Production, characterization, and epitope mapping of a monoclonal antibody against genotype VII Newcastle disease virus V protein

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Abstract

Newcastle disease virus (NDV) V protein is crucial for viral interferon (IFN) antagonism and virulence, determining its host range restriction. However, little information is available on the B cell epitopes of V protein and the subcellular movement of V protein in the process of NDV infection. In this study, the monoclonal antibody (mAb) clone 3D7 against genotype VII NDV V protein was generated by immunizing mice with a purified recombinant His-tagged carboxyl-terminal domain (CTD) region of V protein. Fine epitope mapping analysis and B-cell epitope prediction indicated that mAb 3D7 recognized a linear epitope ^{152}RGPAELWK^{159}, which is located in the V protein CTD region. Sequence alignment showed that the mAb clone 3D7-recognized epitope is highly conserved among Class II genotype VII NDV strains, but not among other genotypes, suggesting it could serve as a genetic marker to differentiate NDV genotypes. Furthermore, the movement of V protein during NDV replication in infected cells were determined by using this mAb. It was found that V protein localized around the nucleus during virus replication. The establishment of V protein-specific mAb and identification of its epitope extend our understanding of the antigenic characteristics of V protein and provide a basis for the development of epitope-based diagnostic assays.

1. Introduction

Newcastle disease (ND) is one of the most serious infectious diseases of birds causing major economic losses to the poultry industry (Aldous and Alexander, 2001; Dimitrov et al., 2016). Its causative agent, virulent Newcastle disease virus (NDV), belongs to the genus Avulavirus, in the subfamily Paramyxovirinae, family Paramyxoviridae, order Mononegaviriales (de Leeuw and Peeters, 1999). Phylogenetically, NDVs have been classified into two major categories, class I and class II (Czegledi et al., 2006; Gould et al., 2003). Class I NDVs are occasionally isolated from wild aquatic birds and domestic poultry and all but one are nonvirulent (Liu et al., 2009; Mia Kim et al., 2007). Class II NDVs, which were recently subcategorized into 18 genotypes, are genetically and phenotypically more diverse, and exhibit a wider range of virulence (Diel et al., 2012; Dimitrov et al., 2016; Miller et al., 2010; Ramey et al., 2013).

NDV has a negative-sense, single-stranded continuous RNA genome of 15,186, 15,192 or 15,198 nucleotides (nt) that contains six genes in the order 3'-NP-P-M-F-HN-L-5', encoding the six viral proteins nucleoprotein, phosphoprotein, matrix protein, fusion protein, hemagglutinin-neuraminidase and large protein (Yusoff and Tan, 2001). Two additional proteins, V and W, are encoded by mRNAs derived from the P gene via RNA editing (Qiu et al., 2016a; Steward et al., 1993). In the process of P gene transcription, some transcripts have inserts of one or more pseudo-template G nucleotides behind the RNA-editing site, leading to open reading frame (ORF) frameshift. The reported proportions of protein-encoding mRNAs in NDV-infected cells are about 68% for P, 29% for V, and 2% for W (Mebatsion et al., 2001; Qiu et al., 2016a). P, V and W protein shared a common N-terminal moiety of ORF and contained unique C-terminal moiety (Huang et al., 2003; Park et al., 2003a).

The V protein of paramyxoviruses is characterized by a unique cysteine-rich carboxyl-terminal domain (CTD), which binds two zinc atoms (Paterson et al., 1995; Steward et al., 1995) and is important for viral interferon (IFN) antagonism in a

variety of ways (Horvath, 2004b). The V protein of parainfluenza virus 5 (PIV5) and mumps virus (MuV) target signal transducer and activator of transcription 1 (STAT1) for proteasome-mediated degradation through assembly of a degradation complex containing signal transducer and activator of transcription 2 (STAT2), damaged DNA binding protein 1, and cullin 4 A (Didcock et al., 1999; Kubota et al., 2001). The V proteins of Nipah virus and Hendra virus inhibit cellular responses to IFN through binding and cytoplasmic sequestration of both STAT1 and STAT2 (Rodriguez et al., 2002, 2003). Measles virus (MV) V protein inhibits host IFN-induced transcriptional responses by preventing IFN-induced STAT1 and STAT2 nuclear import (Palosaari et al., 2003).

Similar to PIV5, NDV V protein is a structural component of virions and considered an effector for IFN antagonism (Paterson et al., 1995; Steward et al., 1995); however, its underlying mechanism is unknown. Based on reverse genetics, several V-deficient NDV mutants have been recovered, which were much more sensitive to the antiviral effects of IFN (Alamares et al., 2010; Mebatsion et al., 2001; Park et al., 2003a; Qiu et al., 2016b); resistance to IFN is restored when V protein is re-expressed in infected cells (Park et al., 2003a). NDV inhibits IFN through the C-terminal region of the V protein, which promotes degradation of phosphorylated STAT1 and blocks IFN signaling (Huang et al., 2003; Park et al., 2003b; Qiu et al., 2016b).

Due to lack of commercial antibodies against V protein, little is known about structural and antigenic differences of V protein between different NDV strains, nor the detailed IFN antagonism mechanism of NDV V protein. In this study, a recombinant protein containing the CTD domain of NDV V protein was used as an antigen for production of mouse hybridomas that secreted an anti-V protein monoclonal antibody (mAb). Sensitivity and specificity of the anti-V mAb was examined by enzyme-linked immunosorbent assay (ELISA), Western blot (WB) and indirect immunofluorescence assay (IFA). The epitope recognized by the mAb were also identified by WB and ELISA assay. Our study indicated that the mAb we developed could be a useful tool for investigating the antigenic structure and function of NDV V protein.

2. Materials and methods

2.1. Virus, cells and plasmids

NDV strains used in this study, La Sota/46, Mukteswar, Queensland V4, Herts/33, F48E8 and Hitchner B1, were from China Institute of Veterinary Drug Control. Pi/China/SD/2012/132 (designated ND132 in this study), Pi/China/SD/2012/167 (ND167), CN/ZJ-1/00 (ZJ-1), JSD0812 and JS-7-05-Ch (HM) were previously isolated on mainland China (Dai et al., 2014; Qiu et al., 2011). All viruses were maintained in our laboratory (Detailed information on NDV strains is in Table 1). All viruses were propagated in 9- to 11-day-old specific-pathogen-free chicken embryonated eggs as previously described (Gough et al., 1988). Fresh allantoic fluid was harvested from embryonated eggs dead between 24 and 120 h after inoculation and kept at -80 °C. DF-1, HeLa and SP2/0 cells were from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) or RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (FBS, Gibco) at 37 °C and 5% CO2. Prokaryotic expression plasmid pET-28a-ZJ1/VCD encoding the C-terminal domain of ZJ1 and eukaryotic expression plasmid

pFLAG-ZJ1-V encoding the complete V protein of ZJ1 were constructed previously (Qiu et al., 2016b).

