the WNV replicon replication.

4. Discussion

In this study, I attempted to clarify Oas1b mechanism of inhibition of flavivirus replication. Focusing on stem-loops of the 3' UTR of WNV, suppression of OAS1b was observed in replicons lacking SL2 and in conditions of SL2 excess. These results showed SL2 is newly identified as the region of replication inhibitory activity by Oas1b.WNV replicons lacking each SL at the 3'-UTR revealed that the replication inhibitory effect of mOas1b was not observed only when SL2 was deleted. This suggests that mOas1b acts on the SL2 region to inhibit WNV replication. SL2 region is reported to play a role in stacking RNA degrading enzymes, thereby making them resistant to degradation. Due to this resistance to degradation, RNA on the 3' side from SL2 of genome RNA is known to accumulate in cells as sfRNA[2, 4]. sfRNA inhibits interferon induction and interferes with the RNAi response and RNA degradation. Since sfRNAs are closely associated with viral replication, the inhibition of sfRNA function by Oas1b may also cause inhibition of replication. In this study, it was not clear whether Oas1b acts through SL2 on genomic RNAs or on sfRNA that accumulate intracellularly. Although the mechanism of replication inhibition was not clarified, it is possible that mOas1b might bind to SL2 or has unknown functions in inhibiting genome replication via SL2 modifications such as cleavage, degradation, and otherwise change. To clarify the hypothesis, the role of SL2 works in the presence of Oas1b should be further investigated.

When SL2 was expressed in BHK-21 cells, SL2 concentration-dependent recovery of WNV replicon replication was observed in cells expressing mOas1b (Fig. 5B). This suggests that expression of SL2 has the effect of weakening the antiflaviviral effect of mOas1b. This could be due to the competitive inhibition of the expressed SL2 RNA by binding to mOas1b, which prevents mOas1b from inhibiting the replication of the WNV replicon. Since the binding between SL2 and Oas1b was not confirmed in this experiment, the binding should be clarified in future studies.

mOas1b is known to bind to the SL structure of sequences in the NS5 region of WNV, and its binding specificity differs from that of other Oas[6]. The inhibition of flaviviral replication by the OAS has been reported to be independent of RNase L in mOas1b as

well as in several avian OASs[2, 51]. Therefore, avian OASs may target SL2 as well as m*Oas1b*. On the other hand, although the enzymatic activity of identified human and other mammalian OASs has been reported in detail, few reports have focused on their ability to inhibit flavivirus genome replication. The fact that the SL2 of WNV is the site of action of mOas1b may be useful for clarifying the mechanism of the inhibitory effect of OASs of human and other mammals against flavivirus genome replication in future studies.

Although there are some differences in the SL2 sequences, the sequences important for the stem-loop structure are conserved in many flaviviruses, such as JEV, YFV, and TBEV[4]. Since it has been reported that mOas1b can also inhibit the replication of several flaviviruses such as TBEV and JEV[28, 63], the SL2 may be a target of mOas1b in other flaviviruses as well. However, because these experiments were conducted using WNV replicons, it is necessary to confirm the same phenomenon in actual viruses.

In this study, I revealed that Oas1b prevents viral replication by acting through SL2 of WNV replicon. I hope that the mechanism underlying the inhibition of flaviviral genome replication by mOas1b will be elucidated in the future, leading to the establishment of new therapeutics that are effective against many flaviviruses.

5. Summary

Oas is known to play a role in intracellular viral defense and conserved in many animal species. Mouse *Oas1b* is determined by positional cloning to be a gene responsible for the susceptibility to flaviviruses in laboratory mice. The inhibitory effect of Oas on flavivirus replication has been reported to be independent of its enzymatic activity but its mechanism remains unclear. The UTR is conserved among multiple flavivirus species and has been reported to be involved in viral replication and virulence. Since OAS generally binds to double-stranded RNA and also single-stranded RNAs with stem-loop structures, it is hypothesized that SLs in 3'-UTR are the target of mOas1b.

In this study, I measured the inhibitory effect on flavivirus replication by removing and representing the regions constituting the SLs in the 3'-UTR of WNV replicon. The results showed that deletion of SL2 in the 3'-UTR of the WNV replicon resulted in loss of the inhibitory effect of mOas1b on flavivirus replication. Further, an excess SL2 RNA reduced the inhibitory effect of mOas1b on the WNV replication. These results suggest that SL2 of WNV is the target of action of mOas1b in the inhibition of flavivirus

replication.

