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Identification of Antigenically Important Domains in the Glycoproteins of Sindbis Virus by Analysis of Antibody Escape Variants

ELLEN G. STRAUSS,[1]* DAVID S. STEC,[2]† ALAN L. SCHMALJOHN,[2]‡ AND JAMES H. STRAUSS[1]

Division of Biology, California Institute of Technology, Pasadena, California 91125,[1] and Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, Maryland 21201[2]

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To study important epitopes on glycoprotein E2 of Sindbis virus, eight variants selected to be singly or multiply resistant to six neutralizing monoclonal antibodies reactive against E2, as well as four revertants which had regained sensitivity to neutralization, were sequenced throughout the E2 region. To study antigenic determinants in glycoprotein E1, four variants selected for resistance to a neutralizing monoclonal antibody reactive with E1 were sequenced throughout the E2 and E1 regions. All of the salient changes in E2 occurred within a relatively small region between amino acids 181 and 216, a domain that encompasses a glycosylation site at residue 196 and that is rich in charged amino acids. Almost all variants had a change in charge, suggesting that the charged nature of this domain is important for interaction with antibodies. Variants independently isolated for resistance to the same antibody were usually altered in the same amino acid, and reversion to sensitivity occurred at the sites of the original mutations, but did not always restore the parental amino acid. The characteristics of this region suggest that this domain is found on the surface of E2 and constitutes a prominent antigenic domain that interacts directly with neutralizing antibodies. Previous studies have shown that this domain is also important for penetration of cells and for virulence of the virus. Resistance to the single E1-specific neutralizing monoclonal antibody resulted from changes of Gly-132 of E1 to either Arg or Glu. Analogous to the findings with E2, these changes result in a change in charge and are found near a glycosylation site at residue 139. This domain of E1 may therefore be found near the 181 to 216 domain of E2 on the surface of the E1-E2 heterodimer; together, they could form a domain important in virus penetration and neutralization.

In recent years many panels of monoclonal antibodies (MAbs) have been isolated that are specific for the surface proteins of viruses. Of particular interest are antibodies that neutralize infectivity. Mechanisms of neutralization by antibodies are not well understood at present, but in many cases neutralizing antibodies are thought to exert their effect by interfering with virus attachment to susceptible host cells. Some neutralizing antibodies appear to bind directly to the receptor-binding site on the virus, and anti-idiotypic antibodies made to such neutralizing antibodies may function as antireceptor antibodies (4, 9, 46). Other neutralizing antibodies block binding by reacting with variable regions that are close but not identical to the virus receptor-binding site, because the highly conserved binding site is hidden or protected from interaction with antibody molecules (30, 47). Some neutralizing antibodies do not block virus attachment, but appear to prevent penetration in some way (28). In a number of cases, neutralizing MAbs can confer passive protection to disease, indicating that these antibodies react with the virus within the animal and that the epitopes involved are immunologically important in protection against disease (1, 18, 32, 39). The epitopes with which MAbs react have been mapped in a number of ways, including competitive-binding studies, use of truncated peptides produced in bacteria, and study of the reactivity of synthetic peptides made to the amino acid sequence of the surface proteins (12, 17, 29, 33). The last two approaches have been of limited usefulness since many epitopes are at least in part conformational, involving structural features absent in short peptides or involving discontinuous portions of the protein.

A complementary approach for mapping antigenic sites has been to isolate virus variants which are resistant to particular MAbs and to determine the change responsible for the resistance (11, 15, 22, 35, 42, 45, 48). A single-amino-acid change may confer complete resistance to neutralization by an antibody because the amino acid involved is an important if not dominant element of the epitope, even in the case of dispersed or conformational epitopes. Cases have been described, however, in which single-amino-acid substitutions in regions away from the actual antibody-binding site itself have led to escape from neutralization, because the substitution leads to a change in conformation of the epitope (21).

Several panels of MAbs that are reactive with the glycoproteins of Sindbis virus or of other alphaviruses have been isolated (3, 18, 26, 27, 32, 33, 38, 49). As has been the case for polyclonal antisera made to the isolated glycoproteins, antibodies which are specific for glycoprotein E1, the hemagglutinin, may react with both the virus and the isolated protein and may inhibit hemagglutination by virus and hemadsorption by infected cells, but seldom neutralize infectivity. On the other hand, E2-specific antibodies are often neutralizing. To further characterize their MAbs, Stec et al. (40) isolated Sindbis virus variants which were resistant to each of five different E2-specific neutralizing MAbs and to one E1-specific neutralizing MAb. In some cases, variants

* Corresponding author.
† Present address: Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892.
‡ Present address: Disease Assessment Division, U.S. Army Medical Research Institute for Infectious Diseases, Fort Detrick, Frederick, MD 21702.

