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Appropriate amount of W protein of *avian avulavirus 1* benefits viral replication and W shows strain-dependent subcellular localization

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ABSTRACT

In order to confirm the existence of W protein in *Avian avulavirus 1* (AAvV-1) infected cells, two monoclonal antibodies were prepared. The presence of W protein in cells infected with lentogenic genotype II strain La Sota or velogenic genotype VII strain SG10 was confirmed with immunofluorescence and western blotting assays. W_{SG10} localized to the cytoplasm, whereas $W_{La\ Sota}$ localized to the nucleus. The influence of W protein was investigated *in vitro* and *in vivo* with two AAvV-1 strains defective in the W C-terminus. The growth kinetic curves and pathogenicity tests in 3-week-old SPF chickens both showed that the replication abilities of strains with C-terminally deleted W proteins were lower than that of the parental strain. Restoring the appropriate dose of W protein increased the viral titers of these strains. The expression validation and functional exploration of W protein will facilitate our understanding of pathogenic mechanism of AAvV-1.

1. Introduction

Avian avulavirus 1 (AAvV-1) is the causative agent of the highly contagious avian disease Newcastle disease (ND), which is responsible for severe economic losses in the poultry industry (Alexander, 2001). AAvV-1 is a member of the genus *Avulavirus*, within the family *Paramyxoviridae* (Mayo, 2002). The genome of AAvV-1 is a nonsegmented, single-stranded, negative-sense RNA, which is approximately 15 kb and encodes six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin–neuraminidase (HN), and the large protein (L) (Paldurai et al., 2014). Previous research has shown that through the cotranscriptional (mRNA) editing of the P gene with a polymerase stuttering mechanism, two different types of mRNA are produced in addition to the P mRNA, which encode two other nonstructural proteins, V and W (Hausmann et al., 1999; Steward et al., 1993).

RNA editing of the P gene is a common phenomenon in the paramyxoviruses (Kolakofsky, 2016). The P gene has a conserved editing sequence (3′-UUUUU CCC-5′), followed by one or two guanines (G) that are inserted during transcription. Therefore, three types of mRNA, P (with no frameshift), V (with a +1 frameshift), and W (with a +2 frameshift), are produced (Steward et al., 1993). These P-gene-encoded proteins share their amino terminus, but differ in their carboxyl termini (Kumar et al., 2013). The RNA editing frequency of the P gene differs between paramyxoviruses. In *Nipah virus* and bovine parainfluenza virus 3, the proportions of P, V, and W mRNAs are similar, but in the measles virus, the proportions of V and W mRNAs are significantly lower than that of P (Bankamp et al., 2008; Kulkarni et al., 2009; Pelet et al., 1991). The proportions of P, V, and W mRNAs produced in AAvV-1-infected cells are approximately 68:29:2 (Mebatsion et al., 2001). However, recent studies have shown that the percentages of these mRNAs in different AAvV-1 strains differ, depending on the stage of the infection. The frequency of AAvV-1 editing increases significantly during the early stage of infection, and the levels of V and W mRNAs reach their highest at 8–10 h postinfection (hpi) (Qiu et al., 2016a).

The majority of alternative P gene products in the paramyxoviruses have been shown to function as inhibitors of the host innate immune response (Gotoh et al., 2002; Horvath, 2004). They inhibit the JAK/STAT signaling pathway through their common N-terminal domain, which binds to STAT1 and inhibits its phosphorylation (Fontana et al., 2008; Shaw et al., 2004). The P protein, which is the first product of the P gene, is an essential component of the viral RNA-dependent RNA polymerase in AAvV-1 (Hamaguchi et al., 1983, 1985). The V protein, the second P-gene-edited product, is an antagonist of alpha/beta interferon (IFN-α/β) and contributes to viral virulence. The overexpression of the V protein enhances the production kinetics of AAvV-1 and increases the rate of AAvV-1 production (Jang et al., 2010). The IFN-antagonistic activities of the V proteins are consistent with the

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virulence properties of the viruses (Alamares et al., 2010). V protein of AAvV-1 targets phospho-STAT1 degradation to block IFN-α signaling (Huang et al., 2003; Qiu et al., 2016b). The W protein, the third P-gene-edited product, was expressed by constructing a eukaryotic expression plasmid with two G bases inserted at the P-gene editing site (Vaidyanathan et al., 2016). However, there have been no reports of its function.

In this study, the influence of the AAvV-1 W protein was preliminarily investigated. We analyzed its length diversity, demonstrated the presence of W protein in different genotype strains, and identified their different subcellular localization. We used reverse genetics systems to construct two strains with C-terminally-deleted W protein, and showed that the lack of a C-terminus in W affected viral replication to some extent *in vitro* and *in vivo*. Restoring an appropriate concentration of W protein increased the titers of the W-protein-deficient strains. To our knowledge, this is the first report to show that the W protein is present in cells infected with different AAvV-1 genotypes, displays virus-strain-dependent subcellular localization, and influences viral replication.

2. Materials and methods

2.1. Animals and ethics statement

SPF embryonated eggs (9 days old) and SPF chickens (1 day or 3 weeks old) were purchased from Beijing Boehringer Ingelheim Vital Biotechnology Co., Ltd (Beijing, China). BALB/C mice (6 weeks old) were provided by Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). All the animals used in this study were cared for in accordance with the experimental protocols, and all procedures, including the possibility of animal death without euthanasia, were specifically considered and approved by the Animal Welfare and Ethical Censor Committee of China Agricultural University (CAU approval number 1901–08). All experiments were conducted in a biosafety level 2 (BSL-2) laboratory.

2.2. Cells and viruses

A chicken embryonic fibroblast cell line (DF-1), a baby hamster kidney cell line stably expressing T7 RNA polymerase (BSR T7 cells), an African green monkey kidney cell line (Vero), and an SP2/0 mouse myeloma cell line were all obtained from the Diagnostic & Research Center of Livestock and Poultry Epidemic Diseases, China Agricultural University. All cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; Gibco) and were maintained in DMEM with 2% FBS. All cell lines were maintained at 37 °C in a 5% CO_2 incubator (Thermo Forma, Marietta, OH, USA). AAvV-1 strain SG10, identified as a velogenic genotype VII virus, was isolated from an outbreak of ND in chickens. Recombinant AAvV-1 strain rSG10 was generated in our laboratory (Liu et al., 2015). AAvV-1 strains SG10, rSG10, and the lentogenic genotype II strain La Sota were propagated in 9-day-old SPF embryonated chicken eggs after allantoic cavity inoculation.

2.3. Sequence length analysis of the W protein

To analyze the lengths of the W proteins from different AAvV-1 strains, the P genes sequences were downloaded from the GenBank database. After two G bases were inserted at the editing site (5′-AAA AAGGG-3′) of the P gene, the W gene sequence was acquired. After the gene sequence was translated into the amino acid sequence, the number of amino acids in each W protein was counted.

2.4. Detection of RNA editing frequency

The RNA editing frequency was determined as described previously (Qiu et al., 2016a). Briefly, DF-1 cells in 24-well plates were infected with AAvV-1 strain SG10 at a multiplicity of infection (MOI) of 3. After the virus had adsorbed for 1 h, the cells were washed and then incubated with DMEM containing 2% FBS at 37 °C in a 5% CO_2 incubator. The infected cells were collected at 4, 6, 8, 10, 12, 16, and 24 hpi for the detection of the P-gene-derived mRNAs. The mRNAs were selectively reverse transcribed with an oligo(dT) primer (Promega, Madison, WI, USA), according to the manufacturer's protocol. The sequences surrounding the RNA editing site of the AAvV-1 P-gene-derived mRNAs were determined with standard PCR amplification. The RT–PCR products were resolved on agarose gel, subjected to gel extraction (Omega Bio-Tek, Doraville, GA, USA), cloned into the pEASY®-Blunt Cloning Vector (TransGen Biotech, Beijing, China), and used to transform *E. coli* Trans-T1 cells (TransGen). At least 50 clones from each time point were collected for sequencing (TsingKe, Beijing, China). An alignment of sequences was constructed with the MegAlign program of the DNASTAR software suite version 7.1 (DNASTAR, Madison, WI, USA).

2.5. Construction of W protein expression plasmids

The P gene ORF was PCR-amplified from AAvV-1 strains SG10 and La Sota, and then inserted into the mammalian expression vector pCMV-Myc to construct pCMV-P/SG and pCMV-P/La, respectively. The V and W gene ORFs were gotten through site-directed mutagenesis with the addition of one or two nontemplate G residues after the conserved RNA editing motif (5′-AAAAAGGG-3′) in the P gene ORF. The PCR products were purified and then recombined into the pCMV vector with the Seamless Assembly Cloning Kit (Invitrogen, Carlsbad, CA, USA). *Escherichia coli* Trans109 cells (TransGen) were transformed with the recombined products. All other plasmids were confirmed with DNA sequencing (TsingKe), and the positive plasmids were designated pCMV-V/SG, pCMV-W/SG, and pCMV-W/La. We constructed plasmids pCMV-WN/SG and pCMV-WC/SG based on pCMV-W/SG, and these expressed only the NTD or CTD of the W protein, respectively.

2.6. Production of mAbs against AAvV-1 W protein

To produce anti-W mAbs, the W protein CTD sequences were PCR-amplified from pCMV-W/SG and pCMV-W/La. After double digestion with *Xho*I and *Eco*RI, the PCR products were inserted into the expression vector pET-32a(+) with an N-terminal histidine (His) tag. The CTDs of the W proteins from SG10 and La Sota were expressed separately as fusion proteins with 6-His tags in *E. coli* BL21(DE3) (TransGen), as described in the pET System Manual. Cultures were grown at 37 °C to an optical density at a wavelength of 600 nm (OD_{600}) of around 0.6 in LBA medium (1 L of medium contained 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 100 mg ampicillin). Bacterial expression was then induced with the addition of isopropyl-β-D- thiogalactoside (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 1 mmol/L and incubation at 30 °C for 5 h. The expression of the recombinant proteins was confirmed with western blotting using an anti-His mAb, and the proteins were purified with affinity chromatography on a nickel–nitrilotriacetic acid column (CWBIO, Beijing, China), according to the manufacturer's instructions. The purified proteins were emulsified with an equal volume of Freund's complete adjuvant (Sigma) and injected subcutaneously into 6-week-old BALB/c mice at a dose of 100 μg, followed by three booster shots at 2-weekly intervals. The mice then received another booster, via intraperitoneal injection, of 50 μg of recombinant protein without adjuvant 3 days before fusion. The splenocytes were collected from the mouse with the highest antibody titer and fused with SP2/0 mouse myeloma cells using 50% polyethylene glycol 1500 (Sigma). Hypoxanthine–aminopterin–thymidine (HAT) medium (Sigma) was used to select the hybridomas. The antibody titer of the cell supernatant was detected on an ELISA plate coated with the W protein CTD fused with a glutathione S-transferase tag. The cells from positive wells were subcloned three times with the limiting

dilution method. The immunoglobulin (Ig) subclass of the mAbs was determined with a mouse mAb isotyping kit (Southern Biotechnology Associates Inc., Birmingham, AL, USA). The supernatant fluid of the hybridomas was harvested and designated 'mAb-SWC' or 'mAb-LWC'.

