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Antigenic Structure of Envelope Glycoprotein E1 of Hog Cholera Virus

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Envelope glycoprotein E1 (gp51 to gp54) is the most antigenic protein of hog cholera virus or classical swine fever virus (CSFV). Four antigenic domains, A to D, have been mapped on E1 with a panel of monoclonal antibodies (MAbs) raised against CSFV strain Brescia. The boundaries of these domains have been established by extensive studies on binding of MAbs to transiently expressed deletion mutants of E1 (P. A. van Rijn, E. J. de Meijer, H. G. P. van Gennip, and R. J. M. Moormann, J. Gen. Virol. 74:2053–2060, 1993). In this study, we used neutralizing MAbs of domains A, B, and C to isolate MAb-resistant mutants (MAR mutants) of CSFV strain Brescia and Chinese vaccine strain ("C"). The E1 genes of MAR mutants were cloned in a eukaryotic expression vector, and the effects of MAR mutations on epitopes were studied with a panel of 19 MAbs by immunostaining of COS1 cells transiently expressing these mutant E1s. Except for the MAR mutation Cys→Arg at position 792, which abolished binding of all MAbs of domains A and D, amino acid substitutions affected only MAbs belonging to the same domain as the MAb used to select the MAR mutant. However, a MAR mutation in a particular domain did not per se abolish binding of all MAbs recognizing that domain. Furthermore, MAR mutants possessed conservative as well as nonconservative amino acid substitutions. To investigate the significance of a secondary structure for the binding of MAbs, all cysteine residues in the N-terminal antigenic part of E1 were mutated to serine. We found that the cysteines at positions 693 and 737 were essential for binding by MAbs of domains B and C, whereas those at positions 792, 818, 828, and 856 appeared to be essential for the binding of most MAbs of domains A and D. These results fully comply with the previously proposed two-unit structure of the N-terminal half of E1. One unit consists of antigenic domains B and C, whereas the other unit consists of the highly conserved domain A and domain D. We conclude that the first six cysteines are critical for the correct folding of E1. A model of the antigenic structure of E1 is presented and discussed.

Hog cholera virus or classical swine fever virus (CSFV), bovine viral diarrhea virus (BVDV), and border disease virus belong to the genus *Pestivirus* of the *Flaviviridae* family (6). Hog cholera is a disease of pigs, bovine viral diarrhea is a disease of cattle, and border disease is a disease of sheep. Pestiviruses are structurally and antigenically closely related; infection with one virus induces, to various extents, antibodies that cross-react with other members of the *Pestivirus* genus (13). Nevertheless, most monoclonal antibodies (MAbs) directed against structural proteins discriminate between CSFV and BVDV strains (5, 17, 28, 29, 34).

The genome of pestiviruses is a positive-stranded RNA molecule of about 12.5 kb (14, 19). The genomes of five pestivirus strains have been cloned and sequenced up to now: three BVDV strains, Osloss (18), NADL (3), and SD-1 (4); two CSFV strains, Alfort (11) and Brescia (16). A single large open reading frame flanked by two small noncoding regions has been found in the genomes. The open reading frame is translated into a hypothetical polyprotein of approximately 4,000 amino acids. Mature proteins are probably released by virus- and cell-encoded proteases. The complete genomic organization of BVDV has been determined (35, 36, and references therein). Of CSFV, only the genes encoding the structural proteins capsid C (p14) and envelope glycoproteins E1 (gp51 to gp54), E2 (gp44 to gp48), and E3 (gp31) have been mapped (15, 20, 23, 24).

MAbs directed against structural envelope glycoproteins E1 and E2 of CSFV strains Brescia and Alfort have been isolated (28, 29, 31, 33). Although MAbs directed against E2 can contribute to neutralization of the virus (28, 31), the immunogenic response against E1 alone is sufficient for protection. Vaccination of pigs with a recombinant pseudorabies virus which expresses E1 of CSFV strain Brescia or with immunoaffinity-purified E1, expressed in insect cells, protects swine against hog cholera (9, 27).

Four antigenic domains, A to D, have been identified on E1 of CSFV strain Brescia with a panel of 13 MAbs (30). On the basis of neutralization and conservation, domain A is divided in subdomains A1, A2, and A3 (34). Subdomains A1 and A2 are conserved in more than 90 CSFV strains, but only MAbs of subdomain A1 are neutralizing. None of the epitopes of subdomain A3 and domains B, C, and D are conserved, and MAbs against only B and C neutralize virus (Fig. 1) (30). Recently, epitopes were mapped with transiently expressed deletion mutants of E1 of CSFV strain Brescia (see Fig. 4) (25). Furthermore, four conserved and neutralizing MAbs directed against E1 of the Chinese vaccine strain (strain "C") (1) were allocated to subdomain A1. Two nonconserved MAbs directed against E1 of strain "C" have been classified as domain B/C-like MAbs by the mapping of their epitopes on chimeric E1 proteins of strains Brescia and "C" (26).

Variants of BVDV which escape neutralizing MAbs have been isolated (17). Mutations were found in the N-terminal half of glycoprotein gp53, the homolog of E1 of CSFV, in

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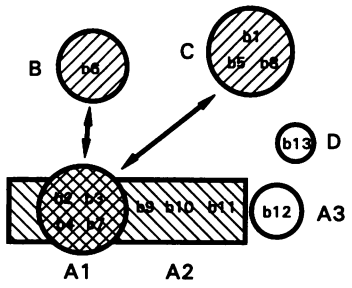

FIG. 1. Representation of four antigenic domains (A to D) on E1. Antigenic domains are defined by 13 MAbs (b1 to b13) directed against envelope protein E1 of CSFV strain Brescia (30). ↔, synergism; ⊘, conserved; ⊘, neutralizing; ⊗, conserved and neutralizing; ○, nonconserved and nonneutralizing.

agreement with the established topographical location of epitopes on the latter protein.

