Identification of B Cell Epitopes in the MSG1 Protein of *Mycoplasma suis*

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Mycoplasma suis (*M. suis*) is an extracellular bacterial organism that attaches to and causes deformity and damage to porcine red blood cells. *M. suis* glyceraldehyde-3-phosphate dehydrogenase-like protein 1 (MSG1), a membrane-associated adhesion protein, plays a major role in *M. suis* attachment and infection of porcine erythrocytes. In order to identify the epitopes in MSG1 protein of *M. suis*, recombinant MSG1 (rMSG1) expressed in *Escherichia coli* Top10 was purified with affinity chromatography and used to immunize BALB/c mice to prepare and screen monoclonal antibodies (MAbs). Western blot results showed that 1C10, 2F10, 4G10, and 10E9 can specifically react with recombinant MSG1 and *M. suis*. Moreover, 23 truncated fragments of MSG1 were amplified and cloned into pET-32a vector and induced by IPTG. Different recombinant truncated proteins were used to identify B cell epitopes in the rMSG1 protein. Epitope mapping revealed that MAbs 1C10 recognizes the linear epitope D²⁹¹THGSVF²⁹⁷; MAbs 2F10 recognizes the linear epitope L²⁵¹CLKI²⁵⁵; and MAbs 4G10 and 10E9 recognize the linear epitope I²⁶⁸KDGENE²⁷⁴. The alignment of MSG1 epitope sequences with that of different *M. suis* strains accessed on NCBI showed that one epitope is highly conserved in *M. suis* strains. This research is the first to examine the epitopes in MSG1 of *M. suis* and demonstrate the variations of epitopes.

Introduction

Mycoplasma suis IS AN EXTRACELLULAR BACTERIAL organism that attaches to and causes deformity and damage to porcine red blood cells.⁽¹⁾ This bacterium mainly infects pigs and is responsible for a disease referred to as infectious anemia.⁽²⁾ Acute clinical porcine infectious anemia can cause hemolytic anemia and death in young piglets, sows' pre-partum or at weaning, and feeder pigs under stress.⁽³⁾ In addition, chronic infection is associated with reproductive inefficiency in sows and an increased incidence of respiratory and enteric disease and reduced body weight gain in feeder pigs.^(3,4) *M. suis* can also infect humans, although people infected by *M. suis* are symptomless.^(5,6)

Adherence of *M. suis* to the surface of porcine erythrocytes is important to its pathogenicity.⁽⁷⁾ Eight *M. suis*-specific antigens have previously been identified by immunoblot analysis.⁽⁸⁾ Among these antigens, *M. suis* glyceraldehyde-3-phosphate dehydrogenase-like protein 1 (MSG1), which is similar to bacterial glyceraldehyde-3-phosphate dehydrogenases (GAPDHs), was identified as the most important adhesion protein of *M. suis*. The similarity of MSG1 with GAPDHs from other pathogenic mycoplasmas ranged from 52.6% to 54.5% at the amino acid sequence level.⁽⁹⁾ In addition, vaccination with recombinant MSG1 protein (rMSG1) elicited strong immune responses in pigs⁽¹⁰⁾; however, little is known about the antigenic epitopes present in this protein.

Recombinant MSG1 can be used as coating antigen to detect the antibody against *M. suis* with indirect ELISA and blocking ELISA.^(11,12) In this study, we developed monoclonal antibodies (MAbs) against rMSG1 and used them to identify antigenic epitopes in rMSG1.

Materials and Methods

Bacterial strains, plasmids, cells, animals, and other reagents

M. suis crude extracts were obtained from experimentally infected pigs, as previously described,⁽⁸⁾ with some modifications. Briefly, splenectomized pig was infected with *M. suis* and confirmed by microscopic examination of peripheral blood smears stained with acridine orange and PCR for 16S rRNA. When the pig was in bacteremia, blood was collected from the vena cava anterior and diluted at a 1:1 ratio with aseptic Alsever's solution (111 mM glucose, 27.2 mM sodium citrate, 71.8 mM sodium chloride, pH 6.1). Blood cells were sedimented by low-speed centrifugation at 300 g for 15 min at room temperature and plasma and buffy coat were discarded. The pellet consisting of *M. suis*-laden erythrocytes was suspended in phosphate-buffered saline and incubated at 56°C for 10 min. *M. suis* cells in the supernatant were sedimented by high-speed centrifugation at 25,000 g for 120 min at 4~8°C. The resulting pellet of *M. suis* was re-suspended in sterile 0.15M NaCl and stored at -20°C until use. Blood sample