2.7. Construction of full-length chimeric SG10 and La Sota antigenomic cDNAs

The construction of full-length antigenomic cDNAs of AAvV-1 strains SG10 (pOK-rSG10), La Sota (pOK-rLa Sota), and their helper plasmids encoding the NP, P, and L proteins (pCI-NP, pCI-P, and pCI-L, respectively) has been described previously (Liu et al., 2015; Yu et al., 2017b). To construct a recombinant cDNA clone lacking the unique C-terminus of the W protein, a point mutation was introduced into the P gene, causing the W protein to terminate after the editing site without affecting the V protein. Briefly, nucleotide 'A' of P_{419} was changed to 'T' with a PCR primer. The PCR products were then recombined into pOK-rSG10 using the Seamless Assembly Cloning Kit. The full-length clones lacking the C-terminus of W were designated pOK-rSG10-ΔW_C and pOK-rLa Sota-ΔW_C, respectively. pOK-rSG10-ΔW_C was then used as the backbone from which to construct a recombinant cDNA clone containing the W ORF in which editing site 'AAAAAGGG' was changed to 'AAGAAAGG' (Huang et al., 2003) or with the EGFP gene inserted between the P and M genes (Qiu et al., 2016b). Briefly, the W ORF with a mutation at the editing sites or the EGFP ORF was amplified with the appropriate primers and engineered to contain the AAvV-1 gene-start and gene-end signal sequences. The products were then inserted between the P and M genes (positions 3161–3162) of the pOK-rSG10-ΔW_C or the pOK-rLa Sota-ΔW_C clone using the Seamless Assembly Cloning Kit, according to the "rule of six". The resulting cDNA clones containing the W or EGFP gene were designated pOK-rSG10-ΔW_C/W, pOK-rSG10-ΔW_C/EGFP, pOK-rLa Sota-ΔW_C/W or pOK-rLa Sota-ΔW_C/EGFP respectively. The mutation or inserted fragment newly introduced into each clone was sequenced to exclude the possibility of PCR-introduced errors.

2.8. Recovery of the virus from cDNA

The recombinant viruses were recovered by the cotransfection of BSR T7 cells with the full-length cDNA plasmid and the three helper plasmids, as described previously (Liu et al., 2015; Yu et al., 2017b). The expression of EGFP was detected 24 h after transfection with inverted fluorescence microscopy. At 4 days posttransfection, the cell culture was harvested after repeated freeze–thaw cycles. The supernatant was then injected into 9-day-old SPF embryonated chicken eggs through the allantoic cavity. After incubation for 4 days, the allantoic fluid was harvested and screened with a hemagglutination (HA) assay. The rescued recombinant virus was sequenced after RNA extraction and PCR.

2.9. Virulence of recovered viruses

The virulence of the recombinant viruses was determined with standard pathogenicity tests for AAvV-1: MDT in 9-day-old embryonated SPF chicken eggs and ICPI in 1-day-old SPF chicks (Alexander and Swayne, 1998). Briefly, for the MDT test, a series of 10-fold dilutions of infected allantoic fluid was made in sterile PBS. Five 9-day-old eggs were inoculated with 0.1 ml of each dilution via the allantoic cavity and incubated at 37 °C for 4 days, with inspection every 12 h. The time at which the mortality of each embryo was first observed was recorded. The highest dilution yielding 100% mortality was considered to be the minimum lethal dose. The MDT was determined as the mean time (h) required for the minimum lethal dose of the virus to kill all of the inoculated embryos. For the ICPI test, 10 1-day-old SPF chicks were inoculated via the intracerebral route with 0.05 ml of a 1:10 dilution of fresh allantoic fluid infected with each virus. The birds were monitored for clinical symptoms and mortality every 24 h for 8 days. At each observation point, the birds were scored as follows: 0, normal; 1, sick; and 2, dead. The ICPI was the mean of the scores per bird per observation point over the 8-day period.

2.10. Viral growth kinetics

The viral titers of the recombinant strains were determined and expressed as the 50% tissue culture infective dose ($TCID_{50}$)/0.1 ml and the 50% embryo infectious dose (EID_{50})/0.2 ml (Reed and Muench, 1938). The growth kinetics of the recombinant strains were determined under multiple-cycle growth conditions in various cell types. Vero and DF-1 cells in triplicate wells of 12-well culture plates were infected with the viruses at an MOI of 0.01. After the viruses had adsorbed to the cells for 1 h, the cells were washed twice with PBS and then incubated with DMEM containing 2% FBS at 37 °C in a 5% CO_2 incubator. The culture supernatants were collected at 12-h intervals until 72 hpi. The viral titers in the collected supernatants were measured with limiting dilution using the endpoint method and expressed as $TCID_{50}$, as described previously (Yu et al., 2017b). Because strains rLa Sota and it recombinant strains could not infect Vero cells, we only measured their growth curves on DF-1. All experiments were performed in triplicate.

2.11. Plaque formation

The virus was serially diluted and used to inoculate BSR T7 cells in six-well plates. After the virus had adsorbed to the cells for 1 h, the inoculum was removed and the cells were washed twice with PBS. An overlay medium containing 2% FBS and 1% agar was added. After the agar had solidified, the plates were placed upside down at 37 °C in a 5% CO_2 incubator for 4 days. The agar was then removed and crystal violet was used to stain the plates. The plaque sizes were measured with the GNU Image Manipulation Program, version 2.8 (https://www.gimp.org/).

2.12. Pathogenicity assessment in 3-week-old chickens

The pathogenicity of strains rSG10 and rSG10-ΔW_C was determined in chickens. In total, 75 3-week-old SPF chickens were assigned randomly to three groups of 25 birds each (15 for sampling and 10 for clinical observation). Each group was inoculated with one of the four viruses at 10^4 EID_{50} per bird via the oculonasal route. PBS was used as the negative control. The birds were observed daily and scored for clinical signs for 14 dpi, as described previously: 0, healthy; 1, sick; 2, wing drop, paralysis, torticollis, or lack of coordination; 3, prostration; 4, death (Jin et al., 2017). Survival was monitored until 10 dpi. At 1, 3, and 5 dpi, three birds from each group were euthanized for the analysis of tissue damage, and the samples were collected in two parts. One part (trachea, lung, spleen, proventriculus, duodenum, and cecal tonsil) was fixed in 10% neutral-buffered formalin for histopathology, and the other part (spleen, proventriculus, duodenum, cecum, and tonsil) was used for viral titration. For histopathology, all the fixed tissues were routinely embedded in paraffin, and 5 μm sections were cut for hematoxylin and eosin staining, and then examined for lesions with light microscopy. For viral titration, the tissue samples were homogenized in PBS containing antibiotics and DF-1 cells were inoculated with the supernatant after it was serially diluted 10-fold, with duplicate wells per dilution. The viral titers were determined as described above and the $TCID_{50}$ per 10 mg of tissue was calculated.

2.13. Western blotting analysis

Total cell protein lysates were extracted from the transfected or infected cells with the ProteinExt® Mammalian Total Protein Extraction Kit (TransGen). The total protein concentration was determined with a BCA Protein Assay Kit (CWBIO). The cellular proteins were separated

with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Germany). Each PVDF membrane was blocked with 5% (w/v) skim milk and 0.1% Tween 20 in Tris-buffered saline (TBST) for 2 h at room temperature, and was then incubated overnight at 4 °C with a primary antibody. The following antibodies were used in the experiments: SG10 or La Sota hyperimmune serum was used to detect HN protein; mAb-SWC and mAb-LWC were used to detect W protein; and anti-β-actin mAb (Cell Signaling Technology, USA) was used to detect the intracellular parameters. After the membranes were washed three times with TBST, they were incubated with corresponding horseradish peroxidase (HRP)-conjugated anti-chicken or anti-mouse antibodies (Bioss Biotechnology, Beijing, China), diluted 1:10,000, for 1 h at room temperature. HRP was detected with Western Lightning Chemiluminescence Reagent (CWBIO).

2.14. Indirect immunofluorescence assay

BSR or D-F1 cells were seeded and transfected with W-protein-expressing plasmids or infected with AAvV-1, as described above. At 36 h posttransfection or 24 hpi, the cells were fixed and permeabilized in ice-cold methanol and then incubated with anti-W mAb or anti-AAvV-1 serum at 37 °C for 1 h. After the cells were washed three times with PBS, they were incubated with fluorescein-isothiocyanate-conjugated goat anti-mouse or rabbit anti-chicken IgG secondary antibody (Bioss) for 1 h at 37 °C. After a further three washes with PBS containing 0.2% Tween 20, the cells were observed with fluorescence microscopy.

2.15. Cellular localization of proteins

Plasmids expressing EGFP-fused proteins were constructed by inserting the EGFP gene before the N-terminal ORF of the target protein in pCMV-Myc, pCMV-W/SG, pCMV-WN/SG, pCMV-WC/SG, and pCMV-W/La. After the sequences were confirmed, we designated these plasmids pCMV-EGFP, pCMV-EGFP-W/SG, pCMV-EGFP-WN/SG, pCMV-EGFP-WC/SG, and pCMV-EGFP-W/La, respectively. Vero cells were seeded on cell slides in 12-well plates, and when they had grown to 70% confluence, they were transfected with 1000 ng of each individual plasmid described above. Cells expressing the fluorescence-fused proteins were rinsed with PBS, fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.25% Triton X-100 in PBS for 5 min, and then counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma) to detect the nuclei. Fluorescent images were obtained with a Leica TCS SP8 fluorescence microscope (Germany). The images were analyzed and merged with the Adobe Photoshop 7.0 software.

2.16. AAvV-1 minigenome system and dual luciferase assay

The AAvV-1 minigenome assay was performed as described previously (Yu et al., 2017a). Briefly, BSR T7 cells were seeded in 24-well culture plates. At 80% confluence, the cells were cotransfected with the AAVV-1 minigenome pAAvV-1-MG plasmid (340 ng) carrying the FLuc reporter gene, using Lipofectamine 2000 (Invitrogen), together with the helper plasmids PCI-NP (340 ng), PCI–P (170 ng), and PCI-L (170 ng) and a *Renilla* luciferase reporter gene (RLuc, 10 ng). Based on PCI–P, a point mutation ('A' to 'T') was introduced at position 419 of the P gene to construct plasmid PCI–P_{419}. Minigenome experiments were performed to verify whether the changed point affected the function of the P protein in the replication complex. On the basis of this detection system, cells were cotransfected with three different doses (100, 500, or 1000 ng) of plasmid PCMV-W/SG and the minigenome plasmids. The total amount of transfected DNA was kept constant by adding the empty pCMV-Myc vector when necessary. At 30 h posttransfection, the cells were washed twice with PBS, and 100 μl of lysis buffer (Promega) was added. The cells were vigorously mixed for 15 min, and 20 μl of lysate from each well was analyzed with a dual luciferase assay kit (Promega) to determine the luciferase activity. The unit of relative luciferase activity was defined as the ratio of FLuc activity to RLuc activity. Luciferase expression was measured in triplicate in each experiment, and three separate experiments were performed. The remaining cell lysates were collected to detect the protein expression with western blotting.