In this study, we used eight neutralizing MAbs directed against subdomain A1 and domains B and C of E1 of CSFV strain Brescia to isolate escape variants or MAb-resistant (MAR) mutants of strains Brescia and "C." The effects of amino acid substitutions on binding of MAbs were determined by immunostaining cells transiently expressing E1 derived from these MAR mutants. Furthermore, we studied the involvement of disulfide bonds in the structure of epitopes by substitution of serines for cysteines in the N-terminal half of E1.

MATERIALS AND METHODS

Isolation of MAR mutants of CSFV strains Brescia and "C." MAR mutants of strains Brescia and "C" were selected essentially as described previously (31). Dilutions of CSFV strains Brescia or "C" in growth medium were mixed with a neutralizing MAb and incubated for 1 h at 37°C. Suspensions of PK15 cells were infected with virus-MAb mixtures and grown on microtiter plates for 4 days. Supernatants of cell cultures were collected and stored at −70°C, and the monolayers were immunostained with a horseradish peroxidase conjugate of the MAb used for selection. In order to establish the presence of MAR mutants, a second immunostaining was carried out with polyclonal anti-CSFV serum conjugated with horseradish peroxidase. MAR mutants were isolated from culture supernatants of monolayers exhibiting more cells immunostained with the polyclonal antiserum than with the MAb used for selection. Occasionally, the selection with neutralizing MAb was repeated to enrich for a particular MAR mutant. Mutant virus was isolated from culture supernatant after two passages in PK15 cells without selection. Virus stocks were titrated, and, after 4 days, infected monolayers were immunostained both with anti-CSFV serum and the MAb used for selection. In this way, virus titers of each isolate were obtained on the basis of reactivity with the MAb used for selection and with anti-CSFV serum. Virus stocks that had a 10,000-fold-higher titer with the anti-CSFV serum were considered to contain a MAR mutant. Double mutants were isolated by the same procedure followed by a selection with a second neutralizing MAb.

Cloning and sequence analysis of E1 genes of MAR mutants. SK6 cells were infected with MAR mutants and incubated for 24 h at 37°C. Hereafter, total cellular RNA was isolated essentially as described previously (2). To amplify sequences encoding the antigenic part of E1, RNA was transcribed to cDNA by reverse transcriptase and amplified (PCR) by standard methods (39 cycles; 94°C, 1 min; 55°C, 1 min; 72°C, 1 min) with primers A (5′-AGATTGGATCCTAAAGTATTAA GAGGACAGGT-3′) and B (5′-AGATTGGATCCGAAT TCTGCGAAGTAATCTGa/tGTGG-3′). The amplified DNA fragments were used to replace similar fragments of expression vector pPRb9, which contains the E1 gene of strain Brescia, or pPRc34, which contains the E1 gene of strain "C" (26). To this end, amplified DNA fragments of MAR mutants of strains Brescia were digested by *Nhe*I and *Bgl*II and ligated into pPRb9. MAR mutants of strain "C" were digested by *Nhe*I and *Afl*II and ligated into pPRc34 (Fig. 2A). The nucleotide sequences of cloned fragments were determined by double-stranded DNA sequencing (T7 kit; Pharmacia LKB).

Site-directed mutagenesis of Cys codons. Plasmids pPRb9, pPEh13, and pPEh10 have been described previously (25) (Fig. 2B). In pPEh13, a region directly downstream of the *Nhe*I site has been deleted. In pPEh10, a region directly downstream of the *Bgl*II site has been deleted, whereby the *Bgl*II site was not restored. PCR was used to mutate codons of cysteine to codons of serine. The Cys-693 codon, in the vicinity of the *Nhe*I site, was mutated by amplification of the *Nhe*I-*Bgl*II fragment of pPRb9 with mutation primer P-Cys-693 overlapping both the Cys-693 codon and the *Nhe*I site (Table 1). The *Nhe*I-*Bgl*II fragment, including the Cys→Ser mutation, was cloned in pPRb9.

Site-directed mutagenesis of five other Cys codons within the *Nhe*I-*Bgl*II fragment was carried out by two rounds of amplification essentially as described previously (12) (Fig. 2B). The first round of amplification consisted of two simultaneously executed PCRs. In one PCR, the *Nhe*I-*Bgl*II fragment of pPEh10 was amplified with primers A and B. The other PCR was carried out with pPEh13 as template DNA, primer B, and one of the mutation primers which are listed in Table 1. PCRs were carried out under standard conditions for 35 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 1 min). Amplified fragments were purified by agarose gel electrophoresis and freeze-queeze. One percent of each purified fragment (one fragment with the deletion of pPEh10 and one fragment with the desired Cys→Ser mutation) was mixed and subjected to a second PCR. The second amplification was carried out under the same conditions with primer A and primer C (5′-CACTTACCTA Ta/gGGGTAGTGTGG-3′), which is complementary to a region deleted in pPEh10 (Fig. 2B). In order to clone mutated Cys codons, amplified fragments were digested with *Nhe*I and *Bgl*II, separated by agarose gel electrophoresis, purified by freeze-queeze, and ligated into pPRb9 as described for MAR mutants. The nucleotide sequences of the amplified fragments, including introduced mutations, were checked by double-stranded sequencing of entire *Nhe*I-*Bgl*II fragments. All cloning procedures were carried out essentially as described previously (21). Restriction enzymes and DNA-modifying enzymes were purchased commercially and used as described by the suppliers. Plasmids were transformed and maintained in *Escherichia coli* DH5α (8).