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TABLE 1. PRIMERS SET FOR AMPLIFICATION OF OVERLAPPING AND TRUNCATED SEGMENTS OF *MSG1* GENE

Fragment	Primer sequence (5' to 3')	Fragment	Primer sequence (5' to 3')
F2: 1-180aa	5' AGTGGTACCATGACCATCCACAAAG 3' 5' CTTGAATTCTTAGCCGCTGTTAATGCCG 3'	F14: 292-337aa	5' TAAGGTACCACCCATGGTAG 3' 5' CGGGAATTCCTACAGGCTGATG 3'
F3: 136-337aa	5' CAGGGTACCAAAACCGTTGTGT 3' 5' CGGGAATTCCTACAGGCTGATG 3'	F15: 233-298aa	5' GCCGGTACCAAACTGGATGGTATT 3' 5' CCCGAATTCCTAGTCGAAAACGCT 3'
F4: 225-337aa	5' CTAGGTACCCGTTATTCCCG 3' 5' CGGGAATTCCTACAGGCTGATG 3'	F16: 233-297aa	5' GCCGGTACCAAACTGGATGGTATT 3' 5' CCCGAATTCCTAGAAAACGCTACC 3'
F5: 225-314aa	5' CTAGGTACCCGTTATTCCCG 3' 5' CCCGAATTCCTATGCATACAGT 3'	F17: 233-296aa	5' GCCGGTACCAAACTGGATGGTATT 3' 5' CCCGAATTCCTAAACGCTACCA 3'
F6: 233-277aa	5' GCCGGTACCAAACTGGATGGTATT 3' 5' CATGAATTCCTACGCCAGGGTTTC 3'	F18: 225-272aa	5' CTAGGTACCCGTTATTCCCG 3' 5' GCGGAATTCCTATTACCATCTT 3'
F7: 264-299aa	5' CACGGTACCATCAACGAAGCGAT 3' 5' CCCGAATTCCTAGCTGTGCGAAAAC 3'	F19: 248-299aa	5' TAAGGTACCCCTGGTGTGACCTGT 3' 5' CCCGAATTCCTAGCTGTGCGAAAAC 3'
F8: 268-314aa	5' CTGGGTACCATCAAAGATGGTG 3' 5' CGCGAATTCCTAGGTTTCGTTTTTC 3'	F20: 251-299aa	5' ATAGGTACCCCTGTGTCTG 3' 5' CCCGAATTCCTAGCTGTGCGAAAAC 3'
F9: 269-314aa	5' CTGGGTACCAAGATGGTGA AAAAC 3' 5' CCCGAATTCCTATGCATACAGT 3'	F21: 252-299aa	5' CGGGGTACGTTGTCTGAAAAT 3' 5' CCCGAATTCCTAGCTGTGCGAAAAC 3'
F10: 233-275aa	5' GCCGGTACCAAACTGGATGGTATT 3' 5' CGCGAATTCCTAGGTTTCGTTTTTC 3'	F22: 196-258aa	5' ATAGGTACCCGACAGCCCGCACA 3' 5' CGCGAATTCCTAGCTTTTGTGTG 3'
F11: 233-274aa	5' GCCGGTACCAAACTGGATGGTATT 3' 5' GGCGAATTCCTATTTCGTTTTCACCATCT 3'	F23: 196-255aa	5' ATAGGTACCCGACAGCCCGCACA 3' 5' CCCGAATTCCTAGATTTTTCAGACAC 3'
F12: 233-273aa	5' GCCGGTACCAAACTGGATGGTATT 3' 5' GGCGAATTCCTAGTTTTCACCATCT 3'	F24: 196-254aa	5' ATAGGTACCCGACAGCCCGCACA 3' 5' CCCGAATTCCTATTTCAGACAC 3'
F13: 291-337aa	5' GAAGGTACCCGATACCCATGGTAG 3' 5' CGGGAATTCCTACAGGCTGATG 3'		

from the non-infected pig was processed in the same manner as negative control. *Escherichia coli* strains Top10 and Rosetta2, pET-32a(+), and Ni-NTA agarose were purchased from Invitrogen (Carlsbad, CA). The recombinant plasmid, pBAD-msg1 was previously constructed by our laboratory.⁽¹²⁾

Mouse myeloma cells (SP2/0) cells were grown in RPMI 1640 medium supplemented with 20% fetal bovine serum (Invitrogen). Female BALB/c mice, 4–6 weeks of age, were purchased from the Laboratory Animal Centre of Nantong University (Nantong, China). Animal experiments were approved by the

FIG. 1. Schematic of MSG1 protein showing expression constructs of MSG1 fragments and their interaction with MABs in indirect ELISA analysis. +, positive; -, negative.