TABLE 1. Primers for sequencing the glycoproteins

Protein[a]	Primer[b]	3′ base	cDNA sequence (5′ to 3′)	5′ base	%G+C
E3	6	8454	CATGGCCGTTACACAAACGAG	8474	52
E3-E2	RD1	8616	CCGTCTTCGTTTTCTTCG	8633	52
E2 (41–46)	RD2	8749	TATTGTGGTATGCGTATG	8766	50
E2 (94–100)	RD3	8911	AATCGATGTTTCCTATGA	8928	39
E2 (129–134)	9	9014	GTTCTATTTTGGTTTTAAGC	9033	33
E2 (158–163)	RD4	9105	TTTCTTTGTTGACGTCCG	9122	30
E2 (228–233)	RD5	9314	GATATTCTCGCTGGTTTG	9331	44
E2 (256–262)	3	9396	GTAAACGGAAAGTTCAACTA	9415	44
E2 (284–290)	RD6	9487	TTAATCTATGTCTGGTGA	9504	35
E2 (350–356)	8	9686	TGTGGCTTTATCATGTCGTAAT	9706	33
E2 (386–391)	RD8	9789	AATACACGGACATTTCGC	9806	33
6K (1–6)	RD9	9900	CTTTGCAAGTGGCTCTGG	9918	44
6K (22–26)	20	9961	TCAACACGTATGGAAA	9976	37
E1 (15–21)	23	10107	ATATTCCGTGAACAACTTT	10125	32
E1 (58–64)	13	10238	TTTTTAGTTTACGACGCCGA	10257	40
E1 (130–135)	26	10449	TTTCATCCTGACGCATAA	10466	39
E1 (175–180)	14	10585	TAGTATTCCAGCAATAGGTA	10604	35
E1 (240–245)	24	10781	AACTCTACACCTTTTTG	10797	35
E1 (287–292)	19	10923	AAATAGTCCTGTAGTCTA	10940	33
E1 (371–376)	22	11191	CCCTTCTTCTGTTGTAC	11207	47
3′ untranslated	25	11394	TTACTAGGCTGGTCGTT	11410	47

[a] Location of the primer in the protein. Amino acid residues in parentheses are numbered from the beginning of the protein.
[b] Primers with the prefix RD were kindly supplied by R. K. Durbin (7). Other primers were synthesized in our facility.

showed resistance to other MAbs in addition to the one with which they were selected. Their results demonstrated the existence of at least two independently mutable epitopes on E2 and showed that isolates sequentially selected with more than one antibody acquired multiple resistance. To facilitate the analysis and rule out exogenous mutations unrelated to antigenic reactivity, Stec et al. (40) then isolated revertants of several of these antigenic escape variants that were once again susceptible to neutralization by the original antibody. To localize the changes responsible for altered antibody reactivity, we have sequenced relevant regions of the genomic RNA of the parental AR339 strain of Sindbis virus and of 12 variants (either singly or multiply resistant), as well as four revertants. A preliminary description of some of these results has appeared (42).

MATERIALS AND METHODS

Virus strains. The parental Sindbis virus from which antigenic variants were isolated was the AR339 strain, obtained from Joel Dalrymple. The virus was plaque purified three times in Vero cells before isolation of antibody escape variants. It is slightly different from the AR339 strain supplied by the American Type Culture Collection (ATCC), as described in Results. The heat-resistant strain of Sindbis virus (HR) was originally isolated from the AR339 strain by Burge and Pfefferkorn (2). In 1972, large-plaque (LP) and small-plaque (SP) strains of Sindbis virus HR were separated in our laboratory. The complete sequence of Sindbis virus HRSP RNA has been reported (41), as well as the sequence of the structural-protein region of HRLP (24). The isolation of Sindbis virus AR339 variants resistant to MAbs and of revertants that were again sensitive to the selecting MAb has been described previously (40).