2.2. Expression and purification of recombinant protein

The His-tagged CTD region of V protein (VCD) from strain ZJ1 was prepared, purified and quantified according to previous reports (Qiu et al., 2016b). The pET-28a-ZJ1/VCD was transformed into Escherichia coli. (E. coli.) BL2l and induced at 37 °C for 8 h with 1 mM isopropyl-β-D-thiogalactoside. Bacteria were harvested by centrifugation at 5000g and washed for three times in phosphate buffered saline (PBS). Pellets were resuspended in buffer A (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mM phenylmethylsulfonyl fluoride) supplemented with lysozyme (0.4 mg/mL) and DNase I (10 μg/mL). After 30 min with gentle shaking, bacteria were sonicated on ice. After centrifugation at 13,000 g for 20 min at 4 °C, pellets were collected and solubilized overnight with gentle shaking at 4 °C in 50 mM Tris-HCl (pH 8.0) containing 8 M urea, 0.5 M NaCl, 5 mM 2-mercaptoethanol and 5 mM imidazole. Supernatant containing solubilized inclusion bodies was collected after centrifugation at 12,000 g for 15 min. Purified recombinant protein was harvested from supernatants using Ni-NTA His•Bind Resin (Novagen, Madison, WI, USA) according to the manufacturer's instruction. Recombinant His-tagged VCD (His-VCD) protein was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and WB with anti-His (Sigma-Aldrich, St. Louis, MO). Protein concentration was determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA).

2.3. Immunization of mice and establishment of hybridomas

Six-week-old female BALB/c mice (SPF grade) were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (China). The His-VCD protein was emulsified with an equal amount of Freund's complete adjuvant (Sigma-Aldrich, USA) and subcutaneously immunized the mice in the abdomen with 50 μg His-VCD protein for each mouse. Four weeks after priming, mice were boosted four times in 2-week intervals by intraperitoneal injection of 100 μg His-VCD protein per mouse. Five days after last injection, spleen cells were collected from immunized mice, washed twice with RPMI 1640 medium, and mixed and fused with logarithmically growing SP2/0 myeloma cells in a ratio of 5:1 in the presence of polyethylene glycol 4000 (Sigma–Aldrich, St. Louis, MO, USA). Treated cells were suspended in HAT (RPMI 1640 medium containing 20% FBS, 100 mg/mL streptomycin, 100 IU/mL penicillin, 100 mM hypoxanthine, 16 mM thymidine, and 400 mM aminopterin), and plated into 96-well tissue culture plates at 1 × 105 cells per well in 200 μL media. After cultivation at 37 °C at 5% CO2 for 10 days, medium was detected for anti-VCD antibodies by indirect ELISA with His-VCD protein. Positive hybridoma cells were subcloned though a limited dilution method several times until monoclonal hybridoma cells were established following standard procedures. Hybridoma cells were cultured in the abdominal cavity of liquid-paraffin-primed BALB/c mice to obtain ascitic fluid. Globulin fractions were precipitated with 2 M (NH4)2SO4 and purified by gel filtration with Sephacryl S-200 HR (GE Healthcare UK Ltd.). MAb clones were tested for immunoglobulin class/subclass using the SBA Clonotyping System (SouthernBiotech, USA).

2.4. Indirect enzyme-linked immunosorbent assays

Purified ZJ1-VCD protein or fresh allantoic fluid for distinct NDV strains treated with an equal amount of 0.1% SDS at 100 °C for 10 min were used as detection antigens.

Microtiter ELISA plates with 96 wells were coated with 1:200 dilutions of prepared allantoic fluid or 1 ng/μL purified ZJ1-VCD protein in 100 μL carbonate buffer solution (CBS) per well. After cultivation at 4 °C overnight, ELISA plates were washed three times with PBS containing 0.05% Tween-20 (PBST) and blocked with 300 μL/well 5% skim milk powder in PBST for 2 h at 37 °C. Hybridoma cultured medium or antibodies were plated into coated ELISA plates, 100 μL per well, and incubated at 37 °C for 1 h. After washing three times with PBST, plates were incubated with 1:6000 diluted horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz, CA, USA) for 1 h at 37 °C. To each well, 100 μL 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich, USA) was added and absorbance was measured at 450 nm in a microplate reader (Synergy 2, BioTek).

2.5. Specificity of mAbs for NDV genotypes

DF-1 cells were cultured in 6-well plates, washed 3 times with PBS, and incubated with NDV strains at multiplicity of infection (MOI) 5 in 600 μL DMEM per well at 37 °C for 30 min. Supernatants were discarded and cells cultured in DMEM containing 2% FBS (Gibco). At indicated time points, cells were washed thoroughly and subjected to IFA and WB assays with anti-VCD mAbs.

2.6. Western blots

Cells harvested at indicated time points were washed three times with PBS and lysed with RIPA buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% v/v NP-40, 1% w/v sodium deoxycholate, 0.1% w/v sodium dodecyl sulfate, 1 mM phenylmethanesulfonyl fluoride, 1 mM Na3VO4, 1 mM NaF, 0.15 μM aprotinin, 1 μM leupeptin, and 1 μM pepstatin). Lysates were incubated at 100 °C for 10 min after addition of 5× SDS-PAGE loading buffer (250 mM Tris-HCl, pH 6.8, 10% SDS, 0.5% bromophenol blue, 50% glycerol, 5% β-mercaptoethanol), and proteins separated by SDS-PAGE on 10% polyacrylamide gels before transferring to nitrocellulose membranes (Millipore, Billerica, MA, USA). Membranes were blocked overnight at 4 °C in Tris-HCl buffer solution (TBS) containing 5% skim milk, then for 2 h with anti-VCD mAb, anti-NP mAb(Zhan et al., 2014) or anti-FLAG (positive control). After incubation with 1:6000 diluted HRP-goat anti-mouse IgG (Santa Cruz) in TBS for 1 h at room temperature, blots were visualized using an enhanced chemiluminescence detection system (Thermo Fisher Scientific, Waltham, MA, USA).

