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Identification of a Novel Linear B-Cell Epitope in the M Protein of Avian Infectious Bronchitis Coronaviruses

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This report describes the identification of a novel linear B-cell epitope at the C-terminus of the membrane (M) protein of avian infectious bronchitis virus (IBV). A monoclonal antibody (MAb) (designated as 15E2) against the IBV M protein was prepared and a series of 14 partially-overlapping fragments of the IBV M gene were expressed with a GST tag. These peptides were subjected to enzyme-linked immunosorbent assay (ELISA) and western blotting analysis using MAb 15E2 to identify the epitope. A linear motif, ^{199}FATFVYAK^{206}, which was located at the C-terminus of the M protein, was identified by MAb 15E2. ELISA and western blotting also showed that this epitope could be recognized by IBV-positive serum from chicken. Given that 15E2 showed reactivity with the ^{199}FATFVYAK^{206} motif, expressed as a GST fusion protein, in both western blotting and in an ELISA, we proposed that this motif represented a linear B-cell epitope of the M protein. The ^{199}FATFVYAK^{206} motif was the minimal requirement for reactivity as demonstrated by analysis of the reactivity of 15E2 with several truncated peptides that were derived from the motif. Alignment and comparison of the 15E2-defined epitope sequence with the sequences of other coronaviruses indicated that the epitope is well conserved among chicken and turkey coronaviruses. The identified epitope should be useful in clinical applications and as a tool for the further study of the structure and function of the M protein of IBV.

***Keywords*:** avian infectious bronchitis virus, monoclonal antibody, linear B-cell epitope, epitope mapping, membrane protein, coronavirus

The RNA genomes of coronaviruses, which range from 26 to 32 kb in length, are the largest viral RNA genomes. Coronaviruses cause diseases of the upper and lower respiratory tracts, gastroenteritis, and central nervous system infections in a number of avian and mammalian hosts including humans (Masters, 2006). Coronaviruses belong to the family *Coronaviridae*, which is a member of the order *Nidovirales*, and are classified into three different antigenic groups on the basis of their genetic and antigenic relationships. However, divergent coronaviruses have been identified recently in bats and wild carnivores, which suggests that the taxonomy should be revised (Dong *et al.*, 2007). Avian infectious bronchitis virus (IBV) is the prototype member of the group 3 coronaviruses. As is the case for other coronaviruses, IBV contains four major structural proteins: the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins. The S glycoprotein is a large type I transmembrane glycoprotein that is responsible for receptor binding and membrane fusion (Hofmann *et al*., 2004). It consists of the N-terminal S1 and C-terminal S2 subunits, which are generated during post-translational cleavage of S. The N protein is a phosphoprotein that interacts with the viral RNA genome to form a long, flexible, helical nucleocapsid. The E protein is a minor virion component that possesses ion channel activity that is important for virus entry and assembly.

The M protein is the most abundant constituent of coronaviruses and gives the virion envelope its shape. It is a multispanning membrane protein with a small, N-terminal domain that is located on the exterior of the virion, or in the lumen of the endoplasmic reticulum. This ectodomain is followed by three transmembrane segments and a large C-terminus that comprises the major part of the molecule. This latter domain is situated within the interior of the virion or on the cytoplasmic face of intracellular membranes (Masters, 2006). M proteins are moderately well conserved within each coronavirus group, but they are quite divergent across the three groups. The region of the M protein that is most conserved among all the coronaviruses is a segment of approximately 25 residues that encompasses the end of the third transmembrane domain and the start of the endodomain; a portion of this segment even retains homology to its torovirus counterpart (den Boon *et al.*, 1991). The ectodomain, which is the least conserved part of the M protein, is glycosylated. For the M protein of IBV, the entire ectodomain was found to be protease sensitive. However, at the other end of the molecule, no more than 20~25 amino acids could be removed from the C-terminus by protease treatment (Rottier *et al.*, 1984; Cavanagh *et al.*, 1986). These results suggest that almost the entire endodomain of M is associated tightly with the surface of the membrane or that it

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has an unusually compact structure that is refractory to proteolysis (Masters, 2006).

Specific interactions between proteins, such as between an antibody and its antigen or between an enzyme and its inhibitor, are mediated through the molecular recognition of subsites on both binding partners. Antigenic epitopes are well-characterized subsites that mediate the interaction between an antibody and its antigen. B-cell epitopes are antigenic determinants that are recognized and bound by membrane-bound receptors on the surface of B lymphocytes (Baggio *et al.*, 2002). They can be classified into two types: linear (continuous) epitopes and conformational (discontinuous) epitopes. Linear epitopes are short peptides that correspond to a contiguous amino acid sequence within a protein (Barlow *et al.*, 1986; Langeveld *et al.*, 2001). In contrast, conformational epitopes are composed of amino acids that are not contiguous in the primary sequence, but are brought into close proximity within the folded protein structure. It is believed that a large majority of B-cell epitopes are discontinuous (Walter, 1986) and many methods have been used to identify B-cell epitopes (Roggen, 2006).

Characterization of the location of viral protein epitopes and their degree of conservation is important to allow the antigenic structure and virus-antibody interactions to be understood at the molecular level. It is also very important for vaccine design and other clinical applications. Until now, mapping of the antigenic epitopes of IBV structural proteins has focused mainly on the S and N proteins. The S1 glycoprotein induces the production of virus-neutralizing antibodies that show cross-reactivity in an ELISA and cell-mediated immune (CMI) responses (Ignjatovic and Galli, 1994, 1995). Several antigenic epitopes that induce the production of virus-neutralizing antibodies have been mapped within the hyper-variable region of S1 at amino acid residues 24∼61 and 132∼149, and also outside the hyper-variable region at amino acids 291∼398 (Niesters *et al.*, 1987; Kusters *et al.*, 1989; Koch *et al.*, 1990; Kant *et al.*, 1992; Moore *et al.*, 1997; Johnson *et al.*, 2003). All the epitopes identified were dependent on conformation (Lenstra *et al.*, 1989; Kant *et al.*, 1992), and the main tool that was used to map antigenic epitopes on S1 was MAbs. Two antigenic regions have been identified within S2. They are located near the N-terminus of the subunit between amino acids 546∼577 of the intact S protein (Kusters *et al.*, 1989; Lenstra *et al.*, 1989). The N protein induces high titers of antibodies that show cross-reactivity in an ELISA and also CMI responses (Ignjatovic and Galli, 1994; Seo *et al.*, 1997). B-cell epitopes have been mapped to the C-terminal portion of N (Seah *et al.*, 2000), and a region between amino acid residues 78∼94 was identified that induces a T-cell response (Boots *et al.*, 1991). In addition, several other previously-unknown antigenic epitopes on the S and N proteins of IBV have been identified in a recent study (Ignjatovic and Sapats, 2005). However, little is known about the ability of the M protein of IBV to induce antibodies that show cross-reactivity between different serotypes, and antigenic epitopes in the M protein have not been identified.

