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Characterization of the Antigenic Structure of Herpes Simplex Virus Type 1 Glycoprotein C through DNA Sequence Analysis of Monoclonal Antibody-Resistant Mutants

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Earlier studies of a group of monoclonal antibody-resistant (*mar*) mutants of herpes simplex virus type 1 glycoprotein C (gC) operationally defined two distinct antigenic sites on this molecule, each consisting of numerous overlapping epitopes. In this report, we further define epitopes of gC by sequence analysis of the *mar* mutant gC genes. In 18 *mar* mutants studied, the *mar* phenotype was associated with a single nucleotide substitution and a single predicted amino acid change. The mutations were localized to two regions within the coding sequence of the external domain of gC and correlated with the two previously defined antigenic sites. The predicted amino acid substitutions of site I mutants resided between residues Gln-307 and Pro-373, whereas those of site II mutants occurred between amino acids Arg-129 and Glu-247. Of the 12 site II mutations, 9 induced amino acid substitutions within an arginine-rich segment of 8 amino acids extending from residues 143 to 151. The clustering of the majority of substituted residues suggests that they contribute to the structure of the affected sites. Moreover, the patterns of substitutions which affected recognition by antibodies with similar epitope specificities provided evidence that epitope structures are physically linked and overlap within antigenic sites. Of the nine epitopes defined on the basis of mutations, three were located within site I and six were located within site II. Substituted residues affecting the site I epitopes did not overlap substituted residues of site II, supporting our earlier conclusion that sites I and II reside in spatially distinct antigenic domains. A computer analysis of the distribution of charged residues and the predicted secondary structural features of wild-type gC revealed that the two antigenic sites reside within the most hydrophilic regions of the molecule and that the antigenic residues are likely to be organized as β sheets which loop out from the surface of the molecule. Together, these data and our previous studies support the conclusion that the *mar* mutations identified by sequence analysis very likely occur within or near the epitope structures themselves. Thus, two highly antigenic regions of gC have now been physically and genetically mapped to well-defined domains of the protein molecule.

There are at least seven virally encoded envelope glycoproteins of herpes simplex virus (HSV) (1, 4, 25, 34) which are also present in the membranes of infected cells. Glycoprotein C (gC) is one of the most immunogenic glycoproteins and represents a major target antigen for antiviral immune responses. gC elicits high-titer complement-dependent neutralizing antibodies (5, 12, 31) and is a major target antigen for cytotoxic T lymphocytes (6, 10, 24, 32). gC is also a serotype-specific antigen, since the majority of antibodies prepared against gC encoded by HSV type 1 (HSV-1 gC-1) do not cross-react with gC encoded by HSV-2 (gC-2) (11). gC is not essential for the production of active virus particles in cell cultures (35), although recent evidence suggests that it may play a role in virus attachment (D. Wudunn and P. Spear, personal communication). gC also has the ability to bind the C3b complement component (7), which can interfere with the alternate pathway for complement activation (8) and thus inhibit both complement-mediated cytolysis of HSV-infected cells and complement-dependent virus neutralization (29, 30). These findings suggest that gC plays a role in viral pathogenesis in human hosts, although animal studies have not provided consistent evidence for a direct role for gC in neuropathogenesis (23, 36).

Because of the important role of gC in type-specific antiviral immunity, studies to define its antigenic structure by using genetic, immunologic, and biochemical techniques were undertaken. Our approach was to generate a large panel of monoclonal antibodies which were used to select a library of neutralization escape mutants referred to as monoclonal antibody-resistant, or *mar*, mutants of gC (17). Marlin et al. (28) described the neutralization resistance patterns of 22 *mar* C mutants when tested against a panel of 30 gC-specific monoclonal antibodies. Nine gC epitopes were operationally defined on the basis of the unique reactivity patterns observed. These epitopes were placed into two groups, or antigenic sites, each composed of distinct but antigenically related epitopes. Site I consisted of three epitopes, and site II contained six epitopes. The sites were shown to be topographically distinct by the finding that only antibodies which recognized the same antigenic site were mutually competitive for antigen binding in antibody competition assays. The physical locations of the two antigenic sites were approximated by analysis of the immunoprecipitation patterns of site I- and site II-specific antibodies tested against a series of carboxy-terminal truncated gC polypep-

