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Fine Mapping of a Foot-and-Mouth Disease Virus Epitope Recognized by Serotype-Independent Monoclonal Antibody 4B2

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VP2 is a structural protein of the foot-and-mouth disease virus (FMDV). In this study, a FMDV serotype-independent monoclonal antibody (MAb), 4B2, was generated. By screening a phage-displayed random 12-peptide library, we found positive phages displaying the consensus motif ETTXLE (X is any amino acid (aa)), which is highly homologous to ^{6}ETTLLE^{11} at the N-terminus of the VP2 protein. Subsequently, a series of GST-fusion proteins expressing a truncated N-terminus of VP2 were examined by western blot analysis using the MAb 4B2. The results indicated that the motif ^{6}ETTLLE^{11} of VP2 may be the minimal requirement of the epitope recognized by 4B2. Moreover, a 12-aa peptide ^{2}KKTEETTLLEDR^{13} was shown to be the minimal unit of the epitope with maximal binding activity to 4B2. Alanine-scanning analysis demonstrated thatThr^{7}, Thr^{8}, and Leu^{10} are the functional residues of the 4B2 epitope Glu^{6} and Leu^{9} are required residues, and Glu^{11} plays a crucial role in the binding of MAb 4B2. The fine mapping of the epitope indicated that MAb 4B2 has the potential to be used in FMDV diagnosis.

***Keywords*:** foot-and-mouth disease virus, monoclonal antibody, phage display, VP2, epitope mapping

Foot-and-mouth disease (FMD), an economically important and highly contagious disease affecting cloven-hoofed animals, is caused by the foot-and-mouth disease virus (FMDV), which belongs to the genus *Aphthoviruses* within the *Picornaviridae* family (Grubman and Baxt, 2004). FMDV has a single-stranded positive RNA of about 8,500 nucleotides. The virus exists as seven distinct serological types (O, A, C, Asia 1, SAT 1, SAT 2, and SAT 3), and multiple subtypes have arisen during the evolution of the virus (Bachrach, 1968). Due to the error-prone nature of the RNA-dependent RNA polymerase, FMDV has a very high mutation rate during replication (Drake and Holland, 1999). Therefore, FMDV populations are believed to exist as a group of related but non-identical genomes known as quasispecies (Domingo *et al.*, 2002). Thus, many antigenic and genetic variants have emerged during virus circulation in the field. Antigenic diversity is one of the major obstacles inthe development of type-independent assays for FMDV diagnosis.

The non-enveloped icosahedral capsid of this virus is composed of 60 copies each of four structural proteins, VP4, VP2, VP3, and VP1. VP1 is the most variable structural protein, VP2 and VP3 are relatively conserved, and VP4 is highly conserved between the serotypes (Jackson *et al.*, 2003). Antigenic sites of VP2 have been determined in FMDV serotype A (Thomas *et al.*, 1988; Saiz *et al.*, 1991), type O (Barnett *et al.*, 1989, 1998; Kitson *et al.*, 1990) and type Asia1 (Marquardt *et al.*, 2000). Although amino acids (aa) 1-33 of VP2 are capable of enhancingthe neutralizing antibody response elicited by the VP1 peptide (Wang *et al.*, 2007), this domain was shown to be of low antigenic potential on the virus particle because the N-terminus of the VP2 protein is located at the bottom of the threefold axis of symmetry of the virus capsid (Lea *et al.*, 1994; Curry *et al.*, 1997).

Previous studies showed that highly conserved linear epitopes exist on the N-terminus of the VP2 protein (Freiberg *et al.*, 2001; Yang *et al.*, 2007). However, fine mapping of the epitope to determine the minimal requirement of the epitope for monoclonal antibody (MAb) binding, theminimal unit of the epitope with the maximal binding activity as well as contributions of individual amino acids to the epitopes at the N-terminus of the VP2 protein needs to be performed. In this study, we generated a FMDV serotype-independent Mab, 4B2. Using a 12-mer random peptide phage display, GST-fusion protein expression and western blot analysis, we located the epitope recognized by MAb 4B2 on the N-terminus of the FMDV VP2 protein and finely mapped the epitope. Moreover, the relationship between MAb 4B2 and the previouslydescribed MAbs is also discussed. Since the linear epitope recognized by MAb 4B2 is highly conserved, this antibody can be used to develop a diagnostic kit for FMDV detection.

Materials and Methods

Viruses and cells

Serotype O FMDV O/YS/CHA/05 (GenBank accession no., HM008917) belongs to the PanAsia lineage serotype Asia1 FMDV Asia1/YS/CHA/05 (GU931682) isolated during the pandemic inChina in 2005 (Valarcher *et al.*, 2005). Serotype A FMDV A/KT/58 (AJ131665) and serotype O FMDV isolates O/Tibet/CHA/99 (AJ539138), O/GD/86 (AJ131468), O/Akesu/58(AF511039), and O/HLJOC12/03/CHA (DQ

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119643) were used for indirect immunofluorescence assay (IFA). The baby hamster kidney cell line BHK-21 was maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum (Gibco-BRL, USA).