In this study, we identified an antigenic epitope of the M protein of IBV for the first time and investigated the conservation of this epitope among coronaviruses. This could provide the basis for understanding of the structure of the M protein and for the development of immunity-based prophylactic, therapeutic, and diagnostic techniques for the control of the avian infectious bronchitis coronavirus.

Materials and Methods

Viruses used and their propagation in specific-pathogen-free embryonated eggs

The IBV strain CK/CH/LHLJ/04V was used for the preparation and identification of the MAbs, and *in vitro* neutralization. A further eight IBV strains (Liu *et al*., 2006a, 2006b) were used for western blotting analysis with the chosen MAb. The IBV field strains and commercially-available vaccine strains represented different IBV serotypes, based on S1 gene analysis and comparison with their prototype (Liu *et al*., 2006a, 2006b, 2007, 2009) (Table 1). All the IBV strains were propagated once in 9- to 11-day-old specific-pathogen-free (SPF) embryonated chicken eggs and the presence of viral particles in the allantoic fluids of the inoculated eggs was confirmed using a negative contrast electron microscope (JEM-1200 EX, Japan Electronics, Japan) as described previously.

Fertile SPF White Leghorn embryonated chicken eggs were obtained from the Laboratory Animal Center, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China.

Gene cloning and construction of recombinant expression vectors

First, the entire M gene was amplified by PCR from IBV strain CK/CH/LHLJ/04V as described below, cloned into the pMD-18T vector (TaKaRa, China), and sequenced. Then the truncated M gene fragments (Fig. 1) that were used in

Table 1. The IBV strains used in this study

Strain	Year	Type	Reference
CK/CH/LSC/99I	1999	CK/CH/LSC/99I-type	Liu *et al.* (2006a)
tl/CH/LDT3/03	2003	tl/CH/LDT3/03-type	Liu *et al.* (2005)
CK/CH/LSD/05I	2005	variant	Liu *et al.* (2008a)
CK/CH/LHLJ/04V	2004	LX4-type	Liu *et al.* (2006a)
CK/CH/LDL/97I	1997	CK/CH/LDL/97I-type	Liu *et al.* (2009)
ck/CH/LHB/08I	2008	variant	unpublished
CK/CH/LHN/00I	2000	Australia-associated	Liu *et al.* (2006a)
IBN	Vaccine	Mass-type	Liu *et al.* (2006b)
H120	Vaccine	Mass-type	Liu *et al.* (2006b)

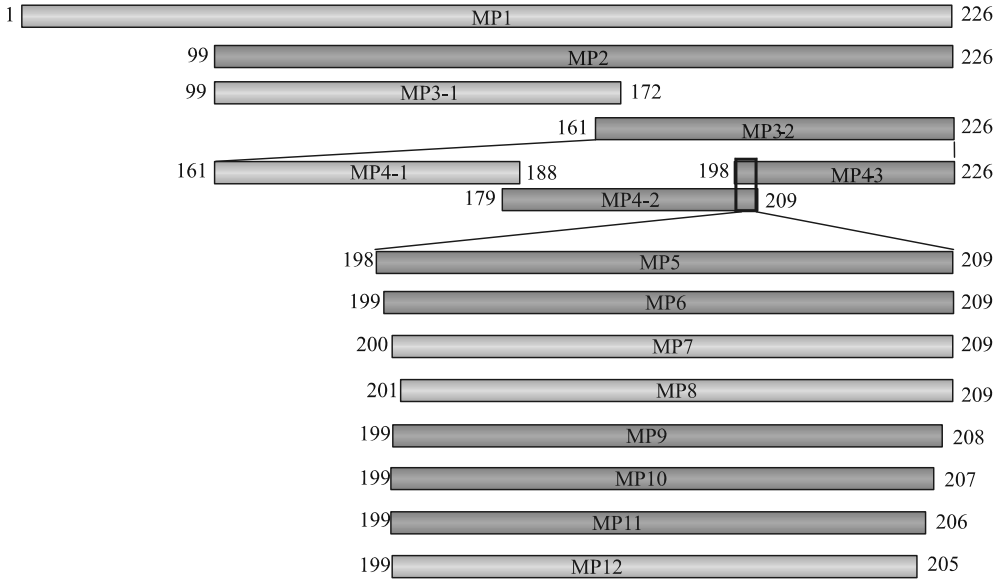

Fig. 1. Schematic diagram showing the truncated fragments derived from the M protein of IBV strain CK/CH/LHLJ/04V and their relative positions. Letters represent the amino acid positions of the M protein. The names of the peptides are the same as in Table 2. The bars represent peptides of the truncated M proteins. The peptides that were negative in western blotting and ELISA with MAb 15E2 are shown in gray and the peptides that were positive in western blotting and ELISA with MAb 15E2 are shown in gray.

this study were amplified from the above-mentioned recombinant vector, cloned into pMD-18T and sequenced. Finally, these gene fragments were subcloned into the pGEX-6P-1 vector (Pharmacia, Belgium) and a collection of expression clones that corresponded to the entire M gene or the truncated fragments were constructed. The primers for the MP1 fragment, which covered the entire M gene, were designed using the consensus nucleotide sequence of the M gene from IBV strain LX4 (Liu *et al*., 2008b) and two restriction enzyme sites, *Eco*RI and *Hin*dIII, were introduced into the sense and negative-sense primers, respectively. The primers for amplification of the truncated fragments were designed using the CK/CH/LHLJ/04V M gene sequence and two restriction enzyme sites, *Eco*RI or *Bam*HI and *Hin*dIII or *Sal*I, were introduced into the sense and negative-sense primers, respectively. The sequences and locations of the primers