In addition to mOas1b, other OASs with inhibitory effects on flavivirus replication have been reported. SL2 may also be a site of action for these OASs. Since the sequence of SL2 is conserved in many flaviviruses, the SL2 may be a target of mOas1b in other flaviviruses as well. I hope that the mechanism of inhibition of genome replication of flaviviruses by mOas1b will be elucidated in the future, leading to the establishment of new therapeutics that are effective against many flaviviruses.

6. Tables and figures

Table 3. Primers for the construction of WNVrep lacking SLs and cloning SL2.

Name	Direction	Primer sequence
WNVrepΔSL2	Reverse	CAGTCCTCCTGGGGTTCTCCTCAAAATTTTCTTAACTA
WNVrepΔSL2	Forward	ACCCCAGGAGGACTGGGTGAAC
WNVrepΔSL3	Reverse	TCTGACATTGGGCTTGGCTTTGTTCACCCAGTC
WNVrepΔSL3	Forward	AAGCCCAATGTCAGACCACG
WNVrepΔSL4	Reverse	GGGCTTTGAAGTTACAACAT
WNVrepΔSL4	Forward	GTAACTTCAAAGCCCTGCCCCAGGAGGACTGG
SL2- *Cla*I	Reverse	AGTCCATCGATAAAAAATTGAGTCGCAGGCAGCAC
*Bam*HI-SL2	Forward	GTAACGGGATCGAAAGTCAGGCCGGGAAG

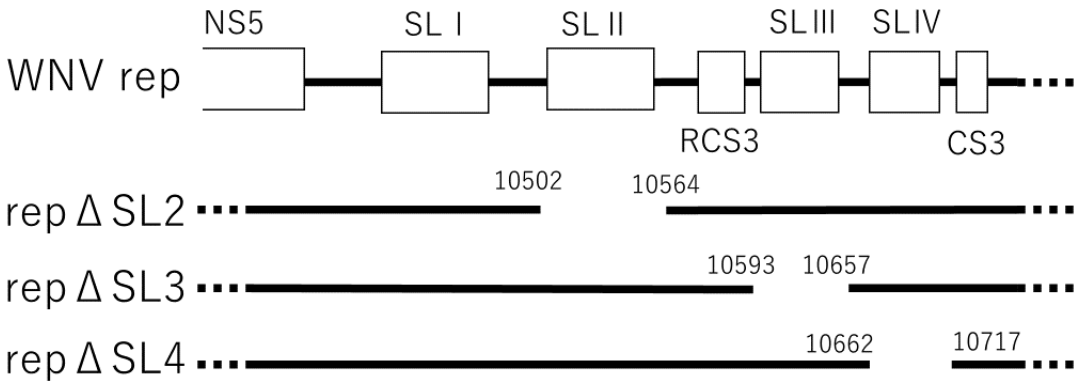

Fig. 4A. The deleted region of the 3'-UTR of the viral replicon. The numbers represent RNA base pairs in WNV NY99 genome. NS: non-structural protein, SL: stem-loop structure, RCS: repeated conserved sequence, CS: conserved sequence[29, 39)].

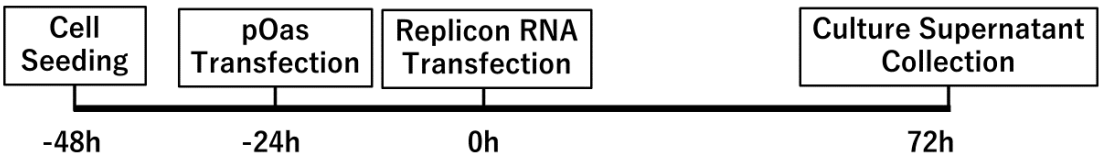

Fig. 4B. Timeline of experiments using cultured cells to measure the antiflaviviral activity of mOas1b. Cells were transfected with pCAG-mOas1a-IRES-EGFP, pCAG-mOas1b-IRES-EGFP, or the empty vector pCAG-IRES-EGFP as negative control.