RNA preparations. Seed virus stocks were prepared on chicken embryo fibroblast monolayers as previously described (23). For RNA preparation, two or three roller bottles (800 cm^2) were infected with virus at a multiplicity of 10 in phosphate-buffered saline (PBS) (containing Ca and Mg) (6) with 1% fetal calf serum and 1 μg of dactinomycin per ml. After a 1-h adsorption at 37°C, the inocula were removed and replaced with Eagle medium containing 3% fetal calf serum and 1 μg of dactinomycin per ml. After 3 h at 37°C, the medium was replaced with fresh medium lacking dactinomycin, and 1 mCi of [^{3}H]uridine (New England Nuclear) was added to one bottle. At 7 h after infection the monolayers were washed with ice-cold PBS and the cells were scraped from the glass with rubber policemen. The cells were washed with high-salt buffer [0.5 M NaCl, 15 mM $MgCl_2$, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 50 mM piperazine-*N*,*N*′-bis (2-ethanesulfonic acid) (PIPES) (pH 6.5)] and lysed with 1% Nonidet P-40 in high-salt buffer, and the nuclei and unlysed cells were removed by centrifugation (20). The supernatant was made 1% in sodium dodecyl sulfate (SDS), and total cellular RNA was prepared by phenol extraction as previously described (20, 31). After chloroform and ether extractions and multiple ethanol precipitations, the total cellular RNA from three roller bottles was dissolved in 50 to 100 μl of TE (10 mM Tris-HCl [pH 7.6], 0.1 mM EDTA) and stored frozen at −70°C.

RNA sequencing. RNA was sequenced by the dideoxy-chain termination method (31) modified to use reverse transcriptase on RNA templates (10, 51). Avian myeloblastosis virus reverse transcriptase was obtained from Life Sciences, Inc. Primers were deoxyoligonucleotides 16 to 22 nucleotides in length. These primers and their locations in the Sindbis virus genomic RNA sequence are listed in Table 1. Following the sequencing reactions, samples were run on 5% urea-acrylamide gels 80 cm long (37) and autoradiographed without an intensifying screen at −70°C.

Competition assays with intact and disrupted virions. Competitive binding between unlabeled and horseradish peroxidase (HPO)-labeled MAbs was performed as described previously (33), except that Sindbis virus antigens were prepared in two different forms to compare the reactivities of intact and disrupted virions. To affix intact virions to the solid phase, we precoated wells with an antibody (MAb 49 at

1 μg/ml in PBS) known to bind virion surfaces. After the wells were blocked with 0.5% bovine serum albumin (BSA) and rinsed with PBS, 0.25 μg of gradient-purified Sindbis virus AR339 in 0.05 ml of PBS was added to each well and adsorbed for 90 min. Wells were rinsed with PBS, and unlabeled (competing) MAbs and HPO-labeled MAb were added. The results of the competition were evaluated by enzyme-linked immunosorbent assay (ELISA). Particular efforts were made to prevent virion disruption by drying, and the 0.02% Tween in our conventional ELISA wash solutions was omitted from these assays. To compare disrupted virions in parallel ELISA, the same virion preparation at 2.5 mg/ml was incubated in 1% Triton X-100, diluted to 0.25 μg/0.05 ml, and adsorbed directly to wells. The unlabeled antibodies used in competition assays contained approximately 1 to 5 mg of MAb per ml of ascitic fluid; HPO-labeled MAbs were used at a concentration about 10-fold above their endpoints, i.e., at about 2 μg/ml.

Isolation and characterization of variants. The isolation and characterization of the MAbs have been previously described (32, 33). Reactivities of the variants with MAbs were determined by ELISA and neutralization, which gave concordant results (40).