Table 2. Sequences of the primers used in this study

Fragment	Primer equences (5'-3')[a]		Position in M gene[b]	Size of amplified (bp)
	Sense	Negative-sense		
MP1	GAATTCATGTCGAACGGCACGGAAAAT	AAGCTTTTATGTGTAAAGACTACCTACA	1~681	681
MP2	GGATCCATCCAGAGTTTTAGACTCTTT	AAGCTTTTATGTGTAAAGACTACCTACA	295~681	387
MP3-1	GGATCCATCCAGAGTTTTAGACTCTTT	GTCGACTTAAAATATATCTTTAGGCAA	295~516	222
MP3-2	GGATCCAAATGTGAACCAGACCAC	AAGCTTTTATGTGTAAAGACTACCTACA	481~681	211
MP4-1	GGATCCAAATGTGAACCAGACCAC	GTCGACTTAGTATTTCTGCACCATACG	481~564	84
MP4-2	GGATCCGATAGAAGAAACATCTAT	GTCGACTTATACTGACTGTTTGGCATA	529~627	99
MP4-3	GGATCCAGGTTTGCTACATTTGTA	AAGCTTTTATGTGTAAAGACTACCTACA	592~681	90
MP5	TGCAGGGATCCAGGTTTGCTACATTT	GTAATGTCGACTTATACTGACTGTTTGGC	592~627	36
MP6	TGCAGGGATCCTTTGCTACATTTGTA	GTAATGTCGACTTATACTGACTGTTTGGC	595~627	33
MP7	TGCAGGGATCCGCTACATTTGTATAT	GTAATGTCGACTTATACTGACTGTTTGGC	598~627	30
MP8	TGCAGGGATCCACATTTGTATATGCC	GTAATGTCGACTTATACTGACTGTTTGGC	601~627	27
MP9	TGCAGGGATCCTTTGCTACATTTGTA	GTAATGTCGACTTATGACTGTTTGGCATA	595~624	30
MP10	TGCAGGGATCCTTTGCTACATTTGTA	GTAATGTCGACTTACTGTTTGGCATATAC	595~621	27
MP11	TGCAGGGATCCTTTGCTACATTTGTA	GTAATGTCGACTTATTTGGCATATACAAA	595~618	24
MP12	TGCAGGGATCCTTTGCTACATTTGTA	GTAATGTCGACTTAGGCATATACAAATGT	595~615	21

[a] The restriction enzyme sites (*Eco*RI or *Bam*HI and *Hin*dIII or *Sal*I) that were introduced in each primer are underlined. The boxed ATG is the start codon of the IBV CK/CH/LHLJ/04V M gene and the boxed TTAs represent the stop codon of the M gene or stop codons that have been introduced into the negative-sense primers for the M fragments.

[b] The nucleotide positions correspond to those in the sequence of the IBV CK/CH/LHLJ/04V M gene, GenBank accession no. FJ641062.

that were used in this study are shown in Table 2.

To provide the template for PCR, viral RNA was extracted from 200 μl of infectious allantoic fluid using TRIzol reagents (Invitrogen, USA) according to the manufacturer's protocol. Reverse transcription (RT) was performed with M-MLV Reverse Transcriptase (Invitrogen) using the negative-sense primer for MP1 (Table 2). The RT reaction was performed using 20 μl of RNA in a 40 μl reaction volume as described previously (Liu *et al.*, 2008b). The cDNA fragments were amplified from the RT products by PCR as described previously (Liu and Kong, 2004) using the primers mentioned above. The PCR products were purified from agarose gels using a DNA Extraction kit (Boehringer, Germany), cloned directly into pMD-18T following the manufacturer's instructions, and sequenced. The entire M gene and the truncated fragments were isolated from the pMD-18T constructs by digestion with *Eco*RI or *Bam*HI and *Hin*dIII or *Sal*I and inserted into the corresponding sites in the pGEX-6P-1 vector. These recombinant expression constructs were verified by sequencing.

Expression of the fusion proteins and SDS-PAGE

Each of the recombinant expression constructs was transformed into *Escherichia coli* BL21 (DE3) (Novagen, USA). An aliquot of 100 μl of overnight culture was diluted into 10 ml of Luria-Bertani medium. Expression of the fusion proteins was induced when the bacteria had reached a density of 0.6 (OD_{600}) at 37°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.6 mM and the cells were grown for a further 4 h, after which they were pelleted by centrifugation and lysed by sonication. The inclusion bodies were recovered by centrifugation, resuspended in SDS-PAGE buffer, and analyzed on a 10 to 15% polyacrylamide gel. The fusion proteins were detected by staining with Coomassie Blue as described previously (Towbin *et al.*, 1979). To prepare purified proteins, the inclusion bodies were washed once with 50 mM Tris-HCl, pH 8.0, 0.5% glycerol, 1 mM NaCl and then solubilized in 50 mM Tris-HCl, pH 8.0, 0.5% glycerol containing 4 M guanidinium chloride. The inclusion bodies were then analyzed by SDS-PAGE. The bands in the SDS-PAGE gel that corresponded to the expected sizes of the proteins of interest were cut out and crushed, and added to an appropriate volume of sterilized PBS. The proteins were then used for subsequent immunization, western blotting analysis, and ELISAs.

Western blotting analysis

The specificity and reactivity of the MAbs were determined by western blotting using IBV strain CK/CH/LHLJ/04V and eight heterogeneous strains (Table 1). The IBV infectious allantoic fluids were condensed and separated on a 12% SDS-polyacrylamide gel under denaturing conditions. For western blotting, the proteins were transferred to a nitrocellulose membrane as described previously (Towbin *et al.*, 1979). The nitrocellulose membrane was blocked with 5% skimmed milk in TBS (20 mM Tris-HCl; pH 7.5, 500 mM NaC1) for 30 min at room temperature. Strips were incubated for 1 h at room temperature with MAb 15E2, washed with TBS for 20 min at room temperature, and incubated with a goat anti-mouse serum. The strips were washed again and allowed to react with HRP-conjugated sheep anti-mouse IgG for 1 h at room temperature. After extensive washing, the strips were developed with a solution of TBS containing 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate until bands appeared. The reaction was stopped by rinsing the strips with distilled water. Purified glutathione S-transferase (GST) protein was used as a negative control.

The reactivity of the recombinant MP2 protein was determined by western blotting. Cell lysates of *E. coli* that had been transformed with the MP2 expression construct and induced with IPTG were separated on a 12% SDS-polyacrylamide gel. An anti-IBV antibody from chicken was used as the primary antibody with SPF chicken sera as negative controls, and HRP-conjugated goat anti-chicken IgG was used as the secondary antibody. To identify the MAb 15E2 epitope, the truncated recombinant proteins were separated on a 12% SDS-polyacrylamide gel. Mab 15E2 was used as the primary antibody, with a MAb that was unrelated to IBV as the negative control, and HRP-conjugated sheep anti-mouse IgG was used as the secondary antibody. Western blotting was performed as described above.

MAb preparation

Two 8-week-old BALB/c female mice were immunized subcutaneously with IBV CK/CH/LHLJ/04V virus particles mixed with Freund's complete adjuvant. This was followed by two booster immunizations with 50 mg of purified recombinant MP2 protein in incomplete Freund's adjuvant (IFA) at 2 week intervals. Immunized mice were bled randomly 7 days after the first booster immunization and the sera were tested by western blotting using IBV as the antigen. The mouse that showed the greatest antibody response was given a final booster (50 mg of MP2/mouse) without adjuvant, intraperitoneally for 3 consecutive days prior to the day of hybridoma fusion. The protocols for the preparation of MAbs and ascitic fluids have been described previously (Ruf *et al*., 1983; Vilella *et al*., 1983). All the hybridomas were cloned by limiting dilution at least three times. Primary screening of the hybridomas was done by ELISA using the IBV CK/CH/LHLJ/04V virus particles as the coating antigen. The secreting polyclones were subcloned three times by limiting dilution. The class and subclass of the MAbs were determined by using a SBA Clonotyping™ System/HRP kit (Southern Biotechnology Associates, USA).