MAbs production

Six-week-old female BALB/c mice were subcutaneously immunized with 100 μg of the inactivated purified FMDV O/YS/CHA/05 antigen emulsified with an equal volume of adjuvant VG206 (SEPTIC, France). Two boosters of the adjuvant emulsified antigen were given at two-week intervals. Two weeks after the third immunization, the mice were intraperitoneally boosted with 100 μg of antigen alone. Three days later, immunized spleen cells were fused with SP2/0 myeloma cells using 50% (w/v) polyethylene glycol and 10% dimethyl sulfoxide (DMSO) (v/v) (Sigma, USA). Hybridomas were screened by indirect enzyme-linked immunosorbent assay (ELISA) and indirect IFA. The hybridoma-producing MAb was cloned three times by limiting dilution of the cells.Antibody subtype identification was performed using the SBA Clonotyping™ System/HRP kit (Southern Biotech, USA).

Table 1. Complementary oligonucleotides coding for the truncated VP2 N-terminus and alanine-scanning peptides

Name	Peptide sequence	Complementary oligonucleotide
Y15-1	^{1}DKKTEETTLLEDRIL^{15}	5'-gatccGACAAGAAAACCGAGGAGACCACTCTTCTTGAGGACCGCATCCTGTAAc-3' 5'-tcgagTTACAGGATGCGGTCCTCAAGAAGAGTGGTCTCCTCGGTTTTCTTGTCg-3'
Y15-2	^{6}ETTLLEDRILTTRNG^{20}	5'-gatccGAGACCACTCTTCTTGAGGACCGCATCCTGACTACCCGCAACGGGTAAc-3' 5'-tcgagTTACCCGTTGCGGGTAGTCAGGATGCGGTCCTCAAGAAGAGTGGTCTCg-3'
ETTLLE	^{6}ETTLLE^{11}	5'-gatccGAGACCACTCTTCTTGAGTAAc-3' 5' tcgagTTACTCAAGAAGAGTGGTCTCg-3'
TTLLE	^{7}TTLLE^{11}	5'-gatccACCACTCTTCTTGAGTAAc-3' 5'-tcgagTTACTCAAGAAGAGTGGTg-3'
TLLE	^{8}TLLE^{11}	5'-gatccACTCTTCTTGAGTAAc-3' 5'-tcgagTTACTCAAGAAGAGTg-3'
L1	^{5}EETTLLE^{11}	5'-gatccGAGGAGACCACTCTTCTTGAGTAAc-3' 5'-tcgagTTACTCAAGAAGAGTGGTCTCCTCg-3'
L2	^{4}TEETLLE^{11}	5'-gatccACCGAGGAGACCACTCTTCTTGAGTAAc-3' 5'-tcgagTTACTCAAGAAGAGTGGTCTCCTCGGTg-3'
L3	^{3}KTEETLLE^{11}	5'-gatccAAAACCGAGGAGACCACTCTTCTTGAGTAAc-3' 5'-tcgagTTACTCAAGAAGAGTGGTCTCCTCGGTTTTg-3'
L4	^{2}KKTEETTLLE^{11}	5'-gatccAAGAAAACCGAGGAGACCACTCTTCTTGAGTAAc-3' 5'-tcgagTTACTCAAGAAGAGTGGTCTCCTCGGTTTTCTTg-3'
L5	^{1}DKKTEETTLLE^{11}	5'-gatccGACAAGAAAACCGAGGAGACCACTCTTCTTGAGTAAc-3' 5'-tcgagTTACTCAAGAAGAGTGGTCTCCTCGGTTTTCTTGTCg-3'
R1	^{6}ETTLLED^{12}	5'-gatccGAGACCACTCTTCTTGAGGACTAAc-3' 5'-tcgagTTAGTCCTCAAGAAGAGTGGTCTCg-3'
R2	^{6}ETTLLEDR^{13}	5'-gatccGAGACCACTCTTCTTGAGGACCGCTAAc-3' 5'-tcgagTTAGCGGTCCTCAAGAAGAGTGGTCTCg-3'
R3	^{6}ETTLLEDRI^{14}	5'-gatccGAGACCACTCTTCTTGAGGACCGCATCTAAc-3' 5'-tcgagTTAGATGCGGTCCTCAAGAAGAGTGGTCTCg-3'
R4	^{6}ETTLLEDRIL^{15}	5'-gatccGAGACCACTCTTCTTGAGGACCGCATCCTCTAAc-3' 5'-tcgagTTAGAGGATGCGGTCCTCAAGAAGAGTGGTCTCg-3'
Y12	^{2}KKTEETTLLEDR^{13}	5'-gatccAAGAAAACCGAGGAGACCACTCTTCTTGAGGACCGCTAAc-3' 5'-tcgagTTAGCGGTCCTCAAGAAGAGTGGTCTCCTCGGTTTTCTTg-3'
S1	^{2}KKTE<u>A</u>ETLLEDR^{13}	5'-gatccAAGAAAACCGAGGCGACCACTCTTCTTGAGGACCGCTAAc-3' 5'-tcgagTTAGCGGTCCTCAAGAAGAGTGGTCGCGCTCGGTTTTCTTg-3'
S2	^{2}KKTEE<u>A</u>TLLEDR^{13}	5'-gatccAAGAAAACCGAGGAGGCGACTCTTCTTGAGGACCGCTAAc-3' 5'-tcgagTTAGCGGTCCTCAAGAAGAGTCGCGCTCCTCGGTTTTCTTg-3'
S3	^{2}KKTEET<u>A</u>LLEDR^{13}	5'-gatccAAGAAAACCGAGGAGACCGCGCTTCTTGAGGACCGCTAAc-3' 5'-tcgagTTAGCGGTCCTCAAGAAGCGCGGTCTCCTCGGTTTTCTTg-3'
S4	^{2}KKTEETT<u>A</u>LEDR^{13}	5'-gatccAAGAAAACCGAGGAGACCACTGCGCTTGAGGACCGCTAAc-3' 5'-tcgagTTAGCGGTCCTCAAGCGCAGTGGTCTCCTCGGTTTTCTTg-3'
S5	^{2}KKTEETTL<u>A</u>EDR^{13}	5'-gatccAAGAAAACCGAGGAGACCACTCTTGCGGAGGACCGCTAAc-3' 5'-tcgagTTAGCGGTCCTCCGCAAGAGTGGTCTCCTCGGTTTTCTTg-3'
S6	^{2}KKTEETTLL<u>A</u>DR^{13}	5'-gatccAAGAAAACCGAGGAGACCACTCTTCTTGCGGACCGCTAAc-3' 5'-tcgagTTAGCGGTCCGCAAGAAGAGTGGTCTCCTCGGTTTTCTTg-3'

