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Epitope mapping and cellular localization of swine acute diarrhea syndrome coronavirus nucleocapsid protein using a novel monoclonal antibody

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A B S T R A C T

A swine acute diarrhea syndrome coronavirus (SADS-CoV) that causes severe diarrhea in suckling piglets was identified in Southern China in 2017. To develop an antigen that is specific, sensitive, and easy to prepare for serological diagnosis, antigenic sites in the SADS-CoV nucleocapsid (N) protein were screened. We generated and characterized an N-reactive monoclonal antibody (mAb) 3E9 from mice immunized with recombinant N protein. Through fine epitope mapping of mAb 3E9 using a panel of eukaryotic-expressed polypeptides with GFP-tags, we identified the motif ^{343}DAPVFTPAP^{351} as the minimal unit of the linear B-cell epitope recognized by mAb 3E9. Protein sequence alignment indicated that ^{343}DAPVFTPAP^{351} was highly conserved in different SADS-CoV strains and SADS-related coronaviruses from bat, with one substitution in this motif in HKU2-related bat coronavirus. Using mAb 3E9, we observed that N protein was expressed in the cytoplasm and was in the nucleolus during SADS-CoV replication. N protein was immunoprecipitated from SADS-CoV-infected Vero E6 cells. Taken together, our results indicated that 3E9 mAb could be a useful tool to investigate the structure and function of N protein during viral replication.

1. Introduction

Coronaviruses (CoVs) are enveloped, single-stranded, positive-sense RNA viruses in the order *Nidovirales*, family *Coronaviridae* and subfamily *Coronavirinae*, which comprises four genera, *Alpha-*, *Beta-*, *Gamma-*, and *Delta-*CoV. CoVs infect humans and other mammals and birds, causing subclinical or respiratory and gastrointestinal disease (Woo et al., 2012). Swine acute diarrhea syndrome coronavirus (SADS-CoV), also called swine enteric alphacoronavirus, is a newly discovered coronavirus that can cause severe and acute diarrhea and rapid weight loss in piglets younger than 6 days old. From January to May 2017, an outbreak of SADS-CoV led to the deaths of almost 25,000 piglets in Southern China and resulted in significant economic losses (Fu et al., 2018; Gong et al., 2017; Pan et al., 2017; Zhou et al., 2018b).

CoVs contain a very large RNA genome (26–32 kb) (Su et al., 2016). The first two-thirds of the genome code for proteins involved in replication and transcription of viral RNA. The other third codes structural proteins that build up the coronavirion. All CoVs contain a common set of structural proteins: N, spike, membrane and envelope (Lai, 1990). In infected cells, N protein is the most abundant of the viral proteins (He et al., 2004). N protein is involved in structure and RNA synthesis (Almazan et al., 2004; Enjuanes et al., 2006). In the virion, nucleoprotein molecules associate with viral RNA to form the helical nucleocapsid (Nelson et al., 2000). CoVs N protein contains both nuclear localization and export signals (Rowland et al., 2005; Timani et al., 2005; You et al., 2005) suggesting shuttling of the protein between the nucleus and the cytoplasm in virus-infected cells. Nucleolar localization of other coronavirus nucleoproteins is reported in transfected cells (Chang et al., 2004; Shi et al., 2014; You et al., 2005). However, inconsistent data were obtained about the presence of N protein in the nucleus of virus-infected cells with some studies seeing no evidence of nuclear localization (Laude and Masters, 1995).

N proteins from different coronaviruses vary in length and primary sequence. Nevertheless, some motifs with functional relevance are conserved, and N proteins share a three-domain organization according to sequence similarity (Parker and Masters, 1990). Identification of

