Mapping a highly conserved linear neutralizing epitope on gD glycoprotein of bovine herpesvirus type I using a monoclonal antibody

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ABSTRACT. Bovine herpesvirus type 1 (BoHV-1), a member of the Alphaherpesvirinae, causes a variety of diseases, which result in significant economic losses worldwide. Envelope glycoprotein D (gD) of BoHV-1 plays an important role in viral entry into the permissive cells, and protective immune response. The fine mapping epitope on the gD will contribute to the understanding of viral pathogenesis and development of alternative vaccines against various diseases associated with BoHV-1. We previously reported the preparation of a monoclonal antibody (MAb) 2B6, which was raised by a truncated recombinant gD protein, demonstrating a neutralizing activity against BoHV-1 infection in Madin–Darby bovine kidney cells. This study described the identification of a linear B-cell epitope on gD using MAb 2B6. A series of partially overlapping gD proteins with glutathione S-transferase tag were generated to define the epitope recognized by MAb 2B6. The amino acid (aa) sequence 323GEPKPGPSPDADRPE337 was recognized by MAb 2B6 using Western blot with the variedly truncated recombinant proteins. Importantly, this epitope was highly conserved among the typical members of BoHV-1, indicating that the epitope may be utilized in diagnosis of diseases due to BoHV-1 infection. Furthermore, the minimal linear epitope sequence 323GEPKPGP329 on gD recognized by MAb 2B6 was confirmed using single-aa residue deletion mutation in carboxyl terminal. This finding not only contributes to our understanding of gD of BoHV-1 virion but also shows a potential for the development of vaccine candidates and diagnostic techniques.

KEY WORDS: bovine herpesvirus, epitope map, gD, monoclonal antibody
Abdelmagid et al. defined two neutralizing epitopes on gD of BoHV-1, amino acids (aa) 92–106 and 202–213, using type-specific monoclonal antibodies (MAbs) triggered by the recombinant protein [1]. Levings and his colleagues generated bovine MAbs specific for gB, gC, and gD proteins [16]; however, the antigenic determinants recognized by bovine MAbs have not been mapped. To date, the information on the epitopes, including B-cell epitopes on gD of BoHV-1, is scant, and whether additional B cell epitopes on gD exist remains unknown.

Previously, we reported that two predicted antigenic regions on gD protein of BoHV-1, ΔgD1 (aa 20–160) and ΔgD2 (aa 257–344), were chimerically expressed in a bacterial system and BALB/c mice immunized with purified tandem recombinant protein ΔgD1–ΔgD2 to prepare MAbs against gD protein. Then, an MAb against gD protein, termed 2B6, was demonstrated to exhibit neutralizing activity against BoHV-1 using plaque reduction assay [28]. Therefore, the current study aimed to define the epitopes of BoHV-1 gD protein using MAb 2B6. In this study, an epitope on gD protein of BoHV-1 recognized by MAb 2B6 was mapped through expression of overlapping fragments of truncated gD protein in a bacterial system, followed by Western blot test. The data demonstrated that a specific linear B-cell epitope on gD of BoHV-1 that is recognized by MAb 2B6 was mapped to the sequence 323GEPKPGPSPDADRPE337. We also confirmed that this epitope is highly conserved among the classical strains of BoHV-1 published in GenBank, suggesting that it can be a useful tool to explore viral entry and vaccine development and diagnosis in the future.

### MATERIALS AND METHODS

**Preliminary epitope mapping on gD protein**

We previously reported the generation of recombinant ΔgD1–ΔgD2 protein and preparation of MAb 2B6 against the recombinant protein. The reaction of 2B6 with BoHV-1 and its neutralizing activity were validated as described previously [28]. To map the epitope on gD protein recognized by MAb 2B6, three truncated overlapping DNA fragments corresponding to the truncated recombinant ΔgD1–ΔgD2 to prepare MAbs against gD protein. Then, an MAb against gD protein, termed 2B6, was demonstrated to exhibit neutralizing activity against BoHV-1 using plaque reduction assay [28]. Therefore, the current study aimed to define the epitopes of BoHV-1 gD protein using MAb 2B6. In this study, an epitope on gD protein of BoHV-1 recognized by MAb 2B6 was mapped through expression of overlapping fragments of truncated gD protein in a bacterial system, followed by Western blot test. The data demonstrated that a specific linear B-cell epitope on gD of BoHV-1 that is recognized by MAb 2B6 was mapped to the sequence 323GEPKPGPSPDADRPE337. We also confirmed that this epitope is highly conserved among the classical strains of BoHV-1 published in GenBank, suggesting that it can be a useful tool to explore viral entry and vaccine development and diagnosis in the future.