Transfection and immunoperoxidase monolayer assay. Transfection of COS1 cells (ATCC) was carried out as described (25). COS1 cells were grown in Dulbecco's modified Eagle's medium with 5% fetal bovine serum. Subconfluent monolayers in 2-cm^2 wells were washed two times with phosphate-buffered saline (PBS; 8% NaCl, 1.15% Na_2HPO_4, 0.2% KH_2PO_4, 0.2% KCl). Cells were transfected by adding 0.5 ml of medium (without fetal bovine serum) followed by the transfection mixture. Transfection mixtures consisted of 0.2 μg of plasmid DNA in 50 μl of a 1:1 mixture of DNA solution and 12.5-times-diluted Lipofectin (GIBCO BRL) in water. Plasmid DNA was isolated from overnight cultures and purified by

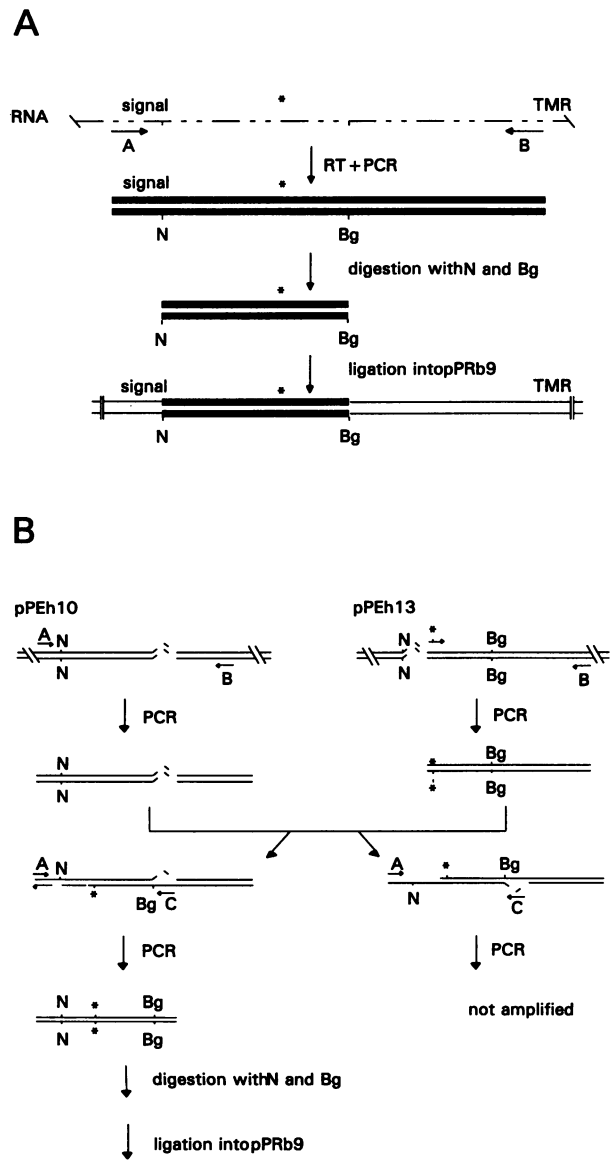

FIG. 2. Schematic representation of the construction of expression plasmids containing mutant E1 genes. (A) Construction of E1 expression plasmids carrying MAR mutations. The broken line symbolizes viral RNA of MAR mutants. Cloning sites are indicated by N (*Nhe*I) and Bg (*Bgl*II). The MAR mutations are indicated by asterisks. RT, reverse transcription. Arrows A and B represent primers used to amplify E1 genes of MAR mutants. (B) Site-directed mutagenesis of Cys codons by two rounds of PCR. Relevant cloning sites are indicated by N (*Nhe*I) and Bg (*Bgl*II). The mutated Cys codons symbolized by asterisks. Deletions in pPEh10 and pPEh13 are indicated by broken lines.

phenol extraction. Medium above the monolayer was gently mixed constantly during the addition of the transfection mixture. After incubation at 37°C overnight, 0.5 ml of medium containing 20% fetal bovine serum was added. Thereafter, incubation at 37°C was continued for another 24 h. Then, transfected monolayers were washed three times with PBS, dried for 45 min at 37°C, and frozen at −20°C for 45 min. Frozen cells were fixed for 5 min with 4% paraformaldehyde in PBS and washed three times with 0.15% NaCl. The immunoperoxidase monolayer assay (IPMA) was performed as described previously (33). When no immunostaining was seen, transfection of plasmid DNA was verified by staining with MAbs directed against other epitopes.

RESULTS

Isolation and cloning of E1 genes of MAR mutants. Eight neutralizing MAbs raised against strain Brescia were used to select mutants of strains Brescia and "C." As expected, most monolayers infected with MAR viruses were negative in immunostaining with the selecting MAb (31). However, after several rounds of selection, some monolayers were still immunostained by the selecting MAb, indicating that some wild-type virus is still present or that although neutralization of the MAR mutant by that particular MAb was abolished, binding was not. Revertants in which some but not all epitopes were restored were also found (data not shown). This suggested that the MAR mutation was not reverted to the original sequence but that other mutations were introduced. This instability of MAR mutants complicates the interpretation of amino acid differences observed in relation to binding by MAbs.

To overcome these complications, we cloned E1 genes of MAR mutants in a eukaryotic expression vector and studied the binding of MAbs to transiently expressed E1 in COS cells. With this approach, a change in MAb binding is directly correlated to amino acid substitutions in E1. We have shown that all epitopes of MAbs directed against E1 of strains Brescia and "C" are located on the N-terminal half of E1, which corresponds to an *Nhe*I-*Bgl*II fragment (25). Therefore, this fragment of wild-type E1 of strain Brescia was replaced by corresponding fragments of MAR mutants of Brescia (Fig. 2A). In a similar way, E1 genes of MAR mutants of strain "C" were studied. Because the E1 gene of strain "C" does not contain the *Bgl*II site, the *Afl*II site further downstream of the antigenic part of E1 was used to replace the *Nhe*I-*Afl*II fragment with corresponding fragments of MAR mutants of strain "C" (see Materials and Methods).

Single amino acid substitutions in MAR mutants of strain Brescia. Amino acid mutations of and IPMA results for MAR mutants of strains Brescia and "C" are summarized in Table 2. In most cases, IPMA results were conclusively negative or positive (Fig. 3A or C, respectively). However, in some cases, staining of mutant E1 transiently expressed in COS cells was very weak and only a few cells were stained (Fig. 3B). The staining of these MAbs is indicated by ± in Table 2. However, this weak staining could be reproduced and was not caused by a low frequency of transfection, as demonstrated by a second immunostaining of the same transfected monolayer with other MAbs (compare Fig. 3B and C). This clearly indicates that mutated amino acid residues in the expressed E1 protein are involved in the binding of MAbs studied.

