K160 in the RNA-binding domain of the orf virus virulence factor OV20.0 is critical for its functions in counteracting host antiviral defense

Guan-Ru Liao\textsuperscript{a}, Yeu-Yang Tseng\textsuperscript{b}, Ching-Yu Tseng\textsuperscript{a}, Ying-Ping Huang\textsuperscript{c}, Ching-Hsiu Tsai\textsuperscript{c}, Hao-Ping Liu\textsuperscript{d}, Wei-Li Hsu\textsuperscript{a#}

\textsuperscript{a}. Graduate Institute of Microbiology and Public Health, National Chung Hsing University, Taichung, Taiwan
\textsuperscript{b}. WHO Collaborating Centre for Reference and Research on Influenza, VIDRL, Peter Doherty Institute for Infection and Immunity, University of Melbourne, Melbourne, Victoria, Australia
\textsuperscript{c}. Graduate Institute of Biotechnology, National Chung Hsing University, Taichung, Taiwan
\textsuperscript{d}. Department of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan

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Abstract

The OV20.0 virulence factor of orf virus (ORFV) antagonizes host antiviral responses. One mechanism through which it functions is by inhibiting activation of the dsRNA-activated protein kinase R (PKR) by sequestering dsRNA and by physically interacting with PKR. Sequence alignment indicated that several key residues critical for dsRNA-binding were conserved in OV20.0, and their contribution to OV20.0 function was investigated in this study. We found that residues F141, K160, R164 were responsible for the dsRNA-binding ability of OV20.0. Interestingly, mutation at K160 (K160A) diminished the OV20.0–PKR interaction and further reduced the inhibitory effect of OV20.0 on PKR activation. Nevertheless, OV20.0 homodimerization was not influenced by K160A. The contribution of the dsRNA-binding domain and K160 to the suppression of RNA interference by OV20.0 was further demonstrated in plants. In summary, K160 is essential for the function of OV20.0, particularly its interaction with dsRNA and PKR that ultimately contributes to the suppression of PKR activation.

Keywords: orf virus, OV20.0; double-stranded RNA; PKR; innate immunity.

Abbreviations: orf virus, ORFV; double-stranded RNA, dsRNA; RNA binding domain,
**Introduction**

Orf virus (ORFV) belongs to Parapoxvirus genus of *Poxviridae* family. Sheep and goats are the nature host of ORFV, and occasionally the infection could spill over to other animal species including humans. Acute pustular lesions around the mouth and nares are the typical clinical signs caused by ORFV infection [1, 2]. OV20.0 is a virulence factor of ORFV; it acts as an antagonist of the interferon (IFN) response via multiple mechanisms [3, 4], including inhibition of dsRNA-activated protein kinase R (PKR) activation by sequestering dsRNA and by physically interacting with PKR [3]. Previous studies identified two isoforms of the OV20.0 protein, a full-length protein and an N-terminal truncated shorter form (sh20) translated from the downstream ATG codon, which share similar function in cell models [3].

Sensing foreign nucleic acid is a crucial step for the initiation of innate immune responses. Importantly, nucleic acids are derived not only from host cells but also from pathogens [5]. In particular, viral infections often lead to the excessive accumulation of double stranded RNA (dsRNA), known as pathogen-associated molecular patterns (PAMPs), originating from replication intermediates of RNA viruses or secondary structures of viral RNA transcripts [6, 7]. For DNA viruses, e.g., vaccinia virus (VACV), the viral RNA transcript of overlapped early genes from opposite orientations hybridizes to form long dsRNA [8, 9]. The dsRNA produced during infection can be detected by the cellular dsRNA sensor of pattern recognition receptors (PRRs), including retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated...
protein 5 (MDA5), and Toll-like receptor 3 (TLR3), which sequentially activates cellular antiviral pathways, resulting in the release of IFN and IFN-stimulated genes (ISG) [5]. Several cellular dsRNA binding proteins (DRBPs) participate in antiviral defense of innate immunity. For example, PKR suppresses host translational machinery through the phosphorylation of translation initiation factor 2α (eIF-2α) to inhibit viral protein synthesis. On the other hand, host cells express adenosine deaminases acting on RNA 1 (ADAR1), one of the DRBPs, to balance cellular IFN homeostasis, which prevents MDA5 from sensing endogenous dsRNA and production of aberrantly high levels of IFN [10].

