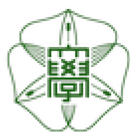
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Analysis of the Relationship Between Enzymatic and Antiviral Activities of the Chicken Oligoadenylate Synthetase-Like

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1 **ABSTRACT**

2

3 The oligoadenylate synthetase (OAS) is well known as an antiviral factor against the flavivirus
4 infection in mammals. It is known that the oligoadenylate synthetase-like (ChOAS-L) protein is
5 only present in the chicken genome. It has been shown in the previous report that the ChOAS-L
6 possesses enzymatic activity to convert ATP into 2'-5'-linked oligoadenylates and antiviral activity
7 against West Nile virus (WNV) replicon. Therefore, this study aimed to investigate the relationship
8 between enzymatic and antiviral activities of ChOAS-L. Eight mutated ChOAS-L proteins were
9 generated using either the site-directed mutagenesis or standard PCR protocol. The wild-typed and
10 mutated proteins were ectopically expressed in HEK293FT to analyze the enzymatic activity, and in
11 BHK-21 and BALB/3T3 cells to analyze the antiviral activity using WNV replicon. The results
12 revealed that all mutated proteins showed no enzymatic activity except for ChOAS-L-AΔUbL2.
13 However, all mutated proteins showed antiviral activity to inhibit the replication of the WNV
14 replicon except for the ChOAS-L-AΔUbL1/UbL2, which showed a partial inhibition compared to
15 the wild-type ChOAS-L-A or other mutated proteins. These results suggest that the ChOAS-L
16 expresses the antiviral activity in a manner independent of enzymatic activity. Our results propose
17 reconsideration of the mechanism of antiviral activity against the flavivirus replication of the
18 ChOAS-L.

19

1 **Introduction**
2

3 Type I interferon secreted from the virus-infected cells triggers and initiates a cascade of
4 expression of the interferon-stimulated genes, which interact with the infectious pathogens through
5 direct or indirect pathways. The well-recognized proteins as an interferon-stimulated factor are the
6 Mx, 2'-5'oligoadenylate synthetase (OAS), and protein kinase RNA-dependent (PKR) (Der and
7 others 1998; Sadler and Williams 2008).
8 The *OAS* gene families have been studied in human, mouse, rat, cattle, pig, dog, horse, and
9 many other living organisms. The four major types of *OAS* genes (*OAS1*, *OAS2*, *OAS3*, and *OASL*)
10 are conserved, whereas some of the other *OAS* genes have been lost or transformed to pseudo-genes
11 during mammalian evolution (Justesen and others 2000; Perelygin and others 2006; Kjaer and
12 others 2009).
13 Activation of the classical OAS/RNase L pathway results in RNA degradation of both viral
14 and cellular RNA including ribosomal RNA within infected cells. Subsequently it inhibits protein
15 translation in these cells, resulting in effective prevention of viral genome replication. The activity
16 of the OAS/RNase L pathway can be monitored by the degradation of ribosomal RNA in
17 virus-infected cells (Silverman 2007).
18 Recently several lines of evidence suggest that there is alternative RNase L-independent
19 pathway, by which some members of the OAS family can prevent viral replication. Human
20 OAS-L/a (HuOAS-L/a) (Rebouillat and others 1998; Hartmann and others 1998) and murine Oas1b
21 (mOas1b) (Elbahesh and others 2010) are devoid of the enzymatic activity and thus incapable of
22 activating RNase L. Nevertheless, both proteins display antiviral activity against West Nile virus
23 (WNV), suggesting that HuOAS-L/a and mOas1b utilize RNase L-independent pathway (Zhu and
24 others 2014; Marques and others 2008; Kristiansen and others 2011; Moritoh and others 2009;
25 Scherbik and others 2007; Kajaste-Rudnitski and others 2006; Perelygin and others 2002).
26 The OAS proteins belong to the nucleotidyle transferase superfamily involved in diverse

1 biological functions. The OAS proteins have the motif of the LxxxP, which seems to be important
2 for the synthetize activity, followed by the P-loop, which is involved in the ATP binding activity, the
3 D-D box, which plays an important role in the Mg^{2+} binding, region of 32-36 residues with high
4 content of lysine and arginine named KR rich region (KR-RR), which is important for
5 oligoadenylate binding and involved in the dsRNA binding (Yamamoto and others 2000), and the
6 CFK domain, which is important for the oligomerization of the small OAS proteins (Ghosh and
7 others 1997b). The OAS-L proteins have OAS unit in the N-terminal, which is fused to
8 ubiquitin-like (UbL) domain containing two repetitive domains, UbL1 and UbL2, in the C-terminal
9 (Eskildsen and others 2003).
10 Up-to-date, the only well characterized avian OAS gene is chicken OAS-like (ChOAS-L)
11 protein, which consists of a single copy gene, locates on chromosome 12, and encodes two
12 alternatively spliced alleles, ChOAS-L-A and ChOAS-L-B. ChOAS-L-B shows 100% identity with
13 its allele ChOAS-L-A except for the partial deletion of 32 amino acids (AA) from the UbL1 (33 AA
14 from 385Ala to 417Cys were substituted with 385Tyr). Interestingly, the ChOAS-L-B possesses the
15 enzymatic activity as well as the ChOAS-L-A (Yamamoto and others 1998; Tatsumi and others
16 2000, 2003).
17 Recently, we reported that the ChOAS-L-A protein possesses antiviral activity against WNV
18 replicon replication (Tag-El-Din-Hassan and others 2012). In addition, Cong and others (2013)
19 reported that the ChOAS-L/RNase L pathway might be involved in anti-avian infectious bronchitis
20 virus (IBV) infection. Moreover, Li and others (2007); Lee and others (2014) indicated that the
21 ChOAS-L/RNase L pathway might be involved in anti-avian infectious bursal diseases virus
22 (IBDV) infection. Therefore, the aim of this research is to examine the relationship between
23 enzymatic and antiviral activities in the ChOAS-L against WNV replicon.
24

25 **MATERIAL AND METHODS**

26

1 ***SITE-DIRECTED MUTAGENESIS***

2 *ChOAS-L-A-FL,* which was *ChOAS-L-A* conjugated with the Flag-Tag DYKDDDDK sequence,
3 was cloned into the pGEM-T easy vector (Promega Corporation, Madison, USA) and used for the
4 generation of eight mutations. The ΔLxxxP domain, ΔP-LooP domain, ΔD-D box domain,
5 KR-RR-K193H (one AA substitution), ΔKR-RR, and ChOAS-L-B (the other allele) were generated
6 by using the QuickChange Lightning Site-Direct Mutagenesis Kit (Stratagene, La Jolla, CA, USA)
7 according to the manufacturer's protocol. The other two mutations, ΔUbL2 domain and
8 ΔUbL1/UbL2 domains, were generated by the standard PCR protocol as follows, denature at 95 °C
9 for 5 min, twenty cycle of denature at 95 °C for 30 sec, annealing at 60 °C for 30 sec, extension at
10 72 °C for 1.5 min, and final extension at 72 °C for 1.5 min. All mutations were confirmed by the
11 DNA sequence analysis. Table 1 shows the primers used to generate these mutations. Figure 1
12 demonstrates the assumed scheme of the mutant proteins.

13 All mutated DNAs conjugated with the Flag-Tag were cut from the pGEM-T easy vector
14 using *Not*I restriction enzyme (Toyobo Co., Ltd. Osaka, Japan) and re-cloned into *Not*I restriction
15 site of the pIRES-EGFP vector (the mammalian expression vector, Clontech Bio. Inc., Shiga, Japan).
16 All cloning reactions were performed using 2x Ligation-Convenience Kit (Wako Nippon Gene Co.,
17 Ltd. Toyama, Japan) according to the manufacturer's protocol. All transformations were done using
18 DH5α *E. coli* competent cells. The large-scaled plasmid purification was performed using
19 $CsCL_2$-ethidium bromide gradient purification method as described previously (Sambrook and
20 Russell 2001).

21

22 ***CELL CULTURE***

23

24 HEK293FT, BHK-21, and BALB/3T3 cells were maintained in Dulbecco's Modified Eagle
25 medium (DMEM) (Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine
26 serum (FBS) and 1% penicillin/streptomycin/glutamine (Gibco/Invitrogen, Carlsbad, CA, USA).

All cell lines were cultured with a passage of every two days in a 5% CO_2 cell culture incubator at 37 °C.