Domain B. E1 genes of two MAR mutants of strain Brescia selected by MAb b6 were cloned and sequenced (pPK04 and pPK12). The cloned fragments carry a single amino acid mutation at position 710; His-710 is changed to Gln in pPK04 and to Leu in pPK12. The binding of MAbs to transiently expressed mutant E1 showed that binding of MAb b6 is abolished, whereas binding of other MAbs directed against E1 of strain Brescia was unchanged. Remarkably, MAb c6, which does not bind to wild-type E1 of strain Brescia (pPRb9), showed a weak positive staining with plasmid pPK12 (His→Leu) but not with plasmid pPK04 (His→Gln). So, position 710 is involved in binding of both MAb b6 and MAb c6; but for binding of MAb b6, a histidine at this position is

TABLE 1. Primers used to mutate Cys codons into Ser codons by site-directed mutagenesis[a]

Primer	Primer sequence
P-Ser-693	5′-CA.CAA.GGC.CGG.CTA.GCC.AGC.AAG.G-3′
P-Ser-737	5′-AAG.GCC.ATC.AGC.ATG.GCA-3′
P-Ser-792	5′-TTC.GGA.CTG.AGT.CCG.TAT-3′
P-Ser-818	5′-TAC.CTA.GTT.AGC.CCA.ATA-3′
P-Ser-828	5′-GTT.ATA.GAG.AGC.ACG.GCA-3′
P-Ser-856	5′-AGA.AGG.GAT.AGT.GTG.ACC-3′
P-Arg-828	5′-ACG.GGT.GTT.ATC.GAT.CGC.ACG.GCA.GTG.AGC-3′
P-Arg-856	5′-CCG.TAC.AGA.AGC.GAT.CGT.GTG.ACC.ACT.ACA-3′

[a] The names of primers indicate the amino acid residues and positions of the substituted cysteines in the open reading frame of CSFV strain Brescia. *Nhe*I and *Pvu*I sites are underlined once, and mutations with respect to the nucleotide sequence of CSFV strain Brescia are underlined twice. The open reading frame of CSFV strain Brescia is indicated.

essential, whereas a leucine at position 710 promotes binding of MAb c6. On the other hand, the reactivity of MAb c6 with E1 of pPK12 was weaker than that with E1 of strain "C" (pPRc34), indicating that other differences in amino acids specific for E1 of strain "C" are also involved in binding of MAb c6.

Domain C. MAR mutants, pPK17, pPK18, pPK19, pPK24, and pPK11, of strain Brescia were selected with MAb b5 and analyzed. One mutant carries a His→Tyr substitution at position 724, three mutants carry a substitution of Asn at 725, and one mutant contains a Met→Ile substitution at position 738. However, the last substitution only partially reduced the immunostaining by MAb b5. We concluded that amino acid residues His-724, Asn-725, and Met-738 are part of the epitope of MAb b5. The mutants were all recognized by the other MAbs, among them other domain C-specific MAbs, indicating

TABLE 2. Results of IPMAs of COS1 monolayers transfected with E1 expression plasmids carrying MAR mutations

Plasmid	Position no.[a]	Mutation	IPMA result[b]																		
			Strain Brescia													Strain "C"					
			B	C			A1				A2			A3	D	B/C		A1			
			b6	b5	b1	b8	b2	b3	b4	b7	b9	b10	b11	b12	b13	c2	c6	c1	c4	c8	c11
pPRb9		None	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+
pPK04	710	His→Gln	−*	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+
pPK12	710	His→Leu	−*	+	+	+	+	+	+	+	+	+	+	+	+	−	±	+	+	+	+
pPK17	724	His→Tyr	+	−*	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+
pPK18	725	Asn→Asp	+	−*	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+
pPK19	725	Asn→Tyr	+	−*	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+
pPK24	725	Asn→Asp	+	−*	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+
pPK07	729	Asp→Gly	+	+	−*	−	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+
pPK08	729	Asp→Gly	+	+	−*	−	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+
pPK28	729	Asp→Gly	+	+	−*	−	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+
pPK15	734	Lys→Glu	+	±	−	−*	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+
pPK11	738	Met→Ile	+	±*	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+
pPK05	833	Pro→Leu	+	+	+	+	−	−	−*	+	−	−	−	+	+	−	−	−	−	+	+
pPK01	834	Thr→Ile	+	+	+	+	−	−	−	−*	−	−	−	+	+	−	−	−	−	−	−
pPK06	837	Arg→Thr	+	+	+	+	+*	+	+	+	+	+	+	+	+	−	−	+	±	+	+
pPK13	823	Thr→Met	+	+	+	+	−	−*	−	+	−	−	−	+	+	−	−	−	−	+	+
	833	Pro→Leu																			
pPK09	792	Cys→Arg	+	+	+	+	−	−*	−	−	−	−	−	−	−	−	−	−	−	−	−
	718	Thr→Ala																			
pPK23	792	Cys→Arg	+	+	+	+	−	−	−*	−	−	−	−	−	−	−	−	−	−	−	−
	718	Thr→Ala																			
pPK29	710	His→Leu	−*	±*	+	+	+	+	+	+	+	+	+	+	+	−	±	+	+	+	+
	738	Met→Thr																			
pPK26.1	710	His→Gln																			
	725	Asn→Asp	−*	−*	+	+	−	−	−*	+	−	−	−	+	+	−	−	−	−	+	+
	833	Pro→Leu																			
pPK26.2	725	Asn→Asp																			
	833	Pro→Leu	+*	−*	+	+	−	−	−*	−	−	−	−	−	−	−	−	−	−	−	−
	858	Thr→Asn																			
pPRc34		None	−	−	−	−	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+
pPK20	833	Pro→Gln	−	−	−	−	−	−*	−	−	−	−	+	−	+	+	+	+	−	+	+
pPK27	833	Pro→Gln	−	−	−	−	−	−*	−	−	−	−	+	−	+	+	+	+	−	+	+

[a] Position of the mutation with respect to the amino acid sequence of CSFV strain Brescia.