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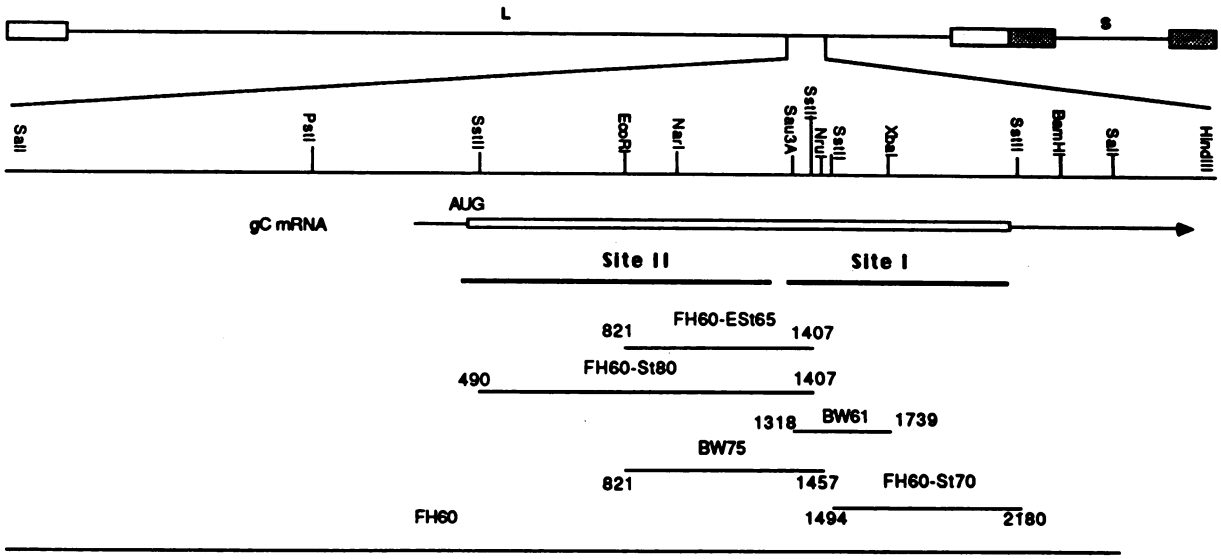

FIG. 1. HSV DNA fragments used for marker rescue of *mar* mutations. The HSV-1 genome is at the top. A *Sal*I-*Hin*dIII fragment containing the complete gC gene is shown with restriction sites. Symbols: □, inverted repeats flanking the long unique element (L); ▦ inverted repeats flanking the short unique element (S); —►, region coding for the gC message; ▭, protein coding region with the approximate location of the translation start site; —, regions covered by the fragments used in the marker rescue experiments (the nucleotides encompassed by these fragments relative to the start of transcription are indicated); ▬, domains for antigenic sites I and II as predicted by Homa et al. (21).

tides synthesized on infection with mutant viruses shown to carry chain-terminating mutations. That study, in combination with biochemical and genetic analyses of these chain-terminating mutants, mapped site I between amino acid residues 298 and 481 and site II N terminal of residue 274 (16, 22, 28).

In this study, the *mar* C mutations of 18 mutants were localized within their respective gC genes by marker rescue, and the appropriate regions were sequenced to identify the specific base pair substitutions. By identifying the genetic alterations which affected the antigenic activities of the mutant gC molecules, the structure and organization of the gC epitopes of sites I and II were precisely mapped to two well-separated regions within the external domain of gC which are predicted to contain hydrophilic β-turn structures. These structures appear to represent the most immunogenic components of the gC molecule of HSV-1.

MATERIALS AND METHODS

Cells, viruses, and monoclonal antibodies. African green monkey kidney (Vero) cells grown in Eagle minimum essential medium supplemented with nonessential amino acids, 100 mg of streptomycin per ml, 100 U of penicillin per ml, and 5% fetal calf serum were used in all experiments. HSV-1 KOS-321 and the *mar* C mutants were isolated and characterized as described previously (16, 21, 28). Virus stocks were prepared by infection at a low multiplicity in Vero cells. The monoclonal antibodies used in this study were described previously by our laboratory (16, 17, 28). The gC gene nucleotide sequence was reported previously (9, 21). The numbering of nucleotides in the gC gene begins with the message cap site. The initiation codon for translation of the gC message is at nucleotide (nt) 381. Amino acid numbering starts with the first residue of the signal sequence in the nascent protein. The nomenclatures for gC-specific monoclonal antibodies and *mar* C mutants were those of Holland et al. (17).