Note: Introduced bases (to form termination codon and overhanging ends of *Bam*HI and *Xho*I after annealing the two complementary oligonucleotides) are shown in lowercase letters. Mutated bases are boxed; mutated residues are underlined.

Indirect ELISA

Plates were coated with 100 μl/well of purified FMDV O/YS/CHA/05 antigen diluted in carbonate-bicarbonate buffer (pH 9.6) for incubation overnight at 4°C, followed by fourwashes with 200 μl/well PBS/0.05% Tween-20 and blocking with 200 μl/well blocking buffer (PBS containing 5% skimmed milk) for 1 h at 37°C. A total of 100 μl/well of the supernatant of the hybridoma was added in duplicate wells, and the plates were incubated for 1 h at 37°C followed by the addition of horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG, Sigma) at a dilution of 1:5,000. After a 1 h incubation at 37°C, 50 μl of substrate o-phenylenediamine dihydrochloride (OPD, Sigma) containing 0.3% H_2O_2 were added, and color was developed for 15 min before stopping with 50 μl/well of 2 M H_2SO_4. Optical density was measured at 492 nm.

IFA

BHK-21 cells in 96-well plates were infected with FMDV O/YS/CHA/05, rinsed with PBS and fixed with icy cold anhydrous ethanol for 15 min at 4°C and air dried. Then 50 μl/well MAb at 1:200 dilutions in PBS was added for 1 h at 37°C. After washing with PBS, 50 μl/well FITC-conjugated goat anti-mouse IgG (Sigma) at a 1:200 dilution was added and incubated for 1 h at 37°C. Plates were washed three times with PBS and observed under an OLYMPUS microscope connected to a Leica DFC 490 digital color camera.

Biopanning

MAb 4B2 was preliminarily purified using a caprylic acid-ammonium sulfate method, followed by purification with the NAb™ Protein G Spin Purification kit (Pierce, USA). The M13 phage display library displaying 12-mer random peptides (Ph.D-12 Phage Display Peptide Library kit, New England Biolabs) was usedto screen the mimicry epitope recognized by MAb 4B2. The affinity selection of the phage clones from the random peptide library was conducted according to the recommended protocol with minor modifications. In the first round of the biopanning, plates were coated with 10 μg/ml of MAb 4B2 in 0.1 M $NaHCO_3$ buffer (pH 8.6) at 4°C for 12 h. The coated wells were washed with Tris-buffered saline (50 mM Tris-Cl pH 7.5, 150 mM NaCl) containing 0.1% Tween 20 (TBST) followed by blocking with 1 mg/ml bovine serum albumin (BSA) in 0.1 M $NaHCO_3$ buffer. The phage library (1.5×10^{11} phages/100 μl) was added to the blocked wells for 1 h at room temperature. Unbound phages were washed off with 10 washes of TBST. The bound phages were eluted by 0.2 M glycine-HCl containing 1 mg/ml BSA (pH 2.2) and immediately neutralized with 1 M Tris-HCl (pH 9.1). The eluent phages were amplified in *E. coli* (ER2738) and were titered on LB/IPTG/Xgal plates for the subsequent rounds of selection. Second and third rounds of selection were performed similarlyto the first round except the concentration of Tween 20 was raised to 0.5%, and the concentrations of MAb 4B2 were reduced to 5 μg/ml and 1 μg/ml, respectively.

For the fourth round of biopanning, 300 ng of purified MAb 4B2 was mixed with the amplified third-round eluate (5×10^{11} phages) and incubated for 20 min at room temperature. Then 50 μl of protein G-Agarose suspension (Gibco-BRL, USA) was added,and the incubation was continued for 15 min at the same temperature. The incubation mixture was centrifuged (3,000×g for 30 sec), and the supernatant was discarded while the pellet was washed 10 times with TBST. The final pellet was resuspended in 1 ml of 0.2 M glycine-HCl containing 1 mg/ml BSA (pH 2.2). The final eluted phages were plated, and individual clones were picked randomly for phage ELISA and DNA sequencing.