DRBP is defined as the protein harboring the dsRNA binding domain (RBD), also known as dsRNA binding motif (dsRBM) [11]. The functional domain of RBD is commonly composed of a compact αββα structure in which the two α-helices are packed against a three-strand anti-parallel β-sheet [12, 13]. RBD shows strong affinity to dsRNA, but not dsDNA, single-stranded RNA or DNA-RNA hybrids [14, 15]. This protein-RNA interaction is likely due to the conformation fitness between two molecules rather than being sequence-specific. The interaction relies on the accessibility of ribonucleotides in the A-form nature of the dsRNA helix and RBD of DRBPs [16-18]. Systematic analysis of different canonical RBDs has revealed three important regions for dsRNA interaction: region 1 in helix α1, region 2 in the loop joining the β1 and β2 strands, and region 3 at the N-terminal tip of helix α2 [19-21]. These three regions function as an interface responsible for dsRNA contacts. Among these three regions, several conserved residues and structures have been found to participate in dsRNA binding. For instance, the side chain of glutamic acid (E) in helix α1 in region 1, the GPxH motif in the β1-β2 loop, and positively charged residues in the KKxAK motif at the beginning of helix α2 are highly conserved in many RBDs, and their contributions to dsRNA binding have been proposed.
Over 30 DRBPs have been discovered in cells and viruses [24, 25]. Cellular DRBPs regulate multiple pathways, including innate immunity, development, translation, RNA editing, and RNA stability in cells [26]. Many viruses encode DRBPs such as influenza virus NS1, Ebolavirus VP35, VACV E3, and ORFV OV20.0 protein [3, 27]. Viral DRBPs contribute to counteracting the anti-viral defense. In particular, they commonly serve as IFN antagonists by sequestering viral dsRNA from cellular RNA sensors, and therefore, the antiviral reaction is suppressed to favor viral replication. Furthermore, most viral DRBPs are virulence factors. For instance, influenza virus with a deficient NS1 gene induces more IFN during infection and shows an attenuated phenotype [28].

In this study, several conserved residues in RBD of OV20.0 were identified, and the importance of these residues on biological functions of OV20.0 was further examined. Furthermore, a key residue, namely K160, which had a great impact on PKR activation and dsRNA binding was identified, and its novel role in regulating RNA silencing was evidenced in a transgenic plant system.

Materials and Methods

Cell. Human embryonic kidney 293T cell (293T) was cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% of penicillin-streptomycin. Cells were maintained at 37°C with 5% CO₂.

Plasmids. 3x FLAG tagged OV20.0 with desired point mutation expressing plasmids were generated as follows: DNA fragment containing CMV enhancer, CMV promoter and OV20.0 sequences were amplified from OV20.0-3x FLAG-pCMV14 [29] and DNA fragment of SV40
poly(A) signal were amplified from p2Luc plasmid by polymerase chain reaction (PCR). Subsequently, two DNA fragments were cloned into pUC19 vector linearized with Hind III and EcoR I by In-Fusion cloning method (Clontech), resulting in the plasmid, namely OV20.0-pUC19-3x FLAG. For the following sub-cloning, the OV20.0 sequence with a newly introduced Sph I site (at residue of 167 without changing amino acid sequence) was firstly generated by site-directed mutagenesis. Subsequently, two strands of oligos containing K160A, K161A, and R164A mutation were annealed and cloned into Sac II and Sph I linearized OV20.0 plasmid. OV20.0 with F141A were generated by site-directed mutagenesis using PCR from pUC19-OV20.0-3x FLAG plasmid. Cloning of OV20.0-GFP plasmid was as previously described [3]. GFP fragment was amplified from pEGFP plasmid (Clontech) and cloned into EcoR I and Sal I sites of pUC19-OV20.0-K160A, resulting in plasmid, designated OV20.0-K160A-GFP.

Binary OV20.0 expressing plasmids used in plant system: DNA fragments of OV20.0 and 3xFLAG were amplified from OV20.0-pUC19-3xFLAG construct by PCR. The two fragments were fused by overlap extension PCR. The overlapped OV20.0 sequence with additional BamH I and Kpn I recognition sequences at the 5’ and 3’ region of OV20.0, respectively, was then cloned into Xba I and Xho I sites of binary plasmid, pBI-EGFP, resulting in the plasmid pBI-OV20.0-3xFLAG. The OV20.0 with K160A mutation or C-terminus deletion (ΔC) was amplified from pUC19-OV20.0-K160A-3x FLAG, and subcloned into BamH I and Kpn I sites of pBI-OV20.0-3x FLAG, resulting in the plasmids pBI-K160A-3xFLAG or pBI-ΔC-3xFLAG, respectively.