2.17. Detecting the effect of overexpressed W protein on viral replication

DF-1 cells were cultured in 24-well plates and then transfected with pCMV-Myc or pCMV-W/SG. At 24 h posttransfection, the cells were infected with SG10 at an MOI of 1. At 4, 8, and 12 hpi, the total RNA was extracted from the cells with TRIzol Reagent (Invitrogen), according to the manufacturer's instructions, and then 500 ng of total RNA was converted to first-strand cDNA with the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). A reference gene, β-actin, was used as the internal control for quantitation, and gene expression was quantified as previously described (Kint et al., 2015). The expression levels of the target gene encoding NP was normalized to that of the internal β-actin control and the fold change in expression was calculated with the $2^{-\Delta\Delta CT}$ method. We also used another method to study the effect of W protein overexpression on viral replication. DF-1 cells were cultured in 24-well plates and then transfected with pCMV-Myc, pCMV-W/SG, or pCMV-W/La. At 6 h posttransfection, the cells were infected with rSG10-ΔW_C or rLa Sota-ΔW_C at an MOI of 1. The cell cultures were harvested at 24 hpi and their $TCID_{50}$ was determined as described above. All reactions were conducted in duplicate.

2.18. Poly(I:C) stimulation and gene expression analysis

Poly(I:C) sodium salt (Sigma) was dissolved in nuclease-free water and stored at −80 °C. DF-1 cells were cultured in 12-well plates and transfected with 1000 ng of each of the target-protein-encoding plasmids: pCMV-V/SG, pCMV-W/SG, pCMV-WN/SG, pCMV-WC/SG, or pCMV-W/La. After transfection for 24 h, 1000 ng of poly(I:C) was added to each well. Wells containing empty-vector (pCMV-Myc)-transfected cells, with or without poly(I:C) stimulation, were included as controls. After 16 h, the total RNA was extracted and qPCR was performed as described above. The expression levels of the target genes encoding IFN-α and IFN-β were normalized to the expression of the internal β-actin control, and the fold change in expression of each gene was calculated with the $2^{-\Delta\Delta CT}$ method. All reactions were performed in duplicate.

2.19. Statistical analyses

All data were analyzed with the GraphPad Prism software version 6.0 (GraphPad Software Inc., San Diego, CA, USA). All values are expressed as the means ± SD of three independent experiments. Student's *t* test and one-way ANOVA were used to evaluate the significance of differences, which is indicated, from least to most significant, as: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3. Results

3.1. Length diversity in W protein

To determine the most common length of the W protein and whether its length is related to the AAvV-1 genotype, 286 P gene sequences of different AAvV-1 genotypes were collected and inserted two G bases at the editing site of the P gene to get the W open reading frame (ORF). When the ORFs of the W proteins were compared, they varied in length from 135 to 231 amino acids, with predominant lengths of 179 and 227 amino acids (Table 1). The length of the W protein did not correlate with the AAvV-1 genotype.

Table 1
Numbers of isolates of different AAVV-1 genotypes with specific W protein lengths[a].

Group	Number of isolates with W protein length																						
	No. isolates	135aa	137aa	147aa	152aa	155aa	156aa	174aa	177aa	179aa	181aa	183aa	187aa	196aa	203aa	221aa	225aa	226aa	227aa	230aa	231aa
Class I	48		1		1			1				28					6				11
Class II																					
I	17			11					1		1								8		
II	19									14		4				1					
III	7									1			1						5		
IV	7													7							
V	20					2				18											
VI	56	2	3			1			6	30	1			1					12		
VII	44					1			10	2				1	2			1	26	1	
VIII	4						2												2		
IX	14		13																1		
X	8									8											
XI	2						1							1							
XII	5			2		3															
XIII	18	7								1					2				8		
XIV	1			1																	
XV	8								2	2					2				2		
XVI	1																		1		
XVII	1																		1		
XVIII	2																	2			
	286	9	17	14	1	7		3	1	19	76		2	32	1	10	6	1	6	3	66	1	11

[a] Sequence data were obtained from the National Center of Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/nuccore/)

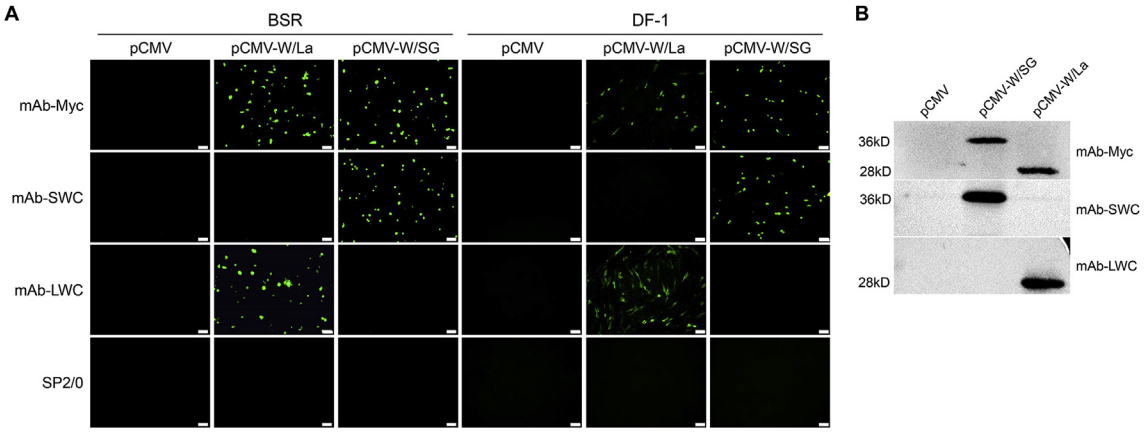

Fig. 1. Confirmation of the monoclonal antibodies against the W proteins of SG10 and La Sota. (A) BSR or DF1 cells were transfected with pCMV-Myc, pCMV-W/SG, or pCMV-W/La. At 36 h posttransfection, the cells were analyzed with an indirect immunofluorescence assay with monoclonal antibodies mAb-SWC and mAb-LWC. Antibody directed against the Myc label was used as the positive control, and SP2/0 cell supernatant was used as the negative control. Scale bars in the figure represent 50 μm. (B) DF1 cells were transfected with pCMV-Myc, pCMV-W/SG, or pCMV-W/La. At 36 h posttransfection, the total cellular proteins were extracted and detected with western blotting using monoclonal antibodies directed against Myc, SWC, and LWC.

3.2. Preparation of monoclonal antibodies (mAbs) mAb-SWC and mAb-LWC

After antigen preparation, the immunization of mice, cell fusion, and the screening of hybridomas with an enzyme-linked immunosorbent assay (ELISA), we obtained two mAbs, designated mAb-SWC and mAb-LWC, directed against the C-terminal domain (CTD) of the W protein of strains SG10 and La Sota, respectively. Subtype identification showed that the heavy chains of both mAbs were immunoglobulin G1 (IgG1) and the light chains were the κ subtype. An indirect immunofluorescence assay was performed to detect the strain specificity of the two mAbs. DF-1 and BSR cells were transfected with plasmids expressing the W protein, the assay was conducted 36 h after transfection, and the fluorescence was observed with an inverted fluorescence microscope. The cells transfected with the W protein of strain La Sota or SG10 were detected with an anti-Myc mAb. The W protein of the La Sota strain could only be detected with mAb-LWC, and not with mAb-SWC, whereas the W protein of strain SG10 was only detectable with mAb-SWC, and not with mAb-LWC. The supernatant of SP2/0 cells was used as the negative control (Fig. 1A). A western blotting analysis was conducted to confirm the strain specificity of the two mAbs. DF-1 cells were transfected with plasmids expressing the W proteins, and 36 h after transfection, the total protein was extracted and analyzed. An antibody directed against the Myc label detected the W proteins of strains SG10 and La Sota at around 36 and 28 kDa, respectively. Antibody mAb-SWC only detected the W protein of SG10, and mAb-LWC only detected the W protein of La Sota (Fig. 1B). These results are consistent with the immunofluorescence assay results. The indirect immunofluorescence assay and western blotting results confirmed that the two mAbs specifically detect the W protein of either AAvV-1 strain SG10 or La Sota.

3.3. Detecting W protein in AAvV-1-infected cells

The RNA editing frequency of AAvV-1 strain SG10 was determined in the early stage of infection. In total, 413 individual clones, collected at different time points after infection, were successfully sequenced. The nucleotide sequences of all the clones were identical, except for the one or more G residues inserted into the RNA-editing site. The proportions of P, V, and W mRNAs differed over time (Table 2). The proportion of W mRNA ranged from 3.1% to 12.6%, with an average value of 8.0%, which was higher than the widely accepted value of 2.4% (Mebatsion et al., 2001) and close to the W RNA editing frequency of La Sota during the early stage of infection, for which an average value of 9.41% has been reported (Qiu et al., 2016a).

An indirect immunofluorescence assay was conducted to detect the W protein after infection. At 24 hpi, cells infected with La Sota (Fig. 2A) or SG10 (Fig. 2B) were treated with hyperimmune serum, mAb-LWC, or mAb-SWC, and their fluorescence was observed. No fluorescence was observed in the negative control when the SP2/0 cell supernatant was used as the primary antibody. The results were consistent in BSR and DF-1 cells. These findings indicate that the W protein can be detected with our mAbs in AAvV-1-infected cells. To confirm that the protein detected by the indirect immunofluorescence assay was the W protein, a western blotting analysis was performed. In La-Sota-infected DF-1 cells, the target protein was detected at around 28 kDa (Fig. 2C), and in SG10-infected cells, the target protein was detected at around 36 kDa (Fig. 2D). The expression of W protein gradually increased after infection. No band was detected at these same positions in uninfected cells. These data suggest that W protein is expressed in cells infected with different AAvV-1 genotypes.

3.4. Recovery of recombinant chimeric viruses

To investigate the function of the AAvV-1 W protein, a series of recombinant chimeric viruses was constructed and rescued. To selectively block the expression of the unique C-terminus of the W protein of SG10, a rSG10 mutant expressing a truncated W protein lacking its CTD, designated rSG10-ΔW_C, was generated. One nucleotide was changed in the P gene to create a stop codon in the W protein without affecting the V ORF, but altering one amino acid in the P protein (Fig. 3A). To reversely verify the role of the W protein and exclude any possible effect of the point mutation in the P gene, we generated two strains expressing the W protein in the rSG10-ΔW_C backbone. The W ORF of SG10 was inserted into rSG10-ΔW_C between the P and M genes, as an extra transcriptional unit (Fig. 3B). The enhanced green fluorescent protein (EGFP) gene was inserted into the same site of rSG10-ΔW_C to exclude any possible effect of inserting an extra transcriptional unit (Fig. 3B). These two recombinant viruses were designated rSG10-ΔW_C/W and rSG10-ΔW_C/EGFP, respectively. All three strains were viable, and could be rescued with a reverse genetics system. After the viruses were passaged seven times in 9-day-old specific-pathogen-free (SPF) chicken eggs, they were amplified with reverse transcription–polymerase chain reaction (RT–PCR) and sequenced, which confirmed the correct structure of each mutation and insertion (data not shown). Using the same strategy, we rescued rLa Sota-ΔW_C, rLa Sota-ΔW_C/W and rLa Sota-ΔW_C/EGFP based on a rLa Sota reverse genetic system (Fig. 3C).

Table 2
Editing frequencies of P gene in DF-1 cells after infection with AAVV-1 strain SG10.