Phage ELISA

For the phage ELISA, ninety-six-well plates were coated with 100 ng of purified MAb 4B2 (100 μl/well Mab in 0.1 M $NaHCO_3$, pH 8.6) overnight at 4°C. To rule out the interference of the MAb constant region as well as the BSA, MAb 4A8, and BSA were used as irrelevant negative controls. The coated wells were blocked for 2 h at room temperature, and then the phages (10^{10} pfu/100 μl/well) diluted in blocking solution were added. The plates were incubated for 1 h at room temperature followed by washing 10 times with TBST. The phage clone was detected using horseradish peroxidase (HRP)-conjugated anti-M13 MAb (GE Healthcare, USA) at the 1:5,000 dilution. The reaction was quantified using the ABTS [2, 2'-azinobis (3-ethylbenzthiazolinesulfonic acid)] substrate, and the absorbance at 405 nm was measured.

Construction of expression plasmidsand GST fusion expression in *E. coli*

Complementary oligonucleotides (Table 1) coding for the truncated N-terminus VP2 protein and alanine-scanning peptides were synthesized, annealed and cloned into expression vector pGEX-6p-1 (GE Healthcare) previously digested with *Bam*HI and *X*hoI. All the resulting recombinant plasmids were validated by restriction analysis and nucleotide sequencing. Expression plasmids were transformed into

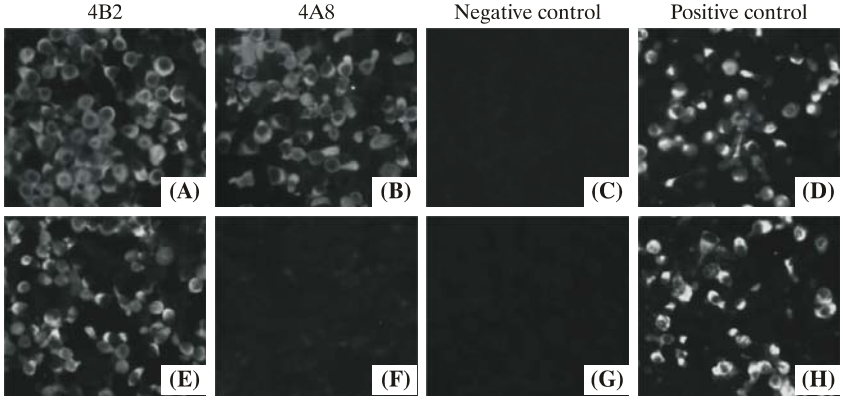

Fig. 1. BHK-21 cells infected with FMDV O/YS/CHA/05 (A-D) and FMDV Asia1/YS/CHA/05 (E-H). Healthy BALB/c mice serum were used as a negative control; BALB/c mice antiserum raised against inactivated whole virus was used as a positive control.

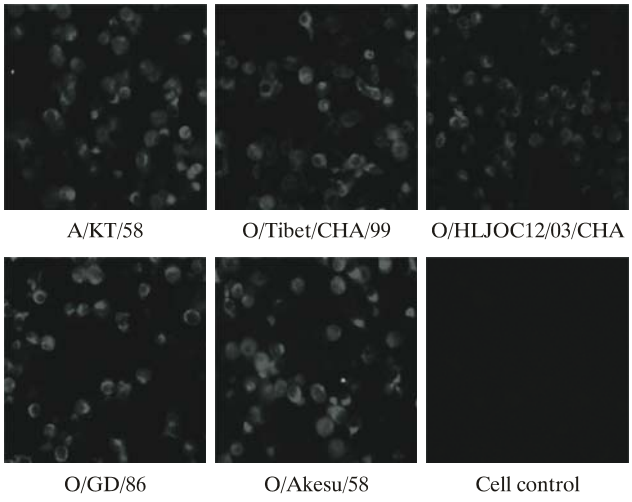

Fig. 2. BHK-21 cells infected with type A and type O FMDV. BHK-21 cells infected with serotype A FMDV A/KT/58, serotype O FMDV isolates O/Tibet/CHA/99(AJ539138), O/GD/86(AJ131468), O/Akesu/58(AF511039), and O/HLJOC12/03/CHA(DQ119643) were used to test the MAb 4B2 reactivity, and normal BHK-21 cells were used as a negative control.

BL21 (DE3) competent cells, followed by the addition of 1 mM isopropyl-D-thioga-lactopyranoside (IPTG; GE Healthcare) for induction.

SDS-PAGE and Western blot

Approximately equal amounts of each GST fusion protein were-subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% SDS-PAGE). The gel was either stained with Coomassie blue staining solution or electrophoretically transferred to a nitrocellulose membrane. After blocking the membrane with 5% nonfat milk in PBS overnight at 4°C, we incubated the membrane with MAb 4B2 (diluted 1:2,000 in PBS) at 37°C for 1 h, washed it three times with PBST, and probed itwith a 1:5,000 dilution of HRP-conjugated goat anti-mouse IgG (Sigma) at 37°C for 1 h. The reactivity was visualized with the substrate 3, 3'-diaminobenzidine (DAB; Sigma).

Results

Generation of MAbs

Two MAbs against FMDV O/YS/CHA/05, 4A8 and 4B2, were generated. Isotype determination showed that both MAbs were of the IgG1/κ-type subclass. IFA showed that MAb 4A8 bound to FMDV O/YS/CHA/05 but not Asia1/YS/CHA/05, whereas MAb 4B2 boundto both FMDV Asia1/YS/CHA/05 and FMDV O/YS/CHA/05 (Fig. 1). In addition, Western blot analysis indicated that MAb 4B2 recognized a linear epitope (data not shown). Moreover, we further tested the reactivity of MAb 4B2 to FMDV isolates (Fig. 2), and the Mab showed positive reactivity with all the FMDVs. These results indicated that MAb 4B2 was a serotype-independent MAb.