RESULTS

Characterization of the MAbs used in this study. Schmaljohn et al. (32, 33) have isolated and characterized panels of MAbs to Sindbis virus which contained both neutralizing and nonneutralizing antibodies. For this study we have chosen six neutralizing antibodies reactive with glycoprotein E2 (MAbs 49, 50, 23, 18, 30, and 51) and one neutralizing antibody reactive with glycoprotein E1 (MAb 33). In a previous study with virions disrupted by drying onto an ELISA plate, all six of these anti-E2 antibodies were found to form a single interference group, in that all six competed with MAb 49 for binding, whereas MAb 33 formed a distinct interference group (33). We wished to test for competition between these antibodies by using intact virions, that is, under conditions similar to those that apply during neutralization. For this purpose we used a capture ELISA in which intact virions (AR339 strain) were first captured on the plate by binding to fixed MAb 49 and then the ability of various MAbs to compete with HPO-labeled MAbs for binding to these intact virions was determined. Virions disrupted with 1% Triton X-100 and dried onto the plate were used for comparison. The results are shown in Fig. 1. Several points can be made from these results. (i) All six E2-specific MAbs form a single interference group. This is particularly striking when intact virions are used. With disrupted virus, MAbs 51 and 30 compete inefficiently with MAbs 49, 50, and 23. (ii) The E2-specific MAbs compete with the E1-specific MAb 33 for binding to intact virions but not to disrupted virions. The converse is not always true. MAb 33 does not compete with MAb 49 or 50, competes inefficiently with MAb 23, but competes reasonably well with MAb 18 when intact virions are used. With disrupted virions, MAb 33 competes only with MAb 18. (iii) MAb 38, included as a control, is an E1-specific antibody that binds only to disrupted virions, in which it blocks binding of MAbs 18 and 33 but not of the others tested.

We conclude that all six E2-specific MAbs used here bind to closely spaced or overlapping epitopes and that the epitope recognized by the E1-specific MAb 33 is found close to these E2 neutralizing epitopes, at least in intact virus. Interference between MAbs 33 and 18 is maintained even in Triton-disrupted virions, for which it has been shown previously that E1-E2 heterodimers are maintained (25, 50).

Reactivity of the variants with MAbs. By using this panel of neutralizing MAbs, Stec et al. (40) isolated a number of Sindbis virus variants. Each variant is named "v" (for variant) followed by the number of the MAb used for selection. Some variants were the product of sequential rounds of selection; e.g., v23/50 was selected first with MAb 23 and then with MAb 50. (Note that this means that v23/50 and v50/23 have been independently selected for resistance with both MAb 23 and MAb 50.) Revertants are named by indicating which variant was used, followed by "R" and the number of the MAb to which it has been selected to once again be sensitive. Thus v23/50/R23 was the variant resistant to MAbs 23 and 50, selected to again be sensitive to MAb 23. Sindbis virus AR339 was the parental strain used for all variants except v49, which was selected from the HRSP strain (2). Table 2 lists the reactivities of all the variants with a number of MAbs, as measured by ELISA. As stated above, all of the anti-E2 MAbs used to generate resistant variants, as well as the single anti-E1 MAb (MAb 33), neutralized the infectivity of viruses with which they reacted by ELISA (33).

Although the competitive binding experiments indicated that the anti-E2 MAbs used here all react with the same or closely spaced epitopes, these MAbs can be grouped as reactive with two epitopes, termed A and B, on the basis of the cross-reactivity of the resistant variants (Table 2). MAbs 49, 50, and 43 react with epitope A, and variants selected to be resistant to MAb 50 are resistant to all three of these antibodies. However, MAbs 49 and 50, both of which were used to select variants, are not identical, as seen by their differing reactivities with v50, v23/50/R23, and HRSP.

MAbs 23, 18, 30, and 51 react with epitope B. Certain variants, such as v23 and v51, are resistant to all members of the group. Note that all of the independent isolates resistant to MAb 23 had this pattern of reactivity. However, other variants show complex reactivities. v30 is resistant to MAbs 30 and 51, but remains sensitive to MAbs 23 and 18. v23/50/R23 is sensitive only to MAb 23, whereas v50/23/R49 is completely resistant only to MAb 51.

A third neutralizing epitope, termed epitope C, is defined by MAbs R6 and R13 (5). Many of the variants were tested for reactivity to these two MAbs and in all cases were found to be completely sensitive, indicating that epitopes A, B, and C are each distinct. Similarly, selection for resistance to anti-E2 MAbs did not alter the reactivity of any variant with anti-E1 or anti-capsid MAbs.

Variants resistant to MAbs 18, 30, 51, and 49 were selected only once, but multiple independent isolates resistant to MAb 23 (v23, v50/23, and v33/50/23), MAb 50 (v50, v23/50, and v33/50), and the anti-E1 MAb 33 (v33 and v23/33) were obtained (Table 2).