**Further mapping of gD epitope on gD protein**

The schematic diagram of the relative locations of various truncated forms of ΔgD1–ΔgD2 was shown as Fig. 1. The truncated fragments were obtained by polymerase chain reaction amplification or direct annealing using the primer pairs listed in Table 1. Each fragment was cloned into BamHI and EcoRI restriction endonuclease sequences, respectively. The recombinant plasmids were validated by DNA sequencing, and each plasmid was transformed into E. coli BL21 (DE3) competent cells, followed by expression of glutathione S-transferase (GST)-tagged recombinant truncated proteins by addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C for 4 hr or at 20°C overnight. Recombinant E. coli cells were harvested by centrifugation at 10,000 × g for 15 min at 4°C. Expressions of various recombinant proteins were confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot with MAb 2B6 as the primary antibody.

**Fine mapping of gD epitope recognized by MAb 2B6**

Precise mapping of epitope and minimum epitope amino acid on gD protein recognized by MAb 2B6 was performed with the methods described above. Table 2 lists the primer sequences for precise mapping epitope and deletion mutations. Figure 1 shows the schematic diagram of the relative locations of various truncated forms of ΔgD1–ΔgD2.

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**Table 1. Primers used for generation of overlapping fragment in this study**

| Name         | Sequences of the oligonucleotides (5’-3’)               |
|--------------|--------------------------------------------------------|
| 107–252-F    | TACGGATCCTGGTACAAGATCGAGAGCGGG                         |
| 107–252-R    | TTTGGAATTCCGCCCGTGGGGCGATGAAACCA                       |
| 245–342-F    | TACGGATCCTACTGTTCTATGCGGCCACCGGG                       |
| 245–342-R    | TTTGGAATTCCGAGGCTCGGCCAGCTCGG                         |
| 335–369-F    | TACGGATCCTCCGCCCGAAGGGCTGACCAGGGGG                    |
| 335–369-R    | TTGGAATTCCGCGATCGCGAGCTGACGCGAC                     |
| 245–292-F    | ATCGGAATTCTACTGTTCTAGCGCACC                           |
| 245–292-R    | TTGGAATTCCGTCTCGTGTCATCCCTCTGAC                       |
| 285–318-F    | ATCGGAATTCGAGGATGAAAGGGAGACCGAGAC                   |
| 285–318-R    | TTGGAATTCCGCGATCGCGAGCTGACGCGAC                     |
| 311–340-F    | ATCGGAATTCCGAGGTCGACCGACCGAAGCCACGGA                 |
| 311–340-R    | TTGGAATTCCGGCCACGCTGCGGGG                           |

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The conservation of epitope amino acid sequence of gD recognized by MAb 2B6 was analyzed using the gD amino acid sequence of the selected member of alphaherpesvirinae by using the MegAlign of Lasergene package (DNAstar, Inc., Madison, WI, U.S.A.). The amino acid sequences of the selected strains of alphaherpesvirus were aligned by Clustal W method. Of note, the gD amino acid sequence of the selected region of BoHV-4 was excluded in conservation analysis given the unavailability of the gD sequence of BoHV-4 in the GenBank.