The detection of silencing suppressor activity in plants. The effect of OV20.0 RNA silencing was evaluated in transgenic plant Nicotiana benthamiana, line that constitutively
expresses GFP [30]. In brief, agrobacteria carrying GFP alone, or in combination with either HcPro (the silencing suppressor control), or OV20.0, were cultured at 30°C with the addition of 10 mM MES pH 5.6 and 450 μM acetoxyringone for 2 days. The agrobacteria expressing either HcPro, or OV20.0 grown to OD$_{600nm}$=1 was suspended with buffer [0.1 M MgCl$_2$, 10 mM MES pH 5.6 and 450 μM acetoxyringone] and then were mixed with that carrying GFP in a 1:1 ratio, whereas for the GFP control group, the bacteria were adjusted to OD$_{600nm}$=0.5, and infiltrated into the leaves of *Nicotiana benthamiana* 16C line, respectively. The GFP signal was observed at 5 days post agroinfiltration and the image was captured by camera with 530 nm filter (Nikon, Japan).

**Detection of RNA interference in mammalian cell.** Human nasopharyngeal carcinoma HK-1 cells, in which TNFAIP2 (NM_006291) was stably knocked down by expressing a lentivirus-delivered TNFAIP2 shRNA in a pLKO.1-puro backbone (sh-TNFAIP2; target sequence: CGTCTCACCAAAAGGAAGAA) purchased from the RNAi Core Lab, Academia Sinica, Taiwan, were seeded in a 12-well plate one day prior to transfection. HK-1 cells stably expressing pLKO.1-puro Luciferase shRNA (sh-Luc) against the firefly luciferase were used as a control. The empty vector (EV), OV20.0 (WT), or K160A-expressssing construct was transfected into HK-1 sh-TNFAIP2 or sh-Luc stable cell clones by using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific). Cell lysates were harvested at 30 hours post-transfection, and TNFAIP2 protein levels were validated by Western blotting analysis with an antibody specific to TNFAIP2 (H-654, sc-30138, Santa Cruz).

**Immunoprecipitation (IP) with FLAG antibody.** FLAG-IP was previously described [31]. In brief, 1 x 10$^6$ of 293T cells were seeded in the 6 well plate one day prior to transfection. On the next day, transfection was performed by Lipofectamine 2000 (Thermo) following the
manufacturer’s instruction. At 24 hour-post transfection, cells were harvested in lysis buffer [50 mM Tris HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA and 1% Triton X-100] and the cell lysates were incubated with Anti-FLAG M2 Affinity Gel (Sigma-Aldrich, A2220) at 4 °C for overnight. Subsequently, the FLAG beads were washed 6 times with Tris-buffered saline (TBS) [50 mM Tris HCl, with 150 mM NaCl, pH 7.4]. Proteins were eluted by 1x sample buffer and analyzed by western blotting.

**Poly (I·C) pull down assay.** Poly (I·C) pull assay was conducted as previously described [29, 31]. Briefly, poly (I·C) beads (Sigma-Aldrich) were mixed with the cell lysate transiently expressing OVP20.0 and incubated at 4 °C for overnight. The poly (I·C) beads were then thoroughly washed with Wash buffer [TBS with 10% glycerol]. Proteins were eluted by sample buffer and verified by western blot analysis.

**Western blotting.** Harvested cell lysates were mixed with protein sample dye and heated at 100 °C for 10 minutes. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane (GE Healthcare). Transferred membranes were blocked in 5% skimmed milk, followed by immunoblotting with specific antibodies against target proteins. In particular, the antibodies (and corresponding dilutions) were the following: FLAG antibody (1:2,000) (F7425; Sigma-Aldrich), PKR antibody (1:1,000) (ab32052; Abcam), phosphor-PKR (PKR-p) antibody (1:1,000) (ab32036; Abcam), ADAR1 antibody (1:500) (sc-73408; Santa Cruz Biotechnology), actin antibody (1:5,000) (NB600-501; Novus Biologicals), actin of *Nicotiana benthamiana* antibody (1:5,000) [32], EGFP antibody (1:2,000) (YH80005; Yao-Hong Biotechnology, Taiwan), and F1L antibody (1:2000) [33]. Secondary antibodies-conjugated horseradish peroxidase (HRP) was diluted in 5% skin milk and incubated with membranes for 1 hour at room temperature. After 6-time wash with
phosphate-buffered saline with 0.1% tween 20, the PVDF membrane was treated by ECL substrate (Merck Millipore), and the signal was detected by chemiluminescence.

**RBD sequence alignment.** RBD sequences of OV20.0 (ABY41265.1), PKR (XP_011531289.1), PACT (AAC25672.1), and ADAR1 (NP_001102.3) were obtained from GenBank. Multiple-sequence alignment was conducted by the Clustal Omega online service (https://www.ebi.ac.uk/Tools/msa/clustalo/). Note, an asterisk (*) indicates the position which contains fully conserved residue, whereas a colon (:), or a period (.) indicates conservation between groups with strongly or weakly similar properties and that was defined as: roughly equivalent to scoring > 0.5, or the scoring between ≤ 0.5 and > 0 in the Gonnet PAM 250 matrix, respectively.