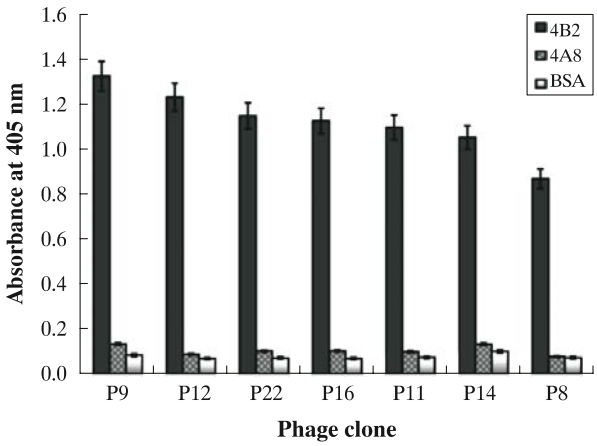

Fig. 3. Detection of binding activity of positive phage clones to MAb 4B2 by ELISA. Microtiter wells were coated with MAbs at 100 ng/well. Phages were added to all wells (10^{10} phages/100 μl/well), followed by the addition of HRP-conjugated anti-phage antibodies. The OD_{405} value was calculated as described in the 'Materials and Methods' section. MAb 4A8 and BSA were used as negative controls.

Phage display of mimicry epitope recognized by MAb 4B2

To determine the epitope recognized by MAb 4B2, biopanning of a phage display 12-mer random peptide library was performed using affinity-purified MAb 4B2. After four rounds of biopanning, individual phage clones were isolated, and the reactivity of these phage clones was assessed by phage ELISA. Thirty-six positive clones were selected for sequencing, and seven different sequences of the phage inserts were obtained (Fig. 3). Sequence alignment of random peptide inserts in the selected positive phage clones revealed a consensus motif ETTXLE (X is any amino acid) (Table 2). Thus, the mimicry epitope recognized by MAb 4B2 contained a core sequence ETTXLE. We then aligned the sequence ETTXLE with the

Table 2. Sequence comparison of random peptide inserts presented in the positive phage clones

Phage	Amino acid sequence of the insert[a]																			Frequency
P9								**T**	**T**	V	**L**	D	E	P	K	Y	S	M	R	11
P12							S	**T**	**T**	K	**L**	**E**	V	S	R	V	L	L		8
P14	T	M	T	K	D	T	N	**T**	**T**	F	**L**	**E**								5
P11							W	S	**T**	R	**L**	D	R	F	P	D	P	L		3
P22							**E**	P	**T**	F	**L**	**E**	S	M	R	A	R	S		4
P16							**E**	D	S	I	**L**	**E**	H	L	F	P	R	P		3
P8						D	**E**	N	**T**	I	**L**	G	S	R	S	F	P			2
Motif[b]							**E**	**T**	**T**	**X**	**L**	**E**								

[a] Conservative amino acid motifs are bold and underlined.
[b] The consensus motif ETTXLE is showed at the bottom, and X is any amino acid.

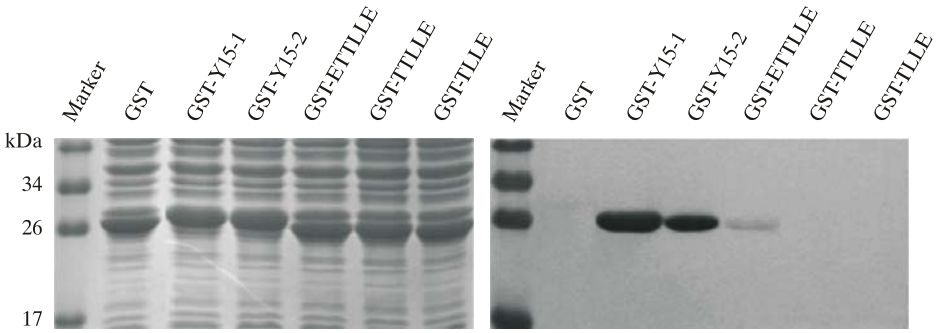

Fig. 4. Reactivity of the GST-fused truncates of the core epitope ^{6}ETTLLE^{11} with MAb 4B2 by western blotting analysis. (A) Approximately equal amounts of *E. coli* cell lysates with the expressed GST-fusion proteins were electrophoresed on 12% SDS-PAGE gells. (B) The fusion proteins were transferred onto a nitrocellulose membrane and probed with MAb 4B2.

sequence of the FMDV O/YS/CHA/05 capsid protein and found a highly homologous motif ^{6}ETTLLE^{11} at the N-terminus of the VP2 protein. Thus, we assumed that the authentic epitope recognized by MAb 4B2 was the 6-aa peptide motif ^{6}ETTLLE^{11}.