RNA sequencing. To determine the changes in these variants that render the virus resistant to the various MAbs, the glycoprotein region of viral RNAs was sequenced directly by using a chain termination method, which has the advantage that the nucleotide recorded at each position is the majority nucleotide and does not arise from minor variants in the population. Total cytoplasmic RNA from infected cells was used as the template, and although the background with such unpurified RNA was higher than with mRNAs selected with oligo(dT)-Sepharose chromatography, the results were acceptable and the presence of rRNA and other RNAs in the preparations stabilized them to long storage. The quality of the sequence ladders was a function of the quantity of

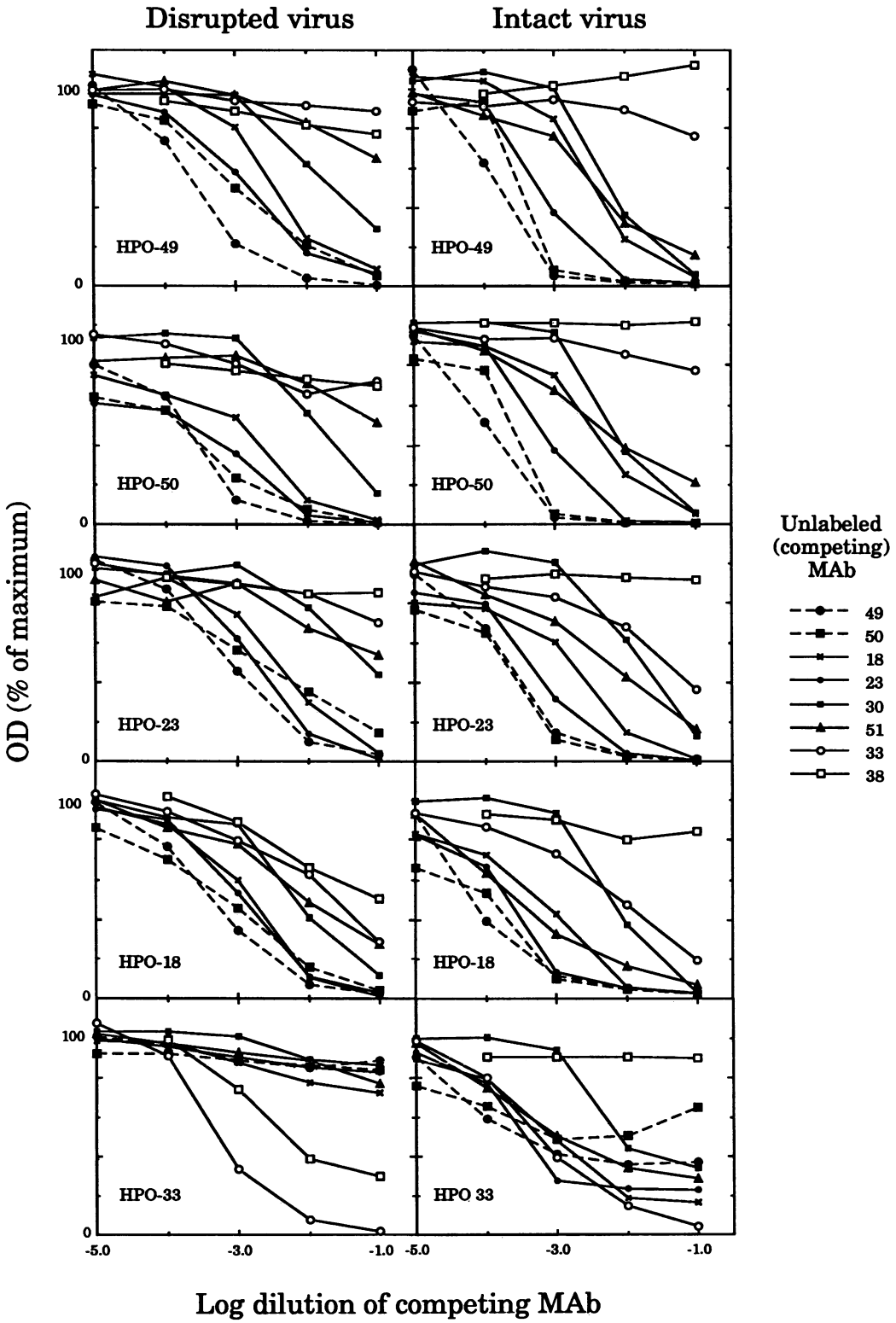

FIG. 1. Competition between unlabeled and HPO-labeled MAbs as assayed with intact or disrupted Sindbis virions. Virions were affixed to ELISA plates either by antibody-mediated capture of intact virions or by direct adsorption of Triton X-100-disrupted virions. Dilutions of unlabeled MAbs (symbols) were added to duplicate wells, followed by the HPO-labeled MAb indicated in each panel. Maximal (diluent only) optical density (OD) values ranged from 0.45 to 1.4 and were normalized to facilitate comparisons. All MAbs used neutralize Sindbis virus AR339, except MAb 38, which is one of several E1-specific MAbs shown previously to bind to disrupted (and cell surface) viral proteins but not to virion surfaces (33). Differences in antigenic topology are clearly evident, with apparently greater intimacy between E2 and E1 neutralization epitopes in intact virions than in disrupted particles.