**Confocal microscopy.** 293T cells were transfected with either OV20-GFP, K160A-GFP, or GFP expressing plasmid. To validate the colocalization of OV20.0 and ADAR1, OV20-GFP or K160A-GFP along with ADAR1-mCherry [31] expressing plasmids were transfected into 293T cells. 24 hr post transfection, cells were fixed by 10% formaldehyde for 10 min and followed by Hoechst staining. Images were taken by confocal laser scanning microscope (FV3000, Olympus).

**Results**

**Conserved residues of OV20.0 RBD in dsRNA binding ability**

Binding to dsRNA is a native property of DRBPs which is important for viral DRBPs to antagonize IFN production as evidenced by influenza virus NS1 [34]. Sequence alignment of the RBD sequences of OV20.0 and cellular DRBPs, which interact with OV20.0 [29], indicated several conserved residues spanning the entire RBD. In brief, tyrosine (Y) or phenylalanine (F)
in β1 and β2, respectively, which were not located at regions directly contacting dsRNA, were highly conserved. In region 3, three conserved basic amino acids, including two lysine residues (K160, K161) and one arginine and (R164) were noticed.

Subsequently, the impact of these four residues on dsRNA binding was examined by substitution with alanine (i.e., F141A, K160A, K161A and R164A). The well-known DRBP, PKR, was used as a positive control and PKR interacted with the synthetic dsRNA, poly (I·C) (Fig. 2A). Of note, OV20.0 was translated into two isoforms via the leaky scanning mechanism [24]. Consistently, both full-length and short form (harboring a complete C-terminal RBD) of WT OV20.0 could interact with dsRNA, poly (I·C) (Fig. 2A). Among the four mutations, K161A only mildly reduced the dsRNA binding ability of OV20.0. Of note, OV20.0 with the alanine substitution at F141, K160 or R164, significantly reduced the interaction of OV20.0 with poly (I·C) RNA (p value<0.0001), suggesting that these residues are important for the dsRNA binding activity of OV20.0 (Fig. 2A and 2B).

**The role of K160 of OV20.0 in the interaction and activation of PKR**

It is known that OV20.0 counteracts with the function of PKR via multiple mechanisms including dsRNA sequestering and intermolecular protein-protein interaction [3]. Therefore, we wondered whether these conserved residues could affect the OV20.0-PKR interaction and ultimately reduce the inhibitory effect on PKR activation. First, we investigated the potential impact of these conserved residues on PKR interaction. Although, the interaction of PKR with F141A, K160A, K161A, or R164A of OV20.0 was significantly weaker than that with WT OV20.0, the point mutation of OV20.0 remained its ability to interact with PKR (Fig. 3A). Of note, K160A diminished PKR interaction to a greater extent than other mutants (Fig. 3A). The results from Fig. 2A and 3A indicated that K160 of OV20.0 is an important residue for both
dsRNA binding and PKR interaction. Thus, we further investigated the potential impact of K160 on OV20.0 biofunction. The effect of K160A mutant on PKR activation was firstly evaluated. As shown in Fig. 3B, the PKR phosphorylation level was significantly higher in cells expressing K160A mutant compared with that of WT (Fig. 3B), indicating that K160A partially reduced the inhibitory effect of OV20.0 on PKR activation.

The involvement of K160 in interactions of OV20.0 with other cellular DRBPs and homodimerization

We demonstrated that ORFV OV20.0 interacts with several cellular DRBPs, including PKR and ADAR1 [3, 29, 31]. Hence, in addition to PKR, we further examined the interaction of K160A OV20.0 with ADAR1. As evidenced in IP experiment, ADAR1 were not associated with the K160A mutant (Fig. 4A), and cell distribution of K160A was not colocalized with ADAR1 (supplementary Fig. S1), suggesting that K160 of OV20.0 is the key residue contributing to the interaction of OV20.0 not only with PKR but also with ADAR1.

Formation of dimer is one of the known features of DRBPs. For instance, formation of homodimer is required for the function of PKR [35] and ADAR1 [36]. It has been shown that the C-terminus spanning residues 100-190 of VACV E3, an ortholog of OV20.0, represent the minimal region required for the formation of E3-homodimer [37]. Due to the sequence similarity, it is possible that OV20.0 shares this feature. Thus, it is interesting to investigate this possibility and extend it to the possible impact of K160 on OV20.0 dimerization. Two sets of OV20.0 fusion proteins that were expressed with either a GFP or a FLAG tag were used to monitor homodimerization. In this scenario, the coexistence of OV20.0-GFP fusion proteins by means of FLAG-IP assay would indicate the formation of OV20.0 dimer. As shown in Fig. 4B, the full-length OV20.0-GFP was successfully pulled down by WT OV20.0-FLAG, suggesting the

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The presence of OV20.0 dimer. Both WT- and K160A-FLAG were associated with OV20.0-GFP, but this association was not found in ΔC-FLAG. Collectively, these results showed that homodimerization of OV20.0 requires C-terminal RBD and is independent of K160 (Fig. 4B), while heterodimerization of OV20.0 with other cellular DRBPs relied on the K160 residue (Fig. 4A).