Minimal requirement of the authentic epitope to be recognized by MAb 4B2

To verify the assumption that MAb 4B2 recognized the peptide motif, we expressed two VP2 overlapping peptides ^{1}DKKTEE TTLLEDRIL^{15} and ^{6}ETTLLEDRILTTRNG^{20}, described previously (Freiberg *et al.*, 2001), as glutathione S-transferase (GST) fusion proteins (Fig. 4A) for western blot analysis. The results showed that the fusion proteins GST-Y15-1 and GST-Y15-2 both reacted strongly with MAb 4B2 (Fig. 4B), thus allowing us to locate the epitope recognized by MAb 4B2 between aa residues 1 and 20 at the N-terminus of the VP2 protein.

To determine the minimal requirement for MAb 4B2 recognition, we expressed the VP2 protein N-terminus truncated peptides ^{6}ETTLLE^{11}, ^{7}TTLLE^{11}, and ^{8}TLLE^{11} as GST-fusion proteins in *E. coli*. The fusion protein GST-ETTLLEshowed a weak reactivity with MAb 4B2 by western blot, whereas the further truncated fusions GST-TTLLE and GST-TLLE showed no binding activity with MAb 4B2 (Fig. 4B). These results indicated that ^{6}ETTLLE^{11} is the minimal unit for MAb 4B2 recognition.

Minimal unit of the epitope with the maximal binding activity to MAb 4B2

To investigate the minimal unit of the epitope with the maximal binding activity to MAb 4B2, we expressed a series of GST-fusion proteins with extended aa residues at both the N- and C-terminus of the motif ^{6}ETTLLE^{11}. These GST-fusion proteins were subjected to SDS-PAGE (Fig. 5A) and then tested for reactivity with MAb 4B2 by western blotting. Fusion proteins GST-L1 (^{5}EETTLLE^{11}), GST-L3 (^{3}KTEETLLE^{11}) and GST-R1 (^{6}ETTLLED^{12}) reacted weakly with MAb 4B2 (Fig. 5B), indicating that the binding activity of the core epitope of MAb 4B2 was not increased by adding three residues ^{3}KTE^{5} at the N-terminal or by adding residue Asp^{12} at the C-terminal. Unexpectedly, GST-L2 (^{4}TEETLLE^{11}) had no binding activity to MAb 4B2 (Fig. 5B). In contrast, we observed strong binding activity of GST-L4 (^{2}KKTEETTLLE^{11}) and GST-R2 (^{6}ETTLLEDR^{13}) (Fig. 5B), providing evidence that positively charged residue Lys^{2} or Arg^{13} significantly increased the binding activity of the core epitope recognized by MAb 4B2. In addition, we observed no further increase in the binding activity of fusion proteins GST-R3 (^{6}ETTLLEDRI^{14}), GST-R4 (^{6}ETTLLEDRIL^{15}) and GST-L5 (^{1}DKKTEETTLLE^{11}) to MAb 4B2 compared with that of GST-L4 or GST-R2 (Fig. 5B). Taken together, these results showed that bothL4 (^{2}KKT EETTLLE^{11}) and R2 (^{6}ETTLLEDR^{13}) were the reactive units with strong binding activity to MAb 4B2.

Furthermore, we expressed a 12-aa peptide Y12 (^{2}KKTEE TTLLEDR^{13}) covering both L4 (^{2}KKTEETTLLE^{11}) and R2 (^{6}ETTLLEDR^{13}) as GST-fusion proteins, and we simultaneously

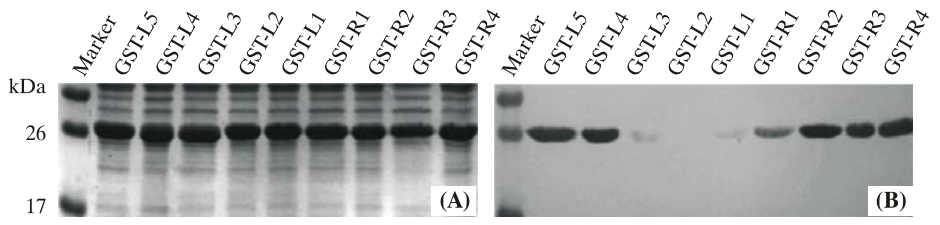

Fig. 5. Reactivity of the GST-fusion proteins expressing the core epitope ^{6}ETTLLE^{11} with extended residues to MAb 4B2 by western blot analysis. (A) Approximately equal amounts of *E. coli* cell lysates of expressed GST-fusion proteins were separated by 12% SDS-PAGE. (B) Western blot analysis corresponding to the fusion proteins shown in (A).

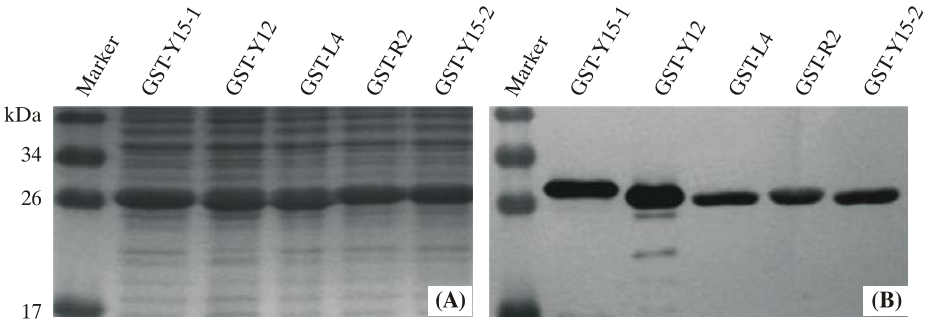

Fig. 6. SDS-PAGE and western blot analysis to determine the minimal unit with the maximal binding activity to MAb 4B2. (A) The GST-fused peptides Y15-1, Y12, L4, R2, and Y15-2 were separated by 12% SDS-PAGE. (B) Western blot analysis corresponding to the fusion proteins shown in (A).

tested the binding activity of GST-Y12 as well as that of GST-Y15-1, GST-L4, GST-R2 and GST-Y15-2 to MAb 4B2. GST-Y12 showed the strongest binding activity by western blotting (Fig. 6B). Thus, the peptide ^{2}KKTEETTLLEDR^{13} was determined to be the minimal unit of the epitope with the maximal binding activity to MAb 4B2.