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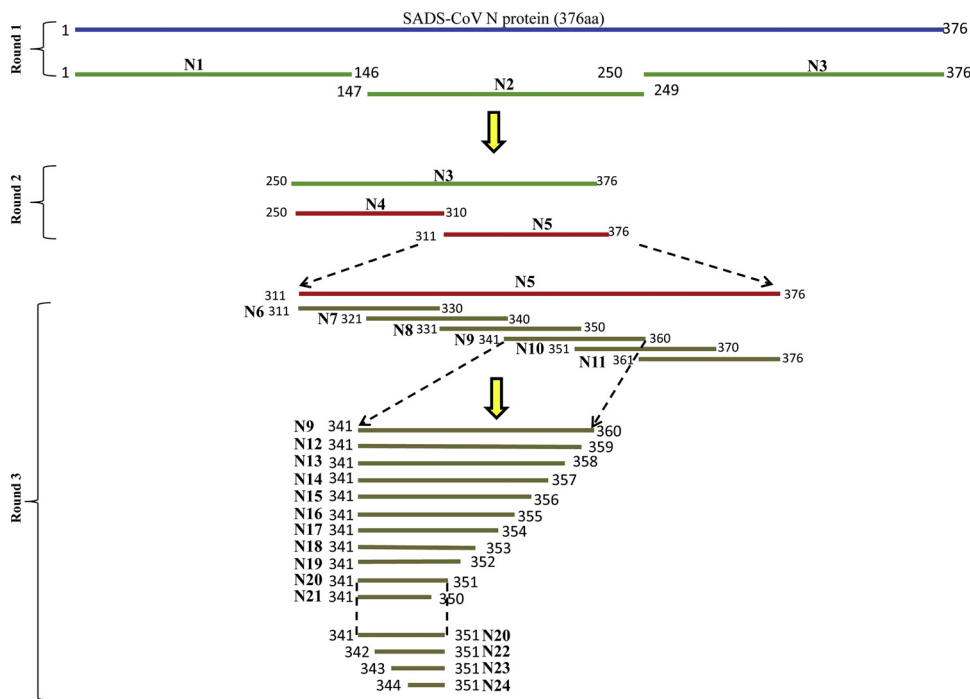
Fig. 1. Schematic representation of SADS-CoV N fragments used for B-cell epitope mapping. Three rounds of N peptides were conducted to investigate epitopes of the generated mAb. The original whole N (376 aa) was marked with blue, the first round was marked with green, the second round was maked with red, and the third round was marked with tawny.

epitopes on the N protein of SADS-CoV has not been reported. In this work, monoclonal antibodies (mAbs) against the SADS-CoV N protein were produced to study the distribution of the nucleocapsid protein in virus-infected cells and identify regions of the protein that may have antigenic, structural and functional properties. The data indicated that the mAb could be useful for investigating the function of N protein.

2. Materials and methods

2.1. Cell lines and viruses

The myeloma cell line SP2/0 and the Vero E6 cell line, maintained in our laboratory, were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) in a humidified 5% CO_2 atmosphere at 37 °C. All culture media were supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, USA) and antibiotics (0.1 mg ml^{-1} streptomycin and 100 IU ml^{-1} penicillin).

2.2. Recombinant protein expression and purification

The N gene of the SADS-CoV genome (GenBank accession No. MF094681) was cloned into prokaryotic expression vector pGEX-6p-1 (Pharmacia, Belgium). Inserts in recombinant plasmids were sequenced and confirmed plasmids were transformed into *Escherichia coli* BL21 (DE3) and induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) over 6 h in Luria-Bertani medium. Expressed fusion proteins were analyzed with SDS-PAGE and detected by staining with Coomassie blue. For preparation of purified proteins, bacterial cultures were harvested and crude lysates subjected to SDS-PAGE. Separated proteins were visualized by soaking polyacrylamide gels in 0.25 M KCl. Bands corresponding to N were excised, homogenized, and added to an appropriate volume of sterile phosphate-buffered saline (PBS). After several freeze-thaw cycles, PBS was separated by centrifugation. Purified proteins were used to immunize mice. Recombinant N protein was identified using mouse mAb against the GST tag (Sigma, USA) that was incorporated into the recombinant protein.

2.3. Production of mAbs against SADS-CoV N protein

Female 6-week-old Balb/c mice were from Beijing Vital River Laboratory Animal Technology Co., Ltd and housed in SPF isolators ventilated under negative pressure. Feed and water were provided ad libitum. The 6-week-old Balb/c mice were immunized with 50 ug purified recombinant GST-N emulsified in complete Freund's adjuvant (Sigma, USA). Booster immunizations were performed in the same manner after 2 weeks, except that protein was emulsified in incomplete Freund's adjuvant. Following booster immunizations, mice were intraperitoneally administered 100 μg recombinant N without adjuvant at 2-week intervals. Mice were euthanized 3 days later and harvested spleen cells fused with SP2/0 cells using standard procedures (Galfre and Milstein, 1981). Fused cells were cultured in 96-well plates and selected in hypoxanthine-aminopterin-thymidine (HAT, Sigma, USA) medium and hypoxanthine-thymidine (HT) medium in sequence. Resulting hybridoma cells were maintained in DMEM containing HT and 10% FBS. Hybridoma supernatants were assayed for N-specific antibodies by western blot and ELISA. Selected positive hybridomas were cloned three times by limiting dilution. Ascites containing N mAbs were prepared from mice injected intraperitoneally with 0.5 ml sterile paraffin oil and hybridomas (10^5 cells/mouse) suspended in DMEM. Titers of mAbs were determined using immunofluorescence assays and antibody subtypes determined using Mouse MonoAb-ID Kits (HRP) (Invitrogen, USA) according to the manufacturer's instructions.

Animal care and all procedures were performed in accordance with animal ethics guidelines and approved protocols. The animal experiments were approved by Harbin Veterinary Research Institute. The animal Ethics Committee approval number is Heilongjiang-SYXK-2006-032.