2.7. Indirect immunofluorescence assays

MAbs were detected in IFA according to previously described procedures (Sun et al., 2017). DF1 cells cultured on glass coverslips were infected with ZJ1 virus at a MOI of 5. Cells were fixed with 4% formaldehyde solution for 10 min at 6, 8, 10, 12, 18, and 24 h post infection (hpi), and permeabilized with 0.25% Triton X-100 for 10 min at ambient temperature. After blocking with PBS containing 3% bovine serum albumin (BSA), cells were incubated with mAb for 1 h at 37 °C. Unbound antibodies were removed with PBST three times and cells were incubated with anti-mouse IgG Alexa Fluor 488 (Zymed-Invitrogen, CA, USA) for 1 h. After staining with 4',6-diamidino-2-phenylindole (DAPI) for 10 min, coverslips were examined using a Zeiss Laser confocal fluorescence microscope (Nikon, JPN).

2.8. Plasmid construction for epitope mapping

To map the epitopes of generated mAbs, a series of eukaryotic plasmids expressing truncated V proteins were constructed. Consecutive truncations were introduced to

the V protein ORF of pFLAG-ZJ1-V by overlapping PCR using PfuUltra II Fusion HS DNA polymerase (Stratagene, Agilent, US). Primers and locations of truncated sequences are in Table 2, Table 3. PCR was 30 cycles of 98 °C 10 s, 58 °C 20 s and 72 °C 8 min. After purification by PCR purification kits (Axygen), PCR products were digested with DpnI (Fermentas) at 1 U/μL at 37 °C for 1 h with inactivation at 80 °C for 10 min. Digested PCR products were directly transformed into E. coli DH5α. All plasmids were identified by sequencing (Sangon Biotechnology, Shanghai, China).

2.9. Plasmid transfection

HeLa cells at 70% confluence, seeded in 6-well plates, were transfected with pFLAG-ZJ1-V and derived recombinant plasmids using FuGENE HD transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. Transfected cells were harvested at 48 h post transfection and subjected to FLAG-tagged V protein detection by WB.

2.10. Polypeptide design and detection

To fine-map epitopes recognized by mAb 3D7, three polypeptides spanning amino acid (aa) 144–167 of V protein were synthesized by GL Biochem (Shanghai, China) were 144SPTSGPTTRGPAELWK159, 147SGPTTRGPAELWKQPGK163 and 152RGPAELWKQPGKTAAS167. A panel of polypeptide mutants was synthesized based on 147SGPTTRGPAELWKQPGK163, in which certain aa residues were replaced by alanine (A) and named S147 A, G148 A, P149 A, T150 A, T151 A, R152 A, G153 A, P154 A, E156 A, L157 A, W158 A, K159 A and Q160 A. Purified His-VCD was the positive control and an irrelevant peptide (aa 40PQGKTKALSTA50 from ZJ1 V protein) was the negative control. Reactivity of mAb 3D7 with each polypeptide was determined by ELISA and WB.

2.11. Bioinformatics analysis

Prediction of aa sequences, alignment of sequences and phylogenetic analysis used the MegAlign program in the Lasergene package (DNASTAR Inc., Madison, WI, USA). V protein sequences of 27 reference NDV strains of different genotypes were from EMBL/GenBank (Table 1). BepiPred-2.0 online software was used to predict sequential B-cell epitopes of NDV V protein (http://www.cbs.dtu.dk/services/BepiPred/). Amino acid sequences of NDV V proteins were sent to Swiss Model (http://swissmodel.expasy.org/) for modelling of a three-dimensional (3D) structure of NDV V protein, which was subjected to DiscoTope 2.0 Server for discontinuous B-cell epitope analysis (Kringelum et al., 2012).

3. Results

3.1. Expression and purification of recombinant NDV ZJ1-VCD protein

A His-tagged form of NDV V protein CTD polypeptide was highly expressed by pET-28a-ZJ1/VCD in E. coli and had a molecular weight of 15 kDa as determined by SDS-PAGE, as predicted (Fig. 1A). Recombinant protein His-VCD was purified through Ni-chelating affinity chromatography under denaturing conditions and confirmed by WB with anti-His, in which a single band with the expected molecular weight of approximately 15 kDa was observed (Fig. 1B). The recombinant protein was harvested and used as the antigen for immunization and detection.

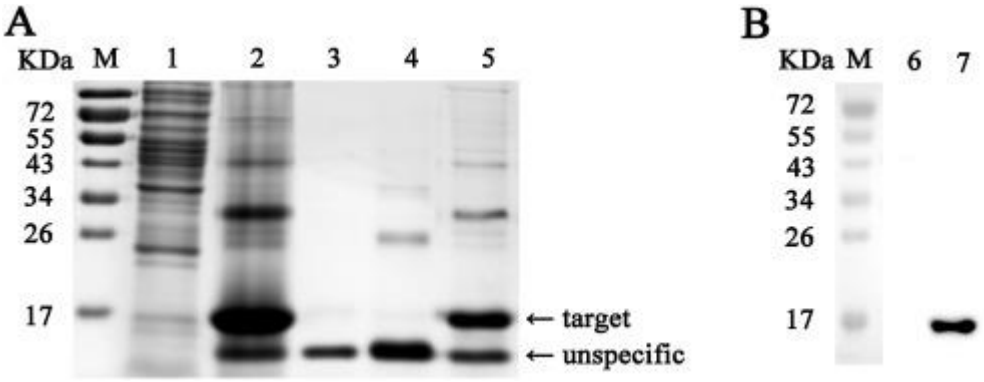

Fig. 1. SDS-PAGE and Western blot assays for the recombinant CTD polypeptide of NDV V protein expressed from pET-28a-ZJ1/VCD in E. coli BL21. (A) SDS-PAGE assay of His-tagged form of the ZJ1 V protein CTD region expressed in E. coli. (B) WB assay of the purified recombinant protein His-VCD using anti-His. M, PageRuler prestained protein ladder; 1 and 6, E. coli BL21 lysate (negative control); 2, total His-VCD expressed from pET28a-VCD; 3, His-VCD expressed in the supernatant; 4 and 7, His-VCD expressed in inclusion bodies; 5, His-VCD purified from inclusion bodies.