[b] Domains on E1 were defined as shown in Fig. 1. MAbs directed against CSFV strains Brescia and "C" are indicated by b and c, respectively. Plasmids pPRb9 and pPRc34 contain wild-type E1 genes of strains Brescia and "C," respectively. Asterisks indicate the MAbs used to select the MAR mutants. Immunostaining was interpreted as negative (−) (Fig. 3A), weak (±) (Fig. 3B), or positive (+) (Fig. 3C).

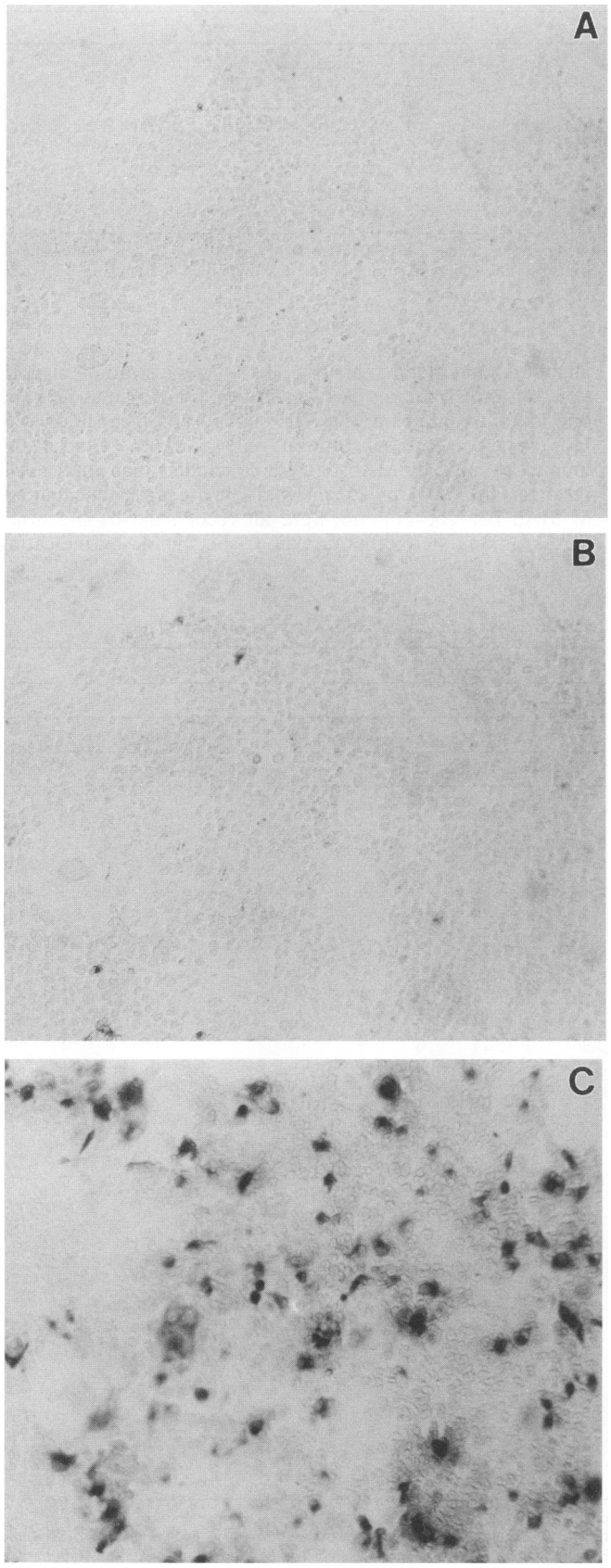

FIG. 3. Successive IPMA results of a transfected COS1 monolayer. The illustrated monolayer was transfected with plasmid pPK12 and immunostained successively with MAb b6 (A), MAb c6 (B), and MAb b4 (C). Immunostaining was interpreted as negative (A), weak (B), or positive (C).

that amino acid residues at positions 724, 725, and 738 are not essential for binding of these MAbs.

Three independent MAR mutants, pPK07, pPK08, and pPK28, each of which carries an Asp→Gly substitution at position 729, were selected with MAb b1. This amino acid mutation also abolished binding of MAb b8. One MAR mutant, pPK15, with a Lys→Glu substitution at position 734 was selected with MAb b8. This amino acid substitution affected binding of all three MAbs, b1, b5, and b8, of domain C, although the staining by MAb b5 was not completely negative. We concluded that amino acid residues at positions 729 and 734 are part of the epitope of MAbs b1 and b8. The latter amino acid residue is also part of the epitope of MAb b5, a finding which confirms the binding of these MAbs to the same antigenic region of E1 (30).

Domain A. Three strain Brescia mutants, pPK01, pPK05, and pPK06, with single amino acid substitutions were selected with neutralizing MAbs b7, b4, and b2, respectively, which are directed against conserved subdomain A1. The amino acid substitutions are located at positions 833 (Pro→Leu), 834 (Thr→Ile), and 837 (Arg→Thr), respectively. Transiently expressed E1 of plasmid pPK05 was not recognized by most of the MAbs of domain A, indicating the importance of Pro-833 for this domain. This importance was also demonstrated by the binding of the four conserved, domain A1-specific MAbs raised against strain "C," c1, c4, c8, and c11. Two of these MAbs, c1 and c4, do not bind to mutant E1 of pPK05, whereas MAbs c8 and c11 still do. Thr-834 seems to be even more important for domain A, since Ile at this position abolished binding of all MAbs of conserved subdomains A1 and A2. One MAR mutant, pPK06, was selected by MAb b2, whereas transiently expressed E1 was still bound by this MAb. This mutant carries an Arg→Thr substitution at position 837. The significance of position 837 for antigenic domain A, however, was demonstrated by the weaker immunostaining by MAb c4 of cells transfected with pPK06. We concluded that amino acid residues at positions 833 and 834 are very important for highly conserved epitopes of domain A.