	Time post infection of SG10							Total
	4 h	6 h	8 h	10 h	12 h	16 h	24 h	
No. sequenced	95	42	64	42	66	49	55	413
P	36	22	36	14	30	29	31	198
	37.9%[a]	52.4%	56.3%	33.3%	45.5%	59.2%	56.4%	47.9%
V	47	18	26	24	31	14	22	182
	49.5%	42.9%	40.6%	57.2%	47.0%	28.6%	40.0%	44.1%
W	12	2	2	4	5	6	2	33
	12.6%	4.8%	3.1%	9.5%	7.6%	12.2%	3.6%	8.0%

[a] Represents the proportion of P, V, or W transcripts in the P gene transcription products.

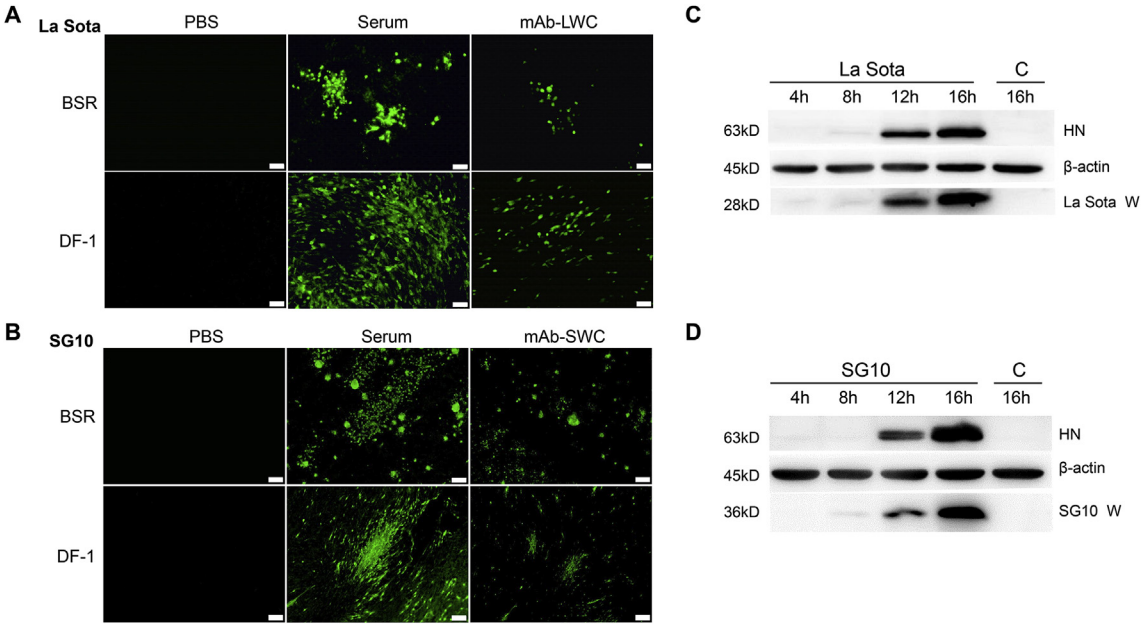

Fig. 2. Detection of W proteins in DF-1 cells infected with AAvV-1. (A) BSR and DF-1 cells were infected with La Sota at an MOI of 0.01. At 24 hpi, the cells were analyzed with an indirect immunofluorescence assay with La Sota hyperimmune serum or mAb-LWC (monoclonal antibody for specific detection of $W_{La\ Sota}$). PBS was used as the negative control. Scale bars in the figure represent 50 μm. (B) BSR and DF-1 cells were infected with SG10 at an MOI of 0.01. At 24 hpi, the cells were analyzed with an indirect immunofluorescence assay with SG10 hyperimmune serum or mAb-SWC (monoclonal antibody for specific detection of W_{SG10}). PBS was used as the negative control. Scale bars in the figure represent 50 μm. (C) DF-1 cells were infected with La Sota at an MOI of 1. At 4, 8, 12, and 16 hpi, the total cellular proteins were extracted and analyzed with western blotting and La Sota hyperimmune serum, mAb-LWC, and antibody directed against β-actin. Uninfected cells were used as the negative control. (D) DF-1 cells were infected with SG10 at an MOI of 1. At 4, 8, 12, and 16 hpi, the total cellular proteins were extracted and analyzed with western blotting and SG10 hyperimmune serum, mAb-SWC, and antibody directed against β-actin. Uninfected cells were used as the negative control.

An indirect immunofluorescence assay was used to detect the W protein after cells were infected with the six strains. At 24 hpi, the specific fluorescence could be seen in all the infected cells. The W protein was detected with mAbs in the rSG10-, rSG10-ΔW_C/W-, rLa Sota-, and rLa Sota-ΔW_C/W-infected cells but not in the rSG10-ΔW_C- or rLa Sota-ΔW_C-infected cells. The fluorescence was more obvious in the rSG10-ΔW_C/W-infected cells than in the rSG10-infected cells (Fig. 4A). We then detected the expression of W protein in DF-1 cells after infection with the eight strains, with a western blotting analysis. At 24 hpi, HN protein was detected in all the infected cells, whereas W protein was only detected in the rSG10-, rSG10-ΔW_C/W-, rLa-Sota and rLa Sota-ΔW_C/W-infected cells (Fig. 4B and C). The W protein expression was higher in rSG10-ΔW_C/W- and rLa Sota-ΔW_C/W-infected cells than in rSG10-and rLa Sota-infected cells. These results verified the identity of the recombinant strains based on their protein levels.

3.5. Biological characteristics of the recombinant viruses

To determine the effect of removing the CTD from the W protein, the virulence of the reconstructed viruses and their parental virus was evaluated with the mean death time (MDT) and the intracerebral pathogenicity index (ICPI). The recombinant viruses based on rSG10 but lacking the CTD of W protein showed slightly longer MDT and lower ICPI than their parental strain (Table 3), but according to the standard used to evaluate the virulence of AAvV-1, the recombinant viruses were still virulent strains. The lentogenic strains rLa Sota and its recombinant viruses showed the same MDT and ICPI. The sizes of the plaques formed by the three mutated rSG10 viruses and their parental strain on BSR T7 cell monolayers did not differ significantly (Fig. 5A and B).

The growth kinetics of the mutated viruses in DF-1 cells were compared using multicycle growth curves. When the growth of strain rSG10-ΔW_C was compared with that of its parental strain rSG10, it showed lower viral titers than rSG10 at 12 and 24 hpi ($p < 0.05$) (Fig. 5C). The viral titer of rSG10-ΔW_C/W was between those of rSG10 and rSG10-ΔW_C at 12 hpi. As expected, the growth characteristics of rSG10-ΔW_C/EGFP were consistent with those of rSG10-ΔW_C. When we constructed the multicycle growth curves on Vero cells, the same growth trends were observed. The viral titers of rSG10-ΔW_C at 24 and 36 hpi were lower than those of rSG10 ($p < 0.05$), and the titer of rSG10-ΔW_C/W was between these values at 24 hpi (Fig. 5D). The

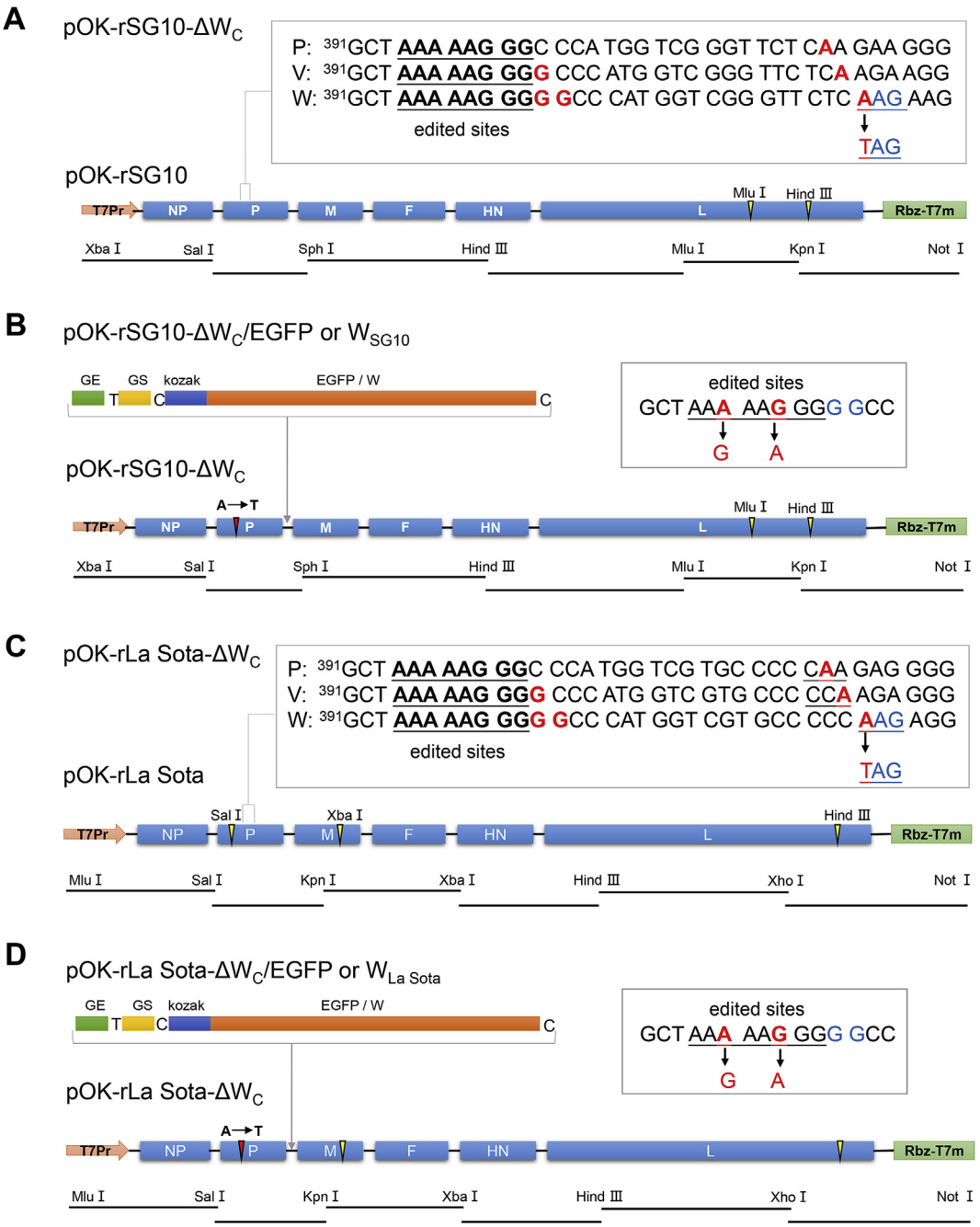

Fig. 3. Construction of recombinant chimeric viruses. (A) Strategy for constructing full-length antigenomic cDNA of rSG10-ΔW_C in which base 'A' at position 419 of the P gene was changed to base 'T', creating a premature stop codon in W protein. (B) Full-length antigenomic rSG10-ΔW_C/EGFP and rSG10-ΔW_C/W cDNAs were constructed based on rSG10-ΔW_C. The enhanced green fluorescent protein (EGFP) gene or the W ORF of SG10 with mutated sites was inserted into rSG10-ΔW_C between the P and M genes as an extra transcriptional unit. (C) Strategy for constructing full-length antigenomic cDNA of rLa Sota-ΔW_C, in which the same mutation point as in rSG10-ΔW_C was introduced. (D) Strategy for constructing full-length antigenomic cDNA of rLa Sota-ΔW_C/W and rLa Sota-ΔW_C/EGFP. W ORF of La Sota or EGFP was inserted into rLa Sota-ΔW_C in the same position as rSG10-ΔW_C.

growth of strains rLa Sota and its recombinant viruses on DF-1 cells was compared, and the titer of rLa Sota-ΔW_C, rLa Sota-ΔW_C/W, or rLa Sota-ΔW_C/EGFP was significantly lower than that of rLa Sota (Fig. 5E). Compared with rSG10-ΔW_C or rLa Sota-ΔW_C, rSG10-ΔW_C/W and rLa Sota-ΔW_C/W showed no significant difference. These results indicate that the lack of the CTD of W protein clearly influenced the level of replication of La Sota, but only affected that of SG10 in the early stages of infection.