3.2. Generation of the mAb 3D7 against NDV V protein

Five hybridoma cell lines were acquired and only one stably produced antibodies that reacted strongly with His-VCD in indirect ELISA and IFA (data not shown). This mAb clone was designated as 3D7. Using a commercially available isotyping kit (Roche), the mAb 3D7 heavy chain was determined to be IgG1 and the light chain was κ. The ascites fluid of mAb 3D7 was produced and purified to the final concentration of 1.5 mg/mL.

3.3. Specificity of the mAb 3D7 for different NDV genotypes

As shown in Fig. 2A, the mAb 3D7 reacted with the recombinant His-tagged V protein expressed by pFLAG-ZJ1-V in DF1 cells. A single band of about 35 kDa was observed, the same as the result with anti-His antibodies. Further, the purified ZJ-1 (class II, genotype VII), JS10 (class I), La Sota/46 (class II, genotype II), Herts/33 (class II, genotype IV) and ND167 (class II, genotype VI) viruses were detected in WB to determine the reactivity of mAb 3D7 with distinct NDV virions. Both P and V protein were detectible in all of the NDV virions using antiserum anti-PNT; however, mAb 3D7 reacted only with the V protein of ZJ1, but not any other strains.

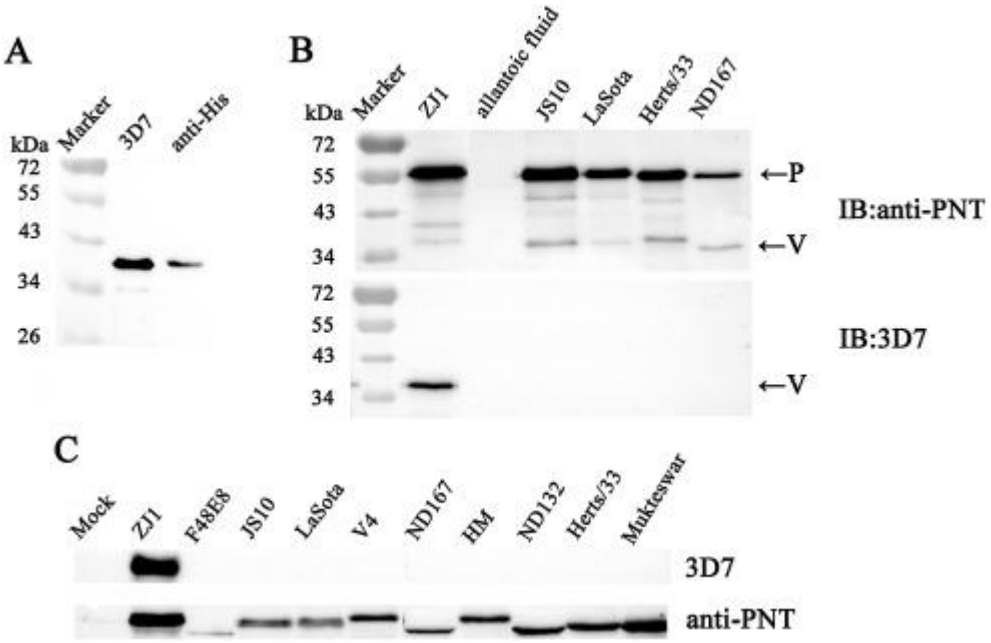

Fig. 2. Reactivity and specificity assay of mAb clone 3D7 by Western blot. (A) WB assay of the His-tagged V proteins expressed by the recombinant plasmid pFLAG - ZJ1-V in DF1 cells using anti-His or mAb 3D7 antibodies. (B) WB assay of V and P proteins contained in different NDV virions using the mAb 3D7 and antiserum anti-PNT. (C) WB assays of the V and P protein expressed in NDV-infected DF1 cells.

This result was confirmed in NDV-infected DF1 cells. Using mAb 3D7, V protein with a molecular weight of 35 kDa was detected only in ZJ1-infected DF1 cells (Fig. 2C), but not any other NDV-infected cells, involving JS10 (class I), V4 (class II, genotype I), La Sota/46 (class II, genotype II), Mukteswar, HM (class II, genotype III), Herts/33 (class II, genotype IV), ND132, ND167 (class II, genotype VI) and F48E8 (class II, genotype IX). As a comparison, P protein were detected in those NDV strains-infected cells, with varying molecular weights of around 55 kDa. This result was probably due to different phosphorylation levels of P protein in different strains (Qiu et al., 2016c).

Indirect ELISA assay was performed and the titer of mAb 3D7 against purified His-VCD protein was 1:3200. The mAb 3D7 never react with all the virus detected except for ZJ1. The titer was 1:800.

3.4. Identification of B cell epitopes recognized by the mAb 3D7

The epitopes recognized by mAb 3D7 was mapped in WBs with NDV V protein and its derived protein mutants. As shown in Fig. 3A, the mAb 3D7 did not react with V protein when aa 132-161 or 152-181 were truncated; by contrast, the truncation of 172-201, 192-221, 212-231 from V protein never influenced the reactivity of 3D7. Subsequent experiments showed that truncated V protein mutants without peptides spanning aa 140-154, 141-155, 142-156, 143-157, 144-158, 145-159, 146-160, 147-161, 148-162, 149-163, 150-164, 151-165, 152-166, 153-167, 154-168, 155-169, 156-170 or 157-171 were not recognized by mAb 3D7 (Table 3 and Fig. 3B). These peptides contain 154PAEL157 as the common aa, suggesting that mAb 3D7 recognized epitope was between aa 140 and 171 and peptide 154PAEL157 was essential.