Indirect ELISA

The specificity of the MAb 15E2 was determined first by indirect ELISA using IBV CK/CH/LHLH/04V virus particles as the coating antigen. Briefly, the virion was sonicated to disrupt the virus, and the protein concentration was determined by Lowry's method. Then, 96-well polystyrene plates (Inotech Diagnostik, Switzerland) were coated with those IBV overnight in 0.1 M carbonate buffer (pH 9.6) at 4°C. The plates were washed with PBST (0.15 M NaCl in 0.1 M sodium phosphate pH 7.4) containing 0.02% Tween 20 and incubated with the MAb 15E2 for 1 h at 37°C. They were then washed again and incubated with an HRP-conjugated secondary antibody. Color development, termination of the assay and absorbance measurements were performed as described previously (Liu *et al.*, 2009). Secondly,

a commercial total antibody ELISA (IDEXX Corporation, USA) was used to evaluate the specificity of MAb 15E2 according to the manufacturer's instructions. The MAb was used to replace the sera samples in this kit and HRP-conjugated sheep anti-mouse IgG was used to replace the goat anti-chicken IgG. The antibody against IBV and SPF sera were used as positive and negative controls, respectively. All assays were performed in triplicate.

The reactivity of the MAb with different truncated recombinant M proteins was determined by ELISA. The purified recombinant proteins were used as coating antigens. The wells in the ELISA plate were coated with 10 μg purified recombinant protein in 100 μl 0.1 M carbonate buffer (pH 9.6) at 4°C for 12 h and blocked with 5% skimmed milk at 37°C for 1 h. After washing three times with PBST, 100 μl of MAb ascitic fluid were added to the wells and incubated at 37°C for 1 h. The plates were washed three times and incubated with HRP-conjugated sheep anti-mouse IgG at 37°C for 1 h. Color development, termination of the assay and absorbance measurements were performed as described previously (Liu *et al*., 2009). All assays were performed in triplicate.

The absorbance measurements were read at 450 nm when using a commercial total antibody ELISA (IDEXX Corporation, USA) according to the manufacturer's instructions and the absorbance measurements were read at 650 nm when HRP-conjugated sheep anti-mouse IgG was used as second antibodies.

In vitro neutralization test

The MAb 15E2 was tested for the presence of IBV-neutralizing antibodies using an SPF chicken embryo-protection assay (Liu *et al.*, 2008b). Two-fold dilutions of the MAb ascitic fluids were mixed with 10^2 50% embryo infectious doses (EID_{50}/0.1 ml) of IBV strain CK/CH/LHLJ/04V, and incubated for 2 h at 37°C. Virus titrations were performed by inoculation of 9-day-old SPF embryonated chicken eggs via the allantoic cavity route (Yachida *et al.*, 1979), and the titers were expressed as EID_{50}. Serial l0-fold dilutions were used for the titrations. Five embryos received 0.1 ml of inoculum at each dilution. The eggs were candled daily and examined for 1 week; those that showed characteristic IBV lesions, such as dwarfing, stunting, or curling of embryos, were recorded as infected by IBV. Ascitic fluid that contained the MAb unrelated to IBV and PBS were used as negative controls for inoculation with IBV strain CK/CH/LHLJ/04V. All assays were performed in triplicate and the statistical significance of the difference between each two groups was determined by Independent-Samples *T*-test by the one-way analysis of variance (ANOVA) (Chen *et al*., 2009).

Comparison of the epitope-containing sequence with those of other coronaviruses

The sequences that contained the epitope were assembled, aligned, and compared with those of other coronavirus strains using the MEGALIGN program in DNAStar. The sequences of the coronaviruses that were used as reference strains for comparison were from the GenBank database with the GenBank accession numbers in Fig. 8.

Results

Characterization of the IBV CK/CH/LHLJ/04V M gene

The open reading frame of the IBV CK/CH/LHLJ/04V M gene contained 681 bp, which encoded a 226-amino acid protein. The sequence of the M gene of strain CK/CH/LHLJ/04V was deposited in the GenBank with the following accession number: FJ641062. Basic Local Alignment Search Tool (BLAST) searches using the M gene revealed that it shared a high degree of nucleotide identity with most of the related Chinese IBV strains. The most closely-related virus was strain CK/CH/LJL/04I (GenBank accession number: EF602452), which shared 99% nucleotide identity with CK/CH/LHLJ/04V in the M gene.

Expression of the recombinant proteins

The expression strategy for the fragments of the IBV M gene is illustrated in Fig. 1. The intact M gene and the truncated fragments were expressed as GST fusion proteins in *E. coli* BL21 (DE3). The intact M protein was not expressed successfully in *E. coli* BL21 (DE3) after induction with IPTG. However, a truncated M protein (MP2) was expressed successfully in *E. coli* BL21 (DE3) and the recombinant MP2 protein reacted with a chicken antibody against IBV in both an ELISA (Fig. 2) and western blotting analysis. The MP2 protein was then used as an immunogen together with the IBV CK/CH/LHLJ/04V virus particles to produce a MAb. In addition, all the truncated fragments were expressed successfully in *E. coli* BL21 (DE3) as shown by SDS-PAGE of the cell lysates after induction.

Production of IBV M MAb 15E2

Two hybridomas that secreted MAbs against the IBV CK/CH/LHLJ/04V M protein were established by cell fusion. One of the MAbs, which was designated as 15E2, reacted with both the truncated MP2 protein and the native IBV CK/CH/LHLJ/04V antigen in western blotting analysis (Fig. 3A). The reactivity and specificity of MAb 15E2 were con-

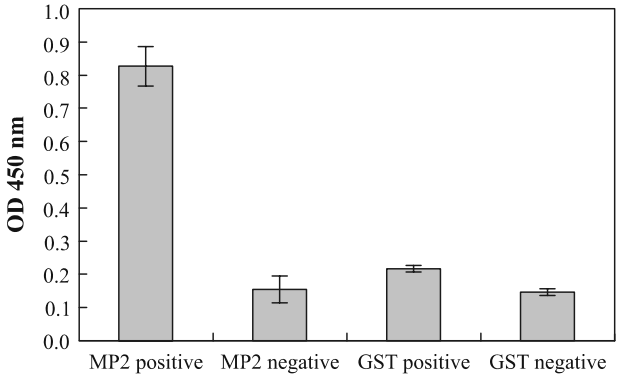

Fig. 2. Reactivity of truncated recombinant MP2 with an anti-IBV antibody. The truncated recombinant protein MP2 was used as the coating antigen in an ELISA and purified GST protein was used as controls. The anti-IBV antibody from chicken was used as the primary antibody and SPF chicken sera were used as negative controls.

