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The chicken 2'-5' oligoadenylate synthetase A inhibits the replication of West Nile Virus.

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Abstract

West Nile virus (WNV) is a pathogen to cause West Nile encephalitis when the infection occurs in the brain. Previous studies in mice identified the 2'-5' oligoadenylate synthetase 1b (*Oas1b*) gene as a determining factor for resistance to WNV infection. In addition, it has been suggested that human OAS1 and OASL are associated with the resistance to the WNV infection. WNV is maintained in nature through a complex life cycle involving wild birds and mosquitoes. Birds are not only susceptible to the WNV, but also act as reservoir hosts, thus participating in the spread of the disease. It has previously been reported that chicken OASL possesses the oligoadenylate synthetase activity. However, until now the antiviral activity of chicken OASL has not been determined. In this study, we investigated the putative antiviral activity of chicken OASL by ectopic expression of this enzyme in mammalian cells and then infecting these cells with WNV replicon. We demonstrate that chicken OASL has an antiviral activity against the WNV. This is the first report to show that chicken OASL is associated with the resistance to the WNV infection.

Key Words: West Nile virus, oligoadenylate synthetase, chicken OASL, mouse *Oas1b*, virus replicon

Introduction

Viral infection of mammalian cells prompts the innate immune system to initiate an antiviral

response. The recognition of the virus triggers several antiviral signaling pathways, including the family of 2'-5' oligoadenylate synthetase (OAS) proteins²⁶⁾. OAS is a double-stranded (ds)

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RNA-dependent nucleotidyl-transferase induced by interferon (IFN). The cloning of *OAS* cDNAs from human, mice, horse, rat, cattle, pig, dog, and chicken elucidated that the four types of genes, *OAS1*, *OAS2*, *OAS3*, and *OASL* (OAS-like) are conserved among them with some differences resulting from the evolutionary changes such as duplication, loss, or transformation to the pseudo-genes^{10,23}. In mice, the *Oas* gene family, mapped on chromosome 5, consists of eight small *Oas1* (*Oas1a* through *Oas1h*), *Oas2*, *Oas3*, and two *OasL* (*OasL1* and *OasL2*) genes^{9,16}. In contrast, the human *OAS* gene family, mapped on chromosome 12, consists of four genes, *OAS1*, *OAS2*, *OAS3*, and *OASL*, all of which are induced by IFN^{16,19,25}. The *OASL* proteins consist of the N-terminal part showing highly conserved amino acids with the other *OAS* proteins, fused to the C-terminal part containing two ubiquitin-like domains.

West Nile virus (WNV) is a single-stranded positive sense RNA virus, a member of the Japanese encephalitis serocomplex group in the arthropod-borne *Flaviviridae*, maintained in an endemic cycle between mosquitoes and birds. WNV can infect horses and humans, which serve as incidental dead-end hosts. WNV shows endemic infection in several regions in the world. After the first isolation of WNV in Uganda 1937, WNV became to be considered as a significant pathogen, because several outbreaks have occurred after it. Furthermore, after the outbreak in Romania 1996, the WNV infection became to be considered as a serious public health threat^{14,17}.

The *OAS*/RNase L pathway is activated after the *OAS* binds dsRNA. The active enzyme converts ATP to diphosphate and 2'-5'-linked oligoadenylates (2-5 (A)) ranging from dimers to 30mers. The only known function of 2-5 (A) is to bind to the latent ribonuclease RNase L and activate it by dimerization. The activated RNase L inhibits the protein synthesis through the degradation of both viral and cellular RNA including ribosomal RNA, which effectively prevents viral replication. Previous studies elucidated that the *OAS*/RNase

L pathway plays a role in many biological functions such as in cell growth and differentiation, human immunodeficiency virus replication, heat shock, atherosclerotic plaque, pathogenesis of type I diabetes, and apoptosis²⁴. The importance of this pathway was confirmed by generating RNase L-deficient mice (RNase L^{-/-}), which allows the replication of picornaviruses as these mice displayed an increased sensitivity toward the encephalomyocarditis virus (EMCV) infection³⁴. In the same way, a recent study investigated the role of the human *OAS* family against dengue virus (Flavivirus) and found that both *OAS1* and *OAS3* possess antiviral activity against dengue virus in an RNase L-dependent manner¹³.