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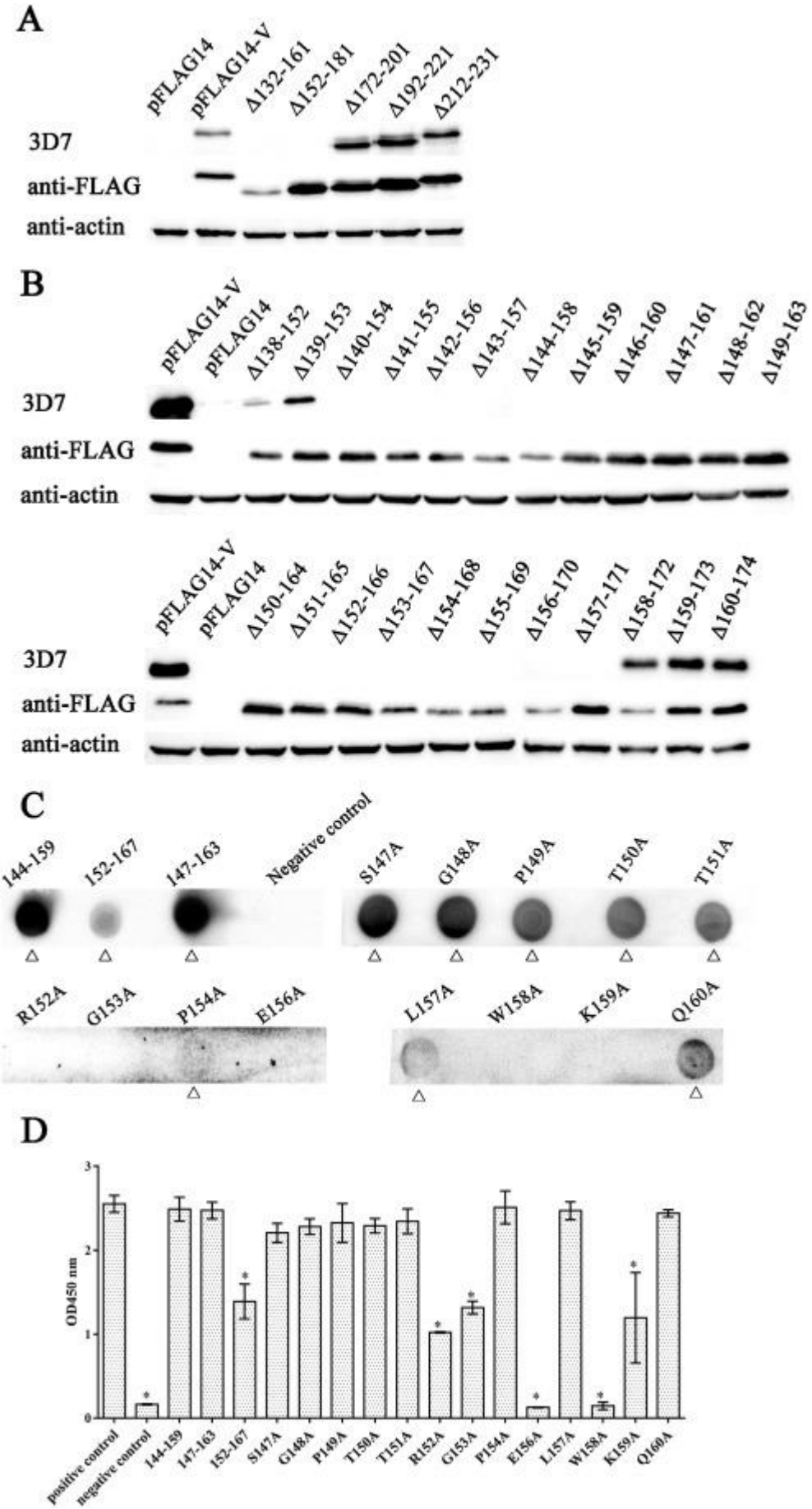
A
pFLAG14
pFLAG14-V
Δ132-161
Δ152-181
Δ172-201
Δ192-221
Δ212-231
3D7
anti-FLAG
anti-actin
B
pFLAG14-V
pFLAG14
Δ138-152
Δ139-153
Δ140-154
Δ141-155
Δ142-156
Δ143-157
Δ144-158
Δ145-159
Δ146-160
Δ147-161
Δ148-162
Δ149-163
3D7
anti-FLAG
anti-actin
pFLAG14-V
pFLAG14
Δ150-164
Δ151-165
Δ152-166
Δ153-167
Δ154-168
Δ155-169
Δ156-170
Δ157-171
Δ158-172
Δ159-173
Δ160-174
3D7
anti-FLAG
anti-actin
C
144-159
152-167
147-163
Negative control
S147A
G148A
P149A
T150A
T151A
R152A
G153A
P154A
E156A
L157A
W158A
K159A
Q160A
D
OD450 nm
positive control
negative control
144-159
147-163
152-167
S147A
G148A
P149A
T150A
T151A
R152A
G153A
P154A
E156A
L157A
W158A
K159A
Q160A

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*Fig. 3. Mapping of the epitope recognized by mAb 3D7. (A) WB detection of a panel of recombinant V protein, in which 30 aa were consecutively truncated in the CTD region. All the V protein mutants and their deleted regions are listed in Table 2. (B) WB detection of a panel of recombinant V protein, in which 15 aa were consecutively truncated in the ORF spanning from aa 138 to 174. All the V protein mutants and their deleted regions are listed in Table 3. (C) Dot blots detection for the mAb 3D7 using synthesized peptides. The labels 144-159, 147-163 and 152-167 indicate the peptides 144SPTSGPTTRGPAELWK159, 147SGPTTRGPAELWKQPGK163 and 152RGPAELWKQPGKTAAS167, respectively. S147 A, G148 A, P149 A, T150 A, T151 A, R152 A, G153 A, P154 A, E156 A, L157 A, W158 A, K159 A and Q160 A indicate the polypeptide mutants based on 147SGPTTRGPAELWKQPGK163, in which certain aa was replaced by alanine. All the positive results are labeled with ⊿ under the blot. (D) Dot ELISA detection for the mAb 3D7 using synthesized peptides. Error bars represent standard deviation. The OD450 value of synthesized peptides were compared with the positive control by using the Student's t-test and the ELISA readings that were significantly different from the positive control are labelled * ($P < 0.05$).*

Fine mapping of the epitope was performed by dot blots and dot ELISAs using three synthesized peptides spanning aa 144-159, 147-163 and 152-167 of V protein. Reactivity of 3D7 with peptide 152-167 in dot blots was impaired compared to peptides 144-159 and 147-163 (Fig. 3C). In dot ELISAs, all three peptides were recognized by 3D7. The OD450 of peptide 152-167 was slightly lower than other peptides (Fig. 3D). This result suggested that aa region 147-159 was involved in formation of the epitope, but only the common aa sequence 152RGPAELWK159 of these peptides was essential. A panel of point mutations was introduced into the synthesized peptides. Removal of residues at R152, G153, E156, W158 and K159 blocked recognition by 3D7 in dot blots; while the residue removal at S147, G148, P149, T150 and T151 did not influence the reactivity with 3D7. Besides, the mutation of P154 and L157 compromised mAb reactivity. Similar results were observed in dot ELISA results, the ELISA readings of 3D7 with the peptide mutant R152 A, G153 A, E156 A, W158 A and K159 A were significantly lower than the positive control. In addition, residue K159 was not recognized by the mAb in dot blot assays but was detectible in dot ELISA assays (Fig. 3C and D).