We further examined whether alteration of K160 residue affects cellular distribution of OV20.0. OV20.0-GFP localized in both cytoplasm and nucleus, within distinct subnuclear domains (Fig. 5, top row). Surprisingly, with K160A substitution, K160A-GFP expressed throughout the cell, which share a similar pattern to GFP control (Fig. 5, middle and bottom row, respectively).

**K160 is critical for suppression of RNA silencing mediated by OV20.0 in the plant system**

Mérai and Silhavy et al. proposed that sequestering dsRNA from its cellular counterpart could be a general strategy for plant RNA viruses to suppress RNA silencing mechanism [38, 39]. Although similar phenomenon was also observed in animal viruses in mammalian cells, two different groups had reported contradictory results that indicated VACV E3 may or may not inhibit RNAi in mammalian cells [40, 41]. As aforementioned, K160 of OV20.0 plays an indispensable role in dsRNA binding (Fig. 2), we thus further investigated whether OV20.0 acts as a bona fide dsRNA sequester in vivo simply by means of a plant model. Initially, whether full-length OV20.0, as a DRBP, could possibly interfere the RNA silencing was determined. As shown in Fig. 6A, GFP gene infiltration reduced the intrinsic expression of GFP from the transgenic plant. Noticeably, co-infiltration of OV20.0 restored GFP expression to a similar extent as in the positive control (PC), the silencing suppressor (i.e. HcPro). Subsequently, the effect of K160 on suppression of silencing activity was further investigated. To address this
issue, the agrobacteria carrying GFP was co-infiltrated with either WT, ΔC (OV20.0 with C-terminal RBD deletion), or K160A of OV20.0 into 16C leaves. Unlike that in WT OV20.0 and the positive control of the suppressor (PC), the GFP signal in the ΔC filtrated region was depleted to a similar level as those infiltrated with GFP control alone. Noticeably, the GFP signal in the region infiltrated with K160A was comparable to the region expressing ΔC (Fig. 6B). The overall GFP expression in infiltrated leaves was further validated by immunoblot analysis. The result showed that the GFP level in OV20.0 was higher than that in both ΔC and K160A (Fig. 6C). The effect of OV20.0 in RNA silencing was also investigated in a mammalian system. As indicated in Fig. 6D, the expression of TNFAIP2 (also named B94) was decreased in HK-1 cells stably expressing shRNA that targets TNFAIP2, as compared with HK-1 cells stably expressing control shRNA (sh-luc). In the presence of OV20.0, TNFAIP2 expression was partially restored (Fig. 6D), while this effect was not statistically significant. Collectively, OV20.0 could serve as a viral suppressor of RNA silencing (VSR) in plant systems, and mutation of K160 diminishes this activity.

Discussion

The regulatory role of DRBPs has been extensively described in several cellular biological processes, for instance, innate immunity, DNA repair, and gene expression [26]. Several consensus sequences have been identified in RBDs, and the structural features of DRBPs have been observed to be well conserved during evolution [25, 42]. However, the key residue contributing to the function of ORFV OV20.0 has not yet been depicted. To the best of our knowledge, this study identified, for the first time, the key residue (K160) of OV20.0 responsible for dsRNA binding and the biological processes that are influenced by the K160 of OV20.0.

It has been proposed that the conserved residues on RBDs are responsible for not only
optimization of dsRNA binding but also the maintenance of a stable protein conformation. Indeed, as evidenced by poly (I:C) pull-down assay, the residues F141, K160, and R164, located in the RBD of OV20.0 (Fig. 1) are critical for dsRNA binding (Fig. 2). Among the four amino acids, F141 is located in the proximity of region 2, while the rest of the three conserved residues are within region 3 of the RBD (Fig. 1). Of note, based on the structure simulation of the Drosophila DRBP Staufen, the F32 residue in the RBD3 of Staufen does not fall into the interface of dsRNA binding [12]. Nevertheless, substitution of F141 completely impaired the dsRNA binding activity of OV20.0 (Fig. 2A and 2B), which is consistent with findings that the F32 (corresponding to F141 of in the RBD3 of Staufen protein is essential for dsRNA binding [12]. Moreover, the importance of highly conserved residues with aromatic side chains has been proposed, such as phenylalanine residues (e.g., F127 indicated with a colon in Fig. 1, and F141), which are mapped within the β1 strand and the β2 strand in RBD of OV20.0, respectively. Despite an indirect contact with dsRNA, the aromatic group maintains the optimal orientation of the key positively charged residues for dsRNA binding [19, 22]. In addition, the position of these aromatic rings may possibly contribute to the stability of the overall functional conformation [25].