Recent several lines of evidence suggest that there are several RNase L-independent pathways, by which some members of the *OAS* family can prevent viral replication. The human *OASL* (HuOASL) and murine *Oas1b* (mOas1b) are devoid of enzymatic activity and thus incapable of activating RNase L. Nevertheless, both proteins display antiviral activity if expressed in mammalian cells¹¹. The HuOASL protein, which has a C-terminal domain showing homology to ubiquitin protein, is a unique member of the *OAS* family that lacks the enzymatic activity. However, it possesses antiviral activity against EMCV in a manner requiring the ubiquitin-like domain, but independent of RNase L¹⁵. The comparison studies between mOas1b and HuOASL demonstrate clearly that both of them link to the different RNase L-independent pathways¹¹.

The *OAS* activity was examined and confirmed in the poultry such as chicken, goose, pigeon, and quail^{6,28}, with the highest activity in chicken²⁸. Up to date, the only well characterized avian *OAS* gene was chicken *OAS*-like gene, *ChOASL*. Southern blotting hybridization indicated that the chicken *OAS* are encoded by a single gene, *ChOASL*, which has two alternative spliced forms, *ChOAS-A* and *ChOAS-B*, encoding predicted proteins of 508 and 476 amino acids, respectively. The Expressed Sequence Tag profile of *ChOASL* showed that it was highly expressed in blood,

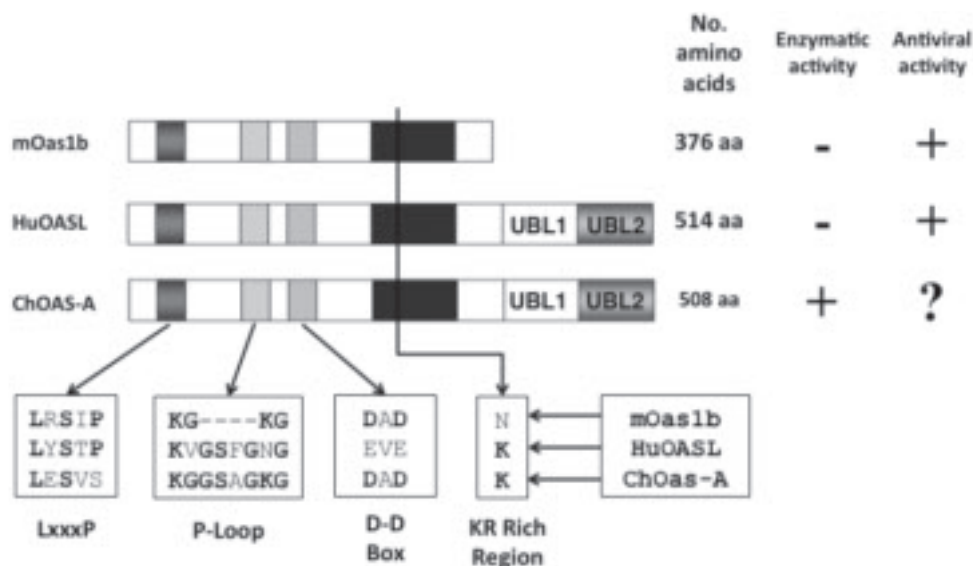


Fig. 1. Schematic diagram of ChOAS-A (accession number, BAB19016), HuOASL (NP_003724), and mOas1b (NP_001077394.1) proteins. The mOas1b protein consists of the OAS domain only, which shows high similarity with the N-terminal OAS domains from both ChOAS-A and HuOASL, both of which are fused to ubiquitin-like protein at the C-terminal. The sequence information under the figure demonstrates the important domains conserved in these proteins, LxxxP, P-Loop, D-box, KR rich region, and UBL domains (UbL1 and UbL2).