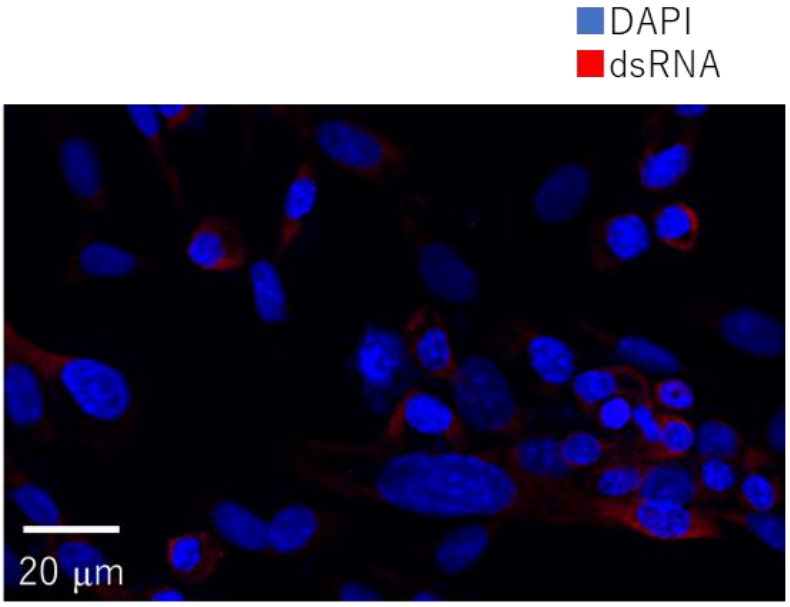

Fig. 4C. Immunofluorescence for dsRNA in BHK-21 cells transfected with WNVrep ΔSL2. dsRNA associated with viral replication was detected in the cytoplasm.

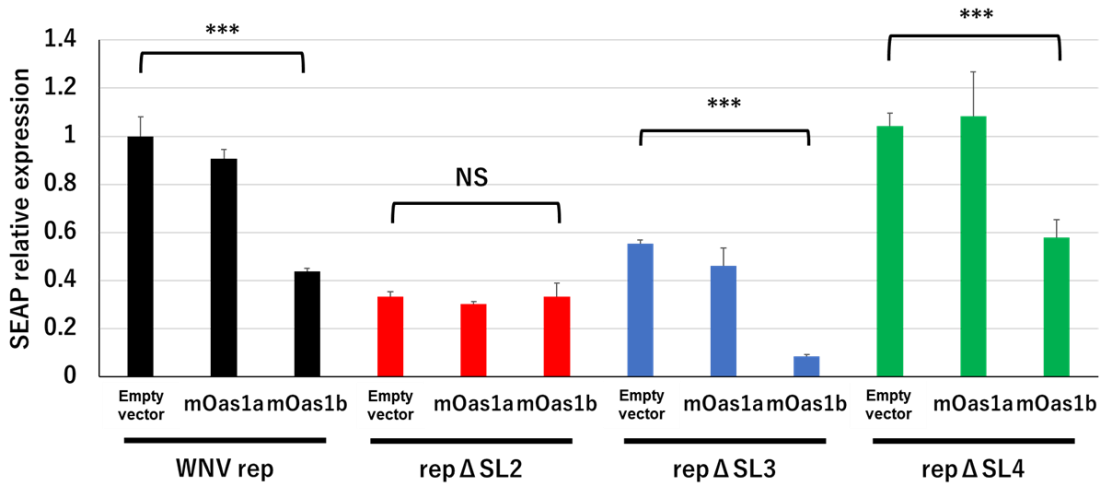

Fig. 4D. Relative amounts of SEAP, a reporter protein transcribed from WNV replicons in the supernatant are shown. Error bars indicate standard errors. *** indicates statistically significant (p<0.001) compared to empty vector.

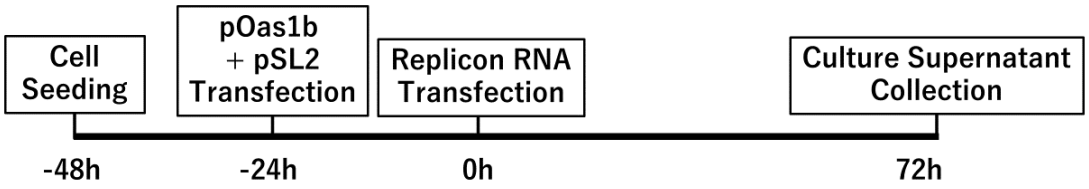

Fig. 5A. Timeline of experiments using cultured cells to measure the inhibitory effect of mOas1b in the presence of excess SL2. Cells were transfected with pCAG-mOas1b-IRES-GFP or the empty vector pCAG-IRES-EGFP as a negative control. pSIN-SL2-hU6 encoding SL2 was transfected into cells at several concentrations simultaneously with pCAG-mOas1b-IRES-EGFP.