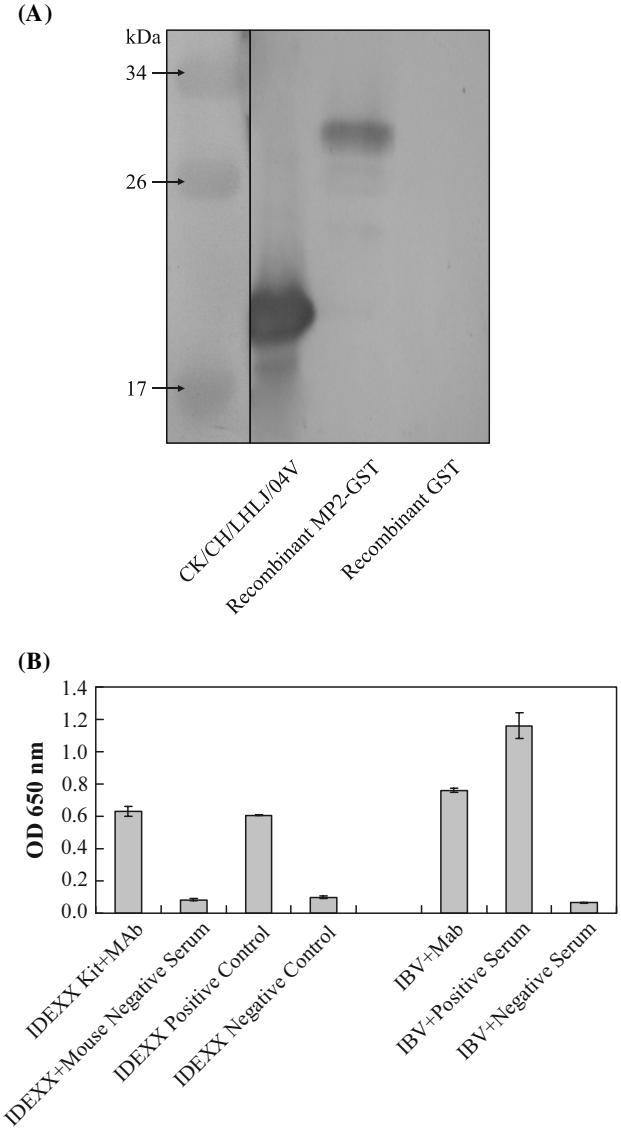

Fig. 3. Reactivity of MAb 15E2 with IBV strain CK/CH/LHLJ/04V by western blotting (A) and ELISA (B). Both IBV strain CK/CH/LHLJ/04V and recombinant MP2-GST protein were probed with MAb 15E2 by western blotting and GST protein was used as a negative control. In the ELISA, both commercial IBV-coated plates from the IBV tested kit (IDEXX Corporation, USA) and plates that had been coated with IBV CK/CH/LHLJ/04V particles were used to test MAb 15E2. SPF chicken sera were used as negative controls.

firmed using a commercial ELISA as well as an ELISA in which whole IBV CK/CH/LHLJ/04V virus particles were used as the coating antigen (Fig. 3B). MAb 15E2, which was IgG1 (K), was used for the additional studies.

In vitro neutralizing activity

Given that no cell lines could sustain the growth of IBV strain CK/CH/LHLJ/04V in this study (data not shown), 9-day-old embryonated eggs were used to evaluate the growth of the virus *in vitro*. An equal dose (10^2 EID_{50}) of the

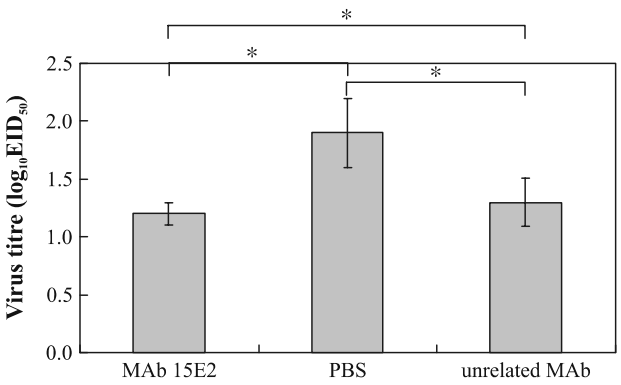

Fig. 4. *In vitro* neutralization test in 9-day-old SPF chicken embryos evaluated by EID_{50}. Ascitic fluid that contained a MAb unrelated to IBV and PBS were used as negative controls for the inoculation with IBV strain CK/CH/LHLJ/04V. All assays were performed in triplicate. No significant differences were found between different groups (*).

CK/CH/LHLJ/04V virus was mixed with unpurified MAb 15E2, a control MAb unrelated to IBV or PBS, and inoculated into the eggs at 37°C for 2 h. The results showed that the virus mixed with PBS had the highest titer of the three groups. However, there was no obvious difference in viral titer between the virus mixed with MAb 15E2 and that mixed with the control MAb (Fig. 4). This indicated that MAb 15E2 may have no virus-neutralizing activity *in vitro* and is a non-neutralizing antibody against the M protein of IBV.

Fine localization of the MAb 15E2-defined epitope

For fine mapping of the epitope of the IBV CK/CH/LHLJ/04V antigen that is recognized by MAb 15E2, the set of above-mentioned GST fusion proteins (Fig. 1) was used together with both western blotting and ELISA. Western blotting results showed that MP4-2 (^{179}PDRRNIYRMVQKYTGDQSGNKKRFATFVYAKQSV^{209}) and MP4-3 (^{198}KRFATFVYAKQSVDSGELESVATGVGSLYT^{226}) were recognized by MAb 15E2, equally. However, MP4-1 (^{161}AKCEPDHLPKDIFVCTPDRRNIYRMVQKY^{187}) failed to react with 15E2. This indicated that the sequence that is shared by MP4-2 and MP4-3 (MP5: ^{198}KRFATFVYAKQSV^{209}) contained the epitope that is recognized by MAb 15E2. To map the epitope in greater detail, a series of truncated peptides that were derived from MP5 were obtained and subjected to western blotting. The results showed that the minimal sequence required appeared to be ^{199}FATFVYAK^{206}, because deletion of F^{198} or K^{205} destroyed the binding of the GST fusion peptides by MAb 15E2 (Fig. 5A). Therefore, this linear site is the MAb 15E2-defined epitope in the M protein of IBV strain CK/CH/LHLJ/04V. The results were confirmed by ELISA using the same truncated peptides as coating antigens (Fig. 5A). Thus, we concluded that the linear B-cell epitope in the M protein of IBV that is recognized by MAb 15E2 can be localized exactly to ^{199}FATFVYAK^{206} (Fig. 5C). This peptide is located on the cytoplasmic side of the membrane (or inside the virion) (Fig. 5D).