liver, muscle, spleen, and brain (<http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Gga.536>). Although the chicken is shown to be resistant to the WNV infection compared to other avian species⁵, the reason for it is not clearly elucidated. In particular, it is not yet demonstrated that the ChOASL possesses the antiviral activity against WNV. The ChOASL protein composed of two units, the OAS and ubiquitin-like (UbL) domains. ChOAS-A possesses the two UbLs, UbL1 and UbL2, whereas ChOAS-B does UbL2 only. Previously it has been reported that UbL is essential for the antiviral activity in HuOASL¹⁵, and for the enzyme stability in ChOAS-B³². Thus, based on the schematic diagram of the mOas1b, HuOASL, and ChOAS-A with comparison of their functions (Fig. 1), we raise the question of whether the ChOAS-A has the antiviral activity against WNV. Therefore, in this study, we investigate the putative antiviral activity of ChOAS-A by ectopically expressing this protein in mammalian cells and using a WNV replicon.

Materials and Methods

Generation of OAS transfectants: The White Leghorn chicken oligoadenylate synthetase A (*ChOAS-A*, accession number: AB037593) cDNA was kindly provided from Dr. K. Hamada (Kyoto Institute of Technology). The *ChOAS-A* was amplified by PCR to include the native translation initiation sequence of *ChOAS-A* with primer pair F/R: ATCGATATGGGGTTGGAGAGCGTGAG and TCAGATCTTATCGTCGTCATCCTTGTAATCGGAGGGCACGCAGCGTCTGG. The sequence coding for the FLAG (FL) peptide YKDDDDK was added to the 3'-terminal and the resultant cDNAs were sub-cloned into the pIRES-EGFP vector (Clontech Bio. Inc., Shiga, Japan) to generate pChOasA-FL-EGFP. For positive control, the *mOas1b* cDNA described in the previous paper¹⁸ was ligated to the same vector (pmOas1b-FL-EGFP). HEK293FT or BHK-21 cells were grown in Dulbecco's modified minimum essential medium (Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/Glutamine (Gibco/

Invitrogen, Carlsbad, CA, USA) at 37°C and 5% CO₂, and passaged every two days. One day before transfection, cells were harvested, counted, and cultured in 100 mm dish with the cell density of 1.5×10^6 cells/dish. After 24 h, the cells were transfected with 10 µg of pIRES-EGFP (empty vector), pmOas1b-FL-EGFP, or pChOasA-FL-EGFP using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After 24 h, the transfection efficiency was monitored by observation of EGFP fluorescence. RT-PCR was performed using ReverTra Ace (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. Western blot analysis was performed using ECL advance (GE Healthcare UK Limited, Little Chalfont, UK) according to the manufacturer's instructions using a FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO, USA).

Assay of the OAS enzymatic activity: The measurement of enzymatic activity was performed as previously described²⁷. In brief, each plasmid was transfected into 1×10^6 HEK293FT cells. After 48 h, each cell fraction was homogenized in 500 µl of ice-cold 300 mM NaCl, 20 mM Tris-HCl (pH 7.5), 10% glycerol, 0.2% Triton X-100, 5 mM β-mercaptoethanol, and 1x protease inhibitors. An aliquot of the 2.5 µl of whole cell lysates was incubated in the reaction buffer [20 mM Tris-HCL, pH 7.4, 20 mM magnesium acetate, 2.5 mM dithiothreitol, 5 mM ATP, 50 µg/ml poly(I): poly(C), and 5 µCi of [α -³²P] ATP (6000 Ci/mmol)] in a final volume of 10 µl for 18 h at 30°C. The synthesized 2–5 (A) products were separated using 20% polyacrylamide-urea (7M) gel, and visualized by the autoradiography with a BAS-2500 Bio-Imaging analyzer (FUJIFILM, Tokyo, Japan).