3.6. Pathogenicity and replication of the recombinant viruses in 3-week-old chickens

The replication and pathogenicity of rSG10 and rSG10-ΔW_C were evaluated in 3-week-old SPF chickens by inoculating each bird with 10^4 egg infective doses (EID_{50}) of viral particles via the eye drop/intranasal (ED/IN) route. At 3 days post infection (dpi), the rSG10-and rSG10-ΔW_C-infected chickens showed clinical signs. Chickens infected with rSG10 began to die at 3 dpi and reached 100% mortality at 6 dpi. However, in the rSG10-ΔW_C-infected group, death was observed until 5 dpi and mortality reached 100% at 6 dpi. The clinical scores did not

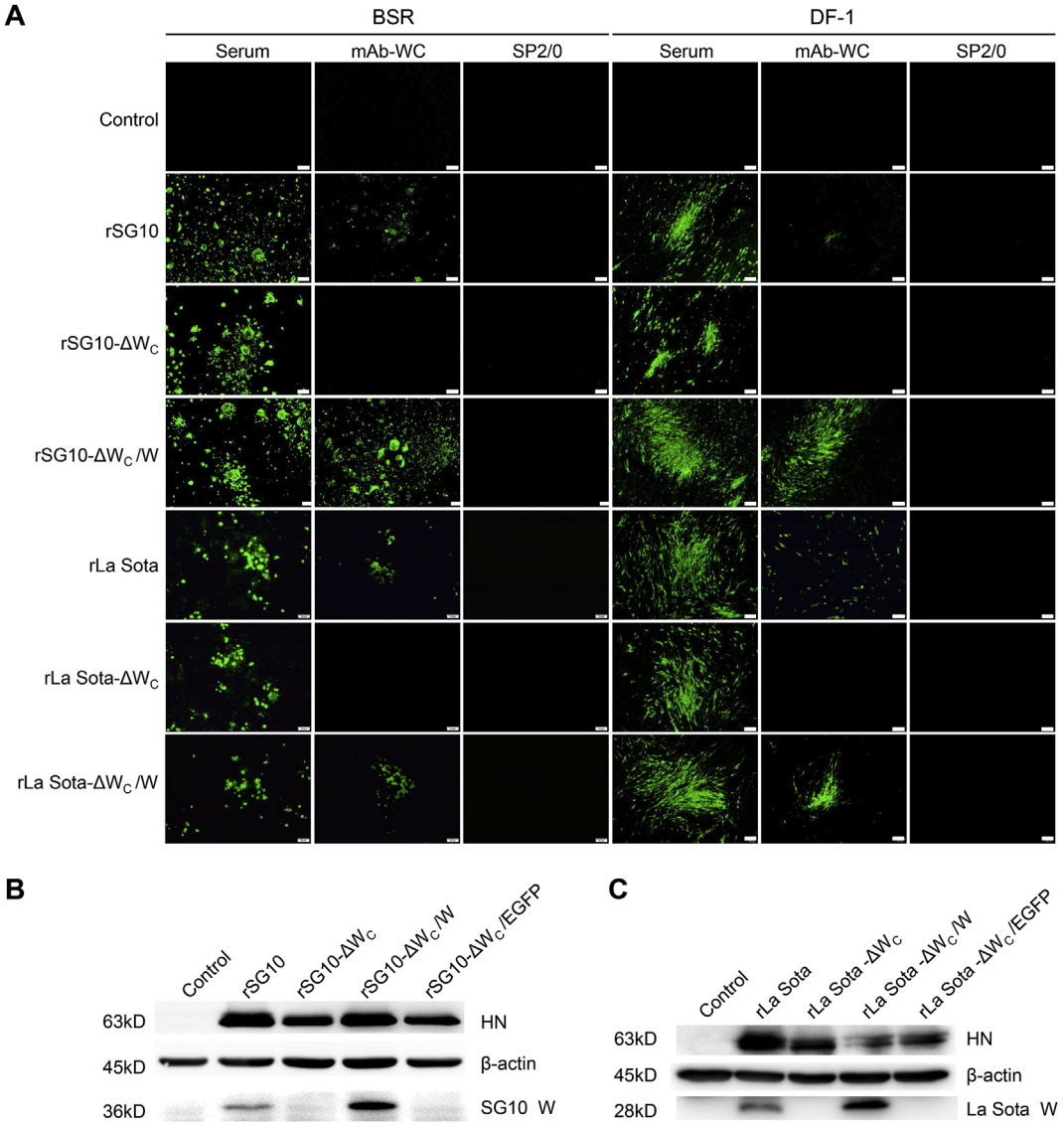

Fig. 4. Confirmation of recombinant chimeric viruses. (A) BSR and DF-1 cells were infected with rSG10, rSG10-ΔW_C, rSG10-ΔW_C/W, rLa Sota, rLa Sota-ΔW_C or rLa Sota-ΔW_C/W at an MOI of 0.01. At 24 hpi, the cells were analyzed with an indirect immunofluorescence assay and hyperimmune serum, mAb-SWC or mAb-LWC. SP2/0 cell supernatant was used as the negative control. Scale bars in the figure represent 100 μm. (B) DF-1 cells were infected with rSG10, rSG10-ΔW_C, rSG10-ΔW_C/W, or rSG10-ΔW_C/EGFP at an MOI of 0.01. At 24 hpi, the total cellular proteins were extracted and analyzed with western blotting and SG10 hyperimmune serum, mAb-SWC, or antibody directed against β-actin. Uninfected cells were used as the negative control. (C) DF-1 cells were infected with rLa Sota, rLa Sota-ΔW_C, rLa Sota-ΔW_C/W or rLa Sota-ΔW_C/EGFP at an MOI of 0.01. At 24 hpi, the total cellular proteins were extracted and detected with western blotting with La Sota hyperimmune serum, mAb-LWC, or antibody directed against β-actin. Uninfected cells were used as the negative control.

Table 3
Biological characteristics of the parental and mutant viruses.

Virus	Virus titer		Pathogenicity	
	EID_{50}/0.2 ml	$TCID_{50}$/0.1 ml	MDT(h) [a]	ICPI score [b]
rSG10	$10^{8.89}$	$10^{7.58}$	48	1.86
rSG10-ΔW_C	$10^{8.17}$	$10^{7.54}$	55	1.73
rSG10-ΔW_C/W	$10^{8.43}$	$10^{7.24}$	55	1.70
rSG10-ΔW_C/EGFP	$10^{8.17}$	$10^{7.31}$	55	1.76
rLa Sota	$10^{8.67}$	$10^{7.88}$	> 120	0.00
rLa Sota-ΔW_C	$10^{8.43}$	$10^{7.78}$	> 120	0.00
rLa Sota-ΔW_C/W	$10^{8.17}$	$10^{7.33}$	> 120	0.00
rLa Sota-ΔW_C/EGFP	$10^{8.36}$	$10^{7.43}$	> 120	0.00

[a] Mean death time (MDT): virulent strains, < 60 h; moderately virulent strains, 60–90 h; avirulent strains, > 90 h.

[b] Intracerebral pathogenicity index (ICPI): virulent strains, 1.50–2.00; moderately virulent strains, 0.70–1.50; avirulent strains, 0.00–0.70.

differ significantly. No obvious clinical signs were observed in the birds inoculated with phosphate-buffered saline (PBS) (Fig. 6A and B). Three chickens from each group were euthanized at 1 dpi, 3 dpi (Fig. 6C), or 5 dpi (Fig. 6D) for virus titration in DF-1 cells and an indirect immunofluorescence assay. At 1 dpi, no virus was detected in any organ of either group. The results at 3 dpi and 5 dpi showed that rSG10 replicated in the proventriculus, spleen, duodenum, and cecum, and that rSG10-ΔW_C showed a lower average virus titer than rSG10 across all tissues, although the difference was not significant because of the individual differences between the chickens. Three chickens from each group were euthanized at 5 dpi for histopathological analysis (Fig. 7). The parental strain rSG10 caused moderate to severe histological changes in the sampled tissues, including: a large number of necrotic lymphocytes, focal necrosis (black arrow), and hemorrhage (black triangle) in the white pulp of the spleen; severe mucosal epithelial necrosis and shedding in the proventriculus (black arrows); serious mucosal damage and intestinal villus fracture (black arrows) in the