Contributions of individual amino acids to activity of the core epitope

To further delineate critical residues ofthe core epitope recognized by MAb 4B2, a panel of GST-fusion proteins expressing mutant peptides of the Y12 (^{2}KKTEETTLLEDR^{13}) was generated (Fig. 7A), and each individual residue of the core epitope was substituted with alanine. Decreased reactivity to MAb 4B2 was observed for GST-S2 (^{2}KKTEEATLLEDR^{13}), GST-S3 (^{2}KKTEETALLEDR^{13}) and GST-S5 (^{2}KKTEETTLA EDR^{13}) by western blotting (Fig. 7B), suggesting that Thr^{7}, Thr^{8}, and Leu^{10} were important residues of the core epitope. GST-S1 (^{2}KKTEAETLLEDR^{13}) and GST-S4 (^{2}KKTEETTAL EDR^{13}) showed the same binding activity to MAb 4B2 as GST-Y12 (^{2}KKTEETTLLEDR^{13}) (Fig. 7B), indicating that residue substitution at both position 6 and position 9 had no effect on the binding activity of the epitope to MAb 4B2. In contrast, the expressed mutant peptide GST-S6 (^{2}KKTEE TTLLADR^{13}) lost all binding activity to MAb 4B2 (Fig. 7B), indicating that the residue Glu^{11} was a critical residue for MAb 4B2 binding.

Core sequence of the epitope is well conserved among seven FMDV isolates

The VP2 sequences of FMDV isolates available from GenBank were aligned with the epitope sequence (data not shown). The core epitope ^{6}TTLLE^{11} was shown to be highly conserved among FMDV strains, except for the Ala-to-Thr substitution at position 7 found in FMDV Asia1/YNBS/58 (GenBank accession no. AY390432) and Asia1/Israel/1963 (AY593797). In addition, residues Phe-, Met- or His-to-Leu substitution at position 9 was found in FMDV Asia1/IND/82/96 (DQ989309), Asia1/IND/52/87 (DQ989313) and SAT3/FMDV/BEC1/65 (AY 593853), respectively. However, we determined that residue substitution at position 7 had less effect on the epitope binding activity of MAb 4B2. In addition, residue substitution at position 9 had no effect on the epitope binding activity of MAb 4B2 (Fig. 7B). Taken together, our results showed that the core epitope recognized by MAb 4B2 was highly conserved among all seven FMDV serotypes.

Discussion

Previous studies have shown that the VP2 protein is immunogenic. Epitopes on the B-C loop (Marquardt *et al.*, 2000) and the N-terminus of the VP2 protein (Freiberg *et al.*, 2001; Yang *et al.*, 2007) of FMDV type Asia1 have been documented. However, fine mapping of the epitope suggested that critical residues of the epitope interact with MAbs and needed further elucidation. In this study, we generated a FMDV serotype-in-

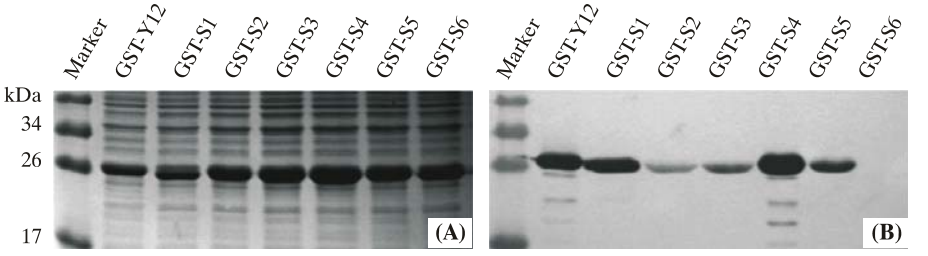

Fig. 7. Alanine scanning to determine key amino acids in the motif ^{6}ETTLLE^{11}. (A) SDS-PAGE analysis of seven GST-fused proteins expressed in *E. coli*. (B) Western blot analysis of the corresponding fusion proteins shown in (A). GST-Y12 (^{2}KKTEETTLLEDR^{13}) was used as a control.