PREPARATION OF CELL LYSATE

Both HEK293FT and BHK-21 cells were cultured in 100-mm dish at the density of 1.5×10^6 cells/dish for 24 h, the cells were transfected with 10 μg of pIRES-EGFP (empty vector as a control) or gene-inserted vector, pChOAS-L-A-FL-EGFP, pΔLxxxP-FL-EGFP, pΔP-LooP-FL-EGFP, pΔD-D-Box-FL-EGFP, pKR-RR-K193H-FL-EGFP, pΔKR-RR-FL-EGFP, pChOAS-L-B-FL-EGFP, pΔUbL2-FL-EGFP, or pΔUbL1/UbL2-FL-EGFP using Lipofectamine 2000® (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The EGFP expression was analyzed at 24 h post-transfection for all transgenes using a NIKON ECLIPSE TE2000E microscope. In respect of HEK293FT cells, at 48 h post-transfection, the cells washed twice with phosphate-buffered saline (PBS), scratched in 1 ml PBS, spin for 5 min at 780 xg, the supernatant was discarded, then, the cells were suspended in Flag-Tag lyses buffer as described previously (Sarkar and others 2005). The HEK293FT cell lysates were used for the Western blotting and enzymatic activity analysis. Transfected BHK-21 cells were lysed at 72 h post-transfection in the Flag-Tag lyses buffer in the presence or absence of protease inhibitors using the same method (Sarkar and others 2005). The BHK-21 cell lysates were kept at -80°C until used for protein stability assay.

WESTERN BLOTTING

The protein concentrations were determined using SmartSpec™ (BioRad) according to the Bradford reagent protocol. Twelve μg proteins of each sample were separated in 10% polyacrylamide-SDS gel electrophoresis on 100 V for 1 h, blotted onto PVDF membrane, and then,

blocked with PBS containing 3% non-fatty milk and 0.1% Tween-20 for 1 h at room temperature. The membrane was incubated at room temperature with mouse-anti FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:2,000 with PBS containing 0.1% Tween-20 (PBS-Tween-20) for 2 h. The membrane was washed 3 times with PBS-Tween-20 for 10 min, and then, the membrane was incubated at room temperature with HRP-conjugated goat anti-mouse antibody diluted 1:5,000 with PBS-Tween-20 for 1 h. The membrane was washed 3 times with PBS-Tween-20 for 10 min. The immunoreactive bands were detected by ECL™ Western Blotting Detection Reagents (GE Healthcare UK Limited, Little Chalfont, UK) according to the manufacturer's protocol.

ASSAY OF ENZYMATIC ACTIVITY

The enzymatic activity of ChOAS-L-A protein and all of its mutants was measured as described previously (Sarkar and others 2005), with a slight modification as follows; an aliquot of 2.5 μl of HEK293FT whole cell lysates was incubated in incubation buffer (20 mM Tris-HCl at pH 7.4, 20 mM magnesium acetate, 2.5 mM dithiothreitol (DTT), 5 mM ATP, 50 μg/ml poly(I):(C), and 5 μCi of [α-^{32}P] ATP (6,000 Ci/mmol) in a final volume of 10 μl for 18 h at 30 °C. The reaction was stopped by boiling for 5 min at 95 °C, followed by adding loading buffer containing 25% formamide, 0.5% bromophenol blue, and 0.5% xylene cyanol.

The synthetized 2'-5' (A) products were run in a 20% polyacrylamide-urea (7 M) gel. The electrophoresis was performed for 4 h on 200 V, and then products were visualized by autoradiography with a BAS2500 Bio-Imaging analyzer (FUJIFILM, Tokyo, Japan).

PROTEIN STABILITY ASSAY

The protein stability assay was performed by incubating the protein samples (12 μg) in the

presence or absence of protease inhibitors at 37°C for 2 h. The incubated samples were subjected to Western blotting as shown above to determine the protein stability.

MEASUREMENT OF INHIBITORY ACTIVITY ON WNV REPLICON REPLICATION

pUC19repWNV/SEAP plasmid containing the WNV genome harboring the secreted alkaline phosphatase reporter gene instead of viral structural genes (Moritoh and others 2011) was propagated in transformed XL10-Gold® Ultracompetent *E.coli* Cells (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. A PureLink® HiPure Plasmid Maxiprep Kit (Invitrogen, Carlsbad, CA, USA) was used for large-scaled purification according to the manufacturer's protocol.

pUC19repWNV/SEAP plasmid was linearized using *FastDigest Xba*I restriction enzyme (Thermo Fisher Scientific, Tokyo, Japan) according to the manufacturer's protocol. The mMESSAGE mMACHINE® SP6 Transcription Kit (Ambion, Austin, TX, USA) was used to synthesize WNV-replicon RNA from the linearized pUC19repWNV/SEAP according to the manufacturer's protocol. Briefly, 1µg of linearized plasmid was incubated in the reaction mixture for 2 h to get the maximum RNA yield as recommended by the manufacture, then the synthetized RNA was recovered using the PureLink® RNA Mini Kit (Ambion, Carlsbad, Ca, USA), divided into 10 µg aliquots, and stored at -80°C until used for the measurement of antiviral activity.

One day before the transfection with the wild type *ChOAS-L-A* and mutant genes, the BHK-21 and BALB/3T3 cells were cultured in a 100-mm plate at the density of 1.5×10^6 cells/plate, and then, 10µg of pIRES-EGFP (empty vector as a control), pChOAS-L-A-FL-EGFP, pΔLxxxP-FL-EGFP, pΔP-LooP-FL-EGFP, pΔD-D-Box-FL-EGFP, pKR-RR-K193H-FL-EGFP, pΔKR-RR-FL-EGFP, pChOAS-B-FL-EGFP, pΔUbL2-FL-EGFP, or pΔUbL1/UbL2-FL-EGFP was transfected using Lipofectamine 2000® (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The EGFP expression was analyzed at 24 h post-transfection using the NIKON ECLIPSE TE2000E microscope.

BHK-21 and BALB/3T3 cells were harvested from non-transfected cells (mock transfection) and from all transfectans. Then, 0.5×10^7 cells were incubated in 500 µl of PBS on ice with 10 µg of WNV-replicon RNA in 4 mm cuvette (Nepa Gene Co., Ltd. Chiba, Japan). The electroporation was performed using Gene-Pulser X Cell, Bio-Rad, with pulses of 1.5 kV at 25 µF and 0.5 kV at 25 µF of infinite resistance for BHK-21 and BALB/3T3 cells, respectively. The live cells were counted and cultured in triplicate in a 6-well plate (3×10^5 cells/well). Supernatants were collected at 24 and 72 h post-electroporation. The inhibitory activity on WNV replicon replication was measured as the secreted alkaline phosphatase (SEAP) activity in the supernatant using Great EscAPe™ SEAP Chemiluminescence Kit 2.0 (Clontech Bio. Inc., Shiga, Japan) and Mithras LB 940 Multimode Microplate Reader according to the manufacturer's protocol.

RNA EXTRACTION AND RT-PCR

Total RNA was extracted from HEK293FT cells at 48 h post-transfection and from BHK-21 and BALB/3T3 cells before electroporation and at 72 h post-electroporation, using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA samples were treated with DNase to remove any DNA contamination using TURBO DNA-*free*™ Kit (Ambion, Austin, TX, USA) according to manufacturer's protocol. Concentration of purified total RNA samples were determined using SmartSpec™ (BioRad), 1 µg of total RNA was used to generate cDNAs using *ReverTra Ace*® (Toyobo Co., Ltd. Osaka, Japan) according to manufacturer's protocol. The RT condition was at 42° C for 60 min and at 99 °C for 5 min. The synthesized cDNA was stored at -20°C until used for the PCR, of which conditions were as follow; denature at 95 °C for 5 min; 35 cycles of denature at 95 °C for 30 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 1.5 min; followed by the final extension at 72 °C for 3 min.