Evaluation of the antiviral activity: To evaluate antiviral activity of the OAS protein, we have selected a BHK-21 cell line, because BHK cells are susceptible to many viruses including WNV and because BHK cells show a high efficiency for the transfection of exogenous genes. In fact, the WNV replicon is well replicated in this cell line. In the previous paper, we have developed an assay system with this cell line to evaluate the

antiviral activity of the murine Oas1b¹⁸. BHK-21 cells were transfected with 10 µg of pIRES-EGFP (empty vector), pmOas1b-FL-EGFP, or pChOasA-FL-EGFP using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 24 h, cells were transfected with 10 µg of WNV replicon RNA conjugated with secreted alkaline phosphatase RNA (repWNV/SEAP) by electroporation using a Gene Pulser Xcell (BioRad) with two pulses of 1.5 kV at 25 µF and infinite resistance. Next, the SEAP activity in the culture medium was detected using a Great EscAPE SEAP Chemiluminescence Kit 2.0 (Clontech Bio. Inc., Shiga, Japan). The repWNV/SEAP is translated as a polyprotein containing SEAP and cleaved into SEAP and viral NS proteins by viral and cellular proteases, resulting in the secretion of SEAP into the culture medium. Statistical comparisons were performed using one-way analysis of variance *F*-test. These values were represented as mean \pm SD and differences with $P < 0.05$ were considered to be significant.

Results

Comparison of the structure and function among mOas1b, HuOASL, and ChOASL: As shown in Fig. 1, ChOAS-A possesses the OAS enzymatic activity, whereas both mOas1b and HuOASL do not. This discrepancy may be due to the difference in the amino acid sequence of the N-terminal part, especially in the domains, LxxxP, P-Loop, D-D Box, and KR-rich region, all of which have been shown to be important for the OAS enzymatic activity. With respect to the antiviral activity, both mOas1b and HuOASL are reported to be positive, whereas it is unknown for the ChOAS-A.

Analysis of the enzymatic activity: Although enzymatic activity of the ChOAS-A was evaluated in the previous study³³, the activity was determined in *E. coli*. Therefore, we transfected ChOAS-A into mammalian cells, HEK293FT, and compared its enzymatic activity with that of mOas1b in the same host cells. After 48 h from the transfection, protein and total RNA were extracted from the

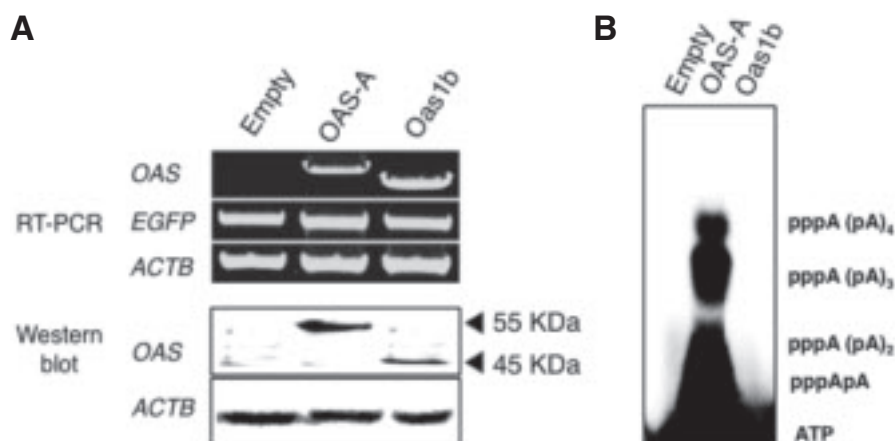


Fig. 2. The enzymatic activity of the ChOAS-A and mOas1b proteins. A) RT-PCR and Western blot from HEK293FT cells 48 h after the transfection with pIRES-EGFP (control), pmOas1b-FL-EGFP, and pChOAS-A-FL-EGFP. B) The enzymatic activity after incubating the cell lysates in the reaction buffer containing radiolabelled [α - 32 P] ATP in addition to poly I:C. The mOas1b showed no enzymatic activity, whereas ChOAS-A showed ability to convert ATP into 2-5 (A) up to tetramer.

cells. The expression of the target genes were confirmed by RT-PCR and Western blot analysis. Both *ChOAS-A-FL* and *mOas1b-FL* genes as well as the *EGFP* gene were sufficiently expressed 48 h after transfection (Fig. 2A).