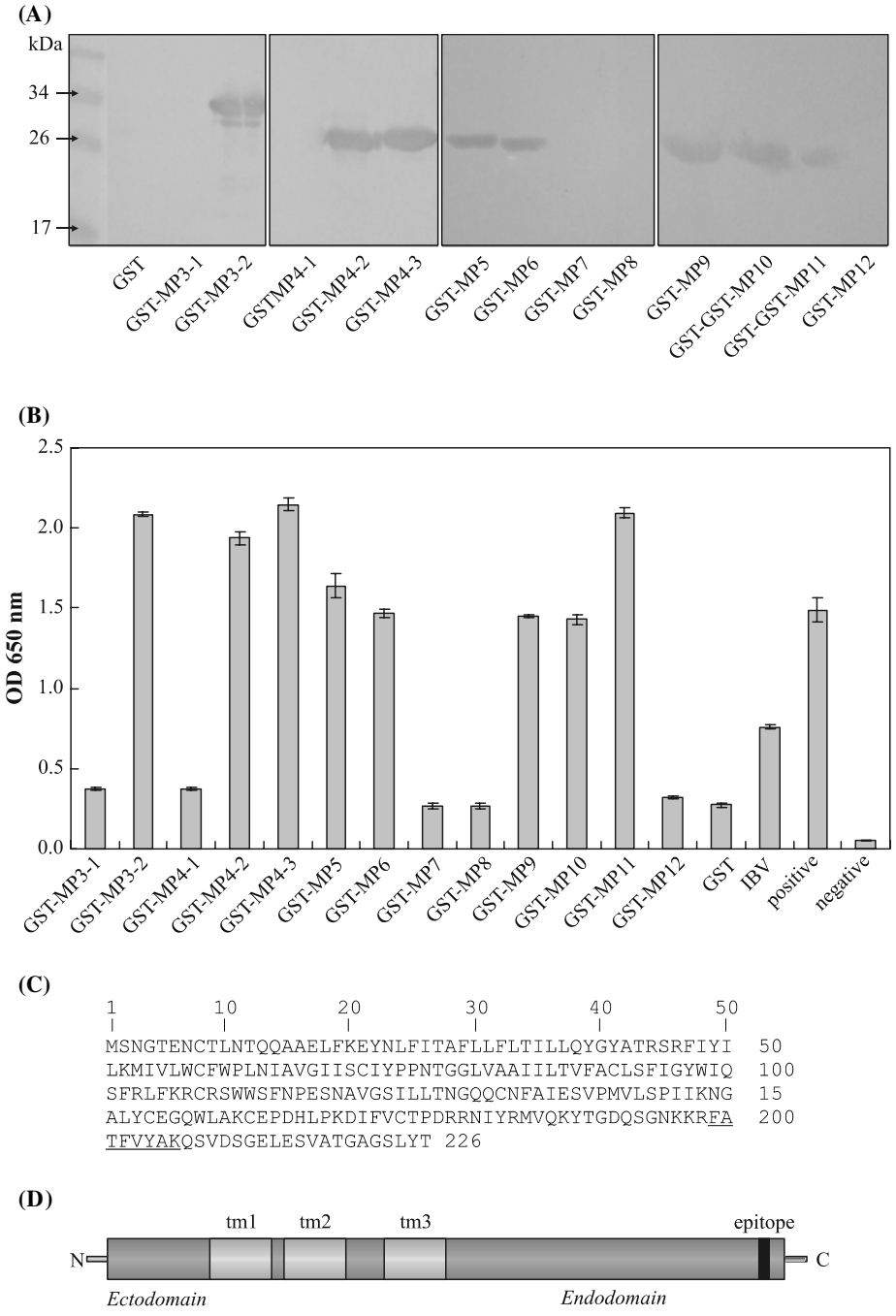

Fig. 5. Fine localization of the MAb 15E2-defined epitope. Reactivity of MAb 15E2 with different truncated recombinant M proteins as determined by western blotting (A) and ELISA (B). The GST fusion proteins were used. The names of the proteins are the same as in Table 1. GST protein was used as a negative control in both the western blotting and indirect ELISA. In addition, IBV CK/CH/LHLJ/04V virus particles were used as the coating antigen, and MAb 15E2, anti-IBV antibody from chickens (positive control), and SPF chicken sera (negative control) were used, as the primary antibodies in the ELISA. The antigenic determinant (underlined) that was recognized by MAb 15E2 was localized exactly to ^{199}FATFVYAK^{206} in the M protein of IBV strain CK/CH/LHLJ/04V (C and D). The linear map of the M protein, which denotes known regions of importance that include the transmembrane (tm) domains, ectodomain, and endodomain, is shown. The location of the epitope identified in this study is also shown.

Reactivity of the identified epitope with an anti-IBV antibody

The peptide that corresponded to the MAb 15E2-defined epitope (^{199}FATFVYAK^{206}) was used as the coating antigen in an ELISA, which demonstrated that this peptide was recognized well by an anti-IBV antibody from chicken (Fig. 6). The peptide showed similar reactivity with the antibody in western blotting analysis, which confirmed further that

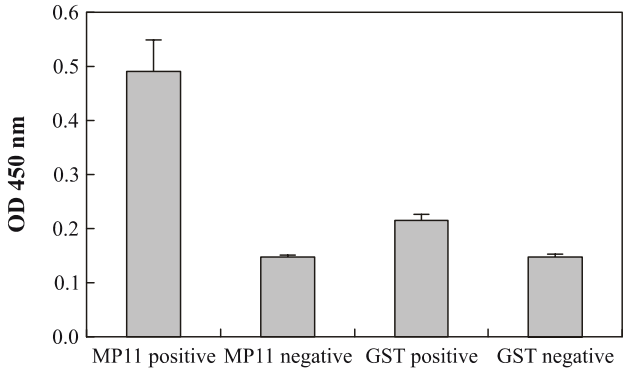

Fig. 6. Reactivity of the identified epitope (MP11: ^{199}FATFVYAK^{206}) with antibodies against IBV. The peptide that corresponded to the MAb 15E2-defined epitope was used as the coating antigen in an ELISA and purified GST protein was used as a negative control. The anti-IBV antibody from chickens was used as the primary antibody and SPF chicken sera were used as negative controls.

