A Single Amino Acid Change within Antigenic Domain II of the Spike Protein of Bovine Coronavirus Confers Resistance to Virus Neutralization

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The spike glycoprotein is a major neutralizing antigen of bovine coronavirus (BCV). Conformational neutralizing epitopes of group A and group B monoclonal antibodies (MAbs) have previously been mapped to two domains at amino acids 351 to 403 (domain I) and amino acids 517 to 621 (domain II). To further map antigenic sites, neutralization escape mutants of BCV were selected with a group A MAb which has both in vitro and in vivo virus-neutralizing ability. The escape mutants were demonstrated to be neutralization resistant to the selecting group A MAb and remained sensitive to neutralization by a group B MAb. In radioimmunoprecipitation assays, the spike proteins of neutralization escape mutants were shown to have lost their reactivities with the selecting group A MAb. Sequence analysis of the spike protein genes of the escape mutants identified a single nucleotide substitution of C to T at position 1583, resulting in the change of alanine to valine at amino acid position 528 (A528V). The mutation occurs in domain II and in a location which corresponds to the hypervariable region of the spike protein of the coronavirus mouse hepatitis virus. Experimental introduction of the A528V mutation into the wild-type spike protein resulted in the loss of MAb binding of the mutant protein, confirming that the single point mutation was responsible for the escape of BCV from immunological selective pressure.

Bovine coronavirus (BCV) is a member of the family Coronaviridae of the order Nidovirales (3) and is closely related to the coronavirus mouse hepatitis virus (MHV). An enteropathogenic virus, BCV causes severe diarrhea in neonatal calves and winter dysentery in adult cattle (13, 29, 31, 33). BCV has also been associated with bovine respiratory disease, which is observed with the most severity in feedlot cattle (18, 29, 34).

An enveloped virus, BCV is composed of five structural proteins and contains a large positive-stranded RNA genome of 31,043 nucleotides (D. Yoo and Y. Pei, VIIIth Int. Symp. Nidoviruses [Coronaviruses and Arteriviruses 2000]). The five structural proteins are the nucleocapsid protein (N; molecular weight, 52,000 [52K]), the membrane associated protein (M; molecular weight, 25K), the small membrane protein (E; molecular weight, 8K), the spike protein (S; molecular weight, 180K), and the hemagglutinin-esterase protein (HE; molecular weight, 65K) (23, 32, 44).

The BCV S protein is a very large membrane glycoprotein of 1,363 amino acids that contains two hydrophobic regions characteristic of type I glycoproteins: one at the N terminus of the protein that functions as a signal sequence and the other at the C terminus that functions as a membrane anchor (25, 32). Electron microscopic studies indicate that the S protein forms the club-shaped structures on the surface of the coronavirus virion (31). For BCV, the S protein is cleaved at amino acid positions 768 and 769 to form two subunits (1): S1 represents the N-terminal half of the S protein and S2 represents the C-terminal half of the protein. The S protein has several important functions including binding of the virus to susceptible cells (4, 6, 22, 28), mediation of membrane fusion (both virus-cell and cell-cell fusion) (6, 35, 36, 42), and induction of neutralizing antibody responses in the host species (10, 17, 22, 24, 37).

For BCV, virus-neutralizing anti-S monoclonal antibodies (MAbs) recognize conformational epitopes in two distinct antigenic sites, A and B, as defined in competitive binding assays (10). While both group A and group B MAbs neutralize BCV in vitro (in cell culture), only group A MAbs demonstrate in vivo virus-neutralizing protective responses in bovine intestinal-loop studies (9). Thus, antigenic site A of the BCV S protein appears to have an important function in the host species.

Previously, mapping studies by proteolysis of antigen-antibody complexes with group A and group B MAbs have demonstrated that the epitopes recognized by both groups of antibodies are located on a 37K-molecular-weight trypsin fragment of the S protein (11). It was proposed that this fragment spans amino acid positions 351 to 621 on the S1 subunit on the basis of an analysis of the fragments generated with three proteolytic enzymes (11, 40). Deletion mapping studies have identified that both group A and group B conformational epitopes consist of two domains located within amino acid residues 324 to 403 and 517 to 720 (40). Since this is in general agreement with the proposed location of the 37K-molecular-weight trypsin fragment, amino acids residues 351 to 403 (domain I) and 517 to 621 (domain II) are thought to contain the critical amino acids of these epitopes (40). In the present study,
to further map the antigenic sites of the S1 protein, we have generated BCV MAb escape mutants, and using these mutant viruses, we have identified an epitope-critical amino acid that occurs in domain II.