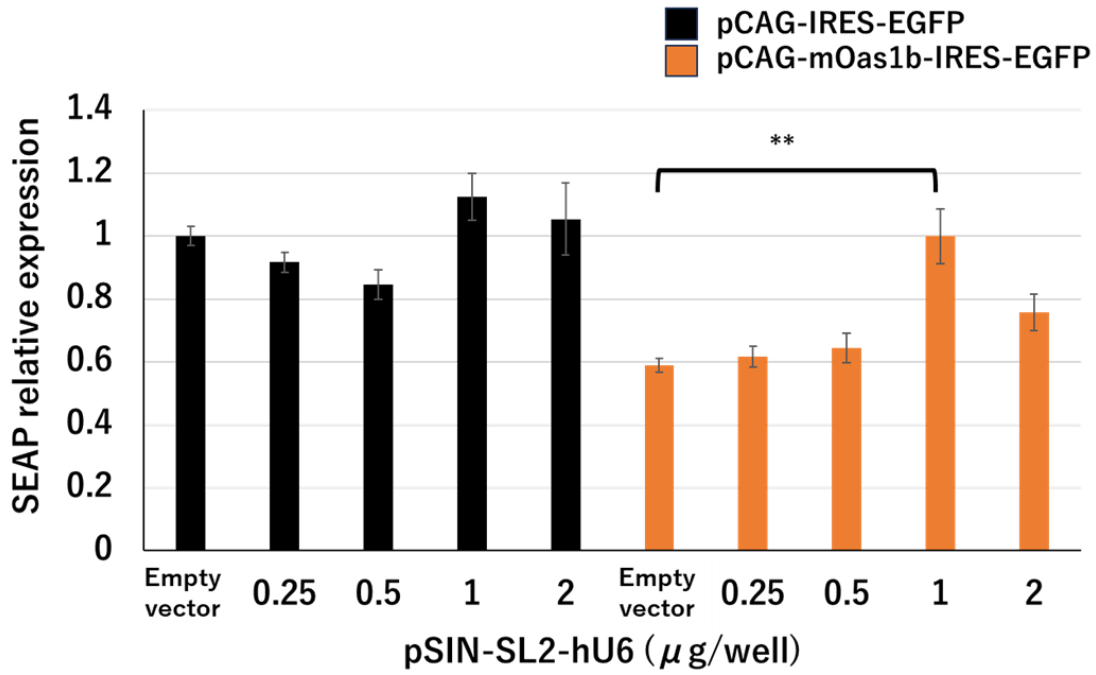

Fig. 5B. Alteration of WNV replication in the presence of SL2. Luminescence intensity by SEAP in the supernatant were measured. ** indicates $p<0.01$, compared with empty vector.

Summary and conclusions

OAS is one of the innate immune proteins induced by virus infection and is known to synthesize oligoadenylate from ATP, activate RNase L, and induce IFN. OAS acts not only as a sensor for foreign RNA but also as an enhancer to eliminate RNAs entering from outside the cell and amplify IFN induction. In addition, OAS has RNase L-independent inhibition of flavivirus replication. Importantly, the previous reports have focused on their enzymatic activity to synthesize oligoadenylates, few have been reported for their ability to inhibit flavivirus replication. In some species, the *OAS* is multiplexed, and enzymatic activity and antiflaviviral function have been segregated for each OAS molecule. In this thesis, I analyzed avian OAS diversity and WNV recognition sites of mouse Oas1b to resolve issues regarding the evolutionary phylogenetic and molecular biological aspects of the virus elimination function of the OAS.