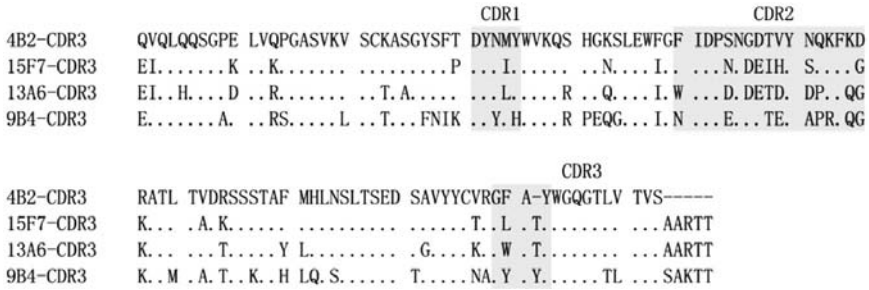

Fig. 8. Alignment of the variable heavy chain sequences of MAbs 15F7, 9B4, 13A6, and 4B2.

dependent MAb 4B2. Using a combined approach of screening phage-displayed 12-mer random peptides, truncated peptide expression as GST-fusion proteins and western blot analysis, we determined the minimal and maximal units of the epitope for MAb 4B2 binding. Importantly, each aa of the core epitopes involved in interactions with MAb 4B2 was determined by alanine-scanning mutagenesis.

Several 4B2-like MAbs F1412SA, 15F7, 13A6 and 9B4 have been generated and identified (Freiberg *et al.*, 2001; Yang *et al.*, 2007). These MAbs recognize conserved epitopes at the N-terminus of the VP2 protein. Among them, F1412SA, 9B4 and 15F7 reacted in an indirect ELISA with the synthesized peptide ^{1}DKKTEETTILEDRIL^{15} but not ^{6}ETTILEDRIITTR NG^{20} (Freiberg *et al.*, 2001; Yang *et al.*, 2007). Our study showed that MAb 4B2 reacted simultaneously with overlapping peptides ^{1}DKKTEETTILEDRIL^{15} and ^{6}ETTILEDRI ITTRNG^{20} expressed as GST-fusion proteins (Fig. 4B). In addition, the complementarity determining region (CDR) of MAb 4B2 differs from that of previous reported MAbs (Fig. 8). Moreover, MAbs F1412SA, 9B4, and 15F7 recognize trypsin-sensitive epitopes, whereas MAb 4B2 recognizes trypsin-resistant epitopes. Taken together, we concluded that the epitope recognized by MAbs F1412SA, 9B4, and 15F7 differed from the epitope recognized by MAb 4B2, although all the epitopes recognized by these MAbs were located atthe N-terminus of the VP2 protein. MAb 13A6 showed similar characteristics as MAb 4B2, including recognizing trypsin-resistant epitopes and reacting with both peptides ^{1}DKKTEETTILEDRIL^{15} and ^{6}ETTILEDRIITTRNG^{20} in an indirect ELISA. Even so, MAb 4B2 differs from MAb 13A6 due to the sequence differences in the CDR3 heavy chain critical for antigen binding (Chothia *et al.*, 1989) (Fig. 8). However, based on previous studies, an overlapping region existsbetween the MAb 4B2-recognized epitope and the MAb 13A6-recognized epitope.

MAb F1412SA failed to react with the SAT3 FMDV BEC1/65 isolate because the residue Leu at position 9 of VP2 was replaced by His (Yang *et al.*, 2007). SAT3 FMDV was assumed to be recognized by the MAbs 9B4 and 15F7, but no experimental results confirmed the hypothesis (Freiberg *et al.*, 2001). In this study, the GST-S4 with Leu^{9}-to-Ala^{9} substitution bound strongly to MAb 4B2 (Fig. 7B), demonstrating that 4B2 can bind to both SAT3 FMDV and type Asia1 FMDV with the VP2 mutation at position 9. In addition, residue-substitutions of Glu^{6}, Thr^{7}, Thr^{8}, and Leu^{10} did not abolish the binding activity of FMDV to MAb 4B2. Moreover, the residue Glu^{11}, which is crucial for MAb 4B2 binding, was observed to be conserved in the FMDV isolates available from GenBank. These results revealed that MAb 4B2 recognizes a more conserved epitope compared to previously documented MAbs.

GST-fusion proteins GST-ETTLLE and GST-L1 (^{4}TEETT LLE^{11}) were shown to bind weakly to MAb 4B2 (Figs. 4B and 5B). In the present study, the motif ^{4}TEETTLLE^{11} was expressed as a GST-fusion protein (GST-L2) to determine the contribution of the residues around the core epitope (5B). Surprisingly, GST-L2 was found to be unable to bind MAb 4B2 by western blot analysis in assays performed three times independently. Although the peptide ^{4}TEETTLLE^{11} contained additional residues Thr and Glu compared with the core epitope ^{6}ETTLLE^{11}, the GST-fusion protein expressing the peptide ^{4}TEETTLLE^{11} (GST-L2) failed to react with MAb 4B2 (Fig. 5B), which cannot be explained by our results.

The majority of polyclonal and monoclonal antibodies against FMDV were serotype specific, which posed a further challenge to FMDV diagnosis. Recently, serotype-independent detection of FMDVhas been achieved under laboratory conditions, and two characterized anti-FMDV monoclonal antibodies were used to evaluate their ability to capture serotype-independent FMDV antigen (Muller *et al.*, 2008). In this study, MAb 4B2 was demonstrated to recognize aconserved epitope located at the N-terminus of the VP2 protein of FMDV strains, and therefore, because it overcame serotype restriction, MAb 4B2 can be used to develop a diagnostic kit for FMDV detection. Moreover, the finely mapped epitopewould facilitate the design of conserved FMDV antigens to generate polyclonal antibodies for antigen capture.

In summary, we finely mapped a conserved epitope located at the N-terminus of the VP2 protein of FMDV. Conservation of the identified epitope may have implications for the development of FMDV-specific diagnostic assays.

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