2.4. Plamid constructions and transfection

The full-length N sequence was amplified from pGEX-6p-N, and cloned into pAcGFP-C1 and pCMV-Myc vectors, respectively. To map epitopes of the generated mAb, a series of polypeptides were expressed (Fig. 1). For the first round, three peptides spanning the SADS-CoV N protein (amino acids 1–146, 147–249, and 250–376) were expressed as green fluorescent protein (GFP) fusion proteins. Fragments encoding

A

SDS-PAGE

B

WB:GST
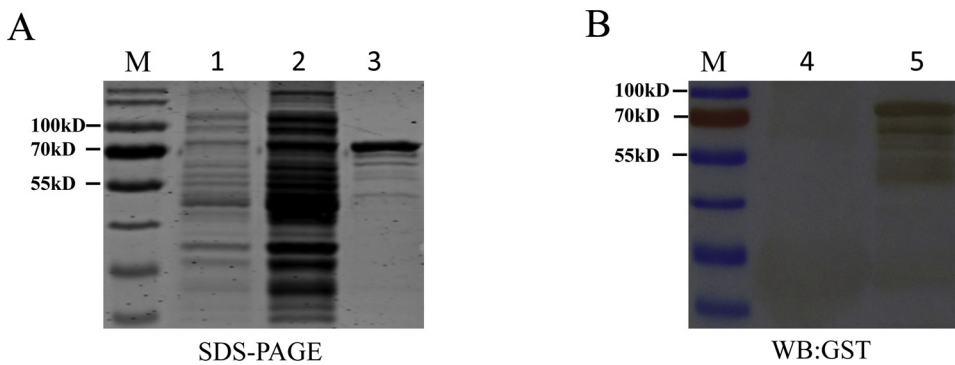
Fig. 2. Analysis of recombinant SADS-CoV N protein by SDS-PAGE (A) and western blot (B) with anti-GST mAb. Lane M: protein molecular weight marker; Lane 1: lysate of pGEX-6p-*N*-transformed *E. coli* BL21 (DE3) before IPTG induction; Lane 2: lysate of pGEX-6p-*N*-transformed *E. coli* BL21 (DE3) after IPTG induction; Lanes 3 and 5: purified recombinant GST-N; Lane 4: lysate of pGEX-6p-1-transformed *E. coli* BL21 (DE3) as negative control. M: Protein marker.

peptide sequences were amplified from pGEX-6p-N and cloned into the pAcGFP-C1 vector for expression. Resulting constructs were transfected into HEK293 T cells for eukaryotic expression using X-tremeGENE HP DNA Transfection Reagent (Roche, Germany) according to the manufacturer's instructions. Cells were harvested at 48 h post transfection. Expression of GFP-fused recombinant proteins was confirmed by western blot using mouse mAb against GFP (Proteintech, China). For the second round, two polypeptides spanning amino acids 250–376 (amino acids 250–310 and 311–376) were designed and expressed as GFP fusion proteins. Coding sequences of the two polypeptides were inserted into pAcGFP-C1 for eukaryotic expression. Recombinant proteins were expressed in HEK293 T cells and confirmed by western blot as described above. For the last round, for peptides N6–N22, pairs of oligonucleotides were synthesized. Each pair of oligonucleotide strands was annealed and cloned into expression vector pAcGFP-C1 for expression as GFP fusion proteins, confirmed by western blot as described above.

2.5. Western blot

Reactivity of mAbs with GFP-fused SADS-CoV polypeptides was analyzed by western blot. Culture lysates containing GFP-fused polypeptides or GFP alone were subjected to 12.5% SDS-PAGE. Proteins were transferred to nitrocellulose membranes, which were blocked with 5% (w/v) skim milk in PBS overnight at 4 °C. Nitrocellulose membranes were incubated with mAb against SADS-CoV N (1:1000 dilution) for 2 h. After washing three times with PBS containing 0.05% (v/v) Tween 20 (PBS-T), membranes were incubated with HRP-conjugated goat anti-mouse IgG (H + L) (1:1000; Li-Cor Biosciences, USA) for 1 h at RT. Membranes were washed with PBS-T and incubated with substrate solution (PBST, 0.05% DAB and 0.006% H_2O_2).

2.6. Immunoprecipitation of SADS-CoV N protein

Immunoprecipitation was as previously described (Zhang et al., 2014). Infected Vero E6 cells were harvested at 48 hpt, washed three times with cold PBS (pH 7.4), and lysed with IP lysis buffer (Thermos, USA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mg/ml protease inhibitor cocktail (Roche, Germany) at 4 °C for 30 min. Lysate supernatant (500 μg) was incubated overnight at 4 °C with 1 μg mAb 3E9. Protein A/G PLUS-Agarose (Santa Cruz, USA) was added to this mixture according to the manufacturer's instructions. After washing four times with lysis buffer, immunoprecipitated proteins were analyzed by western blot using mAb 3E9. A lysate from mock-infected Vero E6 cells was used as a control.