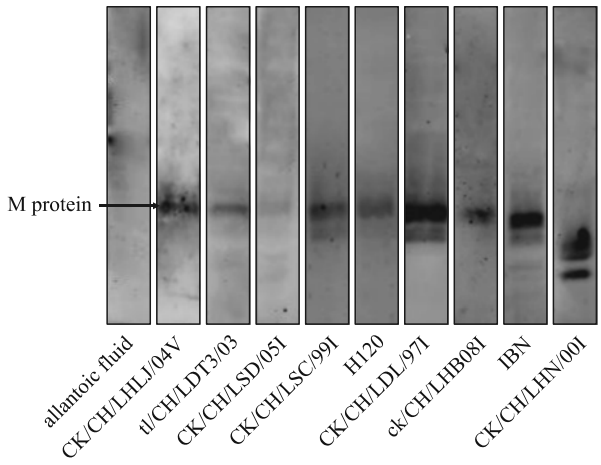

Fig. 7. The reactivity of MAb 15E2 with heterogenous IBV strains was determined by western blotting analysis. Allantoic fluids were used as negative controls in the western blotting.

this epitope had good reactivity.

Cross-reactivity of heterogeneous IBV strains with MAb 15E2

We examined the cross-reactivity of MAb 15E2 with eight IBV strains that represented seven different serotypes. As illustrated in Fig. 7, all the heterogeneous IBV strains showed cross-reactivity with MAb 15E2. For seven out of the eight IBV strains, western blotting of the infected allantoic fluids after SDS-PAGE showed a pronounced band of

(A)

Host	Virus	Strain	MAb 15E2-defined epitope sequence
Chicken	IBV	CK/CH/LHLJ/04V	GDQSGNK-KR**FATFVYAK**QSVDSGELE(EF602452)
Chicken	IBV	Beaudette	GDQSGNK-KR**FATFVYAK**QSVDTGELE(NC_001451)
Chicken	IBV	ArkDPI11	GDQSGNK-KR**FATFVYAK**QSVDTGELE(EU418976)
Chicken	IBV	TW2575/98	GDQSGNK-KR**FATFVYAK**QSVDTGELE(DQ646405)
Chicken	IBV	CU-T2	GDQSGNK-KR**FATFVYAK**QSVDTGELE(U46035)
Turkey	TCoV	MG10 strain	GDQSGNK-KR**FATFVYAK**QSVDTGELE(NC_010800)
Turkey	TCoV	TCoV-ATCC	GDQSGNK-KR**FATFIYAK**QSVDTGELE(EU022526)
Turkey	TCoV	TCoV-540	GDQSGNK-KR**FATFVYAK**QSVDTGELE(EU022525)
Bulbul	Coronavirus	HKU11-934	IESTDDR--D**LAVLIYQG**DRASNAGLH(FJ376619)
Munia	Coronavirus	HKU13-3514	IESTDDR--D**LAVLIYQG**DRSSNAALH(FJ376622)
Thrush	Coronavirus	HKU12-600	ID-TDER--D**FAVLIYQG**DRVSNAGLH(FJ376621)
Beluga Whale	Coronavirus	SW1	ADGSDANVAR**FGTVIYAK**DKYISAGLA(EU111742)

(B)

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GDQSGNKKRFATFVYAKQ---S(EF602452)     IBV CK/CH/LHLJ/04V                ┐
GDQSGNKKRFATFVYAKQ---S(NC_001451)         IBV Beaudette                ├ Group 3
GDQSGNKKRFATFVYAKQ---S(NC_010800)                  TCoV                ┘
LDKVDGVSGFAVYVKSKVGNYR(NC_006852)           MHV JHM      ┐ subgroup 2a ┐
LDRISDTSGFAVYVKSKVGNYR(NC_005147)         HCoV OC43      ┘             ├ Group 2
SQRVGTDSGFAAYNRYRIGNYK(EU371564)       SARS BJ182-12      ┐ Subgroup 2b ┘
KLKASSATGWAYYVKSKAGDYS(NC_002306)              TGEV      ┐ subgroup 1a ┐
QLKATTATGWAYYVKSKAGDYS(NC_007025)             FCoV      ┘             ├ Group 1
SVNASSSTGWAFYVRSKHGDYS(EU581712)   PEDV M_NIAH380_98      ┐ subgroup 1b ┘
SVNSQNSTGWVFYVRVKHGDFS(NC_002645)          HCoV 229E      ┘
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Fig. 8. Alignment of the epitope motif with 11 coronaviruses in group 3 (A) and 7 coronaviruses in group 2 or 3 (B). The epitope sequences are underlined and the amino acid residues in the epitope region that are shared by different coronaviruses are shown in bold. Deleted amino acid residues are represented as '–'. The abbreviations of the viruses are: TCoV (turkey coronavirus), MHV (murine hepatitis virus), HCoV OC43 (human coronavirus OC43), SARS-CoV (severe acute respiratory syndrome coronavirus), TGEV (transmissible gastroenteritis virus), FCoV (feline coronavirus), PEDV (porcine epidemic diarrhoea virus), and HCoV (human coronavirus 229E). The GenBank accession numbers are shown in parentheses.

the expected molecular weight. For IBV CK/CH/LHN/00I, more than one band was observed and their apparent molecular weights were obviously lower than expected.

Alignment of the 15E2-defined sequence with those of other coronaviruses

We have only a limited number of heterogeneous IBV strains that can be used in the cross-reactivity test and we have no other group 3 coronavirus strains in our laboratory. Hence, the equivalent sequences (FATFVYAK) from four heterogeneous strains, which represented four additional IBV serotypes, and seven other group 3 coronaviruses, which infected five different hosts, were aligned and compared. The results showed that nearly all the residues in the MAb 15E2-defined sequence were highly conserved among the chicken and turkey group 3 coronaviruses; residue V^{203} was changed to I^{203} in one of the three TCoV strains (Fig. 8). This indicated that the ^{199}FATFVYAK^{206} sequence represented a conserved epitope on the M protein of chicken and turkey group 3 coronaviruses. However, the homology in this region between chicken and turkey coronaviruses and other animal coronaviruses in group 3 was low.

In order to compare the conservation further, the MAb 15E2-defined epitope and its flanking amino acid sequences were compared with the corresponding sequences from seven coronaviruses from different groups or subgroups within the family *Coronaviridae*. As illustrated in Fig. 8, three residues in the MAb 15E2-defined sequence were found to be highly conserved among the family *Coronaviridae*. The residue A^{200} was highly conserved among all the coronaviruses selected in this study except for human coronavirus 229E, which contained a V residue in this position. Two residues, V^{203} and K^{206}, were highly conserved among all the selected coronaviruses except SARS-CoV which contained N and R residues, respectively, in the corresponding positions. In addition, the residue F^{192} was shared by coronaviruses in group 2 and group 3.