Double and triple amino acid substitutions in MAR mutants of strain Brescia. We selected several MAR mutants with two or three amino acid substitutions (Table 2). Three of these mutants, pPK09, pPK13, and pPK23, which were selected by only one MAb, carry two amino acid substitutions. Comparison of the reactivity of the double mutant pPK13 and the single mutant pPK05 suggests that only the amino acid mutation Thr→Leu at position 833, and not the amino acid mutation Thr→Met at position 823, affected the binding for MAbs specific for domain A. Another possibility, however, is that negative or positive effects of the substitution at position 823 could be masked by the substitution at position 833. Two MAR mutants, pPK09 and pPK23, which were isolated by MAbs b3 and b4, respectively, carry two identical amino acid mutations. One substitution, Cys→Arg at position 792, is located within domains A and D (25). The other amino acid substitution, Thr→Ala at position 718, is located within the map of domains B and C. The latter mutation did not affect immunostaining of E1 with MAbs of these domains (b1, b5, b6, and b8). Consequently, the Cys→Arg substitution at position 792 has to be the

TABLE 3. Results of IPMAs of COS1 monolayers transfected with E1 expression plasmids carrying mutated Cys codons[a]

Plasmid	Position no.[b]	Mutation	IPMA result[b]															
			Strain Brescia												Strain "C"			
			B	C			A1				A2			A3	D	A1			
			b6	b5	b1	b8	b2	b3	b4	b7	b9	b10	b11	b12	b13	c1	c4	c8	c11
pPK30	693	Cys→Ser	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
pPK31	737	Cys→Ser	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
	696	Asp→Gly																	
pPK32	792	Cys→Ser	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
pPK33	818	Cys→Ser	+	+	+	+	±	+	+	−	−	−	−	−	−	−	−	+	+
pPK34	828	Cys→Ser	+	+	+	+	−	±	+	−	−	−	−	−	−	−	−	−	−
pPK35	856	Cys→Ser	+	+	+	+	−	±	−	−	−	−	−	−	−	−	−	±	−
pPK36	828	Cys→Arg	+	+	+	+	−	±	−	−	−	−	−	−	−	−	−	−	−
	827	Glu→Asp																	
pPK37	856	Cys→Arg	+	+	+	+	−	±	−	−	−	−	−	−	−	−	−	±	±
	854	Arg→Ser																	

[a] Position number at which the mutation occurred in CSVF strain Brescia.

[b] Domains on E1 were defined as shown in Fig. 1. MAbs directed against CSFV strains Brescia and "C" are indicated by b and c, respectively. Immunostaining was interpreted as negative (−) (Fig. 3A), weak (±) (Fig. 3B), or positive (+) (Fig. 3C).

cause of the negative immunostaining with all MAbs of domains A and D. This confirms our suggestion that Cys-792 is essential for the conformation of domains A and D (25).

One double mutant, pPK29, isolated after consecutive selection with MAbs b5 and b6, carries the substitutions His→Leu at position 710 and Asn→Asp at position 725. These substitutions are identical to those found in single mutants selected with these MAbs (compare pPK04 and pPK18 with pPK29). Apparently, the effects of double mutants in different antigenic domains are identical to those of single mutants.

MAR mutants with three amino acid substitutions were selected with MAb b4 after a first selection with a mixture of MAbs b5 and b6. From the virus stock, we obtained two different clones, designated pPK.1 and pPK.2. Mutant E1 of pPK.1 contains three amino acid substitutions, His→Gln at position 710, Asn→Asp at position 725, and Pro→Leu at position 833, which were also found in single mutants (pPK04, pPK05, and pPK18, respectively). Again, the effects of the three single amino acid substitutions are identical to those of the triple mutant. Mutant pPK.2 carries, besides two amino acid substitutions which were found earlier, a Thr→Asn substitution at position 858. This substitution is located within the map of domain A. Keeping in mind the effect of the single substitutions, amino acid positions 725 (pPK18) and 833 (pPK05), on binding of MAbs, we presume that the substitution Thr→Asn at position 858 causes a negative immunostaining with MAbs b7, b12, b13, c8, and c11.

MAR mutants of strain "C." Two E1 genes of MAR mutants of strain "C," pPK20 and pPK27, were analyzed. Both MAR mutants were selected by MAb b3 and carry the same nucleotide mutation, resulting in the amino acid substitution Pro→Gln at position 833. This amino acid substitution abolished binding of all MAbs of domain A, except b11, c1, c8, and c11. Interestingly, reciprocal binding patterns were observed for MAbs b7, b11, and c1 to E1 of pPK20 or pPK27 compared with those to E1 of pPK05. E1 of pPK05, which originates from strain Brescia, carries an amino acid substitution, Pro→Leu, different from that of mutant E1 of pPK20 and pPK27 at the identical position (position 833). However, because of other amino acid differences between the E1 sequence of strains Brescia and "C" in the conserved part of domain A, a direct correlation between the difference in the mutation at position 833 and the differences in binding of MAbs b7, b11, and c1 cannot be made.

Cys→Ser substitutions in the N-terminal half of E1. Nine cysteines in the C-terminal half of E1 can be deleted without loss of binding of MAbs (25). Thus, these cysteines are not involved in the formation of epitopes located in the N-terminal half of E1 containing six cysteines. Our data demonstrated the crucial role of Cys-792 for detection of transiently expressed E1 by MAbs of domains A and D (Table 2). In order to study the role of the other cysteines in the N-terminal half of E1 on the binding of MAbs, we changed the Cys codons in this region to Ser codons by site-directed mutagenesis. The effects of these Cys→Ser mutations on the binding of MAbs are shown in Table 3.