2.7. Immunofluorescence assays

Vero E6 cells seeded in glass-bottomed cell culture dishes (80–90% confluence) were inoculated with SADS-CoV strains at 10^5 TCID$_{50}$/dish and fixed with prechilled absolute methanol at -20 °C for 30 min at 48 hpi. After washing with PBS, cells were incubated with mAb 3E9

(diluted 1:500) and incubated at 37 °C for 2 h. Cells were washed with PBS and incubated with donkey anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody (1:1000) conjugated with Alexa Fluor Plus 488 (Thermo Fisher, USA) at 37 °C for 1 h. Cells were washed three times with PBS and DNA stained with 4′, 6-diamidino-2-phenylindole (DAPI, Sigma, USA) at room temperature for 30 min before washing with PBS. Images were captured with a confocal laser scanning microscope (Zeiss).

2.8. Sequence analysis and homology modeling of N protein epitopes

To analyze the conservation of the identified epitope among SADS-CoV reference strains, the epitope sequence and flanking sequences of N protein were compared with nine selected SADS-CoV strains using DNAMAN software (Lynnon BioSoft Inc., USA). Alignment analysis was performed for the defined epitope and corresponding regions of associated coronavirus strains using the DNASTAR Lasergene program (DNASTAR Inc., USA).

3. Results

3.1. Expression and purification of recombinant SADS-CoV N protein

Complete N protein was expressed as a GST-fusion protein. Recombinant protein GST-N was expressed in *E. coli* and had an expected molecular weight of approximately 70 kDa (Fig. 2A). Since the recombinant protein was predominantly insoluble in inclusion bodies, we purified it by excising GST-N protein from SDS-PAGE gels. We determined the purity of prepared recombinant GST-N using SDS-PAGE (Fig. 2A). Western blot showed that purified GST-N protein was recognized by anti-GST-tag mAb (Fig. 2B). The results indicated that purified recombinant GST-N had reactivity that was suitable for immunization. We also detected several bands smaller than GST-N protein, suggesting that small amounts of C terminally truncated proteins are cleaved from the full-length fusion protein by endogenous *Escherichia coli* proteolytic processes.

3.2. Production and characterization of SADS-CoV N protein-specific mAb

Hybridomas were screened by testing supernatants by SADS-CoV N-specific indirect ELISA. One hybridoma cell line secreting antibodies specific to SADS-CoV N protein was selected and subcloned three times by limiting dilution. Isotype determination showed that the N-specific mAb 3E9 was subclass IgG1/κ-type. The specificity of mAb 3E9 was tested using western blot and cell lysates infected with transmissible gastroenteritis virus (TGEV), porcine deltacoronavirus (PDCoV), porcine epidemic diarrhea virus (PEDV), or SADS-CoV. Hybridoma 3E9 mAb proved to be strictly SADS-CoV specific (Fig. 3A). To further determine mAb specificity, cell transfected with pCMV-Myc-N plasmid were analyzed by western blot using mAb 3E9 as the primary antibody. MAb 3E9 specifically reacted with eukaryotically expressed N protein

WB:3E9

IP: mAb 3E9

Input
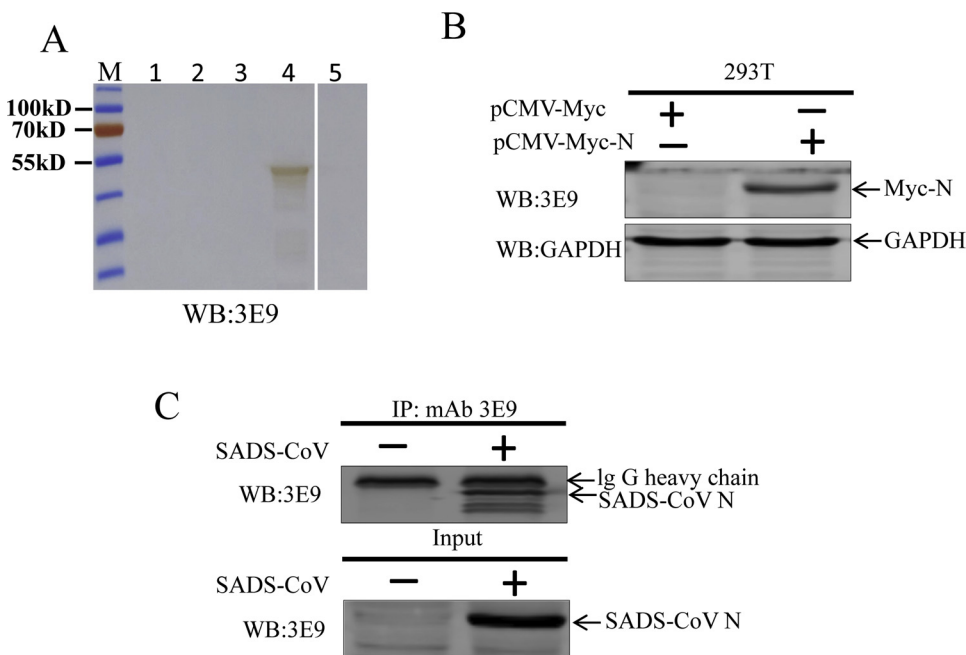
Fig. 3. Application of mAb 3E9 in immunological assays and techniques. (A) Specificity of SADS-CoV mAb 3E9 by western blot. ST cells were infected with TGEV and PDCoV. Vero E6 cells were infected with PEDV and SADS-CoV. Cell lysates were incubated with anti-N mAb 3E9. Lane 1: TGEV; Lane 2: PDCoV; Lane 3: PEDV; Lane 4: SADS-CoV; Lane 5: cell lysates infected with SADS-CoV incubated with negative serum for negative control. M: Protein marker. (B) Specific reactivity of N-specific mAb with eukaryotic-expressed N. Western blot of 293 T cells transfected with eukaryotic recombinant plasmids pCMV-Myc-N or empty vector pCMV-Myc. (C) Immunoprecipitation and western blot of N protein in mock-infected and SADS-CoV-infected Vero E6 cells.