LOCALIZATION OF THE CHOAS-L-A AND MUTATED PROTEINS

1

2 BHK-21 cells were cultured in 96 wells plate (2×10^4 cells/well) for 24, and cells were
3 transfected with 0.1 µg of *pIRES-EGFP* or p*ChOAS-L-A-FL-EGFP* as well as all mutated genes
4 using 0.4 µl Lipofectamine 2000® according to manufacturer's protocol. At 48 h post-transfection,
5 cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and washed
6 twice with 1x washing buffer (PBS containing 0.1% Tween-20). The cells were then permeabilized
7 with 0.1% Triton X-100 in PBS for 5 min at room temperature, washed twice with 1x washing
8 buffer, and followed by blocking in 10% horse serum in PBS for 30 min at room temperature. The
9 mouse-anti FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO, USA) diluted to 1:1000 with the
10 blocking solution was added to wells, incubated for 1 h at room temperature, and followed by
11 washing for three times (5 min each) with 1x washing buffer. The diluted secondary antibodies
12 (Alexa Fluor 568 goat anti-mouse IgG, Invitrogen, Oregon, USA) in 1x PBS were added to wells
13 and incubated for 1 h at room temperature, followed by washing for three times (5 min each) with
14 1x washing buffer. Nuclei counterstaining was performed by incubating cells with DAPI in PBS for
15 5 min at room temperature, followed by washing cells two times (5 min each) with 1x washing
16 buffer, and then the fluorescence images were captured using BZ-X700 All-in-One Florescence
17 Microscope, KEYENCE (Osaka, Japan).

18

19 ***RNASE-L ACTIVATION ASSAY***

20

21 The RNase-L activation assay for ChOAS-L-A protein and all of its mutated proteins was
22 performed using the florescence resonance energy transfer assay (FRET assay) as described
23 previously (Thakur and others 2005) with a slight modification as follows; an aliquot of 2 µg of
24 BHK-21 whole cell lysates transfected with *pIRES-EGFP* as well as p*ChOAS-L-A-FL-EGFP* and
25 all mutated genes was incubated in 50 µl of 1x incubation buffer (25 mM Tris-HCl at pH 7.4, 0.1 M
26 KCl, 10 mM $MgCl_2$, 50 µM ATP, 7 mM β-mercaptoethanol, and 180 nM FRET probe for 0, 5, 10,

15, 30, 45, 60, 90, and 120 min at 21°C. As a negative control, lysates of BHK-21 and HEK293FT non-transfected cells were used, whereas as a positive control, BHK-21 and HEK293FT non-transfected cells stimulated with 3,000 IU/ml of IFN-α2b (INTRON® A, MSD K.K., Tokyo, Japan) for 24 h were used. The fluorescence was determined using the Infinite M200 PRO plate reader, Tecan (Mannedorf, Switzerland).

STATISTICAL ANALYSIS

All experiments were done three times independently. Collected data were subjected to the one-way analysis of variance (ANOVA) using a SPSS 16.0 software. All values were represented as means ± SD. Statistical differences were analyzed using Tukey HSD test. Values of $p < 0.05$ and $p < 0.01$ were considered to be significant.

RESULTS

ENZYMATIC ACTIVITY OF THE MUTATED CHOAS-L-A

Protein and total RNA were extracted at 48 post-transfection from HEK293FT cells transfected with the *pIRES-EGFP* (empty vector as a control), wild-type *ChOAS-L-A* and each mutated *ChOAS-L-A* genes. The expression of all genes were confirmed by RT-PCR and Western blotting.

All mutated genes as well as *EGFP* and *Actb* were expressed at 48 h post-transfection (Fig. 2A). As shown in Fig. 2B, the cells transfected with pChOAS-L-A-FL-EGFP, pChOAS-L-B-FL-EGFP, and pΔUbL2-FL-EGFP showed enzymatic activity to convert ATP to 2'-5' (A) with mono-, di-, tri-, and tetramer, whereas the cells transfected with pIRES-EGFP (the empty vector), pΔLxxxP-FL-EGFP, pΔP-LooP-FL-EGFP, pΔD-D-Box-FL-EGFP,

1 pKR-RR-K193H-FL-EGFP, pΔKR-RR-FL-EGFP, and pΔUbL1/UbL2-FL-EGFP were incapable of
2 synthesizing 2'-5' (A).
3
4 ***ANTIVIRAL ACTIVITY OF THE MUTATED CHOAS-L-A***
5
6 We did not select chicken-derived cells as host cells for measurement of inhibitory activity on
7 WNV replicon replication to exclude the effect of endogenous ChOAS-L. BHK-21 cells were
8 selected, because this cell line is known to harbor many viruses. BALB/3T3 cells were also selected
9 as cells devoid of mouse Oas1b, which is a flavivirus-specific antiviral factor. Total RNA was
10 extracted from BHK-21 and BALB/3T3 control cells (Mock and pIRES-EGFP transfection), and
11 cells transfected with wild-type *pChOAS-A-FL-EGFP* and mutant genes before the measurement of
12 antiviral activity. The RT-PCR results showed that *ChOAS-L-A* and its mutated genes were all
13 successfully expressed (Figs. 3A and 4A).
14 The SEAP expression level was measured in the culture supernatant of BHK-21 cells
15 transfected all genes at 24 and 72 h post-electroporation of the WNV-replicon RNA. All mutants
16 significantly (p<0.01) showed antiviral activity to reduce the SEAP activity compared to that of the
17 controls, both ChOAS-L-A and ChOAS-L-B showed the highest antiviral activity followed by other
18 mutants. The KR-RR-K193H was significantly ($p<0.05$) different from ChOAS-L-A, ChOAS-L-B
19 or ΔLxxxP (Fig. 3B). Although ΔUbL1/UbL2 showed antiviral activity, its extent was partial when
20 compared to that of ChOAS-L-A, suggesting that UbL domain is necessary for the antiviral activity.
21 However, the result that ChOAS-L-B, partial deletion of UbL1, and ΔUbL2 showed almost full
22 antiviral activity suggest that a single copy of UbL domain is satisfactory to retain the antiviral
23 activity.
24 The same trend was seen in the BALB/3T3 cells, all mutants significantly ($p<0.01$) showed
25 antiviral activity to reduce the SEAP activity compared with control groups. Moreover, the
26 ΔKR-RR was significantly different from ChOAS-L-A ($p<0.01$), ChOAS-L-B ($p<0.05$) and

ΔLxxxP ($p<0.05$) (Fig. 4B).

Although the WNV-replicon replication level is much lower than those in the BHK-21 cells. The reason for this difference is unknown; however, the BHK-21 cells were reported to have characteristics to allow the efficient replication of WNV (Shi and others 2002; Rossi and others 2005; Moritoh and others 2011). Although BALB/3T3 cells might possess resistance to the WNV infection compared to the BHK-21 cells, antiviral activities of ChOAS-L-A and its mutants in BALB/3T3 cells were almost the same as that in BHK-21 cells, suggesting that the results were not dependent on the cell types.

STABILITY OF CHOAS-L-A AND MUTATED PROTEINS

Twelve-µg whole cells lysate of BHK-21 cell transfected with *pChOAS-L-A-FL-EGFP* or other mutated transgenes were incubated on 37°C for 0 and 2 h in the presence or absence of proteases inhibitors in the lysis buffer. Then, the incubated protein samples were used for the western blot analysis. The obtained results reveled that in the presence of the proteases inhibitors in the lysis buffer all protein samples were remained protected. However, in the absence of the proteases inhibitor in the lysis buffer, ChOAS-L-A, ΔLxxxP, ΔP-LooP, ΔD-D, KR-RR-K193H as well as ΔKR-RR proteins showed stability up to 2 h of incubation on 37°C (Fig. 5). On the other hand, ChOAS-L-B (partial deletion in the UbL1), ΔUbL2 and ΔUbL1/UbL2 proteins showed instability after incubation period. This result indicates the importance of the UbL domains for the Ch-OAS-L protein stability.