Marker rescue and DNA sequencing. Viral DNA was prepared by the method of Goldin et al. (13). Large-scale preparation of plasmid DNA was done by the method of Birnboim and Doly (2) as modified by Maniatis et al. (26). Minipreparations of plasmid DNA were isolated by the boiling method of Holmes and Quigley (19), as described by Maniatis et al. (26). The calcium phosphate precipitation protocol described by Homa et al. (20) was adapted from the method of Graham and van der Eb (14) for use in cotransfection marker rescue experiments. Linearized DNA of the rescue plasmids was individually cotransfected into Vero cells with viral DNA extracted from the *mar* C mutants. Progeny virus from the cotransfections were plated on Vero cells for plaque formation and screened by the immunoperoxidase black-plaque assay described by Holland et al. (18). Black plaques contain recombinant virus in which wild-type antigenic activity has been restored, indicating positive rescue. The fragments of the wild-type gene used in the marker rescue experiments to localize the *mar* mutations within the gC gene are depicted in Fig. 1. The recombinant plasmids pFH60-St80, pFH60-ESt65, pFH60-St70, and pFH60, which contain different segments of the wild-type gC sequence inserted in pBR322, were constructed as described by Holland et al. (16). pFH60-St80 carries an *Sst*II fragment (nt 490 to 1407) which encompasses the 5′ coding sequence of the gC gene. pFH60-ESt65 carries an *Eco*RI-*Sst*II piece (nt 821 to 1407) which spans the middle region of the gene, and pFH60-St70 contains an *Sst*II fragment (nt 1494 to 2180) at the 3′ end of the gC coding sequence. pFH60 has a 3.6-kilobase *Sal*I fragment insert (map coordinates 0.620 to 0.645) which includes the full coding sequence of the gC gene as well as some 5′-flanking sequences. The pBW plasmids were constructed by inserting pieces of the wild-type gC gene into M13 vectors. pBW61 has a *Sau*3A-*Xba*I fragment (nt 1318 to 1739) in the *Acc*I-*Xba*I sites of M13 mp18, while pBW75 contains an *Eco*RI-*Nru*I insert (nt 821 to 1457) in the M13 mp8 vector. pBW75 covers the middle region of the

TABLE 1. Results of marker rescue experiments as percentages of black plaques[a]

Mutant DNA	% Black plaques from rescue fragments:						
	FH60-St80	FH60-ESt65	BW75	BW61	FH60-St70	FH60	None
Site II							
C16.1	1.7				0		0
C9.6	2.0				0		0
C13.1	2.0				0		0
C10.3	3.5	0			0		0
C13.2	3.5				0		0
C7.1	2.0	0		0		1.2	0
C9.1	2.2	0		0		1.0	0
C16.2	1.4	0			0		0
C17.2	0.2	0					0
C17.3	4.0				0		0
C3.1	0.5	0			0		0
C10.1			0.6	0		2.0	0
Site I							
C11.1			1.8	0			0
C14.1			0.01		0	2.8	0
C15.1			0.1	0		6.3	0
C2.1			0	0.5			0
C4.4			0	0		1.8	0
C15.4			0	0		0	0

[a] The recombination frequency is expressed as the percentage of black recombinant plaques in the total number of plaques. If no value is given, marker rescue was not attempted.

coding sequence and overlaps with the pFH60-St80 fragment on its 3′ side. The pBW61 fragment bridges the regions covered by pFH60-St80 and pFH60-St70.

For sequencing, DNA was extracted from mutant virions, and the gC coding sequence from each mutant was purified as a 3.6-kilobase *Sal*I fragment (map coordinates 0.620 to 0.645), extracted by electroelution from an 0.8% agarose gel, and cloned into pBR322. Recombinant clones containing the gC sequences were cut further with different restriction enzymes to yield fragments of appropriate sizes for DNA sequencing. Fragments covering the whole gC protein coding region were gel purified and subcloned into M13 vectors. The sequencing was performed as described by Sanger et al. (33). The nucleotide mix and primer kit were purchased from Pharmacia, Inc. (Piscataway, N.J.). [α-^{35}S]dATP was obtained from Dupont, NEN Research Products (Boston, Mass.).

RESULTS

Physical mapping of *mar* mutations. The physical location of each *mar* C mutation was determined by marker rescue of the mutant phenotype with cloned wild-type sequences spanning different parts of the gC gene (Fig. 1). The appearance of black plaques among the plated progeny indicated that recombination had occurred between the plasmid DNA and the viral chromosome to rescue the mutation to the wild-type sequence. The results of all marker rescue experiments are shown in Table 1.