Institutional Animal Care and Ethics Committee of Nanjing Agricultural University (approval no. IACECNAU20100902).

Expression and purification of recombinant MSG1 protein in *E. coli*

The recombinant plasmid pBAD-msg1 was transformed into Top10 cells (*E. coli*/pBAD-msg1) and induced by arabinose as previously described.⁽¹²⁾ The recombinant MSG1 (rMSG1) was purified by the Ni-NTA purification system and detected by SDS-PAGE and Western blot analysis. Final protein concentrations were determined by NANODROP2000 spectrophotometry (Thermo Scientific, Waltham, MA).

Production and identification of monoclonal antibodies

The specific anti-MSG1 MAbs were developed and screened according to the standard hybridoma technique.⁽¹³⁾ Briefly, His-MSG1 was combined with equal volumes of complete/incomplete Freund's adjuvant (Sigma-Aldrich) at a final concentration of 0.25 mg/mL. Female BALB/c mice (6 weeks old) were then immunized with 0.05 mg of protein at 2-week intervals for 6 weeks. Mouse myeloma cells (SP2/0) were fused with spleen cells from immunized BALB/c mice to generate hybridomas. ELISA plates were coated with purified rMSG1 protein and *E. coli*/pBAD lysate (as the negative control) and incubated with the hybridoma supernatant. The sera isolated from immunized mice and the supernatant of SP2/0 cells were used as positive and negative controls, respectively. Criteria for anti-MSG1 sera were as follows: the OD₄₅₀ of the positive control was greater than 1.0, and the OD₄₅₀ of the negative controls were lower than 0.2. Samples, giving an S/N ratio value higher than 2.1, were considered to be positive.

Western blot analysis

Western blot analysis was used to identify MAbs targeting rMSG1 and whole *M. suis*. Whole *M. suis*, rMSG1, and

E. coli/pBAD lysate (negative control) were subjected to SDS-PAGE and transferred onto nitrocellulose membranes (Pall Corp., Port Washington, NY). The membranes were then incubated with the various purified MAbs, supernatant of SP2/0 cells, and positive and negative rabbit polyclonal antibodies against MSG1. After washing, membranes were incubated with HRP-tagged goat anti-mouse/rabbit IgG secondary antibody and the colorimetric reactions were developed using chemiluminescence luminal reagents (Super Signal West Pico Trial Kit, Pierce, Rockford, IL).

Broad epitope mapping with overlapping MSG1 protein fragments

In order to identify the minimal antigenic epitopes recognized by MAbs, a series of primers (Table 1) were synthesized to amplify different fragments of *MSG1* gene (Fig. 1). PCR products were separately cloned into a pET-32a expression vector with *EcoRI* and *KpnI* sites. The recombinant proteins were expressed in Rosetta 2 strain and identified by SDS-PAGE and Western blotting, and were then used to determine the epitopes recognized by purified MAbs using indirect ELISA and Western blot methods as described above.

Alignment of MSG1 epitope sequences

DNAStar, MegAlign, and Bioedit were used to align the amino acid sequences of identified B cell epitopes with MSG1 of eight different *M. suis* sequences and GAPDH from seven different species accessed at NCBI.

Statistical analysis

Differences in the level of antibodies between different groups were determined by one-way repeated measurement ANOVA and least significant difference (LSD). Differences were thought statistically significant when $p < 0.05$.

FIG. 2. The specific reactions of MAbs 1C10, 2F10, 4G10 and 10E9 with purified rMSG1 protein (A) and *M. suis* extracts (B). (A) Lane 1, purified recombinant His-NSP7 protein of PRRSV; lane 2, Top10/pBAD lysate; lane 3, purified rMSG1 protein. (B) P, polyclonal rabbit anti-MSG1 antibody; N, pre-immune rabbit serum; +, *M. suis* extracts from *M. suis* positive porcine erythrocytes; -, *M. suis* extracts from *M. suis* negative porcine erythrocytes.

FIG. 3. Truncated fragments were expressed and identified by Western blot analysis. Twenty-three N- and C-terminus truncated fragments of MSG1 were constructed and expressed as described in the Materials and Methods section. A polyclonal rabbit anti-MSG1 antibody bound rMSG1 and the truncated proteins from F2 to F23.