but not with samples from empty plasmid pCMV-Myc transfections (Fig. 3B). *in vitro* neutralization tests showed that mAb 3E9 was not a neutralizing antibody (data not shown). Immunoprecipitation assays determined if SADS-CoV N protein could be precipitated from SADS-CoV-infected Vero E6 cells using mAb 3E9. The mAb precipitated SADS-CoV N protein from SADS-CoV-infected Vero E6 cells but not from mock-infected Vero E6 cells (Fig. 3C). We also noticed that the extent of SADS-CoV N cleavage varied with experiments and was determined primarily by the extent of viral infection or viral propagation conditions (e.g., trypsin addition) (Fig. 3C). SADS-CoV N expressed from an expression plasmid (pCMV-Myc-N) yielded only one major band at the expected size of full-length SADS-N (Fig. 3B). The additional smaller bands could be cleavage products based on several coronavirus N proteins identified by immunoblotting (Jaru-Ampornpan et al., 2017; Laude and Masters, 1995).

3.3. Localization of SADS-CoV nucleoprotein in infected or transfected cells

Using confocal laser scanning microscopy of SADS-CoV-infected cells, the site and distribution of the nucleoprotein was examined. N protein was localized predominantly in the cytoplasm in SADS-CoV infected cells (Fig. 4A), consistent with previous findings (Zhou et al., 2018b). In addition, nucleolar localization was also observed in a few SADS-CoV infected cells. To analyze the subcellular localization of N protein, pAcGFP-N (encoding GFP-N) was transfected into Vero E6 cells. GFP-N protein was found in both the nucleolus and cytoplasm of Vero E6 cells (Fig. 4B). These results demonstrated that the N protein localized to a subnuclear structure and the generated mAb 3E9 was specific for SADS-CoV N protein.

3.4. Precise localization of mAb 3E9 epitope

To determine epitopes recognized by mAb 3E9, three rounds of overlapping peptides fused with GFP-tag were designed and expressed in 293 T cells (Fig. 1). MAb 3E9 recognized the entire N protein (376 aa). For the first round, mAb 3E9 reacted with N3 peptide (aa250–376) (Fig. 5A). For the second round, N3 was divided into two GFP-fusion fragments (N4 and N5) and detected with mAb 3E9. The results showed that N5 peptide (aa 311–376) was recognized by the mAb 3E9 (Fig. 5B). Finally, a minimal peptide, ^{343}DAPVFTPAP^{351}, was characterized as the B-cell epitope recognized by mAb 3E9 (Fig. 5C).

3.5. Homology analysis of N epitope in SADS-CoV strains

To evaluate if the linear epitope recognized by mAb 3E9 was conserved among SADS-CoV isolates and SADS-related coronaviruses, we performed sequence alignments with SADS-CoV N. Epitope ^{343}DAPVF-TPAP^{351} was highly conserved among SADS-CoV isolates and SADS-related coronaviruses from bats. However, we found a substitution in the region in bat coronavirus HKU2 in a comparison with the epitope in SADS-CoV isolates (Fig. 6A). In addition, we analyzed homologous sequences for the defined epitopes in 11 alphacoronaviruses. The identified epitopes had low identity among the 11 alphacoronaviruses, indicating that the epitopes were specific for SADS-CoV and SADSr-CoV.