In addition to the aforementioned phenylalanine residues, the KKxAK motif within region 3 (Fig. 1) of RBD is also highly conserved among DRBPs. These three lysine residues contact the phosphodiester backbone of RNA strands across the major groove of the dsRNA helix [25]. Similarly, OV20.0 harbors this conserved motif (KKxAR, at residues 160-164), despite one alteration at residue 164 where the third lysine (K) is replaced by arginine (R). McMillan et al. showed that the substitution of one or more lysines with arginine or glutamine did not affect the binding ability of RBDs [22]. Therefore, without a canonical KKxAK motif, OV20.0 retains
dsRNA binding ability (Fig. 2A). It has been shown that the amide proton of the first lysine could form a direct hydrogen bond with a nonbridging oxygen atom of the RNA backbone [25]. This is in agreement with our data showing that OV20.0 with a mutation at the K160 residue nearly lost the entire dsRNA binding ability (Fig. 2A), indicating the importance of the first lysine.

DRBPs interact with dsRNA and associate with DRBPs, resulting in the formation of homodimer or heterodimer, which could mutually modulate the function of the two counterpart proteins [26]. Dimer formation was also demonstrated in PKR; it forms a homodimer and associates with TRBP, PACT and ADAR1 [43-45]. Previous studies have described how ORFV OV20.0 interacts with multiple DRBPs, including PKR, PACT, and ADAR1, and regulates the function of PKR [3, 29, 31]. The results of the current study further revealed that the K160 residue is critical for not only dsRNA binding (Fig. 2A), but also interaction with cellular partners, including PKR and ADAR1 (Fig. 3 and 4A, respectively). Nevertheless, despite of the remarkable reduce in PKR interaction, the K160A mutant OV20.0 only partially diminished the inhibitory effect on PKR activation, as compared with WT (Fig 3B). It was proposed that N-terminal Z DNA binding domain (ZBD) of Vario1a virus E3 is required for PKR inhibition [46]. We proposed that the ZBD located in the N terminus of OV20.0, and regions for dsRNA binding and PKR interaction all contribute to PKR inhibition. Recently, the two residues (R35 and R46) of influenza virus NS1 in RBD were identified as crucial signatures for PKR interaction [47]. As with K160A of the OV20.0 mutant, alteration of amino acids R35 and R46 failed to permit interaction with PKR, and the protein was unable to bind dsRNA, suggesting that the dsRNA binding activity of viral DRBP is controlled by several residues, especially basic amino acids.

Similarly, VACV E3 also utilizes two residues, K167 and R168 (equivalent to K160 and K161 of OV20.0), for dsRNA binding and PKR interaction [48]. Of note, in addition to PKR, K160
contributes to the interaction of OV20.0 with ADAR1 (Fig. 4A), suggesting an essential role of K160 in the intermolecular association of OV20.0 with its partners, including dsRNA, and the DRBPs identified herein. However, as shown in Fig. 4B, K160 is not responsible for the formation of OV20.0 homodimer. This phenomenon was also observed in the model of influenza NS1 [47]. Mutagenesis at R35 or R46 of NS1, the key amino acid for dsRNA binding, did not affect its dimerization, indicating that dimerization of interacting partners or itself could be regulated by independent residues.

The two OV20.0 interacting cellular proteins, PKR and ADAR1, mainly localize in nucleus and cytoplasm, respectively. Moreover, the association of OV20.0 with the two DRBPs redirects the subcellular distribution of these proteins [29, 31]. As shown in Fig 5, mutation at K160 had a distinct expression pattern from that of WT OV20.0; in particular, the subnuclear punctate structure was only observed in WT OV20.0. Of note, the particular subnuclear domains represent the co-localization of OV20.0 with ADAR1 (supplementary Fig. S1) [31]. Considering that K160A failed to interact with ADAR1 (Fig 4A), it strengthens our previous finding that the cellular distribution of OV20.0 relies on protein-protein interaction.