TABLE 2. Reactivities of antigenic variants with MAbs

Virus variant	Reactivity[a] with Anti-E2[b] MAb:									Reactivity[a] with Anti-E1 MAb 33	Reactivity[a] with Anti-C MAb 3
	Epitope A			Epitope B				Epitope C[c]			
	49	50	43	23	18	30	51	R6	R13		
AR339	100	100	100	100	100	100	100	100	100	100	100
v18	100	100	100	<50	<1	<1	<1	ND[d]	ND	100	100
v30	100	100	100	100	100	<1	<1	ND	ND	100	100
v51	100	100	ND	<1	<1	<1	<1	ND	ND	100	100
v50	<50	<1	<1	100	100	100	100	100	100	100	100
v23	100	100	100	<1	<1	<1	<1	100	100	100	100
v33	100	100	100	100	100	100	100	100	100	<1	100
v23/50	<1	<1	<1	<1	<1	<1	<1	ND	ND	100	100
v50/23	<1	<1	<1	<1	<1	<1	<1	ND	ND	100	100
v33/50	<1	<1	<1	100	ND	ND	100	100	100	<1	ND
v23/33	100	100	ND	<1	<1	<1	ND	100	100	<1	100
v33/50/23	<1	<1	<1	<1	<1	<1	<1	ND	ND	<1	100
v23/R23	100	100	100	100	100	100	100	100	100	100	100
v23/50/R23	<50	<1	<1	~80	<1	<1	<1	100	100	100	100
v50/23/R49	100	100	<50	<50	<50	<50	<1	ND	ND	100	100
v50/23/R50	100	~80	~80	<1	<1	<1	<1	ND	ND	100	100
HRSP	100	~80	ND	<1	<1	<1	<1	100	100	100	100
v49	<1	<1	ND	<1	<1	<1	<1	ND	ND	100	100

[a] Reactivities are indicated in percentages as follows: 100 indicates reactivity equal to that of the AR339 parent; ~80 indicates reactivity reduced 10 to 50%, presumably as a result of lower avidity; <50 indicates reactivity reduced more than 50%; <1 indicates a strain completely resistant to the antibody. Reactivities were determined by ELISA, as described previously (40).

[b] E2 epitopes A, B, and C are separably mutable sites as determined from the patterns of cross-resistance.

[c] R6 and R13 are two MAbs characterized by Olmsted et al. (19).

[d] ND, not determined.

specific RNA present and of the oligonucleotide primers used. Only oligonucleotide primers with a G+C content of less than 45%, containing no mismatches, gave acceptable results (10). A summary of all of the amino acid changes found in the variants and their revertants is presented in Table 3.

Variants resistant to the E1-specific MAb 33. Only a single neutralizing monoclonal antibody, MAb 33, was found that reacted specifically with the E1 glycoprotein (33). Two independent resistant variants have been selected with this antibody, v33 (the multiple variants v33/50 and v33/50/23 were derived from v33) and v23/33. A schematic diagram of the E1 glycoprotein that indicates the regions sequenced for each of the variants is shown in Fig. 2. The locations of the differences between the parental AR339 strain and each of the variants are indicated. Also indicated on this figure are sequence differences between the parental AR339 strain used to generate the variants, the ATCC AR339 strain (16), and the HRSP strain (41).

Both v33 (and its derivatives) and v23/33 had only a single change in E1, and both resulted in changes in Gly-132, but to different amino acids. The sole difference within E1 between the v33 series and the parental strain is the change from G to A at nucleotide 10458, which results in the change from Gly-132 to Arg (GGA to AGA). v23/33 has a change in a different nucleotide in the same codon: G-10459 is replaced with A, resulting in the change from Gly-132 to Glu (GGA to GAA). Both changes thus result in replacement of the uncharged Gly with a charged residue.