4. Discussion

SADS-CoV is a newly identified virus in south China in 2017 (Zhou et al., 2018b). All affected pigs show acute vomiting and severe watery diarrhea, similar to clinical signs caused by porcine deltacoronavirus, porcine epidemic diarrhea virus and transmissible gastroenteritis virus. The mortality rate of the virus is more than 35% in swine that are less than 10 days old causing serious economic losses to the swine industry (Pan et al., 2017). Although SADS-CoV N protein is instrumental for diagnosis of SADS-CoV (Wang et al., 2018; Zhou et al., 2018a), its biology remains unknown. CoV N protein facilitates template switching and is required for efficient transcription (Zuniga et al., 2010). To understand the multiple functions of SADS-CoV N protein and elucidate the mechanism of SADS-CoV replication, mAbs against this protein are needed. The availability of specific antibodies against SADS-CoV N protein might also facilitate further studies on viral biosynthesis.

The development of fast, easily operated diagnostic methods for SADS-CoV is important for disease control. Coronavirus N proteins are important structural proteins in virus assembly and are main targets for development of immunity-based prophylactic, therapeutic, and diagnostic techniques. Although diagnostic methods using SADS-CoV nucleotides have been developed using PCR and DNA sequencing of species-specific N genes (Pan et al., 2017; Zhou et al., 2018b), no differential diagnostic methods are reported for virus protein detection. To obtain diagnostic mAbs for clinical applications, in this study, we generated mAb 3E9 against a species-specific antigenic N protein of SADS-CoV. We found that the mAb reacted specifically against native SADS-CoV N protein (Figs. 3A and 4) and recombinant N protein
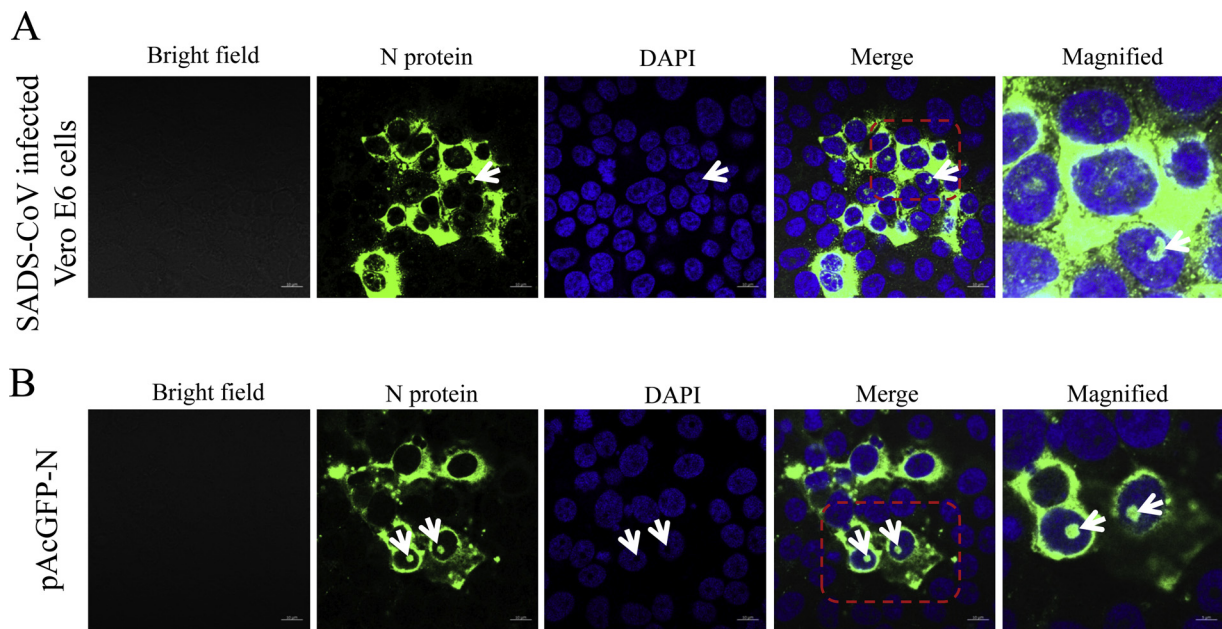
Fig. 4. Subcellular localization of SADS-CoV N protein. (A) Detection by indirect immunofluorescence in cells infected with SADS-CoV. (B) Localization using pAcGFP-SADS-CoV-N in transfected Vero E6 cells. N protein is magnified in merged images. Arrow, nucleolus (No).

(Fig. 3B). Mapping epitopes of viral proteins and defining the degree of conservation of identified epitopes may facilitate our understanding of antigenic structures and virus-antibody interactions. This information is useful for clinical applications. Although CoV N proteins are major structural proteins, epitopes on SADS-CoV N protein have not been reported. To study specificity in more detail and finely map the epitope bound by mAb 3E9, a series of truncated N proteins was generated for peptide scanning. An epitope recognized by mAb 3E9 corresponding to aa 343–351 (DAPVFTPAP) in the SADS-CoV N protein was identified (Fig. 5).