RNASE-L ACTIVITY ASSAY

Two-µg protein of whole cell lysate of BHK-21 cells transfected with pIRES-EGFP, pChOAS-L-A-FL-EGFP, and other mutated genes, as well as control groups (HEK293FT and BHK-21 non-transfected cells with- and without IFN treatment as positive and negative control,

1 respectively), were used to perform FRET assay. RNase L activation detection were performed on 0,
2 5, 10, 15, 30, 45, 60, 90 and 120 minutes post-incubation in the cleavage buffer.
3 The obtained results showed that, in all time point analysis the HEK293FT/IFN expresses
4 high significant (p<0.01) fluorescence level than all protein samples, which indicates that there are
5 high activity of RNase L in HEK293FT/IFN cells (Fig. 6). On the other hand, the ChOAS-L-A
6 protein as well as its mutated proteins showed the same expression as the negative control groups
7 (non-transfected BHK-21 cells). Regarding BHK-21/IFN treated cells, it was statistically
8 insignificant for the 0, 5, 10, 15, and 30 minutes' post incubation. However, starting from 45
9 minutes' post-incubation, both BHK-21/IFN and HEK293FT/non-transfected showed similar
10 significant (p<0.05) increase in the fluorescence activity (Fig. 6). This increasing in the
11 fluorescence activity continued highly significant (p<0.01) on 60, 90 and 120 minutes'
12 post-incubation. In contrast, pIRES-EGFP, ChOAS-L-A as well as all mutated proteins expressed
13 almost the same fluorescence activity as for the negative control (BHK-21/non-transfected) with
14 insignificant differences (Fig. 6). These results showed that the ChOAS-L-A protein and all of its
15 mutated protein are incapable of activate the RNase L in BHK-21 cells.
16
17 **DISCUSSION**
18
19 ChOAS-L-A protein, a member of the OAS-L protein family characterized by the N-terminal
20 OAS unit (similar to small OAS protein) comprises the motif of the LxxxP followed by the P-Loop,
21 D-D box, region of 32-36 residues with high content of lysine and arginine, and finally the CFK
22 motif. This small OAS unit fused into the C-terminal UbL unit, composed of two repetitive UbL
23 domains, UbL1 and UbL2 (Eskildsen and others 2003). Recently, Tag-El-Din-Hassan and others
24 (2012) reported that the ChOAS-L-A possesses both enzymatic and anti-WNV activities.
25 Therefore, in the current study, eight *ChOAS-L-A* mutants were generated by using site-directed
26 mutagenesis protocol or ordinary PCR protocol, in which five mutations were introduced in the

1 domains of the OAS unit and the other three mutations were in the UbL domains, to investigate the
2 relationship between enzymatic and antiviral activities of ChOAS-L-A.
3 The results revealed that the ΔLxxxP domain mutation resulted in a complete lose of the
4 enzymatic activity (Fig. 2-B). Similarly, Ghosh and others (1997a) found that the deletion of 1 to 9
5 or 1 to 23 residues from the N-terminal of mouse 9-2 OAS protein resulted in a 100% loss of
6 enzymatic activity. Moreover, the substitution of three conserved amino acids in this motif, L3H,
7 P7Q, A8D, and P7Q/A8D still retain the ability to bind the dsRNA as well as the wild type;
8 however, the enzymatic activity of L3H and A8D showed 30 and 18% of the wild type, respectively,
9 and both P7Q and P7Q/A8D completely lose the enzymatic activity. Further, to confirm the
10 importance of the proline at position 7, Ghosh and others (1997a) generated several substituted
11 proteins, P7Q, P7F, P7Y, P7E, P7K, P7L, and P7T and examined enzymatic activity. All mutations
12 resulted in complete loss of the enzymatic activity compared with the wild type. However,
13 Bandyopadhyay and others (1998) reported that the P7Q mutation showed full enzymatic activity,
14 when the protein expressed in baculovirus/insect cell system. The current study showed that the
15 ΔLxxxP domain mutation did not affect the inhibitory effects on WNV-replicon replication. The
16 SEAP activity in BHK-21 and BALB/3T3 cell expressing ΔLxxxP domain mutant protein was
17 significantly decreased as well as the wild type (ChOAS-L-A) in comparison with the control
18 groups (Fig. 3-B and 4-B). These findings suggest that the LxxxP domain is critically required for
19 the synthetase activity, whereas it is not required for the antiviral activity of the ChOAS-L protein.
20 The ΔP-LooP domain mutation shows no enzymatic activity (Fig. 2-B). Similarly, Yamamoto
21 and others (2000) examined several mutations in the P-Loop domain of the 42 KDa MuOAS protein,
22 G62A/G63A, K67M, and K67R, all of which mutations resulted in a significant decrease in the
23 enzymatic activity, 0, 2, and 30% of the wild type, respectively. In addition, the K59E mutation in
24 porcine OAS1 resulted in a lose of enzymatic activity (Hartmann and others 2003). With respect to
25 the antiviral activity, the results in the current study shows that the ΔP-LooP mutant protein in both
26 BHK-21 and BALB/3T3 cells resulted in significant inhibition in the WNV-replicon replication

(Figs. 3-B and 4-B). These results show that the P-LooP domain is critically essential for the enzymatic activity, whereas it is not for antiviral properties of ChOAS-L-A protein.

The ΔD-D box domain mutation resulted in a complete loss of enzymatic activity (Fig. 2-B). This result agrees with Yamamoto and others (2000), in which the substitution in D-D box, D76N/D78N, resulted in a 100% loss of enzymatic activity compared with the wild type protein. The same result was obtained by Sarkar and others (1999), in which the enzymatic activity of human p69 OAS was lost, when the D481A and D408A/D410A mutations in D-D box were introduced. In contrast to the enzymatic activity, the ΔD-D box mutation did not affect the antiviral ability of the ChOAS-L-A protein in this report (Figs. 3-B and 4-B). This finding confirms that the D-D box domain is critically important for the enzymatic activity, whereas it is not important for the antiviral activity of ChOAS-L-A protein.

The KR-RR domain mutations in the current study, the mutations either induced by amino acid substitution or complete domain deletion, resulted in a 100% loss of enzymatic activity (Fig. 2-B). These results agreed to the results by Yamamoto and others (2000), in which the K200M and K200R mutations of the 42 KDa MuOAS showed a significant reduction in enzymatic activity to 2 and 50% that of the wild type, respectively. Moreover, the double mutations in both P-loop and KR-RR, K67R/K200R, resulted in a decrease in the enzymatic activity to 10% of the wild type. Similarly, Kon and Suhadolnik (1996) investigated the effects of K199R and K199H mutations of the human 40 KDa OAS. They reported that both mutations resulted in a loss of enzymatic activity; however, both mutants retained the ability to bind poly (I):(C). Similarly, Hartmann and others (2003) induced several mutations in porcine OAS1 protein, R194E, R198E, R198M, R194E/R198E, and K203E, and showed that all of these mutant proteins lost enzymatic activity. Furthermore, Sarkar and others (2002) examined several mutations, Y421P, Y421A, R544A, R544Y, and K547A of the p69 HuOAS. All mutations resulted in a severe reduction in the enzymatic activity to 3% that of the wild type except for K547A, 10% of the control. Furthermore, Ghosh and others (1991) induced several deleting mutations in murine 9-2 Oas protein, one of which deleted the KR-RR.

1 They found that the ΔKR-RR mutated protein retains the dsDNA binding ability almost same as the
2 wild type protein. With respect to the antiviral activity, however, we showed that both mutants
3 remained antiviral activity both in BHK-21 and BALB/3T3 cells as well as the wild-typed
4 ChOAS-L-A protein (Figs. 3-B and 4-B). These results suggest that the KR-RR is critical for the
5 enzymatic activity of the ChOAS-L protein, whereas it is not important for the antiviral activity.
6 Moreover, the ΔLxxxP, ΔP-LooP, ΔD-D Box, KR-RR-K193H or ΔKR-RR mutated proteins was
7 unable to activate the RNase L, using FRET assay, during all studied time points and expresses the
8 same level as the negative control groups (Fig. 6). And all of these mutated protein showed stability
9 up to 2 h post incubation in the absence of the proteases inhibitors and remain detectable as 0.0 h
10 incubation (Fig. 5). As well, ΔLxxxP, ΔP-LooP, ΔD-D Box, KR-RR-K193H or ΔKR-RR mutated
11 proteins was localized in to cytoplasm similar to the wild type ChOAS-L-A protein (Fig. 7). Taken
12 together, these results show that all mutations carried out in the active domains of the ChOAS unit
13 results in deactivation of the enzymatic activity (Fig. 2-B). However, all of these mutated protein
14 still retain the antiviral activity to inhibit the WNV-replicon replication (Fig. 3-B and 4-B), and acts
15 almost same as ChOAS-L-A protein (Fig. 5, 6 and 7), suggesting that the antiviral activity of
16 ChOAS-L is independent of the enzymatic activity.
17 Both ChOAS-L-ΔUbL2 and ChOAS-L-B, the second allele of ChOAS-L-A possessing 32 AA
18 deletion in UbL1 generated by the mutagenesis protocol, still possessed the enzymatic activity. This
19 result is consistent with the report by Tatsumi and others (2003), in which the ChOAS-L-B
20 possesses the enzymatic activity. However, the ΔUbL1/UbL2 mutation, complete deletion of UbL
21 domains, did not show the enzymatic activity (Fig. 2-B), which was unexpected result for us. We
22 had supposed that the ChOASL-L-A-ΔUbL1/UbL2 should retain the enzymatic activity, because
23 the OAS unit was retained in this mutant. The full function of UbL domain is not completely
24 uncovered at present. However, in the current study we showed that UbL domain plays an important
25 role in stabilization of ChOAS-L proteins, where, either ChOAS-L-B (possess partial deletion in
26 UbL1), ChOAS-L-A-ΔUbL2 or ChOAS-L-A-ΔUbL1/UbL2 proteins, showed instability when