Results

Production and characterization of monoclonal antibodies against MSG1 protein

In order to determine antigenic epitopes of MSG1, splenocytes from mice immunized against MSG1 were isolated

and fused with mouse myeloma cells (SP2/0) to generate hybridomas. After screening these hybridomas for reactivity against rMSG1 by indirect ELISA, positive hybridoma cells were sub-cloned three times by limiting dilution. Four MAbs targeting MSG1 (1C10, 2F10, 4G10, and 10E9) were obtained. Western blot analysis showed that these MAbs

FIG. 4. Western blot analysis was used to detect bindings of MAbs 1C10, 2F10, and 10E9 with various truncated rMSG1 fragments. MAbs 10E9, 1C10, and 2F10 specifically reacted with proteins containing amino acid sequence I²⁶⁸KDGENE²⁷⁴ (A), D²⁹¹THGSVF²⁹⁷ (B), and L²⁵¹CLKI²⁵⁵ (C), respectively.

specifically reacted with both the purified His-MSG1 protein and crude *M. suis* extracts (Fig. 2). The results revealed that MAbs against MSG1 can be generated and used to identify epitopes in MSG1.

Mapping of epitopes in MSG1

In order to map the minimal sequences of the epitopes recognized by these MAbs, we generated 23 truncated recombinant MSG1 proteins and used them to test the binding specificity of a polyclonal rabbit anti-MSG1 antibody. The polyclonal rabbit anti-MSG1 antibody bound all 23 truncated recombinant proteins and full-length rMSG1 (Fig. 3).

We next examined the specificity of the monoclonal antibodies. Western blot analysis revealed that MAbs 4G10 and 10E9 reacted with rMSG1 fragments F1, F3-8, F10, and F11, all of which contained the amino acids I²⁶⁸KDGENE²⁷⁴. In addition, fragments missing any one of these amino acids (F2, F9 and F12) were not targeted. These data indicated that the amino acid sequence I²⁶⁸KDGENE²⁷⁴ is essential for the binding of these two MAbs (Fig. 4A). MAb 1C10 reacted with fragments F1, F3, F4, F13, F15, and F16, but not with F2 (aa 1-180), F14 (aa 292-337), or F17 (aa 233-296) (Fig. 4B). These data suggest that MAb 1C10 recognized the epitope spanning amino acids D²⁹¹THGSVF²⁹⁷. Finally, MAb 2F10 bound the truncated fragments F1, F3, F4, F18, F19, F20, F22, and F23, but not F2, F21, or F24. These data suggested that MAb 2F10 recognized the epitope spanning amino acids 251-255 (Fig. 4C).

To further identify the antigens targeted by these MAbs, 23 truncated fragments were used as antigens in an ELISA. The OD₄₅₀ values of MAbs 4G10 and 10E9 against F1, F3-F8, F10, and F11 were much higher than the values against F2, F9, and F12 ($p < 0.05$) (Fig. 5A). In addition, the binding of MAb 1C10 to fragments F1, F3, F4, F13, F15, and F16 was significantly higher than that to fragments F2, F14, and F17 ($p < 0.01$) (Fig. 5B). Finally, the OD₄₅₀ values of MAb 2F10 against F1, F3, F4, F18, F19, F20, F22, and F23 were noticeably higher than those against F2, F21, and F24 ($p < 0.05$) (Fig. 5C). These data confirmed the Western blot analysis and disclosed specific antigenic epitopes recognized by these MAbs.

Amino acid differences of identified epitopes in different *M. suis* strains

We next determined the prevalence of these MSG1 epitopes among various *M. suis* strains. To this end, we aligned sequences of MSG1 from eight *M. suis* strains, with GAPDH from seven other species. The amino acid sequence L²⁵¹CLKI²⁵⁵ was highly conserved among all *M. suis* strains. In addition, the epitopes I²⁶⁸KDGENE²⁷⁴ and D²⁹¹THGSVF²⁹⁷ were moderately conserved, except for the amino acid differences at positions 273 (N273S) and 296 (V296I) (Fig. 6). These data confirmed that the antigenic epitopes identified by Western blot and ELISA are highly conserved among MSG1 sequences and highly variable among GAPDH in other species.