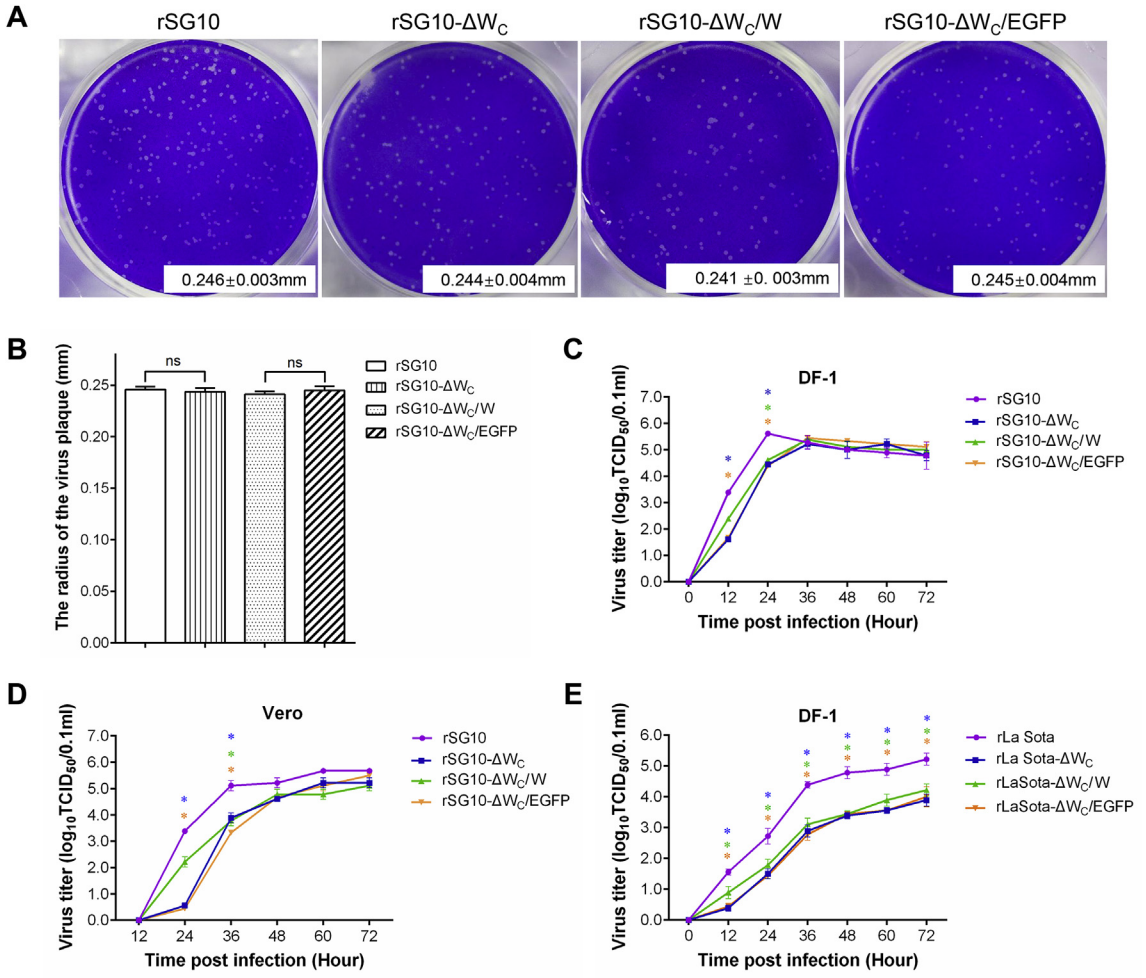

Fig. 5. Growth characteristic of recombinant viruses *in vitro*. (A) Plaque morphologies of rSG10 and its recombinant viruses in BSR T7 cells. (B) Summary of the average radii for 200 plaques of each virus. p values were calculated based on a two-tailed unpaired *t* test (95% confidence levels). "ns", no significant difference. (C, D, E) Multiple-cycle growth kinetics were used to assess the differences in the growth of these viruses. DF-1 (C) or Vero cells (D) were infected with rSG10, rSG10-ΔW_C, rSG10-ΔW_C/W, or rSG10-ΔW_C/EGFP at an MOI of 0.01. (E) DF-1 cells were infected with rLa Sota, rLa Sota-ΔW_C, rLa Sota-ΔW_C/W or rLa Sota-ΔW_C/EGFP at an MOI of 0.01 and assayed as described in the Methods. The color of asterisks represented differences between recombinant strains and their parental strain. p values were calculated with a multiple-samples t test (95% confidence levels). ∗p = 0.01–0.05 was significant.

duodenum; a large area of severe necrosis in the intestinal mucosa and lymphoid cells; inflammatory cells and red blood cells infiltrating the cecal tonsil (black arrows); stenosis in the bronchus, lung cavity, and breathing capillary lumen; numerous exudates in the bronchi (black arrow) and lung cavity, and breathing capillary hemorrhage (white arrow); expansion of the capillary breathing interval and hyperemia (white triangle); interstitial congestion in the lung lobules (black triangle); and severe mucosal shedding and muscle layer exposure in the trachea (black arrows). Compared with rSG10, the mutant strain rSG10-ΔW_C caused less-severe mucosal injury in the proventriculus, duodenum, cecum, and trachea. Congestion and hemorrhage were also observed in the propria of the cecum (white triangle) and lymphocyte infiltration was observed in the propria of the trachea (white arrow). No apparent histopathological changes were observed in any of the tissues from the control group treated with PBS. These results indicate that both strains caused severe tissue damage.

3.7. Subcellular localization of W protein

The W proteins of SG10 and La Sota were fused with EGFP (in the expression plasmid) to observe their subcellular localization in Vero cells. The observed fluorescence indicated that the W protein of SG10 (W/SG) was distributed in the cytoplasm, whereas the W protein from La Sota (W/La) localized in the nucleus (Fig. 8A). Even more interestingly, some of the cells expressing W/SG showed morphological changes. Therefore, W/SG, NTD of W_{SG10} (WN/SG), and CTD of W_{SG10} (WC/SG) fused with EGFP were observed at 6, 12, and 24 h after transfection. WC/SG showed the same subcellular localization as W/SG, and both of them accumulated in the cytoplasm 12 h after transfection. At 24 h, the overexpression of W/SG or WC/SG caused the cells to shrink and lose their normal morphology (Fig. 8B).

3.8. W protein inhibits minigenome system of AAvV-1

To further examine whether the amino acid change in the P protein influenced its function in the viral replication complex, an AAvV-1 minigenome system (pSG10-FLuc) was constructed and a dual luciferase assay was performed. As shown in Fig. 8, when plasmid P_{419}, in which the guanine at position 419 of the P protein is changed to thymine, replaced the original helper plasmid P, no significant differences were observed ($p > 0.05$; Fig. 9A), indicating that this altered amino acid had no effect on the P protein function in viral replication at the minigenome level. We used the same minigenome system to study the ability of the W protein of AAvV-1 to affect the function of the replication complex formed by NP, P, and L. Cells were cotransfected with plasmid DNA expressing the W protein of SG10 and the minigenome

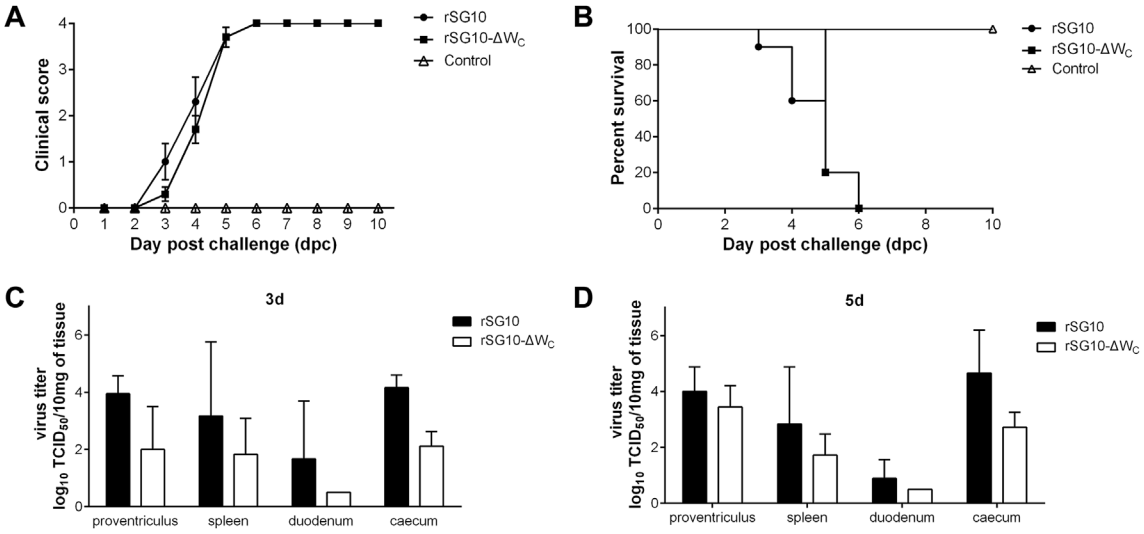

Fig. 6. Pathogenicity and replication of recombinant viruses in 3-week-old chickens. Groups of 25 3-week-old SPF chickens (15 for sampling and 10 for clinical observation) were inoculated with 10^4 EID_{50} of rSG10 or rSG10-ΔW_C via the natural route of infection. (A) Clinical signs in the infected chickens were scored daily based on 10 birds per group (0, healthy; 1, sick; 2, wing drop, paralysis, torticollis, or lack of coordination; 3, prostration; 4, death). Daily mean scores per group are shown. (B) Survival of 3-week-old SPF chickens inoculated with the four viruses, based on 10 birds per group. (C, D) Replication of the four viruses in 3-week-old chickens. Inoculated birds were killed at 3 days post challenge (C) or 5 days post challenge (D). The indicated tissues were collected and the viral titers determined with indirect immunofluorescence in DF-1 cells (n = 3).

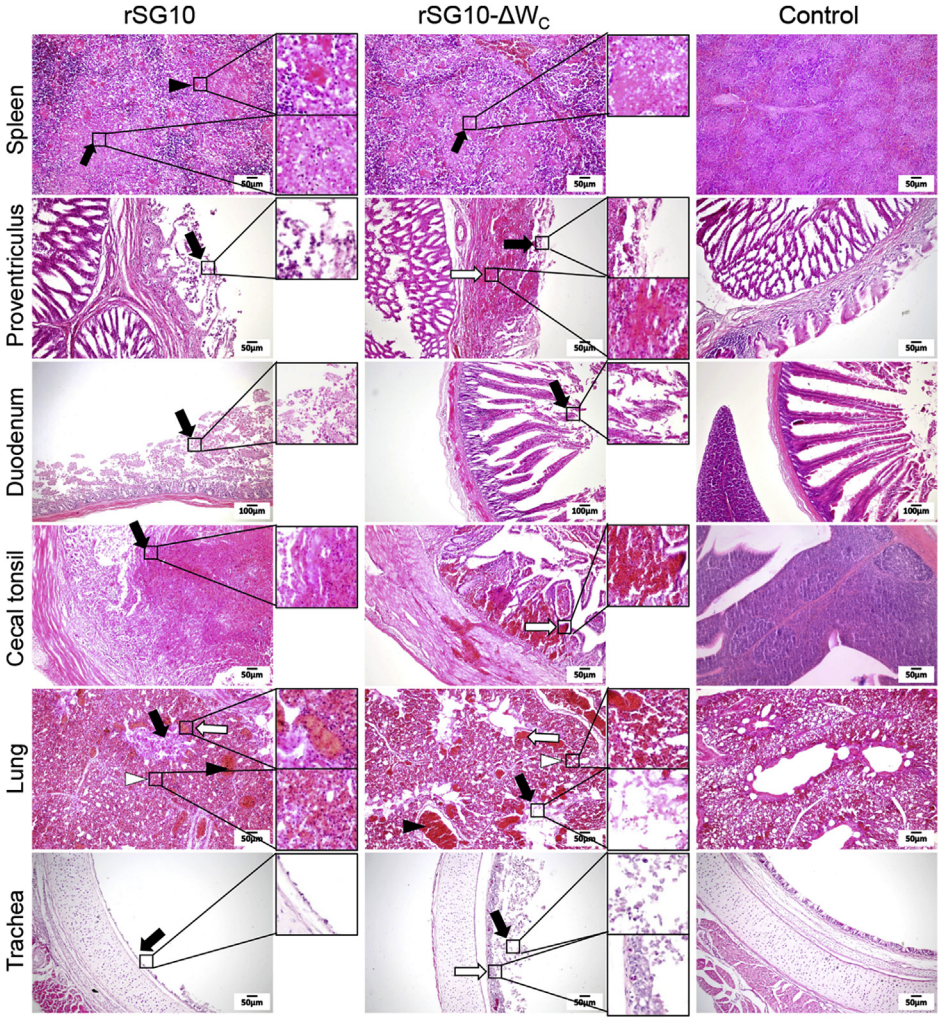

Fig. 7. Tissue histopathology of inoculated 3-week-old chickens. Birds were killed at 5 dpi and their tissues were fixed in formalin, sectioned, and stained with hematoxylin and eosin. The spleen displayed lymphocyte necrosis, focal cellular necrosis (black arrows), and hemorrhage (black triangle). The proventriculus showed severe mucosal epithelial shedding (black arrows), hemorrhage and congestion (white arrows). The duodenum showed villus fracture (black arrows). The cecum had a large area of severe intestinal mucosal necrosis; lymphoid cells, inflammatory cells, and red blood cells infiltrating the cecal tonsil (black arrows); and congestion and hemorrhage in the lamina propria (white arrows). The lung showed stenosis of the bronchus, lung cavity, and breathing capillary lumen; numerous exudates in the bronchi (black arrow), lung cavity, and breathing capillary hemorrhage (white arrow); expansion of the respiratory capillary and hyperemia (white triangle); and congestion (black triangle). The trachea had severe mucosal shedding (black arrows), hyperemia, and lymphocytic infiltration of the lamina propria (white arrow).