HRSP differs from the AR339 used to generate these variants at four positions in E1, amino acids 72, 157, 237, and 399 (Fig. 2). It is interesting that two of these differences are shared with the AR339 strain supplied by the ATCC (16). The AR339 strain was isolated in Egypt in 1953 (43); the sequence data are consistent with the hypothesis that the Dalrymple strain is a lower-passage stock than the ATCC strain and that the substitutions at amino acids 157 and 399 occurred during passage in tissue culture.

A change from A to U at nucleotide 9987, changing Ile-30 to Phe in the 55-amino-acid protein (6K protein), was also found in v33 and its derivatives, but is lacking in v23/33. This change appears to be unrelated to antigenicity and is presumably an extraneous change that was fixed upon plaque purification.

Characterization of variants resistant to neutralizing MAbs that are reactive with E2. Figure 3 shows the regions within E2 sequenced in all of the variants. Several have been sequenced throughout the E2 region. For other variants, when the pattern of alterations became clear, the sequence of E2 has been determined only from the amino terminus through the region in which all of the MAb-selected differences have been found (the heavily shaded domain between amino acids 170 and 220). It seems unlikely that additional alterations outside of the regions sequenced influence the antigenic response of these variants. Table 3 summarizes all of the amino acid changes found for all of the variants, with important changes highlighted in boldface.

Variants resistant to MAb 23. Three independent variants selected to be resistant to MAb 23 and two independent revertants selected to be sensitive once again to MAb 23 have been examined. The resistant variants are v23 and its derivatives v23/50 and v23/33, v50/23 and its derivatives v50/23/R49 and v50/23/R50, and the triple variant v33/50/23. The two independent revertants are v23/R23 and v23/50/R23. In terms of reactivity, we can add another member to this

TABLE 3. Amino acid changes in antigenic variants

Virus variant	Amino acid change in glycoprotein[a]								
	E2							6K, 30	E1, 132
	172	181	184	190	205	214	216		
AR339	G	E	S	K	D	R	K	I	G
v18	(G)	E	S	K	D	R	**I**	ND[b]	ND
v30	(G)	E	**L**	K	D	R	K	I	G
v51	(G)	E	S	K	D	R	**E**	I	ND
v50	G	E	S	**M**	D	R	K	I	G
v23	G	**K**	S	K	D	R	**E**	ND	ND
v33	(G)	E	S	K	D	R	K	F	**R**
v23/50	G	**V**	S	K	D	R	**E**	ND	ND
v50/23	(G)	E	S	**M**	D	R	**E**	ND	ND
v33/50	(G)	E	S	**N**	**G**	R	K	F	**R**
v23/33	(G)	E	S	K	D	R	**E**	I	**E**
v33/50/23	(G)	E	S	**N**	**G**	R	**N**	F	**R**
v23/R23	(G)	E	S	K	D	R	K	ND	ND
v23/50/R23	G	**V**	S	K	D	R	**V**	ND	ND
v50/23/R49[c]	(G)	E	S	K	D	R	**E**	I	ND
v50/23/R50	(G)	**K**	S	K	D	R	**E**	ND	ND
HRSP	R	E	S	K	D	R	**E**	I	G
v49[d]	R	E	S	K	D	**P**	**E**	ND	ND

[a] The position of the residue in the protein is given numbered from the amino terminus of each protein. Important changes are shown in boldface. The nucleotide sequence encoding the amino acids given in parentheses could not be determined unambiguously because of a compression artifact.

[b] ND, not determined.

[c] In addition to reversion to the parental Lys-190, v50/23/R49 has a change in E2 (Gln-71 to Arg). This is the only E2 alteration found for any of the antigenic variants characterized here that occurs outside the variable domain between residues 170 and 220.

[d] The parental virus from which v49 was derived was HRSP, not AR339 as for all other variants studied. Note that the v49 amino acids at positions 172 and 216 of E2 are the same as in the HRSP parent. Two other HRSP-specific differences in E2 (Thr-3 to Ile and Glu-70→Lys) are not shown since in both cases v49 has the sequence of the parental HR. For the same reason the E1 differences in amino acid positions 72, 157, 237, and 399, shown in Fig. 1, are not listed here.

group, HRSP, which (as noted in Table 2) is also resistant to MAb 23 and other MAbs in the epitope B group.