With the exception of *mar* C15.4, each mutant tested was rescued by pFH60. This DNA fragment contained sequences covering the entire gC coding sequence. Physical mapping of the *mar* C15.4 mutation was not possible, because the reaction of the mutant gC with the C15 antibody was not easily distinguishable from that of wild-type gC in the black-plaque assay. For this mutant, it was assumed that the alteration resided in a region of the gC gene where mutations affecting similar epitopes were mapped, a prediction confirmed by sequencing (see below). The mutations of all site II mutants, except the untested *mar* C10.1, were rescued by pFH60-St80. The pFH60-St80 fragment covers the 5′ part of the gC-1 coding sequence between nt 490 and 1407 and accordingly mapped the mutations to that region. The *mar* C10.1 mutation, however, was rescued by plasmid pBW75, which mapped it within the middle region of the gene. This mutation also was rescued by pFH60 but not pBW61, which ruled out the region from nt 1318 to 1457 and narrowed the location of the mutation to between nt 821 and 1318. Attempts were made to rescue many of the site II mutations, including those of *mar* C16.1, C16.2, C9.6, C13.1, C13.2, and C17.3, with pFH60-St70. As expected, no black plaques were observed, since the pFH60-St70 wild-type fragment covers the 3′ part of the gene, which is distal to the predicted location of site II in the 5′ end of the gene. In addition, the mutations of site II *mar* mutants C10.3, C7.1, C9.1, C16.2, C17.2, and C3.1 were not rescued by pFH60-ESt65. Since the pFH60-ESt65 fragment was contained in the pFH60-St80 fragment, the lack of rescue with pFH60-ESt65 suggested that these *mar* mutations lie within the region from nt 490 to 821 and not in the downstream region from nt 821 to 1407, which is covered by both the pFH60-St80 and the pFH60-ESt65 fragments. The mutations in mutants *mar* C7.1 and *mar* C9.1 were not rescued with pBW61; this was consistent with our hypothesis.

The mutations in site I mutants *mar* C11.1, C14.1, and C15.1 were rescued by pBW75, which mapped the mutations to the fragment from nt 821 to 1457. Two of these mutations were not rescued by pBW61, however, making it likely that they were within the segment from nt 821 to 1318. The mutation in *mar* C14.1 was rescued by pBW75, albeit at a low frequency, but not by pFH60-St70, suggesting that the mutation was within the region from nt 821 to 1457, which is covered by the pBW75 fragment. In contrast to other site I mutations, the *mar* C2.1 mutation was localized to the segment in the 3′ end of the gene (nt 1318 to 1739) by rescue with plasmid pBW61. The mutation was not rescued by pBW75, however, which suggested that the mutation might lie in the smaller region, from nt 1457 to 1739. The mutation in *mar* C4.4 was rescued only with pFH60 and therefore was not mapped to a smaller region of the gC gene. Since *mar* C4.4 has the same reactivity pattern against gC-specific monoclonal antibodies as *mar* C2.1 does, this mutation was thought likely to reside in the same area as the *mar* C2.1 mutation.

The low rescue frequency of the *mar* C14.1 and *mar* C15.1 mutations with pBW75 may be attributed to the locations of the mutations relative to the ends of the fragment. As revealed by the sequencing data, the sites of the *mar* C14.1 and C15.1 mutations are only about 150 nt from the 3′ limit of the *Eco*RI-*Nru*I fragment, which may result in a reduced frequency of recombination. Supporting this argument is the finding that both mutations were rescued with high frequency by the 3.6-kilobase *Sal*I fragment in pFH60. Similarly, the small size of the *Sau*3A-*Xba*I fragment used in mapping the *mar* C4.4 mutation may explain rescue failure.

Of 18 mutations, 16 were rescued by a fragment corresponding to a smaller region of the gC gene. The mutations in the *mar* mutant viruses selected by site II monoclonal antibodies were generally localized to the 5′ part of the protein coding region of the gene, while mutations in site I *mar* mutant viruses were mapped to the 3′ region. The results of the physical mapping confirm our previous findings (28) that the locations of the epitopes defined by site II