Table 2. Primers used for fine mapping of epitope in this study

| Name | Sequences of the oligonucleotides (5'-3') | Coding amino acid sequence<sup>a)</sup> |
|------|------------------------------------------|---------------------------------------|
| 311–326-F | ATC<sup>NNNN</sup>GGATCCGAGTCAGACCCCCGAAGCCAACGG | 311ESQTPEANGGAEGEPK326 |
| 311–326-R | TTC<sup>NNNN</sup>GAATTCGTCCTGGGCTGGGGCTGGGGCTGGGGCCGG | 311ESQTPEANGGAEGEPK326 |
| 316–330-F | ATC<sup>NNNN</sup>GGATCCGAGTCAGACCCCCGAAGCCAACGG | 316EANGGAEGEPKPGPS330 |
| 316–330-R | TTC<sup>NNNN</sup>GAATTCGTCCTGGGCTGGGGCTGGGGCTGGGGCCGG | 316EANGGAEGEPKPGPS330 |
| 323–337-F | ATC<sup>NNNN</sup>GGATCCGAGTCAGACCCCCGAAGCCAACGG | 323GEPKPGPSPDADRPE337 |
| 323–337-R | TTC<sup>NNNN</sup>GAATTCGTCCTGGGCTGGGGCTGGGGCTGGGGCCGG | 323GEPKPGPSPDADRPE337 |
| 326–340-F | ATC<sup>NNNN</sup>GGATCCGAGTCAGACCCCCGAAGCCAACGG | 326KPGPSDADRPGWP340 |
| 326–340-R | TTC<sup>NNNN</sup>GAATTCGTCCTGGGCTGGGGCTGGGGCTGGGGCCGG | 326KPGPSDADRPGWP340 |
| 324–337-F | ATC<sup>NNNN</sup>GGATCCGAGTCAGACCCCCGAAGCCAACGG | 324EPKPGPSDADRPE337 |
| 324–337-R | TTC<sup>NNNN</sup>GAATTCGTCCTGGGCTGGGGCTGGGGCTGGGGCCGG | 324EPKPGPSDADRPE337 |
| 323–336-R | TTC<sup>NNNN</sup>GAATTCGTCCTGGGCTGGGGCTGGGGCTGGGGCCGG | 323GEPKPGPSDADR336 |
| 323–335-R | TTC<sup>NNNN</sup>GAATTCGTCCTGGGCTGGGGCTGGGGCTGGGGCCGG | 323GEPKPGPSDADR335 |
| 323–334-R | TTC<sup>NNNN</sup>GAATTCGTCCTGGGCTGGGGCTGGGGCTGGGGCCGG | 323GEPKPGPSDADR334 |
| 323–333-R | TTC<sup>NNNN</sup>GAATTCGTCCTGGGCTGGGGCTGGGGCTGGGGCCGG | 323GEPKPGPSDADR333 |
| 323–332-R | TTC<sup>NNNN</sup>GAATTCGTCCTGGGCTGGGGCTGGGGCTGGGGCCGG | 323GEPKPGPSDADR332 |
| 323–331-R | TTC<sup>NNNN</sup>GAATTCGTCCTGGGCTGGGGCTGGGGCTGGGGCCGG | 323GEPKPGPSDADR331 |
| 323–330-R | TTC<sup>NNNN</sup>GAATTCGTCCTGGGCTGGGGCTGGGGCTGGGGCCGG | 323GEPKPGPSDADR330 |
| 323–329-R | TTC<sup>NNNN</sup>GAATTCGTCCTGGGCTGGGGCTGGGGCTGGGGCCGG | 323GEPKPGPSDADR329 |
| 323–328-R | TTC<sup>NNNN</sup>GAATTCGTCCTGGGCTGGGGCTGGGGCTGGGGCCGG | 323GEPKPGPSDADR328 |
| 323–327-R | TTC<sup>NNNN</sup>GAATTCGTCCTGGGCTGGGGCTGGGGCCGG | 323GEPKPGPSDADR327 |

<sup>a</sup>) Numbers represent the deduced aa position on gD protein of BoHV-1 (GenBank accession number: NC_001847.1).

Analysis of conservation of the epitope of BoHV-1 gD

The conservation of epitope amino acid sequence of gD recognized by MAb 2B6 was analyzed using the gD amino acid sequence of the selected member of alphaherpesvirinae by using the MegAlign of Lasergene package (DNASTAR, Inc., Madison, WI, U.S.A.). The amino acid sequences of the selected strains of alphaherpesvirus were aligned by Clustal W method. Of note, the gD amino acid sequence of the selected region of BoHV-4 was excluded in conservation analysis given the unavailability of the gD sequence of BoHV-4 in the GenBank.
RESULTS