RNA silencing that mediates posttranscriptional RNA decay is a phenomenon triggered by dsRNA and numerous DRBPs. It was regarded as an innate antiviral response initially identified in plants [49]. Nevertheless, a wealth of information has indicated that viruses have evolved multiple mechanisms to counteract such a defense system by expressing viral suppressor of RNA silencing (VSR), as demonstrated both in vaccinia virus and influenza viruses that encode DRBPs which abrogate RNA silencing in Drosophila cells [40, 50]. It was further proven that the RBD of influenza NS1 protein is essential for such a nucleic acid-based antiviral response [50]. The RNA silencing pathway has been identified in many eukaryotic organisms including plants,
fish and mammals, but the initiation mechanisms may not be the same. Several experimental approaches have been well established in plants for the identification of novel VSR encoded by plant viruses and animal viruses [40, 51, 52]. In the present study, OV20.0 remarkably restored the RNA silencing mediated by transgenic GFP in the *Nicotiana benthamiana* 16C line to a similar extent as the positive control of the silencing suppressor, HcPro (Fig. 6). Generally, the RNA silencing process in plants is initiated by production of dsRNA [51]. Moreover, a large number of VSRs in plants have been found to bind siRNA and/or long dsRNA precursors to inhibit siRNA utilization [53]. Consistently, as shown in Fig. 6B, the effect of OV20.0 was markedly reduced when K160 was mutated, indicating the importance of the dsRNA binding ability of OV20.0. In plants, VSRs block the silencing pathway via several mechanisms. For instance, VSRs can suppress the dsRNA production by targeting plant RNA-dependent RNA polymerases (RDRs), which convert aberrant viral single-stranded RNAs (e.g., GFP mRNA in the transgenic 16C line) to dsRNA that is then subjected to cleavage by Dicer-like proteins to produce viral siRNA [54]. Therefore, the inhibitory effect of OV20.0 in the plant 16C line could potentially result from 1) the interaction with long dsRNA substrate that prevents long RNA from being processed and/or 2) the interference with RDR function that mediates the antiviral silencing pathway. Since OV20.0 bearing the K160A mutation and deletion of the C-terminus (ΔC) failed to bind long dsRNA, poly (I-C), it is likely that OV20.0 sequesters the dsRNA from triggering RNA silencing.

Of note, in this study, inhibition of RNA silencing mediated by OV20.0 was clearly demonstrated in a plant model, while OV20.0 showed no impact on shRNA effect in mammalian system (Fig. 6D). This discrepancy phenomenon has been described in other viral proteins. Despite the silencing suppressor role of vaccinia E3 demonstrated in the insect...
system [50], transient expression of vaccinia E3 protein has no impact on the shRNA-induced silencing of STAT3 gene in human 293 cells [41]. Similarly, the inhibitory effect of NS1 on siRNA was shown in plant systems [52, 55], but not in mammalian cells [56]. It was further suggested that the suppression of siRNA in plants system was due to sequestering siRNA by NS1. It coincides with our finding that OV20.0 serves as a potent VSR, while K160 mutant deficient in RNA binding (Fig.3B) failed to restore the expression of GFP in plants (Fig. 6C). As aforementioned, OV20.0 interacts with dsRNA and several cellular factors, including PKR, PACT, ADAR1, it is reasonable to suspect that the net effects of OV20.0 on sequestering siRNA could be greatly diminished due to the complicated intermolecular interactions in mammalian system.

In sum, our study illustrates the significant effect of a single residue (K160) in the RBD of OV20.0 on its versatile functions, including dsRNA sequestration and PKR inhibition. Moreover, the suppressive role of OV20.0 on RNA silencing was demonstrated for the first time. However, this negative regulatory effect was significant in plants but not in a human cell system.

**Author Contributions:** Planned experiments, WLH and GRL; performed experiments, GRL, CYT, HPL, and YPH; provided key experimental reagents, CHT and HPL; analyzed data, WLH, GRL, CYT, and YYT; wrote the paper, WLH, GRL, and YYT; writing-review and editing, WLH, GRL, YYT; visualization, WLH, GRL, and YYT; supervision, WLH; project administration, WLH; funding acquisition, WLH.

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Data Availability: The data that support the findings of this study are available from the corresponding email: whhsu@dragon.nchu.edu.tw] upon reasonable request.

References


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Figures and Figure legends

**Fig. 1.** Sequence analysis of RBDs of OV20.0, PACT, PKR, and ADAR1. Sequences of the RBDs of OV20.0, PACT, PKR, and ADAR1 were aligned. The dashes represent gaps in the alignment. The asterisks indicate fully conserved residues. An asterisk (*) indicates the position that contains a fully conserved residue, whereas a colon (:) or a period (.)
indicates conservation between groups with strongly or weakly similar properties, respectively. The conserved residues tested in this study were highlighted with their corresponding numbers on the top of sequences. The three regions of interaction with dsRNA are also indicated.