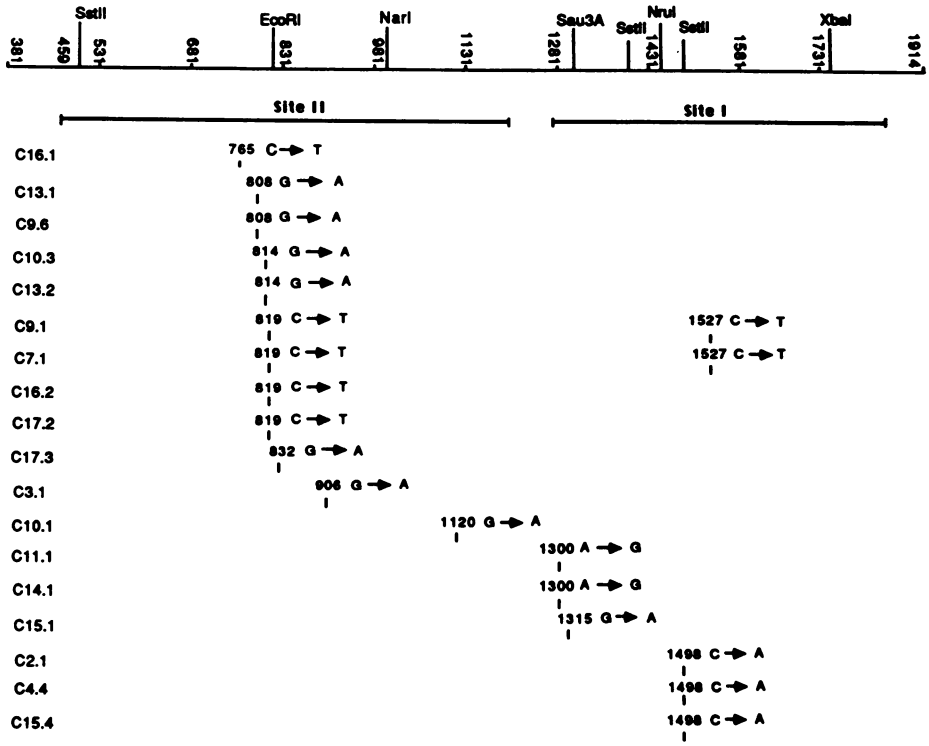

FIG. 2. Nucleotide changes in the *mar* C mutants. A partial restriction map of the protein coding region of the gC gene and the numbering of the nucleotides in the region are shown at the top. The thick solid lines below the map denote the domains for sites I and II as predicted by Homa et al. (21). The position of each mutation, as well as the nucleotide change, is indicated.

monoclonal antibodies are toward the amino-terminal end of the protein molecule, whereas those of site I epitopes are nearer the carboxy terminus of the molecule.

Nucleotide changes in *mar* mutants. The nucleotide substitutions in *mar* C mutant DNA were identified by sequence analysis of the respective gC genes. The *SalI* fragment containing the gC gene was purified from each *mar* mutant and was subcloned into M13 for DNA sequencing. The corresponding wild-type gC gene fragments were sequenced in parallel to ensure the accuracy of sequence reading. Selected regions outside those indicated by marker rescue were sequenced to determine whether other mutations might also be present in the *mar* mutant genes. In the case of *mar* C4.4 and *mar* C15.4, for which the physical mapping data were inconclusive, the 3′ region was sequenced from nt 821 to 2180, where the site I mutations are most likely to reside.

Nucleotide changes were found in all the *mar* mutants (Fig. 2). The locations of the base changes confirmed the predictions of the physical mapping. In mutants that had been rescued successfully, the base changes were situated in the regions implicated by both positive and negative rescue results. Since all of these base changes were the only mutations found within the region identified by marker rescue for each mutant, we concluded that these mutations altered the antigenic properties of the gC *mar* mutants. In two mutants, *mar* C7.1 and C9.1, two mutations were found (Fig. 2). The base change in both mutants at nt 1527 was outside the region implicated by marker rescue data and away from the positions of the rest of the site II mutations. This indicates that the change at nt 1527 was unrelated to the *mar* phenotypes of the two mutants. As previously indicated, marker rescue failed to localize the *mar* C4.4 and *mar* C15.4 mutations to a smaller region of the gC gene. The base changes identified within the sequenced portion of the gC gene of these *mar* mutants were within the predicted domain for antigenic site I. Although these changes are likely to be the cause of the *mar* phenotypes of the two mutants, the possibility that additional base changes elsewhere in the gene exist and contribute to the antigenic alterations cannot be ruled out until these mutant gC genes are completely sequenced.

The nucleotide changes of all site II mutants, except for *mar* C10.1, are situated in a 150-base-pair segment (nt 765 to 906) toward the 5′ end of the gC open reading frame. Nine of these changes cluster within a 24-base-pair region. In agreement with the physical mapping data, the *mar* C10.1 mutation resides in the middle part of the gene, downstream from the other site II mutations. The site I mutations are distributed within a 200-base-pair segment toward the 3′ end of the coding sequence. Among the 18 base changes identified which affect antigenicity, those of *mar* C2.1, C4.4, and C15.4 are transversions, which change pyrimidines to purines. The rest are transitions. Mutants *mar* C9.6, C9.1, C10.3, C11.1, and C14.1 were selected from virus stocks that had been treated with 5-bromodeoxyuridine, which specifically causes transition mutations (16). Our results are consistent with the presence of this expected class of mutation and correlate well with the presence of transition mutations in six gB *mar* mutants (15, 27).