incubated in the lysis buffer in the absence of the proteases inhibitors (Fig. 5), similarly Tatsumi and others (2003) found that ChOAS-L-B showed instability if incubated in the absence of the proteases inhibitors, as well, ChOAS-L-B shows age-dependent expression, where its expression in chicken erythrocytes was decreased by aging, if compared with the ChOAS-L-A protein. Although, only one UbL domain is enough for ChOAS-L-A protein to express the enzymatic activity (Fig 2-B).

Furthermore, ChOAS-L-B, ChOAS-L-A-ΔUbL2 or ChOAS-L-A-ΔUbL1/UbL2 proteins, showed incapability to activate RNase L in FRET assay, and expresses the same level as the negative control (Fig. 6), and localized into cytoplasm as well as the wild type ChOAS-L-A protein (Fig. 7).

As for the antiviral activity, both ChOAS-L-B and ChOAS-L-A-ΔUbL2 mutant proteins still retain and express the antiviral properties to inhibit the WNV-Replicon replication (Fig. 3-B and 4-B). However, the ChOAS-L-A-ΔUbL1/UbL2, which supposed to be completely lose the antiviral activity, surprisingly found to be expressing partial antiviral activity against the WNV-replicon replication (Fig. 3-B and 4-B). The possible reason for this results may be as follows, as it was mentioned before, ChOAS-L-A-ΔUbL1/UbL2 still possess the OAS unit, so that, it showed slightly inhibition of the WNV-replicon replication due to the capability of the OAS unit to bind the dsRNA (step of the viral genome replication) inside the cells. Recently, Ibsen and others (2015) showed that the HuOAS-L-ΔUbL$_{(S)}$ have the ability to bind dsRNA.

Taken together, the obtained results indicating that, however the UbL domains are not participated in the enzymatic activity functions of ChOAS-L protein, it is still required at least one UbL domain to express the enzymatic activity. Moreover, at least one UbL domain is critically essential to express the antiviral activity of ChOAS-L.

Collectively, in the current study, the FRET Assay stands as the connective edge between the enzymatic and antiviral activities of the OAS proteins and explains why the BHK-21 cell line used in this experiment. Where, the lysates containing the ChOAS-L-A or its mutant proteins showed inability to activate the RNase L same as the negative control group (BHK-21/Shame), even if the

1 protein enzymatically active (such as ChOAS-L-A, ChOAS-L-B and ChOAS-L-A-ΔUbL2). The
2 incapability to activate RNase L in this experiment is not related to have or haven't the enzymatic
3 activity, however it is related to the cell line itself where, BHK-21 cell express deficiency in the IFN
4 pathway. Which reflects that the antiviral activity of ChOAS-L-A and all of its mutants except for
5 ChOAS-L-A-ΔUbL1/UbL2, is for sure due to the OAS/RNase L independent pathway.
6 In conclusion, the obtained results suggest that the ChOAS-L expresses the antiviral activity in a
7 manner independent of the OAS/RNase L classical pathway. However, it may be premature to state
8 ChOAS-L functions without investigating other avian OAS family members for enzymatic and
9 antiviral activities not only against WNV but against specific avian pathogens.
10
11

Table 1. Primers used to generate mutations from the wild-typed *ChOAS-L-A* gene and primers for *EGFP* and *Actb* genes.

Name		Direction	Primer sequence	No of AA del.
Mutation	**ChOAS-L-A-FL△LxxxP**	**Forward**	**5’-GATTATCGATATGGGGTCGCGCCAGCTGGAGG-3’**	**5**
		Reverse	**5’-CCTCCAGCTGGCGCGACCCCATATCGATAATC-3’**	
	ChOAS-L-A-FL△P-LooP	**Forward**	**5’-CGTCCTCAAGACCGTCACGGCGCTGCGCAAC-3’**	**8**
		Reverse	**5’-GTTGCGCAGCGCCGTGACGGTCTTGAGGACG-3’**	
	ChOAS-L-A-FL△D-D box	**Forward**	**5’-CGCTGCGCAACAACTCGGTGGTGCTCTTCATCAACTGC-3’**	**3**
		Reverse	**5’-GCAGTTGATGAAGAGCACCACCGAGTTGTTGCGCAGCG-3’**	
	ChOAS-L-A-FL-KR RR-K193H	**Forward**	**5’-CAGAAGAACTTTGTGCACTGGCGCCCTGCTAAG-3’**	**0**
		Reverse	**5’-CTTAGCAGGGCGCCAGTGCACAAAGTTCTTCTG-3’**	
	ChOAS-L-A-FL△KR RR	**Forward**	**5’-CCCCAGCTTCACAGAACTGCCCCCCAAGTATG-3’**	**36**
		Reverse	**5’-CATACTTGGGGGGCAGTTCTGTGAAGCTGGGG-3’**	
	ChOAS-L-B-FL	**Forward**	**5’-GCTACCAGCAGCGCCTGTACACCGAGCCCCAG-3’**	**32**
		Reverse	**5’-CTGGGGCTCGGTGTACAGGCGCTGCTGGTAGC-3’**	
	ChOAS-L-A-FL△UbL2	**Reverse**	**5’-TCAGATCTTATCGTCGTCATCCTTGTAATCCAGCAGCAGCAGCAC-3’**	**92**
	ChOAS-L-A-FL △ UbL1/UbL2	**Reverse**	**5’-TCAGATCTTATCGTCGTCATCCTTGTAATCCTGCACGTCC-3’**	**171**
ChOAS-L-A-FL		**Forward**	**5’-ATCGATATGGGGTTGGAGAGCGTGAG-3’**	
		Reverse	**5’-TCAGATCTTATCGTCGTCATCCTTGTAATCGGAGGGCACGCAGCGTCTGG-3’**	
EGFP		**Forward**	**5’-ATGGTGAGCAAGGGCGAGGA-3’**	
		Reverse	**5’-TTACTTGTACAGCTCGTCCA-3’**	
ActB		**Forward**	**5’-TGATGGTGGGAATGGGTCAG-3’**	
		Reverse	**5’-GAAGGCTGGAAAAGAGCCTC-3’**	

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12

1 **FIGURE LEGENDS**

2

3 Fig. 1. Schematic diagram of the wild-typed ChOAS-L-A and its mutated proteins as follows;
4 deletion mutation, ΔLxxxP, ΔP-Loop, ΔD-D Box, ΔKR-RR, ΔUbL2, and ΔUbL1/UbL2, point
5 mutation; KR-RR-K193H, and the other allele ChOAS-L-B. Each pattern of column represents each
6 domain and the asterisk represents a point mutation.

7

8 Fig. 2. The measurement of enzymatic activity of the wild-typed ChOAS-L-A and its mutated
9 proteins. A) RT-PCR and Western blot of HEK293FT cells at 48 h post-transfection with
10 pIRES-EGFP (control), pChOAS-L-A-FL-EGFP, and each mutated gene. B) The measurement of
11 enzymatic activity. All mutated proteins showed no enzymatic activity except for ChOAS-L-B and
12 ΔUbL2, both of which showed enzymatic activity with the same extent to that of wild-typed
13 ChOAS-L-A in ChOAS-L-B and slightly less activity in ΔUbL2.

14

15 Fig. 3. Antiviral activity of the ChOAS-L-A and its mutated proteins. BHK-21 cells with mock
16 transfection, pIRES-EGFP, pChOAS-L-A-FL-EGFP, and each of its mutated gene. A) RT-PCR
17 using total RNA extracted from BHK-21 cells before and at 72 h after WNV-replicon RNA
18 electroporation. B) SEAP activity at 24 and 72 h post-WNV-replicon RNA electroporation.