The OAS enzymatic activity of the ChOAS-A was compared with that of mOas1b which is shown to be incapable of enzymatic activity. After 18 h of incubation at 30°C, both lysates from the cells transfected with the empty plasmid and the mOas1b showed no enzymatic activity, whereas that of the ChOAS-A could successfully convert the ATP into mono-, di-, tri-, and tetramer 2-5 (A) (Fig. 2B).

Analysis of the antiviral activity: Total RNA was extracted from BHK-21 cells transfected with empty vector pIRES-EGFP (Control), pmOas1b-FL-EGFP, and pChOAS-A-FL-EGFP before and after the electroporation with repWNV/SEAP, and RT-PCR was performed with respect to the *OAS*, *EGFP*, and *Actb* genes. The results show that all genes were expressed with the similar extent between before and after the repWNV/SEAP electroporation (Fig. 3A).

The SEAP activity in the culture medium from each transfectant was determined 24, 48, and 72 hrs after the repWNV/SEAP electroporation. The SEAP activity increased with the time after

the repWNV/SEAP electroporation in the transfectants with the empty vector, whereas the SEAP activity was significantly inhibited in the transfectants with *ChOAS-A-FL* and *mOas1b-FL* genes (Fig. 3B), indicating that the ChOAS-A could inhibit the replication of the WNV genome as well as the mOas1b.

Discussion

One of the most important and responsible domains for the enzymatic activity of OAS proteins is the P-Loop domain, which contributes to the binding of ATP⁴ and shows high conservation among the OAS proteins. The mOas1b protein has a deletion of 4 amino acids in the P-Loop, which may result in the loss of enzymatic activity. However, recent studies have demonstrated that the mOas1b protein possesses antiviral activity against WNV through unknown pathway independent of the OAS/RNaseL pathway^{9,11,18,25}. In the HuOASL, amino acid substitution of G67V occurs in the P-Loop domain, which may result in the loss of enzymatic activity^{4,15}. The amino acid changes are also observed in the D-D box domain, which involves in the binding of Mg²⁺ ions⁴. In the HuOASL, aspartic acids are substituted

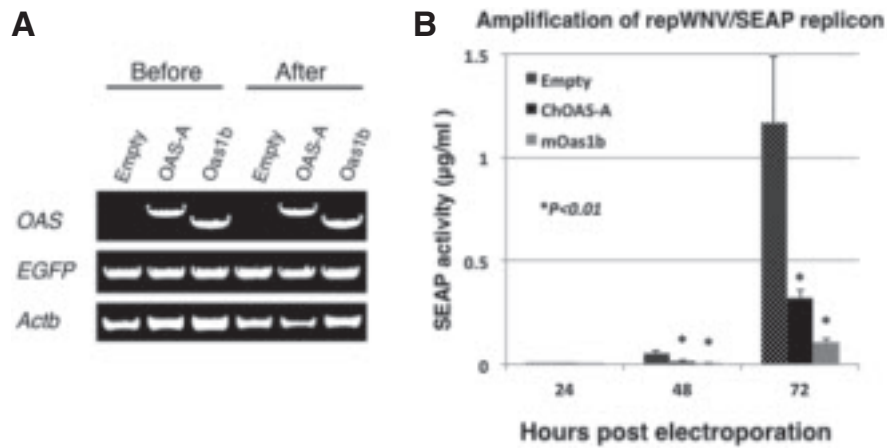


Fig. 3. Antiviral activity of the ChOAS-A and mOas1b proteins. BHK-21 cells were transfected with pIRES-EGFP (control), pmOas1b-FL-EGFP, and pChOAS-A-FL-EGFP. After 24 h, cells were transfected with repWNV/SEAP by the electroporation. A) The results of RT-PCR using total RNA extracted from BHK-21 cells before and after the electroporation of repWNV/SEAP. There were no significant differences in expression level of transgenes before and after the electroporation of replicon. B) SEAP activity analysis at 24, 48, and 72 h after repWNV/SEAP electroporation.