Amino acid substitutions. The amino acid substitutions in the gC mutant molecules (Fig. 3) were predicted from the indicated nucleotide changes in the *mar* C genes (Fig. 2). All the mutations induced amino acid substitutions. Of the changes at the 10 mutated sites which caused antigenic changes, six affected charged residues and nine amino acid substitutions resulted in charge differences (either gain or

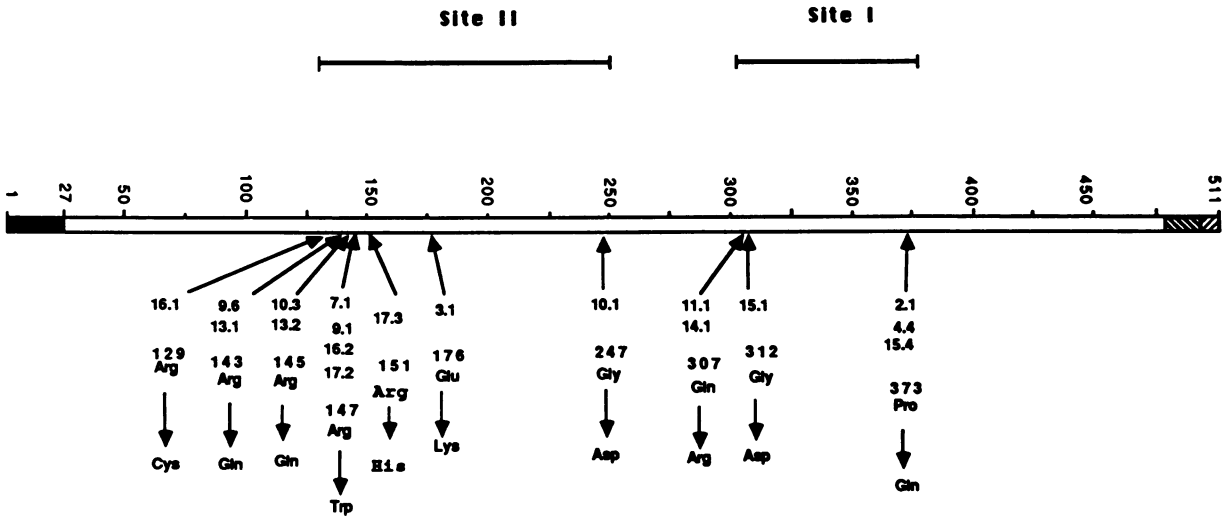

FIG. 3. Predicted amino acid substitutions in the *mar* C mutants. A schematic of the gC molecule is shown. The numbers represent the amino acid residues in the sequence, with 1 as the initiation methionine. Symbols: ■, signal sequence of the protein; □, extracellular domain; ▨, transmembrane region; ▨, intracellular domain. Shown above the map are the predicted regions for sites I and II as determined by the data presented in this report. The positions of the affected residues and the identities of the amino acid substitutions are indicated.

loss of charge). Charged residues are more likely to be surface exposed and are hence more accessible to antibodies. The large number of charged amino acids among the residues identified by the sequence analysis of *mar* mutants is consistent with the role of these residues in the antigenic structure of gC. Five Arg residues were altered in site II, and four were clustered within an 8-amino-acid segment. How these residues are involved in the antigenic activity of gC and how the substitutions affect the physical structure of the protein are discussed below. None of the amino acid substitutions occurred within the predicted asparagine-linked glycosylation sites.

Computer analyses of predicted structural features of gC. Computer analyses were performed to assess the antigenic potential of the regions of gC identified in our studies. Candidate structures for potential antigenicity are β-turn structures flanked by β sheets or α-helical regions with high probabilities for flexibility and surface exposure (37, 38). Computer analysis of the primary sequence of gC with the MSEQ program (3) showed that most of the residues identified as important to the antigenic structure reside at or near one of the two most hydrophilic structures in the molecule, residues 120 to 150 and 300 to 310. The amino acid substitutions in the *mar* mutants often resulted in increases in hydrophobicity at the mutated positions (data not shown). The secondary structure predictions for the gC molecule and for sites I and II are shown in Fig. 4. There are several potential loop structures in both regions which feature β turns flanked by β sheets or α-helical structures. Most of the substituted residues identified in the *mar* mutants are situated in these potential loops. The localization of mutations within these loop structures supports the proposed role of sites I and II as major antigenic domains of gC.

DISCUSSION

Marker rescue and DNA sequencing data for the 18 *mar* mutants presented in this report extend the analysis of the antigenic structure of gC. Mutations in site II *mar* mutants were rescued with DNA fragments spanning the 5′ end of the gC gene, physically mapping the mutations to that region of the coding sequence. Similarly, mutations in site I mutants were physically mapped to the 3′ coding sequence of the gC gene. For the 16 mutations that were marker rescued to a subfragment of the gC coding sequence, a single nucleotide substitution was detected in the region corresponding to the rescuing fragment(s). All site II mutations were located 5′ to nt 1121, and all site I mutations were located 3′ to nt 1299. All mutations were predicted to cause amino acid substitutions in the external domain of the gC molecule. The amino acids affected by site I mutations spanned residues 307 to 373, while those affected by site II mutations spanned residues 129 to 247. Thus, the marker rescue and sequencing data confirm our earlier findings of two nonoverlapping antigenic domains on gC (16, 21, 28) and further define their dimensions (Fig. 3 and 4).