OAS is a gene acquired early in the evolution of organisms, since various animal species possess similar genes. Many avian species have only one *OAS* gene, with both enzymatic activity and flavivirus replication inhibition. However, only ostriches have two *OAS* genes, with OAS1 having only enzyme activity and OASL having only inhibition of flavivirus replication, indicating a division of functions. Such multiplexing and functional segregation of the OAS may provide very useful insights into the evolution of the species and its adaptation to the surrounding environment. In chapter I, I focused on emu, which is closely related to the ostrich, and attempted sequence determination and functional analysis of the emu *OAS* family. The sequencing results showed that emu has two genes of OAS family members, *OAS1* and *OASL*. Their amino acid sequence homology was 80% for OAS1 and 78% for OASL compared to those of ostriches. The regions that are known to be affected in function were almost identical. Functional analysis revealed that the emu OAS family has similar features to those of ostriches, with oligoadenylate synthetic activity and an antiviral function of flavivirus replication inhibition. These findings suggest that emus and ostriches have evolved differently from most birds that have only one *OAS* gene.

The laboratory mouse strain is susceptible to flavivirus infection, whereas the wild-derived mouse strain is resistant, suggesting that there is a gene that determines susceptibility to flavivirus infection, and *Oas1b* has been identified as the responsible gene. Oas1b is proposed to inhibit the replication of the flavivirus by a mechanism different from the OAS/RNase L pathway, but the antiflavivirus mechanism has not been clarified. In chapter II, I attempted to identify the site of action of mOas1b in inhibiting flavivirus replication. In the 3'-UTR of the genomic RNA of flavivirus, there are sequences that are conserved in flavivirus. The stem-loop structure in this sequence makes

RNA degradation resistant by stalling RNA degrading enzymes during RNA degradation. Based on the hypothesis that mOas1b inhibits flavivirus replication by interacting with stem-loops in the untranslated region, I have investigated the effect of removing the RNA sequence constituting the stem-loop in the 3' untranslated region of the WNV replicon. Transfection of WNV replicon RNA with the deleted sequence constituting SL2 in the 3' untranslated region caused no replication inhibition, even in the presence of mOas1b. This suggests that mOas1b acts on SL2 to inhibit WNV replication. When RNA constituting SL2 was expressed in the cells, the inhibition of flavivirus replication was observed to decrease in a concentration-dependent manner. This indicates that RNAs of the sequence constituting SL2 can competitively inhibit the inhibitory effect of mOas1b on flavivirus replication. The mechanism by which mOas1b acts on SL2 could be that mOas1b inhibits genome replication through an unknown function that causes SL2 to cleave, degrade, modify, or otherwise change. SL2 suggested as the site of action of mOas1b in this study may also be a target of mOas1b in other flaviviruses. I hope to clarify the mechanism of flavivirus suppression by mOas1b in the future, which will lead to the establishment of new effective treatments for many flaviviruses.

In conclusion, this study has provided new insights into the viral defenses of OAS. These results will improve our understanding of viral defenses in the host and contribute to the control of viral infections.

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Summary in Japanese

オリゴアデニレート合成酵素(OAS)はRNAウイルスの感染により誘導される自然免疫系を担うタンパク質の一つであり、ATPからオリゴアデニレートを合成し、RNase Lを活性化し、IFNの誘導を行うことが知られている。OASは外来RNAを認識するセンサーであるだけではなく、IFNの誘導を増幅させることにより外来RNAの除去におけるエンハンサーとしての役割を持つ。このオリゴアデニレート合成によるウイルス除去のほかに、OASはRNase Lに依存しないフラビウイルス複製阻害機能を持つことが報告されている。しかし、今までの報告ではオリゴアデニレート合成活性についてはよく調べられているものの、フラビウイルス複製阻害についてほとんど調べられていない。いくつかの動物種において、OAS遺伝子は多重化しており OAS 分子ごとに合成酵素活性とフラビウイルス複製阻害機能の分離が生じている。そこで、本研究ではOASのウイルス排除における分子生物学的な側面と系統学的な分化について明らかにするため、鳥類におけるOASファミリーの多様性とマウスOas1bの作用におけるウエストナイルウイルスの認識部位について解析を行った。