**MATERIALS AND METHODS**

Cells, viruses, and antibodies. The Quebec strain of BCV (8) was propagated in Mardin-Darby bovine kidney (MDBK) cells. MDBK cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum (Cansera, Rexdale, Ontario, Canada). HeLa cells, maintained in Dubboeco's modified Eagle's medium with 10% fetal bovine serum, were used for vaccinia virus propagation. Vaccinia virus expressing T7 RNA polymerase (T7POL) (14) was used for protein expression. Preparation of Mabs was described previously (10), and in the current study group A MAB HB10-4 and group B MAB BB17-4 were used as mouse ascitic fluids.

**Generation of MAB-resistant (mar) mutants.** Neutralizing MAB escape mutants were generated by incubating an equal volume of neat wild-type BCV (~10^8 PFU) and a 1:100 dilution of MAb HB10-4 for 60 min at 37°C. Cells propagated in a 100-mm dish were inoculated with the mixture for 1 h at 37°C. The inoculum was removed and the cells were overlaid with 0.7% agarose in medium supplemented with 10% fetal bovine serum. The cells were incubated at 37°C in the presence of MAb HB10-4. At 3 days of incubation, the cells were stained with neutral red to visualize plaques. Plaques were picked with a Pasteur pipette, washed with medium, and resuspended in 1 ml of medium. The plaque-picking virus was propagated in MDBK cells in the presence of a 1:100 dilution of MAb HB10-4 for three passages until a cytopathic effect was evident. Tenfold dilutions of the passed virus were then incubated with a 1:100 dilution of MAB HB10-4 or without antibody and were propagated in the plaque assay to confirm an MAB resistance phenotype and to generate plaque-purified (subcloned) mutant viruses. Subclones of the escape virus mutant were propagated as described above, tested for the mar phenotype, aliquoted, and stored at –70°C.

cDNA cloning. Cells were infected with mar viruses and incubated for 2 days at 37°C in the presence of MAB HB10-4. Total RNA was extracted from the cells by using TriZol (Gibco BRL, Burlington, Ontario, Canada) according to the manufacturer's instructions. cDNA was synthesized from virus-infected total cellular RNA equivalent to that from approximately 10^6 cells by using SuperScript II RNase H–reverse transcriptase (Gibco BRL) and a primer specific for the S gene of BCV representing nucleotide positions 2256 to 2282 (downstream primer Sm1 [5'-CAAGACATGATCCTGTGGAGATCA-3']). The reverse transcription reaction was carried out for 1 h at 39°C in the presence of 1 mM each dCTP, dGTP, dATP, and dTTP; 50 mM dithiothreitol; 50 mM Tris-HCl (pH 8.3); 75 mM KCl; and 5 mM MgCl2 in a reaction volume of 20 μl. The second-strand DNA was synthesized by PCR amplification with the upstream primer SR954 [5'-CAAGCACATGATCCTGTGGAGATCA-3'] representing nucleotide positions 946 to 970 of the BCV S gene and the downstream primer (Sm1) that was used to make the first-strand cDNA. For PCR, 4 μl of the first-strand cDNA reaction mixture was added to the PCR mixture containing a final concentration of 0.15 μg of the upstream and downstream primers, 20 mM Tris-HCl (pH 8.4), 5 mM MgCl2, 50 mM KCl, each dideoxynucleoside triphosphate at a concentration of 1 mM, and 0.5 U of Vent DNA polymerase (New England Biolabs, Beverly, Mass). The PCR was performed in a thermocycler (Tyler Instrument, Edmonton, Alberta, Canada) for 30 cycles with the following parameters: 94°C for 30 s for denaturation, 62°C for 30 s for annealing, and 72°C for 2.5 min for extension, followed by a 10-min elongation at 72°C after the final cycle. The PCR product was cloned into the Smal site of plasmid pGEM3Z (Promega, Madison, Wis.).