19

20 Fig. 4. Antiviral activity of the ChOAS-L-A and its mutated proteins in BALB/3T3 cells with mock
21 transfection, pIRES-EGFP, pChOAS-L-A-FL-EGFP, and each of its mutated gene. A) RT-PCR
22 using total RNA extracted from BALB/3T3 cells before and at 72 h after WNV-replicon RNA
23 electroporation. B) SEAP activity at 24 and 72 h post-WNV-replicon RNA electroporation.

24

25 Fig. 5. Stability assay of ChOAS-L-A and its mutated protein. Twelve µg of whole BHK-21 cell
26 lysate transfected with pChOAS-L-A-FL-EGFP and all of its mutants, in the presence or absence of

1 the proteases inhibitors, were incubated for 0 and 2 h on 37°C, then used for the western blot. All
2 proteins showed stability on 0 and 2 h post-incubation, however, in the absence of the proteases
3 inhibitors, 2 h post-incubation only the protein with mutation in the UbL domains showed
4 degradation.

5

6 Fig. 6. RNase L activation assay using the florescence resonance energy transfer assay (FRET
7 assay). Two µg protein aliquots of BHK-21 cell lysates transfected with the ChOAS-L-A-FL-EGFP
8 and all of its mutants, were incubated in 50 µl of 1x cleavage buffer with a FRET probe (final
9 concentration 180 ng), for different time points, 0, 5, 10, 15, 30, 45, 60, 90, 120 min at 21°C, then
10 the fluorescence activity was detected. The background fluorescence detected by incubating the
11 FRET probe in the 1x cleavage buffer was subtracted from all reads before statistical analysis.

12

13

14 Fig. 7. Cellular localization of the ChOAS-L-A protein as well as all of its mutant proteins. The
15 BHK-21 cells cultured in 96 well plate, then transfected with the pIRES-EGFP,
16 pChOAS-L-A-FL-EGFP, pΔLxxxP-FL-EGFP, pΔP-LooP-FL-EGFP, pΔD-D-Box-FL-EGFP,
17 pKR-RR-K193H-FL-EGFP, pΔKR-RR-FL-EGFP, pChOAS-L-B-FL-EGFP, pΔUbL2-FL-EGFP, or
18 pΔUbL1/UbL2-FL-EGFP. Forty-eight hours' post-transfection cells were used to perform
19 immunofluorescence protocol. The ChOAS-L-A-FL protein as well as it mutant protein were
20 detected using the mouse-anti FLAG M2 antibody. The Alexa Fluor 568 goat anti-mouse IgG used
21 as secondary antibodies. Nuclei counterstaining was performed by incubating cells with DAPI. Th
22 white par stand for 100 µm.

1

Fig. 1

2

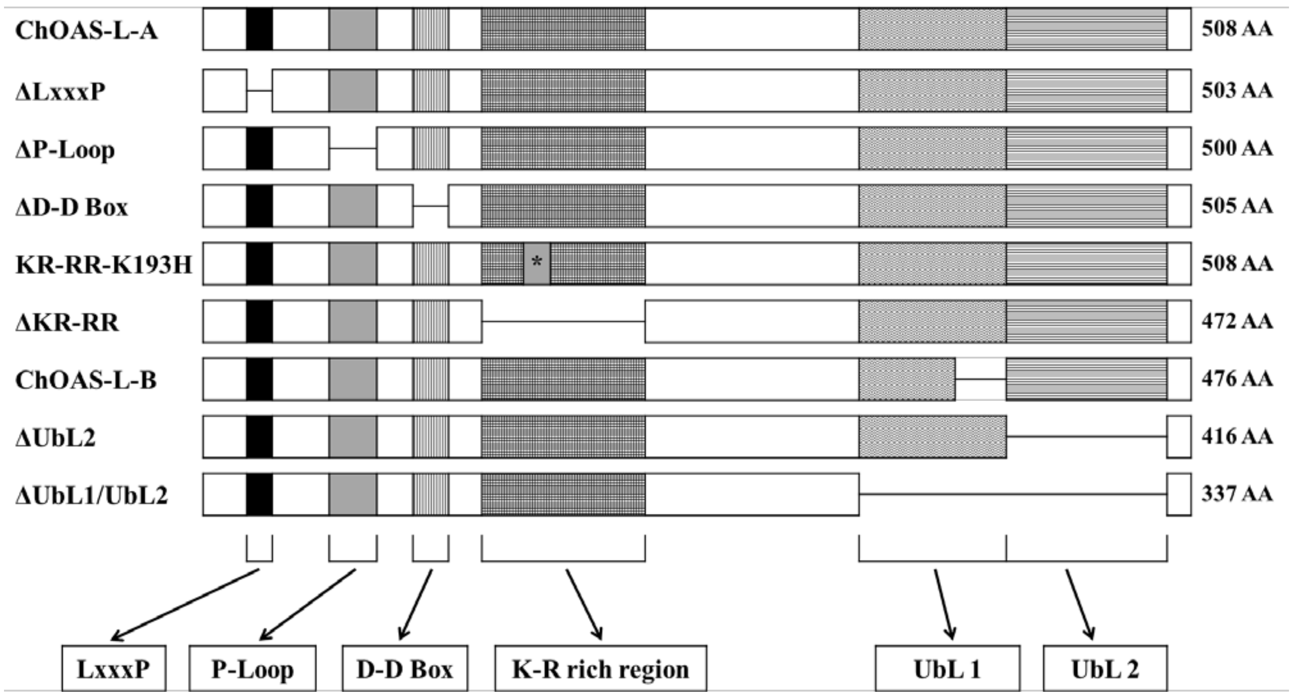

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Fig. 2-A

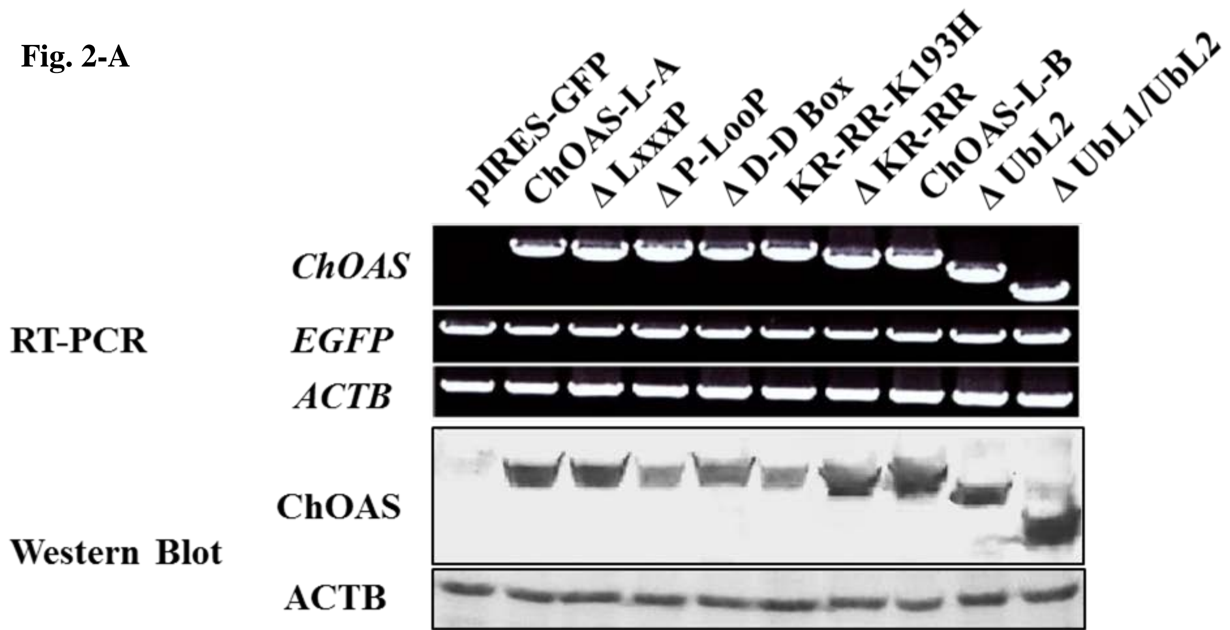

Fig. 2-B

pIRES-GFP ChOAS-L-A ChOAS-L-B Δ LxxxP Δ P-LooP Δ D-D Box KR-RR-K193H Δ KR-RR Δ UbL2 Δ UbL1/UbL2

pppA (pA)4

pppA (pA)3

pppA (pA)2

pppApA

ATP

1 **Fig 3-A**

2

3 Mock transfection | pIRES-GFP | ChOAS-L-A | Δ LxxxP | Δ P-LooP | Δ D-D Box | KR-RR-K193H | Δ KR-RR | ChOAS-L-B | Δ UbL2 | Δ UbL1/UbL2