3.5. Protein modelling and B-cell epitope analysis of NDV V protein

The complete 3D structure of the NDV V protein was modelled according to the crystal structure of its counterpart of PIV5 (Fig. 4). The 3D7-recognized epitope was in the region spanning aa 140–171, all of which was exposed on the surface of V protein. The region 147-159, determined to be recognized by 3D7 were marked in the 3D structure of V protein. The V protein structure displayed that the crucial peptide 154PAEL157 of the 3D7 recognized epitope was not in a same plane with aa 147-151, suggesting that aa 147-151 was not a crucial element for direct mAb binding. The linear B-cell epitopes of the NDV V protein was predicted from the primary protein sequences and the discontinuous B-cell epitopes was predicted based on the 3D structure, all of which covered the identified 3D7-recognized peptide 152RGPAELWK159 (Table 4).

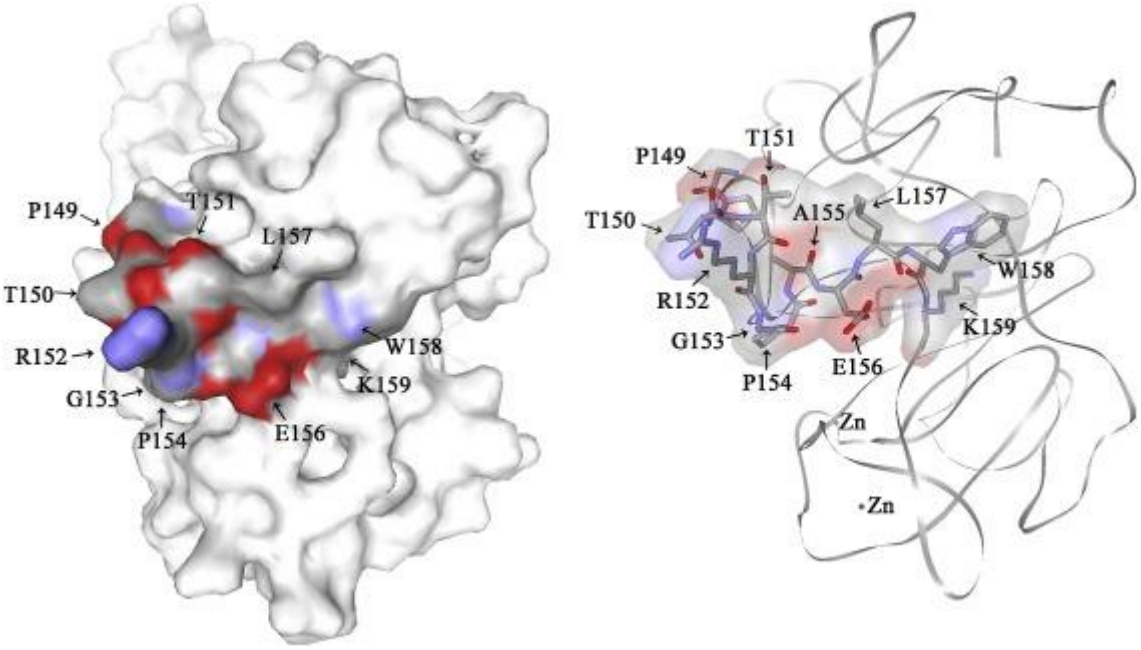

Fig. 4. Relative localization of the identified epitopes in the predicted 3D structure of the NDV V protein. The three-dimensional structure of the NDV V protein was modelled by the online services Swiss Model. The identified mAb 3D7-recognized peptide ^{152}RGPAELWK^{159} and its structurally supporting part is labeled blue and red in the figure. The red areas represent oxygen and the blue areas indicate nitrogen. The model on the left is the predicted V protein structure with the calculated surface structure; while the model on the right displays the atoms of the mAb 3D7-recognized peptide in a NDV V protein backbone without showing the surface structure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Specificity and conservation of the epitope among NDV strains

V proteins from distinct NDV genotypes (Table1) were aligned for analysis. The mAb clone 3D7-recognized epitope 147SGPTTRGPAELWK159 and its surrounding region aa 140–146 and 160–171 were conserved among genotype VII NDV strains; however, the epitope shared low identity among other genotypes (Fig. 5), indicating that these sequences were only conserved in specific genotypes. The epitope recognized by mAb clone 3D7 was not in the zinc finger structure of V protein and was likely not a crucial element for NDV V protein, suggesting it as a potential target for NDV genotype and subgenotype differentiation.