Determination of nucleotide sequence. Sequences were determined in both directions by the dideoxynucleotide chain termination method with a T7 DNA sequencing kit (Pharmacia, Baie d'Urfé, Quebec, Canada) according to the manufacturer's instruction. Approximately 1 to 2 μg of double-stranded DNA denatured in 0.2 N NaOH was incubated with 20 ng of either forward or reverse sequencing primers for 20 min at 37°C, and sequencing reactions for each nucleotide were carried out with T7 DNA polymerase and [α-35S]dATP (specific activity, 50 Ci/mmol; New England Nuclear, Boston, Mass.). The reactions were resolved on an 8 M urea–5% polyacrylamide gel until bromophenol blue dye runoff with an IBI STS-45 sequencing gel apparatus (Kodak). The gel was air dried at 37°C, and the images were visualized by exposure to X-ray film at –70°C.

**Site-directed mutagenesis.** Site-directed mutagenesis was carried out on the template in this work has been deposited in the GenBank database under accession number AF313395.

**RESULTS**

Generation and characterization of mar mutants. Two domains, domains I and II, associated with BCV neutralizing epitopes have previously been mapped to amino acid positions 351 to 403 and 517 to 621 of the S protein, respectively (40). Both domains lie within the S1 subunit of the S protein, and domain II overlaps sequences of the corresponding hypervariable region of the MHV S protein (amino acid positions 456 to 592 in BCV). To dissect the neutralizing epitope recognized by group A MAB HB10-4, mutant BCVs resistant to the neutralizing MAB were generated. From a total of 30 plaques picked from residual virus after incubation of the wild-type BCV with MAB HB10-4, 13 viable viruses were obtained after three passages in cell culture. All of these viable viruses were of the MAB-resistant (mar) phenotype and after incubation with a
S, uncleaved form of the BCV spike protein; S1, N-terminal half of the BCV spike protein.

HBm1 through HBm13, BCV mutants 1, 5, 9, and 13, respectively; S, uncleaved form of the BCV spike protein; S1, N-terminal half cleavage product of the BCV spike protein.

FIG. 1. Immunoprecipitations of BCV mar mutants with MAb HB10-4. MDBK cells were infected with mar mutants and radiolabeled with [35S]methionine. Total cell lysates were prepared and subjected to immunoprecipitation with BCV-specific neutralizing MAb HB10-4. Lanes 1, selecting MAb HB10-4; lanes 2, nonselecting MAb BB7-14; lanes 3, bovine viral diarrhea virus-specific MAb 115. wt, wild-type BCV; HBm1 through HBm13, BCV mar mutants 1, 5, 9, and 13, respectively; S, uncleaved form of the BCV spike protein; S1, N-terminal half cleavage product of the BCV spike protein.

FIG. 2. Electropherogram of sequencing gel for the S1 gene of BCV mar mutants. Arrows indicate sequencing directions. wt, wild-type BCV; HBm1 through HBm13, BCV mar mutants 1, 5, 9, and 13, respectively. Arrowheads indicate the changed nucleotides.
positions 972 to 2160 of the S1 gene only, it was conceivable that mutations other than that coding for amino acid residue 528 may have occurred in other regions of the S1 gene and may be responsible or partially responsible for the loss of antibody reactivity. To exclude this possibility, we introduced the same mutation into the full-length S1 gene of wild-type BCV. By site-directed mutagenesis, the C at nucleotide position 1583 was precisely replaced by T to alter the codon of GCC for alanine to GTC for valine at amino acid position 528 to create the mutant A528V S1 gene. Thus, the mutant A528V S1 protein would be identical to the wild-type full-length S1 protein except for the single amino acid at position 528. Both the wild-type S1 gene and the A528V S1 gene were individually expressed in cells with the T7 vaccinia virus expression system, and the cell lysates were subjected to RIPA with MAb HB10-4 or MAb BB7-14 (Fig. 4). As observed, the A528V mutant S1 protein was precipitated by MAb BB7-14 (Fig. 4, lane 4). In contrast, the mutant protein was not recognized by MAb HB10-4 (Fig. 4, lane 3), although the MAb was able to precipitate the wild-type S1 protein (Fig. 4, lane 2). These results demonstrate that the amino acid change of alanine to valine at position 528 was sufficient to confer resistance to the HB10-4 mar mutants.

The amino acid change of alanine to (the larger) valine is not considered a conservative substitution. According to the PAM250 matrix, a mutation probability index, an alanine-to-valine change occurs in closely related proteins at a frequency similar to that observed for alanine to asparagine, aspartic acid, glutamic acid, or glutamine (7). Changes of alanine to the small amino acids glycine, serine, threonine, and proline occur more frequently. Thus, the alanine-to-valine change may have caused a local or a more extensive disruption in the S1 structure, causing it to be no longer recognized by MAb HB10-4.