4 RT-PCR *ChOAS(s)* *EGFP*

5 *ACTB*

6 Before electroporation | 72h post electroporation

BHK-21 cells

7

8

9

10 **Fig 3-B**

11 **Amplification of repWNV/SEAP replicon**

12

BHK-21 Cell

SEAP activity (µg/ml): 0, 1, 2, 3, 4, 5, 6, 7, 8, 9

**$P<0.01$

*$P<0.05$

NS, **, *, *, *

24 hours | 72 hours

Hours post-electroporation

Mock transfection
pIRES-EGFP
ChOAS-L-A
ΔLxxxP
ΔP-Loop
ΔD-D Box
KR-RR-K193H
ΔKR-RR
ChOAS-L-B
ΔUbL2
ΔUbL1/UbL2

1

2 Fig 6A

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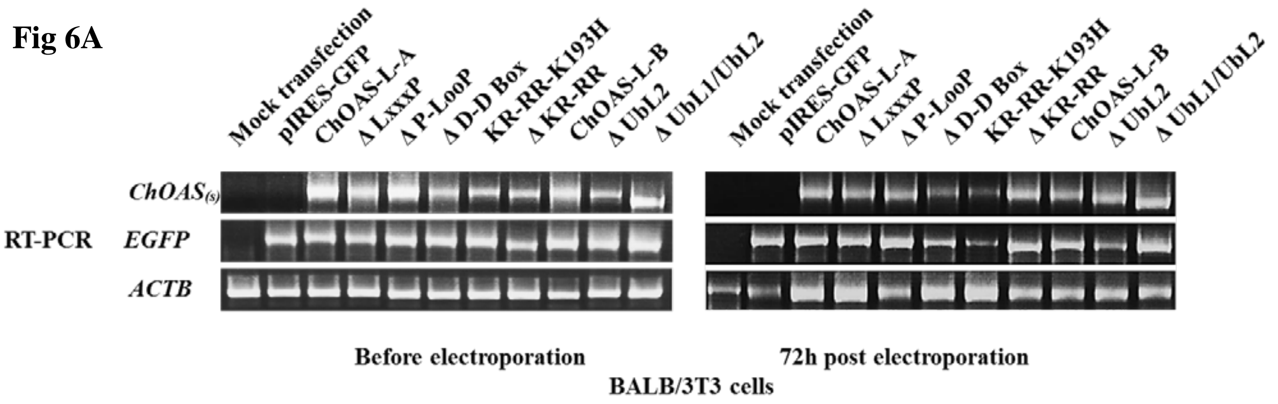

8

Fig 6B

9

10

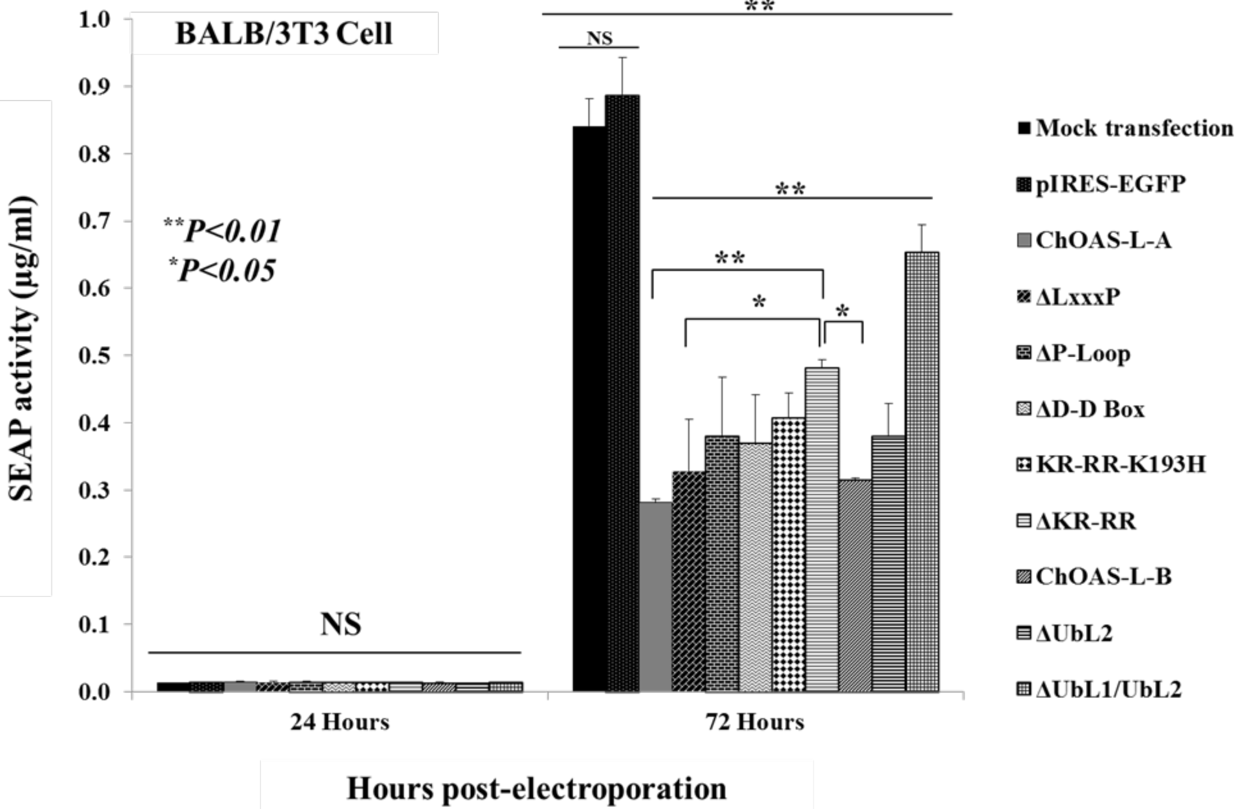

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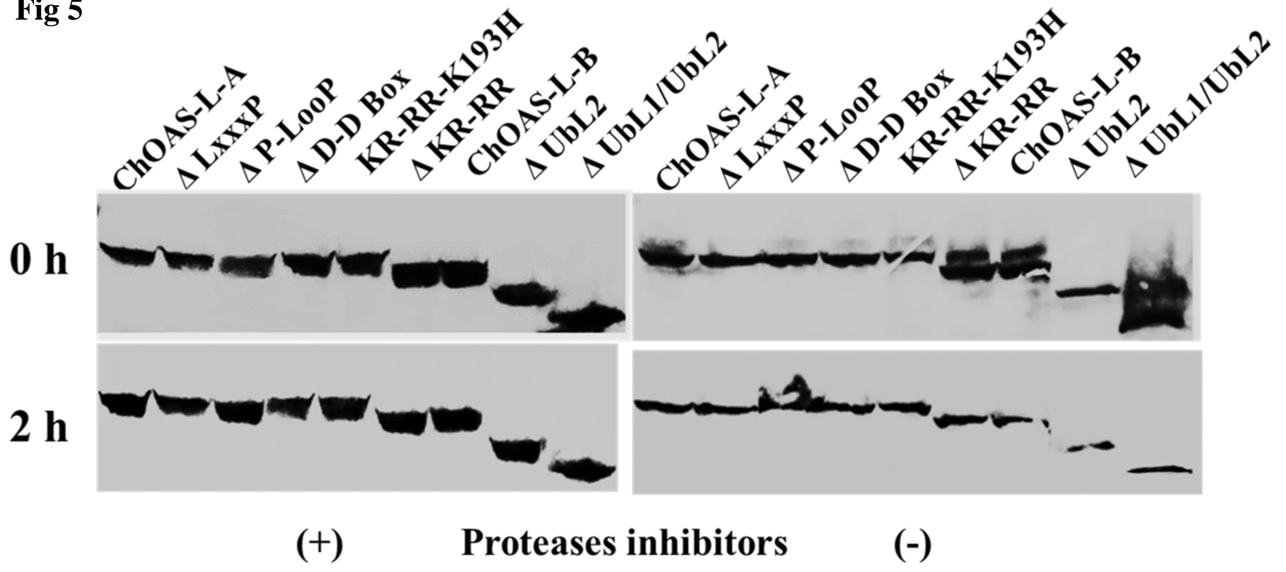
Fig 5