Cys→Ser substitutions at positions 693 and 737 (pPK30 and pPK31, respectively) resulted in negative IPMA results with MAbs of domains B and C, whereas binding of MAbs of domains A and D was not abolished. Obviously, recognition of epitopes of domains B and C depends on Cys-693 and Cys-737, which form, most likely, a disulfide bond essential for these domains (Fig. 4). Cys→Ser substitutions at positions 792 (pPK32), 818 (pPK33), 828 (pPK34), and 856 (pPK35) affected

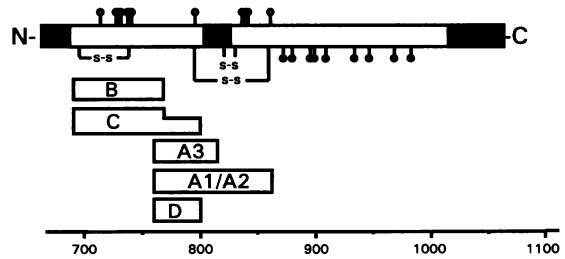

FIG. 4. Schematic representation of CSFV envelope glycoprotein E1. Proposed signal peptide transmembrane region and internal hydrophobic region are shown by closed bars. Positions of MAR mutations and cysteines are indicated by open and closed circles, respectively. Proposed disulfide bonds in the N-terminal half of E1 are shown. The locations of the antigenic domains are indicated by open bars in the lower part. Positions with respect to the amino acid sequence of CSFV strain Brescia are shown at the bottom.

binding of MAbs of domains A and D but not of domains B and C. This indicates that these four cysteines are important for the conformation of domains A and D. We further observed that Cys→Ser substitutions both at positions 792 and 856 and at positions 818 and 828 showed similar effects on the binding of MAbs. The similar reactivities of both pairs of cysteines suggest that disulfide bonds are formed by these pairs of cysteines, one pair consisting of Cys-792 and Cys-856 and the other pair consisting of Cys-818 and Cys-828 (Fig. 4).

On the basis of a comparison of the binding of MAb b4 to E1 proteins of pPK34 and pPK36, it can be concluded that Asp at position 827 or Arg at position 828 or both residues together abolished the binding of MAb b4. Binding of MAbs c8 and c11 did not depend on the presence of cysteine at positions 818 (pPK33), but binding was abolished when Cys-828 was mutated (pPK34 and pPK36). This suggests that a disulfide bond between these cysteines is not essential for binding but that Cys-828 is important for binding of MAbs c8 and c11. A Cys→Ser substitution at position 856 (pPK35) abolished binding of MAb c11 completely, whereas substitutions Arg-854 to Ser and Cys-856 to Arg only reduced binding of MAb c11 (pPK37). Apparently, MAb c11 also binds to position 856, which confirms the proposed complex structure of E1.

DISCUSSION

E1 of CSFV has been found as disulfide-linked homodimers and disulfide-linked heterodimers with glycoprotein E3 (24, 32). Likely, cysteines in the C-terminal half of E1, which contain nine cysteines at positions conserved in pestiviruses, form these intermolecular disulfide bonds. We previously showed that this C-terminal half can be deleted without effect on the binding of MAbs (25). Here we have shown that cysteines located in the N-terminal half of E1 are essential for all identified epitopes. We propose that this antigenic half forms two independent structural units, one consisting of domains B and C and one consisting of highly conserved domain A and domain D (Fig. 5). The structural unit consisting of domains B and C is anchored by a putative disulfide bond between the two most N terminally located cysteines, Cys-693 and Cys-737. The other unit, consisting of domains A and D, is formed by two putative disulfide bonds, one between Cys-792 and Cys-856 and one between Cys-818 and Cys-828. The latter disulfide bond seems to be less important for the structure of domain A. Both structural units contain epitopes of neutralizing MAbs. In order to study the antigenic structure of E1 in more detail, eight neutralizing MAbs directed against this protein were used to select and analyze MAR mutants of CSFV strains Brescia and "C."

The number and nature of amino acid substitutions observed in MAR mutants seem to be limited. On one hand, the same amino acid positions had different amino acid residue mutations after selection with the same MAb, e.g., positions 710, 725, and 833. On the other hand, MAR mutants with the same amino acid substitutions were selected with different MAbs (pPK09 and pPK23). Escape variants of gp53 of BVDV also showed a limited number of amino acid positions that were mutated (17). The amino acid mutations at positions 732, 827, and 839 of gp53 are comparable to the mutations that we found at positions 729, 823, and 834 of E1 of strain Brescia. The limited variability of MAR mutations could be the result of the selection procedure. For instance, the best-growing MAR mutant will be isolated after several passages. Of course, the diversity of mutants in the virus stock is also limited by the codon usage and because substitutions of amino acid residues affecting viability of the virus will never be found.

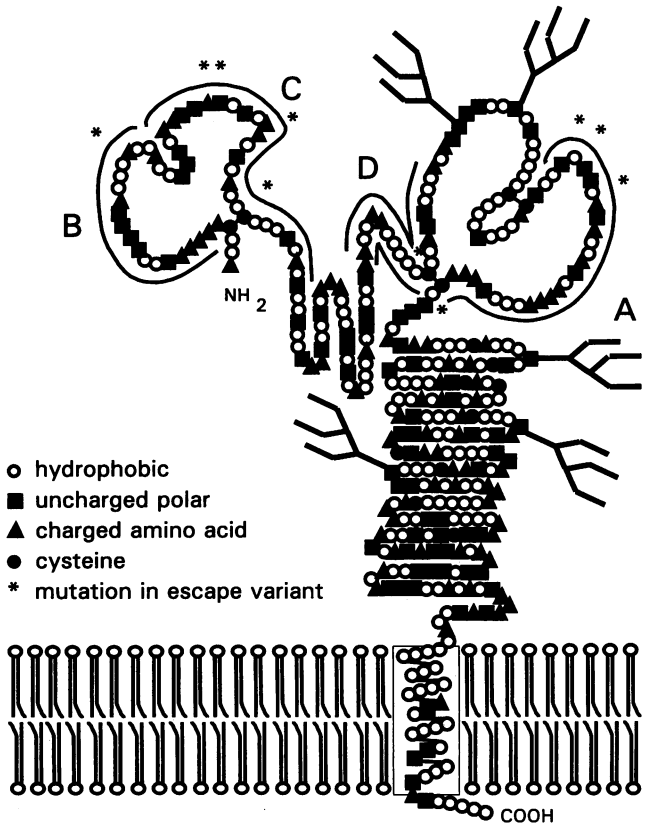

FIG. 5. Proposed antigenic structure of CSFV envelope glycoprotein E1. Amino acids were grouped as hydrophobic (A, G, M, I, L, V, F, W, P), uncharged polar (N, Q, S, T, Y), charged (D, E, K, R, H), and cysteine (C). MAR mutations (*), proposed disulfide bonds in the N-terminal half of E1, antigenic domains, and glycosylation groups are indicated.