with glutamic acids, D81E and D83E, and alanine is substituted with valine, A82V. All alternations in the HuOASL are thought to result in the loss of enzymatic activity. Recently, several lines of evidence have indicated that HuOASL exhibits protection against the viral infection, despite of the loss of enzymatic activity^{7,15}. This suggests that there is another pathway independent of the OAS/RNase L pathway¹⁵. The current study indicated that the enzymatic activity of ChOAS-A was positive, whereas both control and mOas1b-transfected cells failed to synthesize 2-5 (A) from ATP, consistent with the previous reports^{3,31,32,33} (Fig. 2B).

Birds are the primary vertebrate reservoir hosts for WNV. The level and duration of viremia varies with the species. Migratory birds may carry WNV into new areas. Evidence for horizontal transmission was reported during an outbreak in domesticated geese². Many experiments were conducted to determine the immune response, transmission, and clinical symptoms in the poultry such as chickens and turkeys^{5,12,20,30}. Those reports show that the chicken and turkey become viremic; however, the susceptibility decreases rapidly with age, and they develop a high-titered antibody response, which allows them to be used as sentinel animals^{5,12,20}. On the other hand, the

goose showed high susceptibility to the WNV infection. Half of the 2-week-old goslings infected with WNV experimentally were died by the 7th day post infection and the mortality rate reached 75% by the 14th day post infection. Further, the mortality rate of the 8- to 10-week-old goose infected with WNV was reported to be 40%²⁹. Furthermore, it has been suggested that the direct WNV transmission occurs in the multi-aged infected goose flocks. To uncover the importance of the goose as a reservoir bird in the WNV infection cycle, more investigation is necessary^{1,2}. American crows are the most susceptible bird to the WNV infection with 50-100% mortality rate. Until now, the reasons for the differences in the susceptibility/resistance to the WNV infection in the avian species are still unknown. Furthermore, the mechanism of the WNV infection and the antiviral response in birds are still unclear²¹. Most laboratory inbred mouse strains are highly susceptible to the WNV infection, whereas the feral mice are resistant. Many experiments were conducted to identify a gene responsible for the resistance/susceptibility to the WNV infection. Mice carrying the resistant allele (*Flv^r*) are resistant to the flavivirus infection. The *Flv^r* gene was identified as 2'-5' oligoadenylate synthetase 1b (*Oas1b*)^{16,22}. Resistant mice express a full-

length Oas1b protein, while susceptible mice express a truncated Oas1b protein due to a premature stop codon in the genome. The majority of inbred mouse strains used in laboratories are homozygous for the susceptible allele (*Flv^s*).

The HuOASL, lacking the enzymatic activity, has been shown to possess antiviral activity against single-stranded RNA virus such as picornavirus and encephalomyocarditis viruses, notably, this antiviral activity was lost in the truncated mutated HuOASL lacking the C-terminal ubiquitin-like domain¹⁵. The HuOASL also possesses the antiviral activity against hepatitis C virus⁸. Antiviral activity is closely linked to the structure of the HuOASL. Namely, the HuOASL consists of the N-terminal part, which is conserved as the OAS enzymatic domain, and C-terminal part, which is conserved as the ubiquitin-like domain, and both parts are shown to be necessary for antiviral activity. Therefore, the current study aims to investigate the putative antiviral activity of ChOAS-A in comparison with the mOas1b by ectopically expressing these proteins in mammalian cells. We have shown that ChOAS-A shows antiviral activity as well as mOas1b. This result prompts us the question of the relationship between enzymatic and antiviral activities. In fact, the mOAS1b loses the enzymatic activity due to the amino acid substitution in the domains of OAS-conserved N-terminal part and loss of ubiquitin-like domain in the C-terminal part. Nevertheless, mOas1b possesses the antiviral activity. This fact raises the question of whether enzymatic activity is necessary for the antiviral activity of the ChOAS-A. Further investigations, in particular, the elucidation of the relationship between the structure and enzymatic or antiviral activity in the ChOAS-A, are necessary to shed light on this question.

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