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3 **Fig. 7**
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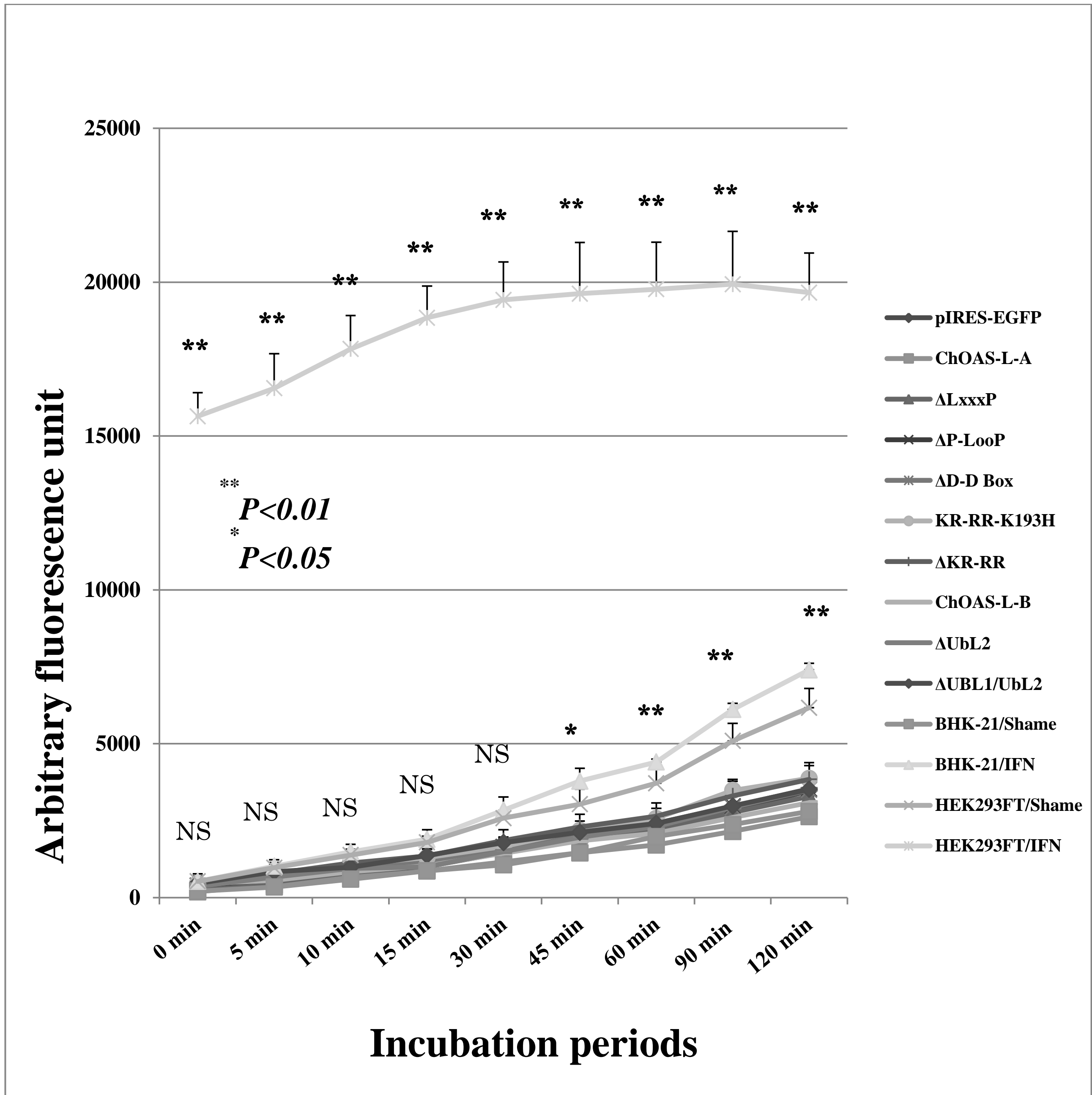

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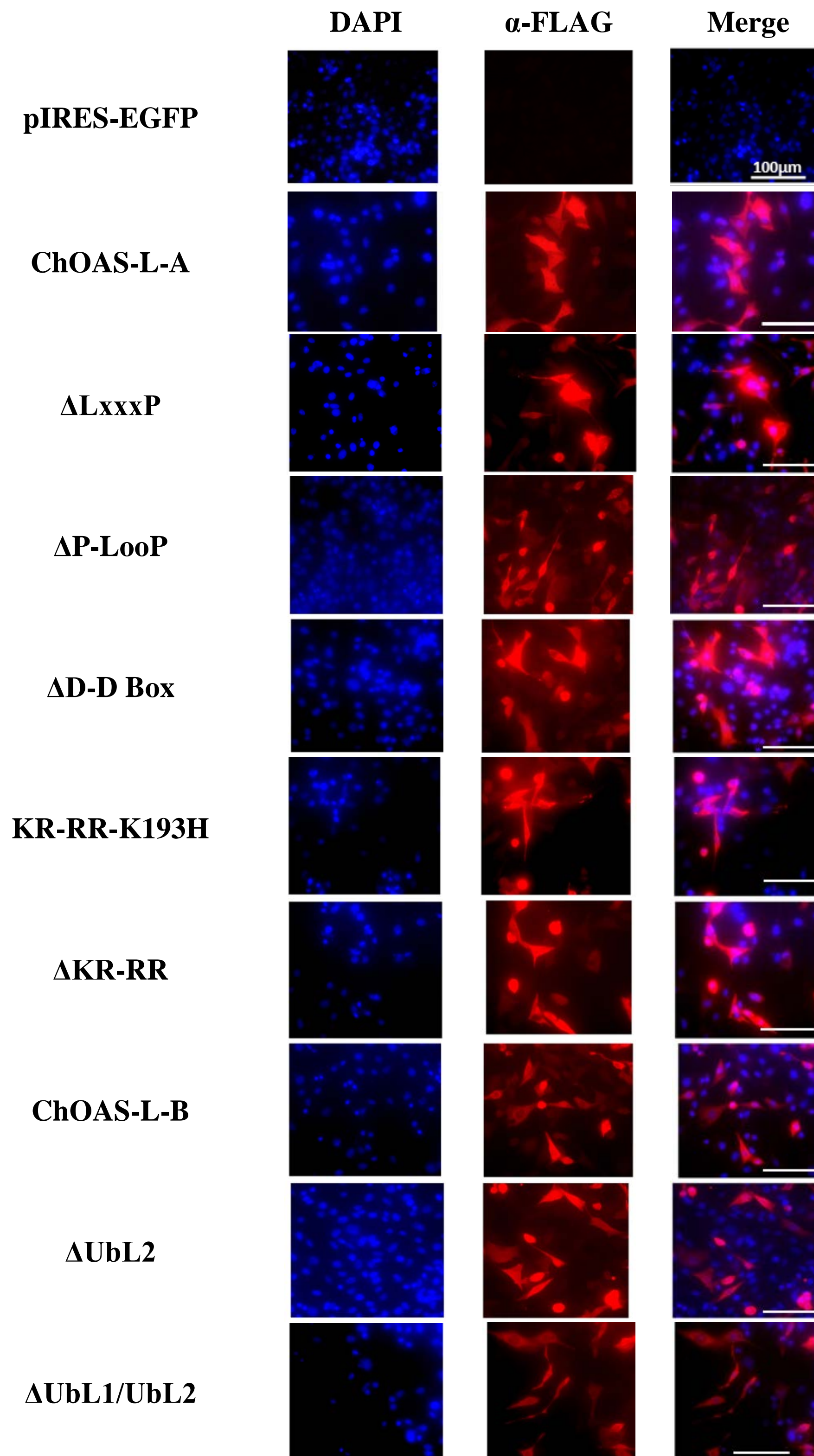
1 **Fig 4**



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