Previous studies found that the NTD and CTD of CoV N proteins are responsible for RNA binding and oligomerization, including in avian infectious bronchitis virus (Fan et al., 2005; Jayaram et al., 2006; Kuo et al., 2013; Spencer and Hiscox, 2006), SARS-CoV (Chang et al., 2006;

Chen et al., 2007), and HCoV-229E (Lo et al., 2013). The NTD and CTD structures have been investigated (Spencer and Hiscox, 2006). The CTDs of CoV N proteins mediate self-association in oligomer formation, making them a good target for mutagenesis research on disrupting CoV N protein self-association and virion assembly (Chang et al., 2005; Yu et al., 2005). In our study, the linear epitope of mAb 3E9 was located in the CTD of the SADS-CoV N protein. The mAb could be used to elucidate the function of this domain.

Sequence analysis demonstrated that epitopes identified in SADS-CoV N were highly conserved among SADS-CoV strains and SADSr-CoVs from *Rhinolophus affinis* and HKU2-CoV from *Rhinolophus sinicus* (Fig. 6a). Despite the structural and functional similarities of the N protein to other alphacoronavirus, the epitope did not share any obvious sequence homology and had low identity (Fig. 6b). The highly
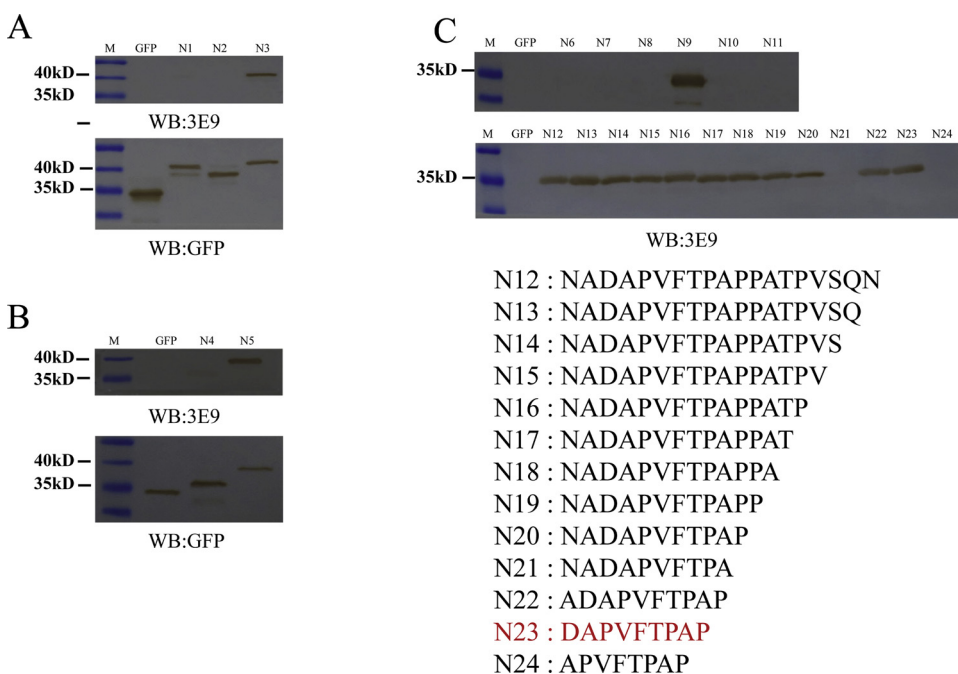

N12 : NADAPVFTPAPPATPVSQN
N13 : NADAPVFTPAPPATPVSQ
N14 : NADAPVFTPAPPATPVS
N15 : NADAPVFTPAPPATPV
N16 : NADAPVFTPAPPATP
N17 : NADAPVFTPAPPAT
N18 : NADAPVFTPAPPA
N19 : NADAPVFTPAPP
N20 : NADAPVFTPAP
N21 : NADAPVFTPA
N22 : ADAPVFTPAP
N23 : DAPVFTPAP
N24 : APVFTPAP

Fig. 5. Identification of B-cell epitopes in N protein by WB. A series of N truncated fragments were cloned into pAcGFP-C1 and expressed as fusion proteins with a GFP tag. For the first round, three peptides spanning the N protein at aa 1–146 (N1), 147–249 (N2), and 250–376 (N3) were expressed. For the second round, peptide aa 250–376 was divided into two peptides, aa 250–310 (N4) and 311–376 (N5), for expression. For the last round, aa 311–376 was divided into six overlapping peptides, then decreased one by one from both ends until the minimal linear epitope was identified. (A) MAb 3E9 recognized the entire N protein (367 aa) and N3 peptide (aa 250–376). (B) In the second identification round, N5 peptide was further minimized. (C) In the last identification round, a minimal peptide [343]DAPVFTPAP[351] was characterized as the B-cell epitope recognized by mAb 3E9. M: Protein marker.