Primary mapping of BoHV-1 gD epitope recognized by MAb 2B6

Previously, we reported the preparation of MAb 2B6 against the ΔgD1–ΔgD2 recombinant tandem protein, representing a reactivity with naïve BoHV-1 by indirect immunofluorescence assay and neutralizing activity [28]. To identify the epitope on gD recognized by MAb 2B6, partially overlapping fragments within ΔgD1–ΔgD2 region were expressed upon GST-tagged fusion proteins in bacterial system, and epitope mapping was confirmed by reaction between recombinant truncated proteins and MAb 2B6 using Western blot. The expression strategy for fragment construction was illustrated in Fig. 1. As demonstrated by SDS-PAGE, each of the three fragments was expressed upon induction with IPTG. Western blot analysis demonstrated that Pep 2 (aa 245–342) reacted with MAb 2B6. Otherwise, the Pep1 (aa 107–252) and Pep3 (aa 335–369) showed no reaction with MAb 2B6 (data not shown). The Pep2 (aa 245–342) was further divided into the three overlapping fragments, which were expressed in GST-tagged fusion proteins (Fig. 2A). Western blot analysis confirmed that the Pep6 (aa 311–340) reacted with MAb 2B6, and the Pep4 (aa 245–292) and Pep5 (aa 285–318) showed no reactogenicity with MAb (Fig. 2B).

Fine mapping of gD epitope recognized by MAb 2B6

For fine mapping of epitope on gD recognized by MAb 2B6, the Pep6 (aa 311–340) was divided into four partially overlapping fragments and expressed in GST-tagged fusion proteins as described above. Western blot analysis demonstrated that the Pep9 (aa 323–337) fusion protein was recognized by MAb 2B6, suggesting that such a 15 amino acid peptide, that is,
323GEPKPGSPDADRPE337, was epitope-recognized by MAb 2B6 (Fig. 2C and 2D). To define the minimum length of the identified epitope, a set of deletion mutations of carboxyl terminal of aa 323–337 was generated and expressed in GST-tagged fusion proteins (Fig. 3A). Western blot analysis validated that the minimal amino acid sequence of the identified epitope was 323GEPKPG329, which was recognized by MAb 2B6 (Fig. 3B).

Analysis of conservation level for the epitope of BoHV-1 gD
To determine whether the epitope 323GEPKPGSPDADRPE337 recognized by MAb 2B6 is conserved among typical alphaherpesviruses, the amino acid sequence of this epitope was aligned with deduced amino acid sequences from selected strains of alphaherpesviruses. As shown in Fig. 4, the linear epitope 323GEPKPGSPDADRPE337 recognized by MAb 2B6 was highly conserved among the selected BoHV-1 strains, including BoHV-1.1 and BoHV-1.2. However, this linear epitope showed moderate or high variability with BoHV-5, pseudorabies virus, caprine alphaherpesvirus, and equine herpesvirus 1.

DISCUSSION
Mapping epitopes of viral structural proteins, including gD of BoHV-1, and defining the degree of conservation of epitopes may facilitate our understanding of the antigenic structure, entry, and pathogenesis of viruses and are also useful for clinical diagnosis and vaccine development [11, 17]. Similar to other enveloped viruses that enter permissive cells, herpesviruses penetrate the cell in a membrane fusion manner. Herpesviruses require at least three receptor binding glycoproteins and in specific cases, an additional glycoprotein, compared with most other enveloped viruses. Herpesvirus gD, gC, and gB interact with various receptors in permissive cells during viral entry. The absence of any one of gD and gB or gH results in severe decline of viral entry if gC is present [22]. As for BoHV-1, pseudorabies virus (PRV), herpes simplex virus type 1 (HSV-1), and HSV-2, gC may be responsible for the initial interaction with cells during viral entry. However, gC is nonessential for viral replication of these viruses, as mutation with gC deletion may rely on other viral envelope proteins to substitute for the missing gC of BoHV-1 or PRV [21, 32]. gD presumably initiates viral binding with cell surface receptors if gC is absent. Of note, gD is insufficient for viral entry, but its involvement with any of cell surface coreceptors may trigger fusion between viral envelope protein and cell membrane to facilitate

Fig. 3. Precise localization of MAb 2B6-defined epitope by SDS-PAGE and Western blot. Panel A, SDS-PAGE; Panel B, Western blot. Reactivity of MAb 2B6 with different GST-fused proteins is determined by Western blot. GST represents glutathione S-transferase.