**Fig. 2. Specific residues critical for dsRNA binding of OV20.0.** (A) Plasmids expressing FLAG-tagged wild-type (WT) OV20.0, OV20.0 mutants including the four mutants bearing single point alteration (F141A, K160A, K161A, or R164A), or the deletion of C-terminal OV20.0 (ΔC) were transfected individually into human 293T cells. The transfected cells were harvested for the poly(I:C) pull-down assay (right panel), and 10% of the whole cell lysate was kept as the input control (left panel). Actin and PKR served as the internal control and the positive control for dsRNA binding, respectively. (B) The results of three independent experiments were quantified and plotted. The interaction of wild-type OV20.0 with dsRNA was arbitrarily set as 1.
presented as the means ± SD from three independent experiments. Lane M, molecular size marker. Note: ns indicates no significance (p>0.05) as compared with WT.

Fig. 3. The K160A residue is critical for the biological function of OV20.0 in PKR interaction and inhibition of PKR activation. (A) Specific residues responsible for the interaction of PKR were monitored in cells transiently expressing FLAG-tagged WT OV20.0 and F141A, K160A, K161A, R164A mutation of OV20.0 or empty vector (EV) by the FLAG-IP assay and western blot analysis with anti-FLAG and anti-PKR antibodies. 10% of the cell lysate serves as the input control. Lane M, molecular size marker. The interaction of wild-type OV20.0 with PKR was arbitrarily set as 1. Values are presented
as the means ± SD from three independent experiments. Student’s t test was applied to evaluate significant differences as compared with WT. **, ***, **** indicated p value <0.01; <0.001; <0.0001, respectively. (B) To further determine the effect of K160A on PKR activation, a set of plasmids expressing GFP, GFP-tagged WT or K160A of OV20.0 were individually transfected into 293T cells for 24 hr followed by poly (I·C) (1 μg/ml) treatment for 4 hr. Total proteins were collected for western blot analysis using antibodies specific to phosphorylated PKR (pPKR), basal PKR (PKR), OV20.0, or actin. The experiments were conducted in three independent repeats. The effect relative to wild-type OV20.0 (WT) was plotted and arbitrarily set as 1 (right panel). Level of phosphorylated PKR was normalized by actin and basal PKR. *, ** indicated p value <0.05; <0.01, respectively.

**Fig. 4. Residue K160 of OV20.0 is important for OV20.0-DRBPs interaction.** (A) Plasmids expressing FLAG-tagged wild-type and the mutants of OV20.0 (K160A, and ΔC), or empty vector (EV), were individually transfected into human 293T cells, followed by the FLAG-IP assay. (B) Dimerization of OV20.0 was determined by co-expression of two sets of OV20.0 constructs, of which OV20.0 was expressed with an FLAG or a GFP-tag fused at its C-terminus, followed by FLAG-IP. The collected pellets and 10% of
the cell lysate were used as the input control. Samples were separately subjected to western blot analysis using corresponding antibodies. Lane M, molecular size marker.

**Fig. 5. Residue K160 of OV20.0 determines its subcellular distribution.** Plasmids expressing GFP-tagged wild-type OV20.0, K160A OV20.0, or GFP were individually transfected into human 293T cells followed by confocal microscopy. Hoechst staining indicated nucleus.
Fig. 6. K160 is critical for suppression of RNA silencing mediated by OV20.0 in the plant system. (A) Leaves of the *Nicotiana benthamiana* 16C line, undergoing systemic RNA silencing of a GFP transgene, were infiltrated with Agrobacteria carrying the GFP gene alone or with either the positive control of silencing suppressor, HcPro (PC) or wild-type OV20.0 (OV20.0). (B) Leaves of *Nicotiana benthamiana* 16C line were infiltrated by Agrobacteria carrying GFP alone, or with either silencing suppressor, HcPro (PC) or wild-type OV20.0 (OV20.0), OV20.0 with C-terminus deletion (ΔC), and OV20.0 bearing the K160A mutation (K160A). The image was captured at 5 days post agroinfiltration. (C) Infiltrated leaves were harvested and subjected to western blot analysis using anti-GFP, anti-actin, and anti-FLAG antibodies. (D) HK-1 cells stably expressing shRNA targeting *TNFAIP2* (sh-B94) or control shRNA targeting a firefly luciferase (sh-luc) were transfected with EV, or constructs expressing WT or K160A of
OV20.0. The effect of OV20.0 on TNFAIP2 expression was monitored by Western blot analysis. The normalized TNFAIP2 level in the presence of OV20.0 proteins relative to EV was plotted and arbitrarily set as 1 (right panel).

Fig. S1. Cellular distribution of K160 and ADAR1. Plasmids expressing ADAR-tagged mCherry was co-transfected with GFP-tagged wild-type OV20.0, or K160A OV20.0 into human 293T cells, followed by confocal microscopy. Hoechst staining (blue fluorescence) indicated nucleus.
Author/s:
Liao, G-R; Tseng, Y-Y; Tseng, C-Y; Huang, Y-P; Tsai, C-H; Liu, H-P; Hsu, W-L

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