5

Majority	IRRSQSQQDLNA**DAPVFTPAP**PATPVSQNP
	• • •
	340 350 360
	• • •
MF094681-SADS-CoV FarmA	. . . .
MF094682-SADS-CoV FarmB	. . . .
MF094683-SADS-CoV FarmC	. . . .
MF094684-SADS-CoV FarmD	. . . .
MF167434-SeACoV	. . . .
MF370205-SeACoV	. . . .
MF094685-SADSr-CoV8462	. . . .
MF094686-SADSr-CoV8495	. . . . I . . . .
MF094687-SADSr-CoV141388	. . . . I . . . .
MF094688-SADSr-CoV162140	. . . .
NC_009988.1_Bat coronavirus HKU2	. . . . V . . . A . . . . R . .

MF094681 SADS-CoV FarmA	IRRSQSQQDLNA**DAPVFTPAP**PATPVSQNP
	• • •
	340 350 360
	• • •
AF353511.1-PEDV strain CV777	TLQQHEEAIYDDVGAPSDVTHANLEWDTAV
HM245926.1-Mink coronavirus	EQ..R-SKSRERA.TPVPTTVS..AENYTD
JQ404409.1-Canine coronavirus	EQ.QRKARSKS.ERVEQEVV.D.LTENYTD
KF272920.1-PEDV strain USA	TQQLNEEAIYDDVGVPSDVTHANLEWDTAV
KF761675.1-PEDV strain China	TQQLNEEAIYDDVGVPSDVTHANLEWDTAV
NC_002306.3-Feline infectious peritonitis virus	DQ.QRRSRSKS..K-KPEELSVTLVEAYTD
NC_002645.1-Human coronavirus 229E	--EM.QHPL..PS.LE.N---.SQTSPATA
NC_018871.1-Rousettus bat coronavirus HKU10	TEKATV.PL..PT-AP--------DFQPAT
NC_028752.1-Camel alphacoronavirus	QGDM.QHPL..PS.QEL------QTSPGTA
NC_028806.1-Swine enteric coronavirus strain	EQ.KRKSRSKS.ERSEQEVV.D.LIENYTD
AJ271965.2-Transmissible gastroenteritis virus	EQ.KRKSRSKS.ERSEQDVV.D.LIENYTD

Fig. 6. Amino acid sequence alignment of identified epitopes in SADS-CoV N protein. (A) N protein amino acid sequence for six SADS-CoV reference strains from swine and five from bat coronavirus. (B) Amino acid sequences from other Alphacoronaviruses that were homologous to SADS-CoV N protein were also aligned. Black dots indicate residues that are exact match. Homologous regions in the various Alphacoronaviruses that correspond to the identified SADS-CoV N epitope are also indicated by black dots. GenBank accession numbers are shown at the beginning.

conserved nature of these epitopes in N would be an advantage in developing technologies for epitope-based diagnoses. MAb 3E9 against SADS-CoV N protein could be used in various assays. For example, mAb 3E9 showed that SADS-CoV N protein localizes to the cytoplasm and also to the nucleolus in infected cells and cells expressing only N protein alone (Fig. 4). During infection, a number of viral proteins interact with the nucleolus (Shi et al., 2017). The interaction of viral proteins with nucleolar antigens may explain why viral proteins have been observed in the nucleolus and the viral exploitation of nucleolar function that leads to alterations in host cell transcription and translation and disruption of the host cell cycle to facilitate viral replication. MAb 3E9 immunoprecipitated N protein from lysates of SADS-CoV infected Vero E6 cells (Fig. 3C). The capacity of mAb 3E9 to immunoprecipitate N protein will promote further studies on interactions of N protein with viral and cellular proteins.

In summary, a specific mAb 3E9 against SADS-CoV N protein was produced and linear B-cell epitopes in the N protein CTD were identified. Subcellular localization of the N protein was observed using mAb 3E9. The mAb was also used to immunoprecipitate N protein from lysates of SADS-CoV infected Vero E6 cells. MAb 3E9 and its epitope identified in this study will be useful for clinical applications and as a tool for further study of SADS-CoV detection and diagnosis. Taken together, our findings provide a solid foundation for further investigations into the antigenic functions of SADS-CoV N protein and the development of diagnostic and therapeutic approaches to SADS-CoV infection.

Authors and contributors

Li Feng, Da Shi and Jingyun Ma conceived and designed the

experiments; Yuru Han and Jiyu Zhang performed the experiments; Yuru Han, Jiyu Zhang, Hongyan Shi, Ling Zhou, Jianfei Chen, Xin Zhang, Jianbo Liu, Jialin Zhang, Xiaobo Wang, Zhaoyang Ji and Zhaoyang Jing analyzed the data; Yuru Han and Jiyu Zhang revised the manuscript; Li Feng, Da Shi, Jingyun Ma, Yuru Han and Jiyu Zhang wrote the paper.

Ethical approval

Funding information

Declaration of Competing Interest

None of the authors has a conflict of interest.

Acknowledgements

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