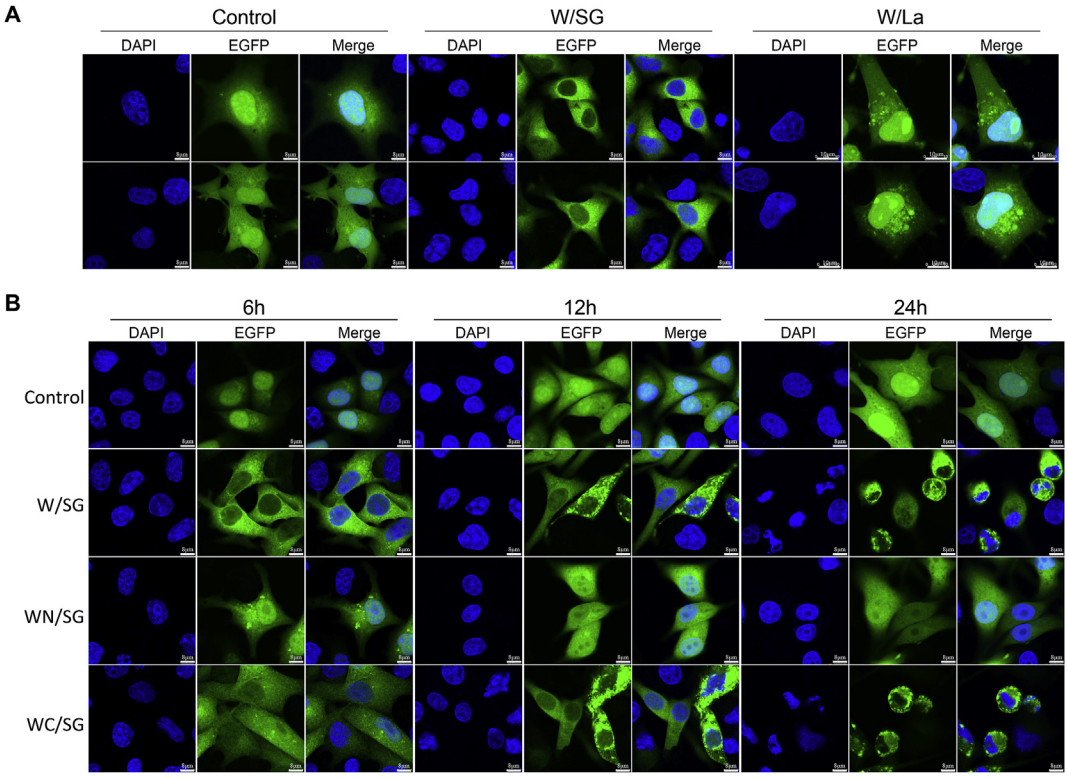

Fig. 8. Subcellular localization of W proteins, and cell morphological changes caused by W protein overexpression. (A) Vero cells were transfected with plasmids expressing the W protein of SG10 or La Sota fused with EGFP. At 12 h posttransfection, the cells were fixed and DAPI was used to stain the nuclei. The subcellular localization of the fused proteins was then observed with laser scanning confocal microscopy. (B) Vero cells were transfected with plasmids expressing the W protein of SG10 or its NTD or CTD fused to EGFP. At 6, 12, and 24 h posttransfection, the cells were fixed and DAPI was used to stain their nuclei. The cell morphological changes caused by W protein overexpression were then observed with laser scanning confocal microscopy. Scale bars in the figure represent 8 or 10 μm.

system. The value for the control, which consisted of plasmids expressing the AAvV-1 minigenome and the N, P and L proteins, was set at 100%. The total amount of plasmid DNA in each transfection was kept constant by the addition of the empty vector. The inclusion of 0.1, 0.5, or 1 μg of plasmid DNA encoding the W protein of SG10 caused replication to decline to approximately 82.5%, 61.4%, and 48.4%, respectively, compared with that of the empty-vector-transfected control. These results demonstrate the dose-dependent (range, 0.1–1.0 μg) inhibitory effect of the W protein on minigenome system of AAvV-1 (Fig. 9B).

3.9. Appropriate amount of W protein promoted replication of viral strains lacking W

To investigate the effects on viral replication of overexpressing the W protein in cells, we first infected DF-1 cells with SG10 24 h after their transfection with W/SG. The NP gene mRNA in the cells was detected with quantitative PCR (qPCR) at 4, 8 and 12 hpi. The mRNA of NP gene in cells transfected with pCMV-Myc at 4 h was set to 100%. The relative amount of NP mRNA was significantly lower in the W/SG-protein-expressing cells than in the control cells transfected with the empty vector (Fig. 9C). We then infected cells at an early point after transfection, using the W-protein-C-terminal-deleted strains rSG10-ΔW_C and rLa Sota-ΔW_C as the challenge strains. The cell cultures were harvested at 24 hpi and their viral titers determined as the median tissue culture infective dose ($TCID_{50}$). The viral titer was significantly higher in the rSG10-ΔW_C-infected cells transfected with 100 or 500 ng of W/SG plasmid than in the control group, but the titer did not improve when the cells were transfected with 1000 ng of W/SG plasmid (Fig. 9D). The rLa-Sota-ΔW_C-infected cells showed the same trend, in that the viral titer was significantly higher when 100 ng of W/La plasmid was transfected than in the control group, but did not improve as the dose increased (Fig. 9E). These results indicate that the appropriate dose of W protein increased the viral titer of the W-protein-defective strains in cells, whereas a high level of W protein expression did not promote viral replication.

3.10. W protein has no effect on IFN-α/β production

To investigate whether the W protein affects the production of type I IFNs, we used the V protein, which is known to have a function antagonistic to type I IFN, and the common N-terminal domain of the V and W proteins, which is known to have no antagonistic function for type I IFN, as the positive and negative controls, respectively. Because the W proteins of SG10 and La Sota differ greatly in length and subcellular localization, we also examined whether their effects on IFN differed. After stimulation with polyinosinic–polycytidylic acid (poly [I:C]), the effects of W/SG, W/La, and WC/SG did not differ significantly from that of the empty-vector control (Fig. 10A and B). The results for V protein and its N-terminal domain were consistent with those in previous studies. Therefore, our findings confirm that the W protein has no effect on IFN-α or IFN-β production in uninfected cells.

4. Discussion

In other paramyxoviruses, such as *Nipah virus*, the expression of W protein in virus-infected cells has been confirmed with specific antibodies (Lo et al., 2009), and this protein inhibits minigenome replication (Sleeman et al., 2008). It is located in the nucleus and acts on IFN signaling by interacting with STAT1 and STAT2, and also inhibits the TLR3 pathway (Audsley et al., 2016; Shaw et al., 2004, 2005). Recent studies have shown that the W protein of *Nipah virus* modulates the

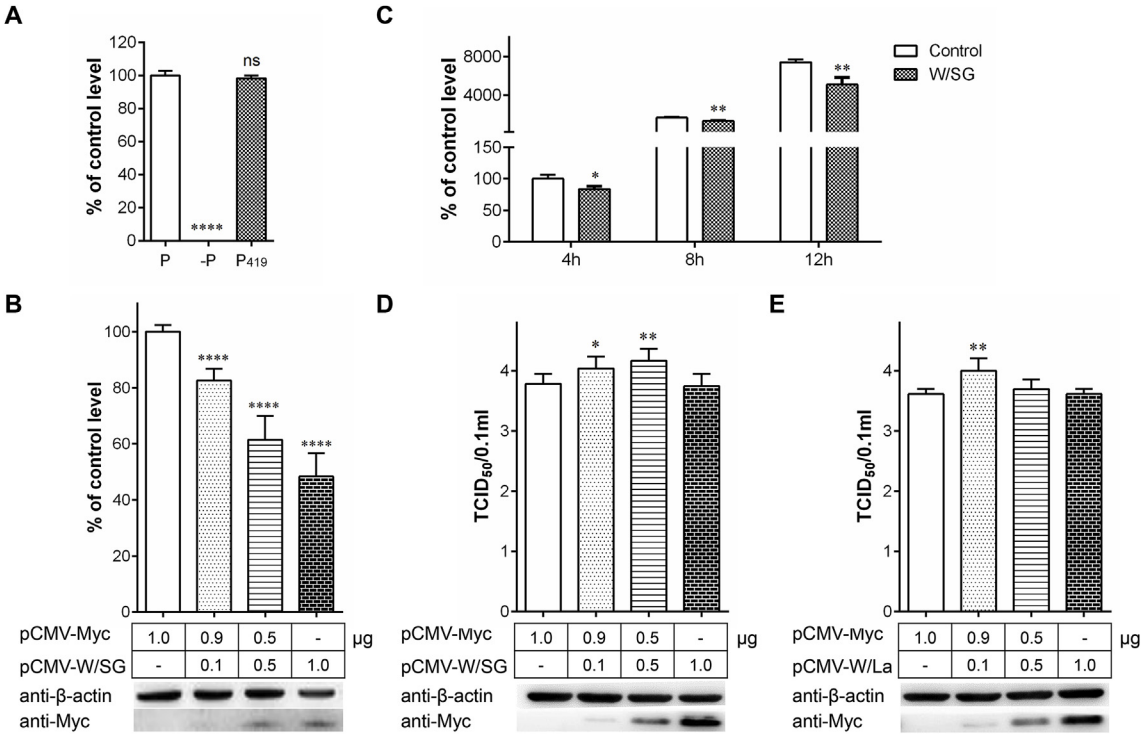

Fig. 9. Relative luciferase reporter gene expression from the AAvV-1 minigenome system (A, B) and the effect of the overexpression of W protein on viral replication (C, D, and E). (A, B) BSR T7 cells were transfected with plasmids, as described in the Materials and Methods. The AAvV-1 minigenome expression of the P_{419} mutant was compared with that of the wild-type AAvV-1 P protein (A). Relative luciferase expression in cells cotransfected with the empty plasmid pCMV-Myc was defined as 100%. Expression of the W/SG mutant was detected with western blotting with an anti-Myc antibody (B). (C) DF1 cells were transfected with plasmid expressing the W/SG protein, and at 24 h posttransfection, the cells were infected with rSG10-ΔW_C at an MOI of 1. Total RNA was extracted at 4, 8, and 12 h after infection. Cells transfected with the empty pCMV-Myc vector were used as the control. The levels of NP mRNA were detected and normalized to those of β-actin mRNA, and the value of the control group at 4 hpi was set to 100%. (D) DF1 cells were transfected with different doses of plasmid expressing the W/SG protein, and at 6 h posttransfection, the cells were infected with rSG10-ΔW_C at an MOI of 1. At 24 hpi, the cell supernatant was harvested and the viral titer determined. (E) DF1 cells were transfected with different doses of plasmid expressing W/La protein, and at 6 h posttransfection, the cells were infected with rLa Sota-ΔW_C at an MOI of 1. At 18 hpi, the cell supernatant was harvested and the viral titer was determined. p values were calculated with a two-tailed, unpaired *t* test (95% confidence levels). "ns", no significant difference; ∗p = 0.01–0.05; ***p = 0.001–0.0001; ****p < 0.0001, n = 3.

inflammatory host immune response in a manner that determines the course of the disease (Satterfield et al., 2015). In AAvV-1-infected cells, W mRNA is produced from the P gene by RNA editing (Steward et al., 1993). However, little is known about the W protein of AAvV-1. In this study, we attempted to figure out if W protein could be detected in cells infected with different AAvV-1 genotypes, and its relationship with the replication of the virus.