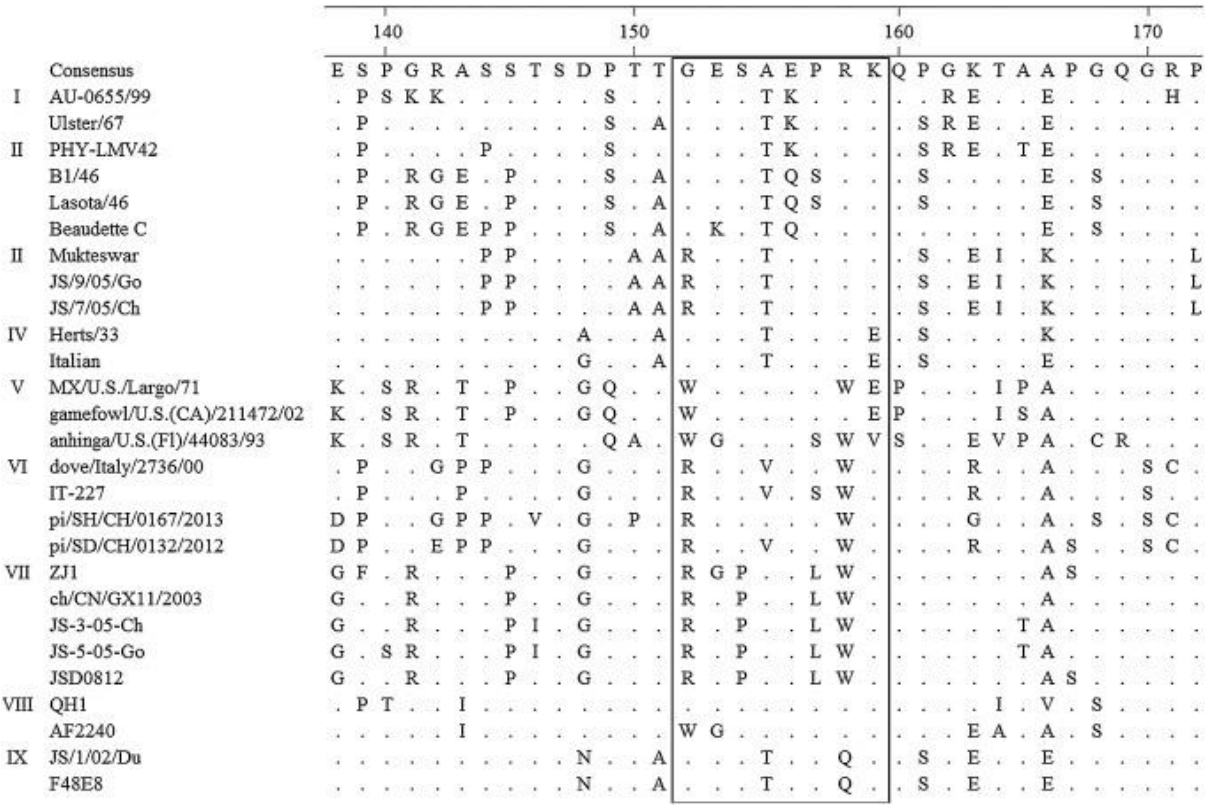

Fig. 5. Alignment of the identified epitope in the V proteins of NDV strains from diverse genotypes. V protein sequences from the 27 reference NDV strains of different genotypes were from the EMBL/GenBank (Table 1). Prediction of amino acid (aa) sequences, alignment of sequences and phylogenetic analysis were performed using the MegAlign program in the Lasergene package (DNASTAR Inc. Madison, WI, USA). The aa sequence that resembled the consensus sequence is indicated by a ".". The region of the mAb 3D7-recognized peptide 152RGPAELWK159 identified in the different NDV strains is boxed in the figure.

3.7. Expression of V protein during genotype VII NDV infection

To determine if mAb clone 3D7 could be used as a tool for immune-detection, dynamic expression of the V protein in NDV-infected cells was surveyed by IFAs and WBs. Firstly, the WB assay of NP protein showed that NDV was propagated in the infected cells. The mAb clone 3D7 reacted with V protein in ZJ-1-infected cells (Fig. 6). V protein was detectable at 6 hpi as early as NP of NDV, suggesting it was likely expressed early during the virus's replicative cycle. Similar results were observed by IFAs (Fig. 7). V protein was initially detected at 6 hpi, scattered in the cytoplasm with dotted distribution. From 8 hpi to 12 hpi, V protein accumulated in the cytoplasm and moved towards the periphery of the host cell nucleus. At 18 hpi, some nuclei of infected cells were surrounded by V protein. A large area of syncytia was observed at 24 hpi, and V protein was observed in the middle of the syncytia surrounded by several host nuclei and starting to dissipate.

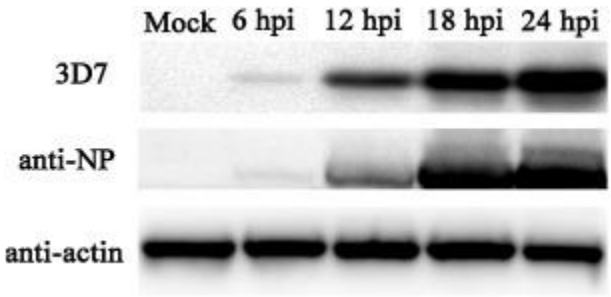

Fig. 6. Dynamic expression of V protein in DF-1 cells infected with the NDV strain ZJ1 by WB. DF-1 cells were infected with NDV ZJ1 at MOI 5 and collected at 6, 12, 18, 24 hpi. Lysates from cells were immunoblotted with the indicated 3D7, anti-NP and anti-actin monoclonal antibodies.

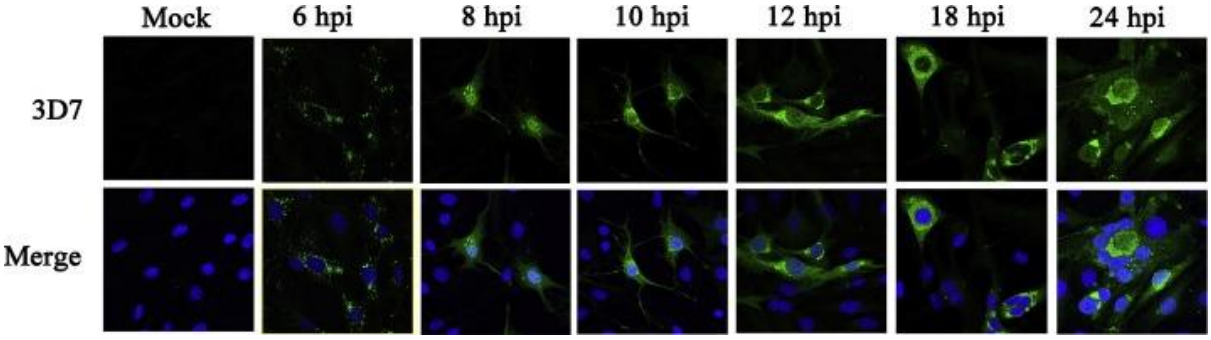

Fig. 7. Dynamic expression of V protein in DF-1 cells infected with the NDV strain ZJ1 by IFA. DF-1 cells were infected with NDV ZJ1 at a MOI of 5 and harvested at 6, 8, 10, 12, 18, 24 hpi. Cells were double stained with the mAb3D7 for V protein and 4',6'-diamidino- 2-phenylindole (DAPI) for nuclei. The upper panel of figures show the V protein (green), while the lower figures show overlapping of V protein (green) and DAPI (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

It has been reported that NDV V protein plays an important role in facilitating virus replication in infected cells via antagonizing cellular IFN signaling (Huang et al., 2003; Park et al., 2003b; Qiu et al., 2016b). The V proteins from distinct NDV strains showed different interferon antagonistic activities (Alamares et al., 2010), albeit the molecular mechanism is unclear since there is little information about the structure and functional domains of NDV V protein. Mapping mAbs binding peptides may shed light on the V protein structure analysis. In this study, a NDV V protein-reactive Mab 3D7 was generated. The reactivity of this mAb was limited to genotype VII strain and could be genotype specific (Fig. 2). To analyze the specificity of mAb 3D7, epitope mapping was performed based on detection of consecutive truncated His-tagged V proteins and synthesized peptides. The results showed that the epitope recognized by mAb 3D7 was located in aa 147–159, and in which peptide 152RGPAELWK159 was essential (Fig. 3).