Five monoclonal antibodies were used to define antigenic site I. Two antibodies, C2 and C4, probably represent sister clones, since they were isolated in the same hybridoma fusion experiment, showed similar isoelectric focusing profiles (17), and were indistinguishable by their reactivities with the panel of *mar* mutants (28). The mutations carried by the *mar* C2.1 and *mar* C4.4 mutants selected by these monoclonal antibodies both contained substitutions of Gln for Pro at residue 373. We conclude that monoclonal antibodies C2 and C4 recognize the same site I epitope, EpC2/4.

The remaining site I antibodies were unique in their isoelectric focusing patterns. Monoclonal antibodies C11 and C14 appear to recognize the same epitope, since *mar* C11.1 and *mar* C14.1 carried identical Arg-for-Gln substitutions at residue 307 and exhibited identical patterns of neutralization with the panel of monoclonal antibodies (28). The analysis of additional *mar* mutants selected by these antibodies could provide evidence that these antibodies recognize different but overlapping epitopes. For this discussion, however, we will consider that antibodies C11 and C14 recognize a single epitope, EpC11/14.

Monoclonal antibody C15 interacts with an epitope, EpC15, defined by residues Gly-312 and Pro-373. The substitution in *mar* C15.1 at residue 312 is close to the EpC11/14 at residue 307. In addition, the *mar* C15.4 alteration at

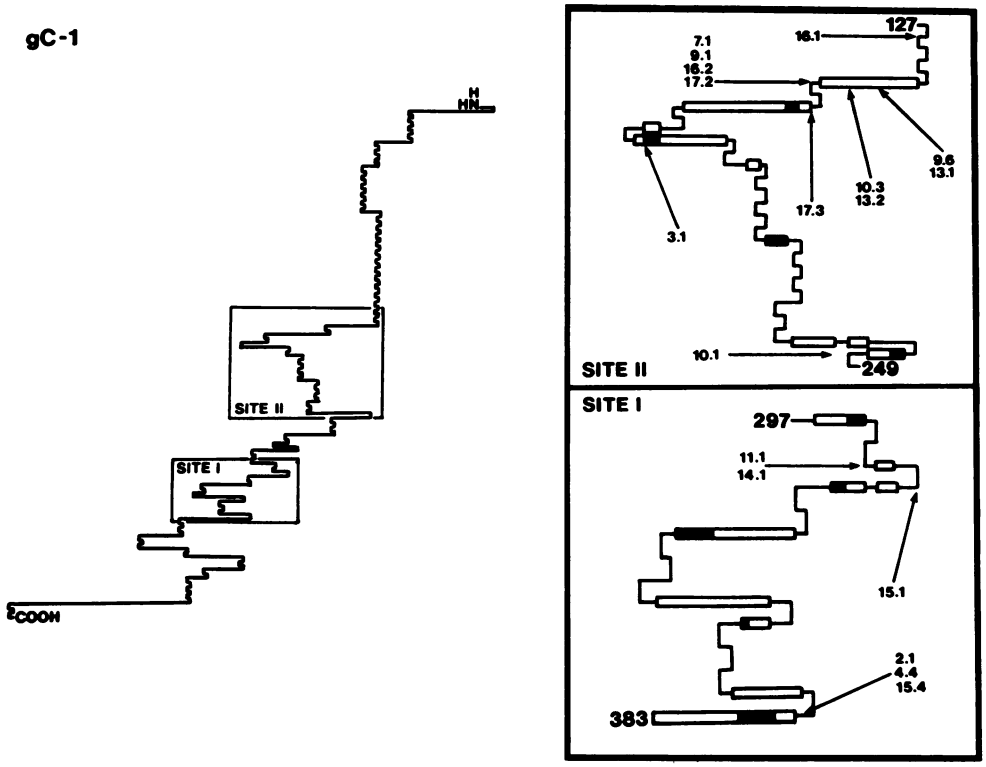

FIG. 4. Secondary structures of sites I and II in gC. A cartoon of the gC-1 molecule as drawn by the computer protein analysis program MSEQ (3) is shown. The boxed regions labeled SITE II (residues 127 to 249) and SITE I (residues 297 to 383) depict the predicted secondary structures of sites II and I, respectively, and show the locations and identities of the mutations within each region. Symbols: ■, α helix; □, β sheet; —, random coil;], β turn.