As expected, most amino acid substitutions resulted in a negative immunostaining by the MAb used for selection. Exceptions were Met→Ile at position 738 (pPK11) and Arg→Thr at position 837 (pPK06). The Met→Thr substitution at position 738 resulted in reduced binding with MAb b5, which indicated that Met-738 is important but not essential for this epitope of domain C. The amino acid substitution at position 837 seemed to reduce neutralization of the mutant virus by MAb b2, but immunostaining of transiently expressed mutant E1 was not affected. Moreover, the reduced binding of MAb c4 showed that amino acid residue 837 is also relevant for binding of other MAbs of subdomain A1.

The Thr→Asn substitution at position 858 creates a potential N-linked glycosylation site (Asn-N-Thr/Ser) in pPK.2. Experiments are in progress to investigate whether glycosylation of Asn or Asn itself at position 858 affects the binding of MAbs b7, b12, b13, c8, and c11. Three MAR mutants, pPK09, pPK23, and pPK13, carry more than one amino acid substitution. The substitutions Thr→Ala at position 718 (pPK09 or pPK29) and Thr→Met at position 823 (pPK13) seem not to affect the detection of transiently expressed E1. It is unknown whether the mutations at positions 718 and 823 are PCR errors or silent mutations or whether they are an additional result of selection for growth in the presence of the neutralizing MAb. It is remarkable, however, that amino acid position 823 in E1, which is comparable to position 827 in gp53, is also mutated in escape variants of BVDV with two substitutions (17).

Nonconserved epitopes on E1 of strain "C" have been mapped as domain B/C-like epitopes with chimeric E1 proteins of strains Brescia and "C" (26). Here we show that the amino acid residue at position 710 is crucial for binding by both MAbs c6 and b6, which restricts the epitope of MAb c6 to domain B of strain "C." Since MAb c2 competes for binding with MAb c6 (31), we conclude that the epitope of MAb c2 is also domain B like. The affinity of MAb c6 for E1 of pPRc34 is higher than that for E1 of pPK12. As both E1 proteins carry Leu at position 710, we suppose that this is due to amino acid residues 705 and 713, which are the only amino acid differences between domains B of strains Brescia and "C" (25). Binding of MAb c2 to E1 of pPK12 is negative, indicating that the specificity of MAb c2 for E1 of strain "C" is determined by amino acid residues at positions 705 and 713.

Conserved epitopes of neutralizing MAbs c1, c4, c8, and c11 have been mapped in subdomain A1 with deletion mutants of E1 of strain Brescia (25). In line with this, MAR mutations in this domain of E1 of Brescia also affected binding of these "C" strain MAbs. Nevertheless, binding of most of these "C" strain MAbs showed reactivities different from those of Brescia MAbs. For instance, binding properties of MAbs c1 and c4 to E1 mutants of strain Brescia were identical to those of MAbs b2, b3, and b4, except for binding of MAb c1 to mutant E1 proteins of strain "C." Likewise, binding profiles of MAbs c8, c11, and b7 to MAR mutants of strain Brescia were identical, but MAb b7, in contrast to MAbs c8 and c11, did not bind to MAR mutants of strain "C." Thus, neutralizing MAbs raised against strain "C" can be grouped in subdomain A1, although these MAbs may have different binding characteristics with respect to the Brescia MAbs of subdomain A1.

Epitopes on E1 of CSFV strains Brescia and "C" have been mapped in the N-terminal half of E1 (25). In this part of E1, an internal hydrophobic region is situated between neutralizing domains B and C and domain A (Fig. 4 and 5). The hydrophobicity of this region is conserved among pestiviruses. A stretch of conserved hydrophobic amino acid residues is also found in envelope glycoproteins of orthomyxoviruses, paramyxoviruses, and retroviruses. Cleavage of precursor proteins, such as hemagglutinin of influenza virus and gp160 of human immunodeficiency virus, at the N-terminal side of this stretch of hydrophobic residues releases the hydrophobic part which thereafter penetrates into the membrane of the cell (7, 10, 22). We speculate that the internal hydrophobic region in E1 is also involved in membrane fusion, although, until now, no cleavage had been demonstrated in this region of E1.

Subdomains A1 and A2 are conserved in more than 90 CSFV field and laboratory strains (34) and are, therefore, expected to be highly resistant to mutation. Surprisingly, however, MAR mutants were easily isolated with neutralizing MAbs directed against this domain. One striking mutation is the Cys→Arg substitution at position 792, which abolished the binding of all MAbs of domains A and D but did not affect the viability of the virus. Apparently, MAR mutants of highly conserved domain A can be isolated in vitro but do not evolve in vivo.

All epitopes of envelope glycoprotein E1 of CSFV are conformational, since binding of a panel of 19 MAbs is dependent on two or more cysteines. Cysteines in E1 are conserved among pestiviruses, suggesting that the structure of this glycoprotein is similar in pestiviruses. The finding that several amino acid substitutions in escape variants of gp53 (17) are located at the same positions as those in E1 variants confirms this suggestion.

We propose that the N-terminal antigenic half of E1 independently forms two structural units, one consisting of domains B and C and one consisting of highly conserved domain A and domain D (Fig. 5). Both units contain epitopes of neutralizing MAbs. Synergism in neutralization has been found for MAbs of domains B or C and domain A but not for MAbs of domains B and C (30). This indicates that the two structural units also function independently. They probably interact with the cellular receptor as suggested for BVDV attachment by gp53 (37). Since antigenically intact E1 can be purified from insect cells in large amounts (9), resolution of the three-dimensional structure of E1 protein by X-ray crystallography and immunological studies of the structural units of E1 can now be undertaken.

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