DISCUSSION

Of the two cleavage products of S, the S2 subunit is highly conserved among coronaviruses. In contrast, the S1 portion generally shows a low level of sequence homology, and in MHV an extensive heterogeneity has been shown to exist. When the amino acid sequence of the BCV S1 protein is compared to those of various strains of MHV, large deletions of 49 and 138 amino acids are identified within the region between positions 456 and 592 in MHV strains A59 and JHM, respectively (23). Similarly, MHV 2 has deletions of 150 amino acids within the same region. In contrast, MHV 4 and the rat coronavirus sialodacryoadenitis virus have only minor deletions of 9 and 12 amino acids, respectively, in this region (26, 43). Thus, the region between positions 456 and 592 in the S protein is considered hypervariable in rodent coronaviruses. The hypervariable region appears to be biologically significant in MHV, and studies have indicated that it acts as a pathogenic determinant. For MHV JHM and MHV 4, two highly neurotropic viruses which produce acute fatal encephalitis in mice,
large deletions and single or multiple point mutations are observed in this region for viral mutants with reduced neurovirulence (15, 26, 38).

The cellular receptor binding region of the MHV S protein occurs distally from the hypervariable region in the first 330 amino acid residues of the protein (22). In contrast, for the enteropathogenic enteritis coronavirus (TGEV) of swine, the cellular receptor binding region occurs in the location on the S protein that corresponds to the hypervariable region of MHV (16) and enteric tropism determinants occur in the N-terminal region (2, 21). Respiratory porcine coronaviruses, which are nonenteropathic variants of TGEV, demonstrate large amino acid deletions in the N-terminal region of the S protein (39).

The S gene of BCV has been sequenced for several cell culture-adapted reference strains and some low-level cell culture-passaged clinical isolates. Although S1 gene sequences appear to be highly conserved among strains and isolates of BCV and sequence deletions or insertions have not been observed, a polymorphic region in the gene is apparent. Low-level cell culture-passaged clinical isolates recovered from diarrheic calves show sequence differences which cluster in the region representing amino acid positions 456 to 592, which corresponds to the MHV hypervariable region. For low-level-passaged respiratory BCV isolates, sequence differences also cluster in this region and in the N-terminal region of the S1 protein in comparison with the locations in enteric BCV strains (5).

For the (polymorphic) region from amino acids 456 to 592, a 6 to 9% variation in amino acid sequence occurs between our reference strain and the enteric and respiratory BCV clinical isolates and a 4 to 6% variation occurs between the enteric and respiratory isolates (5, 25, 27). Amid the clustering of sequence variation in the region, the alanine at position 528 and the surrounding sequence from amino acids 511 to 530 are fully conserved among all BCV strains and isolates sequenced to date, suggesting that functional constraints may exist for this portion of the polymorphic region. The polymorphic region contains 15 conserved cysteine residues, a large number for its size, many of which are likely involved in disulfide linkages and confer a complex structure. Our analysis of the region for respiratory and enteric BCVs reveals that only three amino acid changes occur consistently between the two groups; two of these are conservative substitutions at amino acid positions 510 and 578 (serine and threonine are interchanged). The third change, a nonconservative change between groups, occurs very close to critical residue 528 identified in this study at amino acid position 531, where an aspartic acid or asparagine residue occurs in enteric BCV isolates and a glycine occurs in respiratory BCV isolates.

The BCV S protein binds to sialic acid residues (30), but the cellular receptor protein and viral receptor binding region have not yet been identified. The polymorphic region from amino acids 456 to 592 may be involved in receptor binding, as in TGEV, or may be a pathological determinant like MHV. Sequence differences between respiratory and enteric BCV isolates suggest that tropism determinants may occur in the polymorphic region, perhaps involving residue 531, or in the N-terminal region of the S protein.

It remains to be directly demonstrated if the region of the S protein from amino acids 456 to 592 plays a significant role in BCV pathogenesis in cattle. However, the finding that it harbors a critical amino acid essential for the reactivity with a MAb with demonstrated in vivo neutralizing ability strongly suggests that it has an important biological role in virus-cell interactions. Development of a system which will enable the introduction of specific modifications into the coronavirus genome, such as an infectious cDNA clone, is essential to further study the biological significance in vivo of this and other regions of the BCV S protein.

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