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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Received 29 August 2000/Returned for modification 26 September 2000/Accepted 17 November 2000

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, 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 1 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 (Cancerex, Bethesda, Md.). HeLa cells, maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, were used for vaccinia virus propagation. Vaccinia virus expressing T7 RNA polymerase (vTF7-3) (14) was used for protein expression. Preparation of MAb was described previously (10), and in the current study group A MAB HB10-4 and group B MAB BBT-14 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 (~10^6 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 containing a 1:8000 dilution 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 and were resuspended in 1 ml of medium. The plaque-purified virus was propagated in MDBK cells in the presence of a 1:1000 dilution of MAB HB10-4 for three passages until a cytopathic effect was evident. Tenfold dilutions of the passaged 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 vi- ruses. Subclones of the escape virus mutant were propagated as described above, restet 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 10^5 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 SMr1 [5’-TCTCATCGATGTTTATG-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; 10 mM dithiothreitol; 50 mM Tris-HCl (pH 8.3); 75 mM KCl; and 3 mM MgCl2 in a reaction volume of 20 

Site-directed mutagenesis was carried out on the mar phenotype and after incubation with a 1:100 dilution of MAB HB10-4.

Nucleotide sequence accession number. The sequence reported 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
The S protein (20, 32, 41). Since the S1 proteins of the wild-type BCV, it was apparent that notable deletions of the protein were not involved in changes that resulted in the loss of reactivity with MAb HB10-4. Previously, for some MHV mar mutants, large deletions in the S protein were observed (15, 26).

Identification of substituted amino acid in mar mutants. To identify the change in sequence responsible for the loss of immune reactivity with MAb HB10-4, a portion of the S1 gene of the mar mutants was cloned and sequenced. To minimize misincorporation rates which might occur during the PCR cloning step, a DNA polymerase containing a proofreading activity was used throughout the PCR experiments. Since the domains recognized by MAb HB10-4 were identified within amino acid positions 324 to 720, the nucleotide sequences representing this region were specifically examined in all four mutants. Plaque-purified virus from the original stock of parental wild-type BCV was also cloned and sequenced in parallel with the mar mutants in order to compare the mar sequence directly to the wild-type BCV sequence.

When the S1 sequences of the mar mutants were compared to that of the wild-type BCV, only a single nucleotide substitution was observed, and the same substitution occurred in all four mutant viruses. This change (C to T) occurred at position 1583 of the S1 gene (Fig. 2). The mutation of C to T resulted in the change of the codon for alanine to valine at amino acid position 528 of the S1 protein (Fig. 3A). It is interesting that this mutation occurs within the region of domain II which overlaps the hypervariable region of the MHV S1 protein (Fig. 3A, and B). Furthermore, comparison of the sequence with those of different strains of MHV shows that the mutated codon occurs in a region of sequence that is deleted in MHV JHM and MHV A59. This is consistent with our finding that MAb HB10-4 does not recognize the S proteins of these strains of MHV (unpublished data), although they are classified in the same serogroup with BCV (19).

Experimental introduction of the mutation. Since the sequence of the mar mutants was determined for nucleotide

which were immune precipitated by BB7-14 (Fig. 1, lanes 2), did not show a discernible change in size from that of the wild-type BCV, it was apparent that notable deletions of the protein were not involved in changes that resulted in the loss of reactivity with MAb HB10-4. Previously, for some MHV mar mutants, large deletions in the S protein were observed (15, 26).

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Experimental introduction of the mutation. Since the sequence of the mar mutants was determined for nucleotide
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.

FIG. 3. (A) Structural illustration of the S1 protein of BCV. Arabic numbers indicate amino acid positions. Two antigenic regions that have previously been identified are indicated as domains I and II, respectively. Vertical arrows and underlined boldface characters indicate the substitution. The darkened area at the N terminus depicts a hydrophobic signal sequence. Shaded areas indicate antigenic domains or the hypervariable region. aa, amino acid; wt, wild type. (B) Comparisons of the sequences of the hypervariable regions of various coronaviruses. Dotted lines indicate deletions. BCV, bovine coronavirus (wild-type BCV sequence, GenBank accession number D00662; mar mutant sequence, GenBank accession number AF313395); JHM, mouse hepatitis virus strain JHM (GenBank accession number D00093); A59, mouse hepatitis virus strain A59 (GenBank accession number M18379); SDAV, sialodacryoadenitis rat coronavirus (GenBank accession number AF188193); MHV4, mouse hepatitis virus strain 4 (GenBank accession number S5114).

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,
also cluster in this region and in the N-terminal region of the level-passaged respiratory BCV isolates, sequence differences which corresponds to the MHV hypervariable region. For low-region representing amino acid positions 456 to 592 (27), arrheic calves show sequence differences which cluster in the level cell culture-passaged clinical isolates recovered from diarrheic calves and a polymorphic region in the gene is apparent. Low-BCV and sequence deletions or insertions have not been observed. The polymorphic region from amino acid residues 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 MAab suggests 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 MAab 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.

ACKNOWLEDGMENTS

This study was supported by the Medical Research Council of Canada, Ontario Cattlemen’s Association, and Ontario Ministry of Agriculture Food and Rural Affairs (OMAFRA).

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