OASは様々な動物種がその類似する遺伝子を保有していることが報告されており、生物の進化の序盤で獲得された遺伝子であるといえる。しかし、OASファミリーを構成する遺伝子数は多様であり、マウスやラットは12種のOASを持つが、ヒトやブタは5種のみである。今まで報告された遺伝子ではオリゴアデニレートを合成する酵素活性については詳細に調べられている一方、フラビウイルス複製阻害作用についてはほとんど報告されていない。鳥類の多くは1種類しかOASを持たないことが報告されており、酵素活性とフラビウイルス複製阻害作用の両方の機能を持つことが報告されている。しかし、ダチョウのみが２種類のOASを有しており、OAS1が酵素活性のみを、OASLがフラビウイルス複製阻害作用のみを持ち合わせており、機能の分業化が行われている。マウスにおいても同様の機能の分業化が行われており、Oas1a、Oas1gは酵素活性のみを持ち、Oas1bがフラビウイルス複製阻害作用を持っている。このようなOASの多重化及び機能の分離はその種の進化やとりまく環境への適応を考える点で非常に有益な知見をもたらしうる。

そこで第１章ではダチョウと近縁であるエミューに注目し、エミューのOASファミリーの配列決定及び機能解析を試みた。配列決定の結果、エミューはOAS1とOASLの２種類を持つことが示された。また、そのアミノ酸配列の相同性はダチョウと比べ、OAS1は80%、OASLは78%であった。また、機能に影響があるとされている領域に関してはほぼ一致していた。機能解析を行った結果エミューのOASファミリーはオリゴアデニレート合成酵素活性とフラビウイルス複製阻害の抗ウイルス機構を持ち、ダチョウと同様の特徴を持っていることが分かった。これらのことからエミューとダチョウは1種類のOAS分子のみを有する大部分の鳥類とは異なる進化を遂げた存在であることが推定された。

実験室マウス系統がフラビウイルス感染に感受性があり、野生由来マウス系統は抵抗性であることから、フラビウイルス感染への感受性を定める遺伝子が存在することが示唆されており、その原因遺伝子としてOas1bが同定された。Oas1bにより感受性が変化する病

原体としてウエストナイルウイルス（WNV)、日本脳炎ウイルス（JEV)、黄熱ウイルス(YFV)などがあり、節足動物により媒介され、公衆衛生上問題となるウイルス群である。Oas1b の機能について調べられた結果、Oas1b はオリゴアデニレート酵素活性がなく、OAS/RNase L 経路とは異なる機構によりフラビウイルスゲノムの複製を阻害することが考えられているが、その機序は明らかになっていない。

第 2 章ではマウス Oas1b のフラビウイルス複製阻害作用の作用部位の解明を試みた。フラビウイルスのゲノム RNA の 3'側非翻訳領域にはフラビウイルス間で保存されている配列が存在している。この配列内にはステムループ構造が存在しており、RNA 分解酵素を RNA 分解中に停滞させることにより RNA 分解に抵抗性を持っている。これまでの報告から Oas1b が非翻訳領域に存在するステムループと相互作用することによりフラビウイルス複製阻害効果を発揮すると仮定し、WNV レプリコンの 3'側非翻訳領域に存在するステムループを構成する RNA 配列を除去することによる Oas1b のフラビウイルス複製阻害効果の影響を確認した。WNV レプリコンの 3'非翻訳領域に存在する SL2 を構成する配列を削除した WNV レプリコンの RNA を細胞にトランスフェクションした場合、Oas1b の存在下においても複製阻害は生じていなかった。このことから Oas1b が SL2 に作用することによりフラビウイルスの複製が阻害されることが考えられた。また、細胞内で SL2 を構成する RNA を過剰に発現させた場合、SL2 の濃度に依存して Oas1b によるフラビウイルス複製阻害の減弱が確認された。このことから、SL2 を構成する配列の RNA は Oas1b のフラビウイルス複製阻害作用を競合阻害することが可能であることを示している。Oas1b が SL2 に対して作用するメカニズムとして、SL2 に結合して物理的にゲノム複製を阻害する、あるいは SL2 に対し、切断、分解、修飾などの変化を起こさせる未知の機能によりゲノムの複製を阻害していることが考えられる。また、今回 Oas1b の作用部位として示唆された SL2 は他のフラビウイルスにおいても Oas1b のターゲットとなっていることが考えられる。将来 Oas1b によるフラビウイルス抑制機構が明らかになることで、多くのフラビウイルスに効果的な新たな治療法の確立ができることを願っている。

結論として、今回の研究によりオリゴアデニレート合成酵素のウイルス防御に対する新たな知見を得ることに成功した。これらの結果は、生体のウイルス防御についての理解を向上させ、ウイルス感染症に対する制御に貢献するであろう。