Discussion

The B-cell antigenic epitopes of IBV that have been identified so far are in the S and N proteins and these epitopes induce the production of virus-neutralizing antibodies and protection against virulent viruses (Niesters *et al.*, 1987; Kusters *et al.*, 1989; Lenstra *et al.*, 1989; Koch *et al.*, 1990; Boots *et al.*, 1991; Kant *et al.*, 1992; Ignjatovic and Galli, 1994; Moore *et al.*, 1997; Seo *et al.*, 1997; Seah *et al.*, 2000; Johnson *et al.*, 2003; Ignjatovic and Sapats, 2005). "All coronaviruses contain a large number of copies of an integral membrane (M) glycoprotein that is smaller than the S and N proteins and is required for virus particle formation. It has been reported that the S protein interacts with the transmembrane region of M (Cavanagh, 2007). However, the roles of the M protein in IBV replication, immunogenicity, and pathogenicity need to be investigated further. In this study, we expressed the C-terminal 129 amino acids of the IBV M protein (MP2) and found that this fragment showed good reactivity with antibodies against IBV. This indicated that this recombinant protein had similar antigenicity to the native IBV antigen and could be used as an immunogen for animal immunization. The S and N proteins of coronaviruses, rather than the M protein, are the main protein inducers of the host immune responses (Ignjatovic and Sapats, 1994). Hence, in the present study, we immunized mice first with IBV CK/CH/LHLJ/04V virus particles and then performed two booster immunizations with recombinant M protein to produce MAbs by hybridoma technology. Two hybridomas were established by cell fusion and one of the MAbs (15E2) was found to react specifically with the native IBV CK/CH/ LHLJ/04V particles as well as with the recombinant MP2 protein.

To study the specificity in more detail and finely map the epitope of MAb 15E2, a series of 14 fragments that covered the C-terminus of the IBV CK/CH/LHLJ/04V M protein were expressed with a GST tag and used to screen for the minimal epitope in the IBV M protein. Two methods, western blotting and ELISA, were used to screen for the epitope. It was demonstrated that the minimal sequence ("core sequence") of the MAb 15E2-defined epitope appeared to be ^{199}FATFVYAK^{206}, because deletion of any of the residues from either end of ^{199}FATFVYAK^{206} destroyed the ability of MAb 15E2 to bind. MAb 15E2 was not a neutralizing antibody, as demonstrated by the determination of EID_{50}. The absence of neutralizing activity against IBV may indicate that this region has low immunogenicity or, more probably, that this region is not exposed on the surface of the virion. Indeed, the epitope ^{199}FATFVYAK^{206} is located on the cytoplasmic side of the membrane (or inside the virion) according to the structure of coronavirus M protein. Interestingly, both the ascites that contained MAb 15E2 and that which contained a control MAb unrelated to IBV could "neutralize" IBV CK/CH/LHLJ/04V, as compared with PBS. However, unpurified ascites was used in the present study and it is likely that non-specific components in the ascites rather than MAb 15E2 itself killed the IBV in the *in vitro* neutralization test.

Given that MAb 15E2 lacks neutralizing activity, it is of little importance for the design of potential subunit vaccines to protect against IBV. However, considering the conservation between chicken and turkey coronaviruses, MAb 15E2 and its defined epitope may be useful for diagnostic purposes. It has been proposed that coronaviruses isolated from turkeys (TCoV) and chickens (IBV) should be considered as distinct species (Cavanagh, 2005). The S gene of TCoV strain MG10 showed only 57% identity to different strains of IBV. However, the M genes of the two viruses shared 93% identity (Cao *et al*., 2008; Gomaa *et al.*, 2008). In this study, the epitope that was identified in the M protein of IBV is highly conserved in turkey coronaviruses. Until now, little has been known about the antigenic epitopes within the structural proteins of turkey coronaviruses. The conserved epitope shared by the M proteins of turkey coronaviruses and IBV is of significance because not only are the two viruses of economic importance to the poultry industry, but they also show antigenic and biological similarities when compared with other coronaviruses. In recent years, coronaviruses have been identified from the beluga whale (Mihindukulasuriya *et al.*, 2008) and three different wild birds (bulbul, thrush, and munia) (Woo *et al.*, 2008). Comparative

analysis of their complete genome sequences revealed that they all belong to group 3 of the coronaviruses. However, the coronaviruses from the whale and wild birds were clustered into subgroups 3b and 3c, respectively, by phylogenetic analysis using the chymotrypsin-like protease (3CLpro), RNA-dependent RNA polymerase (Pol), helicase, spike, and nucleocapsid proteins (Woo *et al.*, 2008) and are distantly related to IBV and TCoV, which belong to subgroup 3a. In this study, we found that only two to four residues in the MAb 15E2-defined epitope region of the M protein were shared invariably between IBV and coronaviruses in subgroups 3b and 3c. This was similar to the amino acids shared between IBV and coronaviruses in groups 1 and 2. The roles of the residues in the epitope region that are conserved among different groups and subgroups of coronaviruses require further investigation. Group 3 coronaviruses were also identified in or isolated recently from pheasants (*Phasianus colchicus*) (Cavanagh *et al.*, 2002; Cavanagh, 2005), graylag geese (*Anser anser*), feral pigeons (*Columbia livia*), and mallards (*Anas platyrhynchos*) (Jonassen *et al.*, 2005), and a green-cheeked Amazon parrot (*Amazon viridigenalis Cassin*) (Gough *et al.*, 2006). Due to the fact that the sequence of the M gene in these coronaviruses is not known, we could not determine the degree of conservation of the Mab 15E2-defined epitope sequence in these viruses.

The coronavirus M protein is almost invariably glycosylated on its exposed N-terminal domain. The M proteins of IBV and SARS-CoV contain only N-linked sugars and N-glycosylation is initiated by the polypeptide in the endoplasmic reticulum at asparagine residues (de Haan and Rottier, 2005). In the present study, the molecular weights of the proteins in IBV CK/CH/LHN/00I that reacted with MAb 15E2 were obviously lower than those of the proteins in the other IBV strains selected. The MAb 15E2-defined sequence in strain CK/CH/LHN/00I (GenBank accession no. EF602456) (Liu *et al.*, 2008b) showed a high degree of conservation with that of strain CK/CH/LHLJ/04V (^{199}FATFVYAK^{206}) in the present study. Hence, the bands in strain CK/CH/LHN/00I that react with MAb 15E2 during western blotting should be specific. The discrepancy in the molecular weight of the M protein between CK/CH/LHN/00I and the other IBV strains selected may be related to different degrees of glycosylation within the M protein. However, additional investigations may be required.

In this report, we have used a combination of molecular and immunological techniques to screen MAbs against the native M protein of avian infectious bronchitis coronavirus and to map the MAb 15E2-defined epitope in fine detail. To our knowledge, this is the first report of the mapping of a B-cell epitope in the M protein of avian infectious bronchitis coronavirus. To date, several methods have been used to map antigenic epitopes (Roggen, 2006). The advantage of mapping epitopes via expression in prokaryotes is that, once the subclones have been generated, it is possible to screen a large number of MAbs rapidly. Therefore, the truncated recombinant proteins that were used in this study may be useful tools for mapping other potential epitopes on the IBV M protein in future studies.

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