Discussion

MSG1 was previously shown to be a glycolytic, glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-like pro-

FIG. 5. Reactivity of various MAbs with different MSG1 fragments expressed in *E. coli* by ELISA. (A) MAbs 10E9 and 4G10 specifically react with epitope 268-274aa. (B) MAb 1C10 specifically reacts with epitope 291-297aa. (C) MAb 2F10 binds epitope 251-255aa.

tein. In addition, GAPDH of *M. genitalium* binds to mucin and is characterized as an adhesion factor.⁽¹⁴⁾ Similarly, GAPDH from group A *Streptococci* binds fibronectin, lysozyme, and cytoskeletal proteins such as myosin and actin.⁽¹⁵⁻¹⁷⁾ Despite the fact that MSG1 is a surface protein with similarities to GAPDH, there are only a few reports demonstrating the functions of MSG1 in *M. suis* infection. The *msg1* gene was reported and the immunogenicity and adhesive properties of its protein product were studied with recombinant protein expressed in *E. coli*.⁽¹⁸⁾ However, the antigenic epitopes of MSG1 have not been reported to date.

Monoclonal antibodies are useful and effective tools for mapping antigenic epitopes of immunogenic proteins.⁽¹⁹⁾ In this study, four MAbs recognizing the rMSG1 were developed and analyzed. Western blot studies demonstrated that these MAbs specifically reacted with *M. suis*, suggesting they

	Epitope251-255	Epitope268-274	Epitope291-297
	250	260	270
	280	290	300
Putative adhesion protein G1	LTGSLVDLCLKINKSVSAEEINEAIKDGEN	ETLAYVEDPIVSADIIGDTHGSVF	
Jia ding-1(M.suis)	S----	I.	
Jia ding-2(M.suis)	S----	I.	
Jia ding-3(M.suis)	S----	I.	
Jia ding-4(M.suis)	V.....	I.	
Jia ding-5(M.suis)	V.....	I.	
Jia ding-6(M.suis)	S----	I.	
str.Illinois(M.suis)	V.....	I.	
KI3806(M.suis)			
GAPDH(Sus scrofa)	PNV.V...TCRLE.PAKYDD.KKV.V.QASEGPLKGI.G.T..QV..C.FNS...S.T.		
GAPDH(Escherichia coli F11)	K...VTE.VSILG.K.T...V.N.L.KAT.N--N.SFG.TDEE...S...SHF...		
GAPDH(Mycoplasma hyopneumoniae)	I...F...SVELKSNP.I...QKMREYA....SF..CDE...S...R...I.		
GAPDH(Mycoplasma hyorhinis)	I...F...SVELKSNP.V..V.AEM.RVQ....SFQ.N..QV..S..VNN...I.		
GAPDH(Mycoplasma synoviae)	SVRLE..P.V..L.A.F.KAA....SFKFET.E...S..VNSHY...		
GAPDH(Pasteurella multocida subsp. multocida str. Pm70)	PNV.V...TVNLE.PATYA..CAE...RASENEMKGV.G.T..AV..T.FN.CALT...		
GAPDH(Salmonella enteric)	PNV.V...TVRLE.AATY.Q.KA.V.AAAEGEMKGV.G.T..DV..T.FN.EVCT...		

FIG. 6. Multiple alignments of B cell epitopes between MSG1 of different *M. suis* strains and GAPDH of different species. Identified B cell epitope sequences are underlined. Alignments of the amino acid sequences were made using BioEdit software, and identical amino acid residues are indicated with a dot.

recognized the epitopes on MSG1 of *M. suis*. The epitope mapping confirmed that MAbs 10E9 and 4G10 recognized the same linear epitope I²⁶⁸KDGENE²⁷⁴, 1C10 recognized the epitope D²⁹¹THGSVF²⁹⁷, and 2F10 recognized the epitope L²⁵¹CLKI²⁵⁵.

B cell epitopes are either continuous or discontinuous, resulting from unique protein folding, and are defined as the regions on the surface of the native antigen that could bind to B cell receptors or specific antibodies.^(20,21) Typically, continuous B cell epitopes are short and recognized directly by immunoglobulin. This study revealed that B cell epitopes on MSG1 of *M. suis* are continuous and one epitope was conserved among all *M. suis* strain sequences available on NCBI. Meanwhile, the epitopes I²⁶⁸KDGENE²⁷⁴ and D²⁹¹THGSVF²⁹⁷ were relatively conserved among all *M. suis* strains, except for the N273S and V296I variations in two of the strains. Further studies should be carried out to confirm the relationship between these epitope variations and their pathogenicity.

Conclusions

We generated four MAbs against the MSG1 protein of *M. suis* and identified three MSG1 epitopes (268-274aa, 291-297aa, and 251-255aa). These results contribute to a deeper understanding of the antigenic structure of the MSG1 protein and facilitate development of diagnostic methods for detecting *M. suis* in the blood and tissues.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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