position 373 is identical to the EpC2/4 alteration. However, in immunoprecipitation studies with truncated gC polypeptides, monoclonal antibody C15, along with C11 and C14, immunoprecipitated a protein which terminated at residue 359 but not one carrying frame shift-induced substitutions carboxy terminal to residue 297 (28). In contrast, antibodies C2 and C4 failed to precipitate either polypeptide but did precipitate another gC polypeptide carrying a frame shift at residue 480, a finding consistent with the location of a substitution at residue 373. This suggests that EpC15 exists entirely within the first 359 amino acids of gC and that residue 373 is not required for C15 recognition of gC. Therefore, it is likely that the residue change seen in *mar* C15.4 results in the steric or electrochemical inhibition of antibody binding at a more amino-terminal location, presumably at or near residue 312. We conclude that antigenic site I of gC consists of at least three epitopes, clustered in a restricted region of the carboxy-terminal half of the external domain of the molecule. EpC11/14 and EpC15 are likely to be overlapping, while EpC2/C4 lies nearby. These antibodies are mutually competitive for binding to the intact gC molecule (28), and the *mar* mutant reactivity patterns with these antibodies also indicate that the epitopes are closely related (28). Thus, it might be speculated that residues 307, 312, and 373 are brought into close promixity by the folding of gC, although the immunoprecipitation data described above indicate that the stabilities of EpC11/14 and EpC15 are not strictly dependent on this folding. Nevertheless, alterations in residue 373 can disrupt EpC15 and EpC2/4 but not EpC11/14, suggesting that the integrity of epitopes in site I relies on distinct but localized structures within this segment of the gC molecule.

Seven monoclonal antibodies were used to define the epitopes of antigenic site II. Except for C9 and C17, which had similar isolectric focusing patterns, the other monoclonal antibodies had unique banding patterns and represent distinct clonotypes (26). Thus far, all the epitopes in site II can be distinguished from one another by the residues identified in the *mar* mutants; these residues are likely to represent contact points for the monoclonal antibodies which recognize overlapping site II epitopes. This conclusion is consistent with the distinct but similar reactivity patterns of the antibodies with site II *mar* mutants (28). For instance, while EpC9 and EpC17 share Arg-147 (*mar* C9.1 and C17.2) as an antibody contact point, they are distinguishable by a second component residue in each epitope as derived from the sequence analysis of *mar* mutants C9.6 (Arg-143) and C17.3 (Arg-151).

A striking feature of site II is the clustering of altered residues within a segment of eight amino acids (residues 143 to 151). Four of these amino acids are charged arginines, all of which were substituted in different *mar* mutations affecting this segment. Of the 12 site II mutations sequenced in this study, 9 induce amino acid substitutions within this arginine-rich segment of the gC molecule. Six of the seven monoclonal antibodies recognize the arginines in this 8-amino-acid segment as components of their epitopes, suggesting the presence of multiple, overlapping epitopes. Four monoclonal antibodies, C7, C9, C16, and C17, overlapped in their recognition of Arg-147. Two antibodies, C9 and C13, recognized Arg-143; another two, C10 and C13, overlapped at Arg-145. Two of the remaining *mar* mutations affected residues outside of the 8-amino-acid segment, but these changes were selected by antibodies which also select

changes within the segment. EpC16 is characterized by *mar* C16.1, which is altered at Arg-129, and by the *mar* C16.2 mutation at Arg-147. EpC10 is characterized by the *mar* C10.1 mutation at Gly-247 and by the *mar* C10.3 mutation at Arg-145. Both EpC16 and EpC10 appear to be conformational epitopes, composed of more distant amino acids. The last site II *mar* mutant, *mar* C3.1, characterizes EpC3 with Glu-176 as a binding site. Additional mutants selected by monoclonal antibody C3 should be studied to further define this epitope. It is possible that an association of this epitope with the 8-amino-acid segment would then be demonstrated. On the basis of these findings, we conclude that the 8-amino-acid segment is the antigenic core of site II on the gC molecule.

Computer analysis of most *mar* C mutations failed to produce noticeable effects on the predicted secondary structure of gC. This was also true for most *mar* mutations in the gB glycoprotein (S. Highlander, Ph.D. disseration, University of Michigan, Ann Arbor, 1988). In these cases, it is likely that contact points for antigen-antibody interactions have been disrupted or made sterically undesirable. However, some of the *mar* mutations are predicted to induce structural alterations in gC. The most dramatic change was a consequence of the substitution of residue Arg-147 with Trp within the 8-amino-acid core antigenic structure described above. Secondary structure analysis indicated that this substitution could both delete a β turn and alter a loop structure of site II. This structural change might be the basis of the antigenic alteration affecting EpC7, -9, -16, and -17. The substitution of Arg-143 by Gln in *mar* C9.6 and *mar* C13.1 may extend an existing β sheet in a potential loop structure. Outside this core region, the change of Gly-247 to Asp in *mar* C10.1 may result in the disruption of an α helix which is also part of a potential loop structure. The role of local secondary structure in formation of these epitopes, particularly in the 8-amino-acid segment, will require further analysis by site-directed mutagenesis.

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