At first, we found that the W proteins of different strains are inconsistent in length. It has been reported that the diversity in viral protein lengths is related to different genotypes and influences the biological activities of the proteins or the virulence and pathogenicity of the virus. This is true of the HN protein of AAvV-1 (Jin et al., 2016). Therefore, we determined the predominant length of the W protein and whether its length is related to the AAvV-1 genotype. The predominant

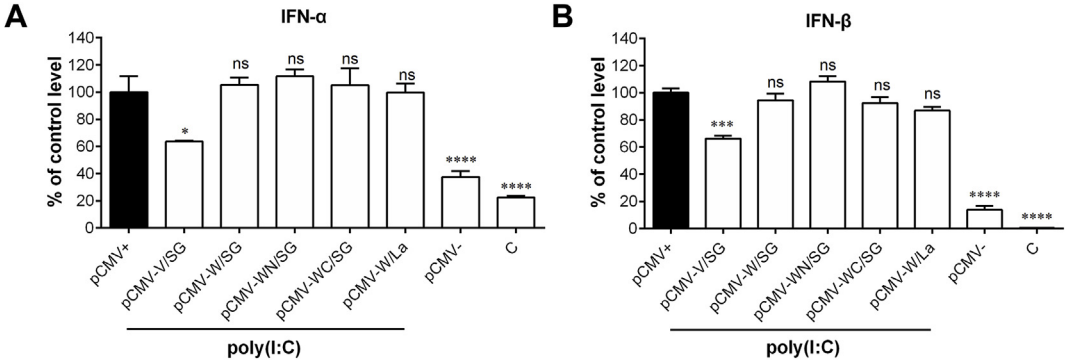

Fig. 10. Effect of W protein on IFN-I, detected with real-time PCR. DF-1 cells were cultured in 12-well plates and transfected with 1000 ng of plasmid encoding each target protein. After transfection for 24 h, 1000 ng of poly(I:C) was added per well. After 16 h, total RNA was extracted and converted to first-strand cDNA. qPCR was then used to detect the expression levels of IFN-α (A) and IFN-β (B). Expression levels of the target genes were normalized to that of the β-actin control. Relative gene expression in cells transfected with the empty pCMV-Myc plasmid and stimulated with poly(I:C) was defined as 100%. The fold change in the expression of each gene was calculated with the $2^{-\Delta\Delta CT}$ method. p values were calculated with a two-tailed unpaired *t* test (95% confidence levels). "ns", no significant difference; ∗p = 0.01–0.05; ***p = 0.001–0.0001; ****p < 0.0001, n = 3.

lengths were 179 and 227 amino acids. However, no relationship between the length of W and the genotype was detected. It is possible that the diversity of W protein lengths in AAvV-1 has arisen from differences in the P protein, and that the generation of a predominant length may be attributable to the conservation of the P gene at the corresponding positions.

The expression of V protein in AAvV-1-infected cells has been detected with antipeptide serum directed against the carboxyl-terminal amino acids of the V protein (Mebatsion et al., 2001; Qiu et al., 2016a). Here, the W protein was detected by specific mAbs in SG10 or La Sota infected cells. And the immunofluorescence assay indicated that W_{SG10} localized in the cytoplasm, whereas $W_{La\ Sota}$ localized in the nucleus. Based on the sequence analysis of the W proteins, we speculate that the difference in localization may depend on the C-terminal region where W_{SG10} was longer than $W_{La\ Sota}$. The overexpression of W_{SG10} or CTD of it also caused the cells to shrink and lose their normal morphology. The clumping of W protein in the cell indicates it may interact with some host proteins to play a certain function, which is worthy of further research. This is the first confirmation of the presence of W protein in cells infected with different AAvV-1 genotypes and their different cellular localization.

AAvV-1 mutants lacking either the unique C-terminal part of the V protein or six nucleotides in the conserved editing site showed markedly reduced titers and clearly attenuated pathogenicity (Mebatsion et al., 2001). As we had confirmed the presentation of W protein in AAvV-1-infected cells, whether the reduction in pathogenicity or virus replication were also related to the lacking of W protein? We rescued recombinant strains rSG10-ΔW_C and rLa Sota-ΔW_C, Strain rSG10-ΔW_C showed impaired replication in the early stage of infection in both DF-1 and Vero cells, and rLa Sota-ΔW_C showed impaired replication at various points in DF-1 cells. These data suggest that the W protein has some biological function that affects viral replication. However, replication of both rSG10-ΔW_C/W and rLa Sota-ΔW_C/W could not restore by inserting the W ORF between the P and M genes, although the expression W was significantly higher than the parental strain. We infected DF-1 cells with SG10 after overexpression of W protein, and the results indicated the viral replication was inhibited. Combined with the overexpression of the W/SG protein caused the cells to shrink and lose their normal morphology and W protein had an inhibitory effect on the function of the viral replication complex at mini-genome level, we propose two explanations of this phenomenon. One is that during the formation of the replication complex, the overexpressed W protein competes with the N-terminal domain of the P protein (Curran et al., 1991; Hamaguchi et al., 1983; Jahanshiri et al., 2005). The other explanation is that the overexpressed W protein causes cell shrinkage and other unknown internal cellular changes. Considering that W constitutes the lowest proportion of the edited products of the P gene, some W protein may be beneficial for viral replication, although large amounts are not. We investigated whether the low-level expression of the W protein promoted viral replication. Cells were transfected with different doses of W protein and infected with strains lacking C-terminal of W protein and the viral titer was detected 24 h after infection. The results indicated that low doses of W protein promoted the replication of viral strains lacking W, whereas higher doses did not. This may explain why the titer of rSG10-ΔW_C/W and rLa Sota-ΔWC/W did not reach or exceed that of rSG10 and rLa Sota. The results of *in vivo* experiments showed rSG10-ΔW_C had a lower viral titers than those infected with rSG10. The rSG10-ΔW_C-infected chickens began to die later than the rSG10-infected chickens, but all the chickens had died by day 6 after infection. When analyzed with MDT and ICPI, the deletion of the C-terminal domain of the W protein had no significant effect on the virulence of these strains. These results indicated that the deletion of C-terminus of the W had no significant effect on the virulence and pathogenicity of the velogenic strain but the appropriate dose of W could promote the viral replication.

Considering we changed the nucleotide at position 419 of the P gene when we removed the C-terminus of the W protein, we used minigenome assays to exclude any possible effect of this site change on the function of the P protein in an SG10 minigenome system. The results indicated that this amino acid change in the P protein did not affect the function of the viral replication complex which is composed of the NP, P, and L proteins, at the minigenome level. Based on the observation of low doses W protein can promote the replication of viral strains lacking W, we believe that the difference in replication between the W protein deletion strains and the parent strain was due to the deletion of the W protein, while not the amino acids functional change of P protein.

The V protein antagonizes IFN and the functional domain responsible for it is located in the C-terminus of the V protein (Park et al., 2003). We investigated whether the W protein affects replication because it also has IFN-antagonizing activity. However, it showed W protein had no effect on the production of IFN-α/β in uninfected cells. These findings suggest that the effect of W protein on viral replication may be independent of its ability to antagonize IFN, but is instead induced by other unknown mechanisms.

The P gene of Measles Virus encodes nonstructural proteins C via the alternative translational initiation in a different reading frame. In early phases of infection C protein facilitates viral transcription to control the formation of nascent nucleocapsid composed of N protein and vRNA. And C protein inhibits MV RNA polymerase activity by binding to the host protein SHCBP1 (Ito et al., 2013). C-knockout has resulted in significant amounts of dsRNAs accumulate and defective interfering RNA (DI-RNA) generation during mutant infection but not in parental virus infection, thereby triggering innate immune responses leading to impaired MV growth (Pfaller et al., 2014). These studies explained why protein C deletion results in impaired replication of the strains but the C protein itself has an inhibitory effect on the minigenome. In our study, W showed the same phenomenon, and the presence of a protein C encoded by the P gene has not been found in AAvV-1. So, we speculate W may have an equivalent role of C protein in avulavirus replication. And W may stimulate the innate antiviral response in infected cells and decrease specific infectivity of progeny virus through a higher relative content of DI particles. It need to be verified in future research.

In a recent study, researchers used the AAvV-1 lentogenic genotype II strain clone 30 to prepare a polyclonal antibody specific for its W protein, and showed that the W protein was present in the clone 30-infected cells and localized to their nuclei. A W-protein-deficient strain was constructed and showed that the W protein was not essential for its replication *in vitro* (Karsunke et al., 2019). The cellular localization of $W_{La\ Sota}$ in the present study is consistent with their results. However, the effect of the deletion of the W protein on viral replication is inconsistent. We compared the strategies used to construct the W-protein-deficient strains. In their study, the V and W proteins were knocked out by deleting 6 nucleotides within the editing site of the P gene, and the V protein was inserted between the P and M genes. Many studies have shown that the V protein significantly promotes the replication of AAvV-1 (Chu et al., 2018; Jang et al., 2010; Mebatsion et al., 2001; Huang et al., 2003). The V protein is just one of the products of P gene editing, but a foreign gene inserted into the noncoding region between the P and M genes expressed the highest level of gene product of all the other insertion sites (Zhao et al., 2015). In that study, the expression of V protein was not detected. It is unclear whether the expression level of V protein in their W-protein-deficient strain was higher than in the parental strain. If it was higher, the effect of V protein in promoting viral replication may have concealed the effect of the deletion of W, impairing viral replication. In our study, we exclude any possible effect of this point mutation in the P gene on the function of the replication complex and confirmed that the appropriate amount of W protein improve viral replication with overexpression experiments *in vitro*.

In summary, we analyzed different lengths of W proteins, confirmed the presence of W protein in two different AAvV-1 genotypes, and found that the two W proteins localized to different subcellular

compartments and the overexpression of W_{SG10} caused the cells to shrink and lose their normal morphology. Appropriate amount of W protein was conducive to viral replication. Therefore, our research offers important finding for the further exploration functions of the AAvV-1 W protein.

Declaration of competing interest

The authors have declared no conflict of interest.

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