Fig. 4. Multiple amino acid sequence alignment of the defined epitope by 2B6. The amino acid position is ruled based on gD sequence (GenBank accession number NC_001847.1). PsRV, represents pseudorabies virus; CaHV, caprine alphaherpesvirus; EqHV, equine herpesvirus 1.
viral entry [10]. However, highly limited information describes how herpesvirus glycoproteins interact during entry. In this study, the mapping of a conserved linear epitope on gD of BoHV-1 may facilitate the study of the role of C-terminus of gD in viral penetration, which will contribute to understanding how BoHV-1 enters permissive cells. To this point, synthesized soluble peptides can be used to test its suppressive activity on BoHV-1 entry to cells.

During viral entry, structural feathers of gD among animal and human herpesviruses are presumed to be highly conserved owing to the highly diverse identity of primary amino acid sequence of gD among alphaherpesviruses. The gD of BoHV-1, which consists of 417 amino acid residuals, shares 28.4, 29.3, and 38.5% identity with HSV-1, HSV-2, and PRV, respectively. In the past decade, the role of gD has been extensively characterized mostly for HSV-1, HSV-2, and PRV instead of BoHV-1. Similar to the gD of HSV-1, the N-terminal hairpin structure, including residues 1–32, is characterized for binding with herpesvirus entry mediator, a member of the tumor necrosis factor receptor family [5, 6]. Furthermore, additional four key amino acid residues, Y38, D215, R222, and F223, bind with another cell receptor, nectin-1, which these amino acid residues locate as a contiguous area on the surface of gD on the basis of a structural model [20]. The linear epitope defined in this study locates in the region close to the C-terminus of gD, where the structure is disorder and highly flexible. The structural models for C-terminus of gD, including BoHV-1, PRV, and HSV, are unavailable, resulting in difficulty in structural analysis. We believe that the epitope identified in this study is useful in functional analysis of C-terminus of gD, especially in viral entry by inhibition of such peptide to BoHV-1 infection [9]. Additionally, the key amino acid residue among minimal linear epitope sequence 322GEPKPGP329 on the gD recognized by MAb 2B6 remains to be defined.

Furthermore, epitope mapping might contribute to candidate vaccine development against diseases associated with BoHV-1 infection in cattle. BoHV-1 creates significant economic losses to cattle industry worldwide, as BoHV-1 acute infection can cause severe damage in the respiratory tract of cattle, which are vulnerable to further fatal secondary bacterial infections [24]. Additionally, BoHV-1 infection can establish latency in neuron system, by which BoHV-1 evades host immune response and may be re-activated under stress conditions. Therefore, immunization with safe and highly immunogenic vaccine is considered an effective strategy to prevent and control diseases associated with BoHV-1 [35]. Although live attenuated BoHV-1 vaccines, including marker vaccines or genetically engineered and modified live deletion mutants such as gE deletion-modified live virus (MLV) BoHV-1 vaccines, offer strong and robust immune protection from BoHV-1 and induce long duration of immunity [27], vaccination of cattle with other live attenuated BoHV-1 vaccines, including MLV vaccine, may result in undesired effects and viral shedding from vaccinated animals [15]. Moreover, MLV vaccines may cause abortions and maybe insufficiently safe for pregnant animals [23]. Therefore, the development of alternative vaccines, such as killed vaccines [26], subunit vaccines [19, 29], or DNA vaccines [18], against BoHV-1 are necessary as effective complementary vaccines against BoHV-1 infection, especially for pregnant cattle. In this study, a new linear B cell-neutralizing epitope on gD of BoHV-1 was verified, suggesting a potential for the development of subunit vaccine or DNA vaccine candidate. Of note, we are currently generating a recombinant subunit vaccine based on multiple epitopes on gD and are aiming to evaluate its efficacy in animal model in the future. Besides, MAb 2B6 recognized the highly conservative amino acid residue, 322GEPKPGP329, suggesting its further application in diagnosis and epidemiological survey in cattle herds, where the diseases associated with bovine respiratory disease complex pose significant threat to cattle industry.

CONFLICTS OF INTEREST. The authors declare no conflict of interest.

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