The epitope (152RGPAELWK159) that we identified in NDV V protein was located at a region after the RNA editing site, which was flanked by two important functional regions, including N-terminal domain of P protein (Karlin et al., 2003; Qiu et al.,

2016c) and CTD (Horvath, 2004b); nevertheless, there is rare information regarding this region of NDV V protein at present. Based on the crystal structure of SV5 V protein (Li et al., 2006), the 3D structure of NDV V protein was established using the primary sequence. The predicted 3D structure provides potential useful structural information about the function and antigenic characteristics of the V protein. The predicted V protein structure displayed that the aa 147-159 of V protein was exposed on the protein surface and the core part peptide 152RGPAELWK159 formed a pocket, which would be recognized by the mAb. Furthermore, the reactivity of peptide 152-167 with 3D7 was weaker than peptide 144-159 and 148-163, suggesting that the region of aa 147-151 structurally contributed to epitope presentation on the V protein surface although it was not indispensable for mAb-epitope interaction, which was supported by the 3D structure of V protein.

One purpose of our study was to determine if V protein could be used as a tool for quick differentiation of genotypes and subgenotypes. Different from their counterparts in other paramyxoviruses, V protein NDVs are reported to be a structural component of virions (Lamb and Kolakofsky, 2002; Steward et al., 1995), which is confirmed by our results (Fig. 2). It suggested that V protein can be used as a detection target for NDV virion. Bioinformatics analysis of the NDV V protein revealed that aa 147-159 of V protein, especially the core peptide 152RGPAELWK159 was exposed on the protein surface and displayed strong antigenicity for B-cell recognition based on the online analysis (Table 4), making it a good target epitope for detection.

The identified and predicted B-cell epitopes were compared (Fig. 5). The identified epitope region aa 147–159 overlapped with predicted epitopes aa 141-148 and aa 150-173. Furthermore, the core peptide 152RGPAELWK159 was totally included in the predicted epitopes aa 150-173. Not only linear epitope but the predicted discontinuous epitope aa 141–163 contained all the region recognized by mAb 3D7, suggesting the peptide would contribute to the formation of conformational epitopes. All the above results indicated that the region of NDV V protein recognized by mAb 3D7 was highly immunogenic.

The mAb only react with genotype VII strain and the sequence alignment indicated that the sequence of aa 140-171 of NDV V protein varied among genotypes but was conserved among NDV strains belonging to the same genotype. Importantly, the 152RGPAELWK159 was conserved in genotype VII NDV strains, suggesting that the mAb clone 3D7 recognized an epitope specific for genotype VII or VIId. Defining conserved epitopes can contribute to the development of epitope-based diagnosis methods. It is widely known that most of the prevalent virulent NDV isolates belong to class II, genotype VII; meanwhile, the class I and class II non-virulent strains are spread worldwide due to live vaccine administration and natural infection (Kim et al., 2007; Ramey et al., 2013). The limited genetic and antigenic diversity of NDV genotypes makes quick diagnosis complicated and difficult (Miller et al., 2010). Since the epitope recognized by mAb clone 3D7 was conserved in the genotype VII NDV strains, it could be a potential targeting site for NDV genotype and subgenotype differentiation. However, the NDV isolates used for detection in this study is limited, one cannot rule out the possibility that there would be some NDV variants with different reactivity with 3D7. More detection is required before it can be used for clinical applications. Since the 3D7-recognized region displayed genotype-specific

conservation, it can be used as an immunogen to establish more genotype-specific mAbs, or directly used for epitope-based genotype differentiation.

It is found in this study that the V protein-specific mAb clone 3D7 could be applied to various assays. The expression levels and cellular movement of V protein during viral replication were determined, since the subcellular localization of V protein during NDV replication has not been previously reported. Dynamic expression of V protein in NDV-infected cells was seen in IFAs and WBs (Fig. 6, Fig. 7). Scattering of V protein in the cytoplasm was initially detectible at 6 hpi, and the protein moved to the periphery of the host cell nucleus in the process of infection. At late stage of infection, a mass of V protein was observed around the nuclei of infected cells. This result showed the subcellular movement of NDV V protein in the process of IFN antagonism. In response to NDV infection, latent cytoplasmic STAT proteins are phosphorylated on tyrosine by the Janus family of tyrosine kinase (JAK) enzymes and form a heterotrimer of phosphorylated STAT-1, STAT-2 and IRF-9. Subsequently, this heterotrimer translocates to the nucleus and binds to cis-acting DNA elements to activate the IFN-I-stimulated antiviral genes (Horvath, 2004a, b; Samuel, 2001). V protein has IFN-antagonist activity in the CTD, which promotes degradation of STAT1 and blocks IFN signaling (Alamares et al., 2010; Park et al., 2003b). Our results showed that V protein tend to accumulate around the nuclei of infected cells, suggesting it might act on STAT-1 protein in the course of nuclear import of phosphorylated STAT-1.

5. Conclusion

The mAb clone 3D7 against NDV V protein was isolated and the mAb recognized epitope was identified to be 152RGPAELWK159. This peptide was located in a region which was varied in sequence among genotypes but conserved in sequence and structure among NDV strains in the same genotype. The generated V protein-specific mAb clone 3D7 could be applied to various assays and helped us to determine the location of V protein during NDV replication in infected cells. These results extend our understanding of the antigenic structure of V protein and the function of V protein during NDV infection. They also provide a foundation for development of novel, epitope-based genotype differentiation of NDV genotypes.

Conflict of interest

The authors declare that they have no competing interests.

Author contributions

CD and XQ conceived and designed the research. JL, WW, TR, CM, and YZ performed the experiments. XQ, CM, CS, ZD, XL and YS analyzed the data. LT, SX, WY, XL, VN, MM and YL contributed reagents/materials/analysis tools. XQ and CD wrote the paper.

Ethical approval

The Animal experiment protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Veterinary Research Institute (SHVRI), Chinese Academy of Agricultural Sciences (CAAS), and the Permit Number is shvri-mo-0124. The Animal experiment was carried out in agreement with the IACUC guidelines set by SHVRI, CAAS.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Data availability

All data generated or analyzed during this study are included in this published article.

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