All of the variants resistant to MAb 23 have changes at Lys-216 of E2. This amino acid is changed to Glu in v23 and its derivatives (v23/33 and v23/50), in the independent isolate v50/23 and its derivatives, and in HR. This changes the charge at this position from positive to negative. Of the two revertants, one (v23/R23) restores the original positively charged Lys and the second (v23/50/R23) inserts a neutral amino acid, Val, at residue 216. Thus, it appears that Lys-216 of E2 is a dominant residue affecting epitope B and that the charge of this residue is important. Note that in v23/50/R23, Val-216 only partially restores the reactivity with MAb 23 and fails to restore activity with MAbs 18, 30, and 51 which also react with epitope B (Table 2).

The change in the triple variant v33/50/23 that renders the virus resistant to MAb 23 is also a change in Lys-216, this time to Asn. This substitution is particularly interesting: Asn at this position creates a new asparagine-linked glycosylation site, Asn-Ile-Thr. As shown in Fig. 4, the electrophoretic mobility of E2 from this variant is significantly altered, suggesting that this site does indeed have an oligosaccharide attached. Steric hindrance due to the bulky carbohydrate moiety presumably prevents v33/50/23 from interacting with MAb 23. A similar situation has been reported by Davis et al. (5), in which a change at Thr-213 created a new glycosylation site and the resulting virus was resistant to their MAb 8, although other MAb 8-resistant variants had amino acid substitutions at Lys-216.

It should be noted that the original MAb 23 variant, v23, contains one additional change, a Glu-to-Lys substitution at amino acid 181. This appears to be a change in the v23 stock that occurred at some time after the original selection, since it is not present in either v23/50 (in which it could have been obscured by MAb 50-specific changes, some of which occur at this position) or v23/33 (in which the second selection was with an anti-E1 MAb). Revertant v23/R23 also has the parental amino acid at residue 181.

Other variants in epitope B. Single variants resistant to MAbs 18, 30, and 51 of epitope B have been examined (Table 3). v18 and v51 have substitutions for Lys-216, as did all of the MAb 23-resistant variants. In v51 the change at position 216 is the same as that in most of the MAb 23-resistant variants, Lys to Glu, and the patterns of cross-resistance of v51 and v23 are identical (Table 2). In v18 the change is from Lys to Ile, and the pattern of reactivity of this mutant with respect to epitope B is essentially identical to that of the revertant v23/50/R23 in which the amino acid at 216 is Val.

It is clear that amino acid 216 is an important element in epitope B and may also be important in epitope A (see below). Five different amino acids have been found at residue 216 in the many variants examined, involving changes in all three nucleotides of the codon. These are summarized in Fig. 5.

v30 is the only epitope B variant affected at an amino acid other than 216, and the pattern of reactivity of this variant differs from those of the others (Tables 2 and 3). The change Ser-184 to Leu renders the variant resistant to MAbs 30 and 51 while remaining reactive with MAbs 23 and 18. This is also the only example in the series of resistant variants studied here in which the substitution does not affect the net charge of the glycoprotein.

Epitope A variants. Variants resistant to MAbs reactive with epitope A gave a more complicated pattern of results than did the epitope B variants. Three independent variants selected to be resistant to MAb 50 were examined: v50 and its derivative v50/23, v23/50, and v33/50 and its derivative v33/50/23. v50 had a change at residue 190, Lys to Met. v33/50 also had a change at residue 190, Lys to Asn, but had a second change in E2 as well, Asp-205 to Gly. Both changes may be important (see below). The third variant, v23/50, had a change at residue 181, Glu to Val. Thus, in each case a change in charge is involved, and residues Glu-181, Lys-190, and possibly Asp-205 are involved in resistance to MAb 50.

Two independent revertants of v50/23 (resistance to MAb 50 as a result of the Lys-190-to-Met change) that reacquired reactivity with MAb 50 were sequenced. v50/23/R50 was selected with MAb 50, whereas v50/23/R49 was selected with MAb 49. In v50/23/R49 a simple reversion back to Lys-190 occurred. In v50/23/R50 reversion to Lys-190 also occurred, but a second change of Glu-181 to Lys was also found. The significance of this second change is unclear, especially since the Glu-181-to-Val change renders the virus resistant to MAb 50.

Results with variants resistant to MAb 49 were also complicated. It was not possible to obtain a variant of AR339 resistant to this antibody, despite repeated attempts. However, v49 was selected by growing HRSP in the presence of MAb 49, which indicated either that the change at position 214 from Arg to Pro, rendering the virus resistant to MAb 49, was tolerated only in the HR background or that one or more

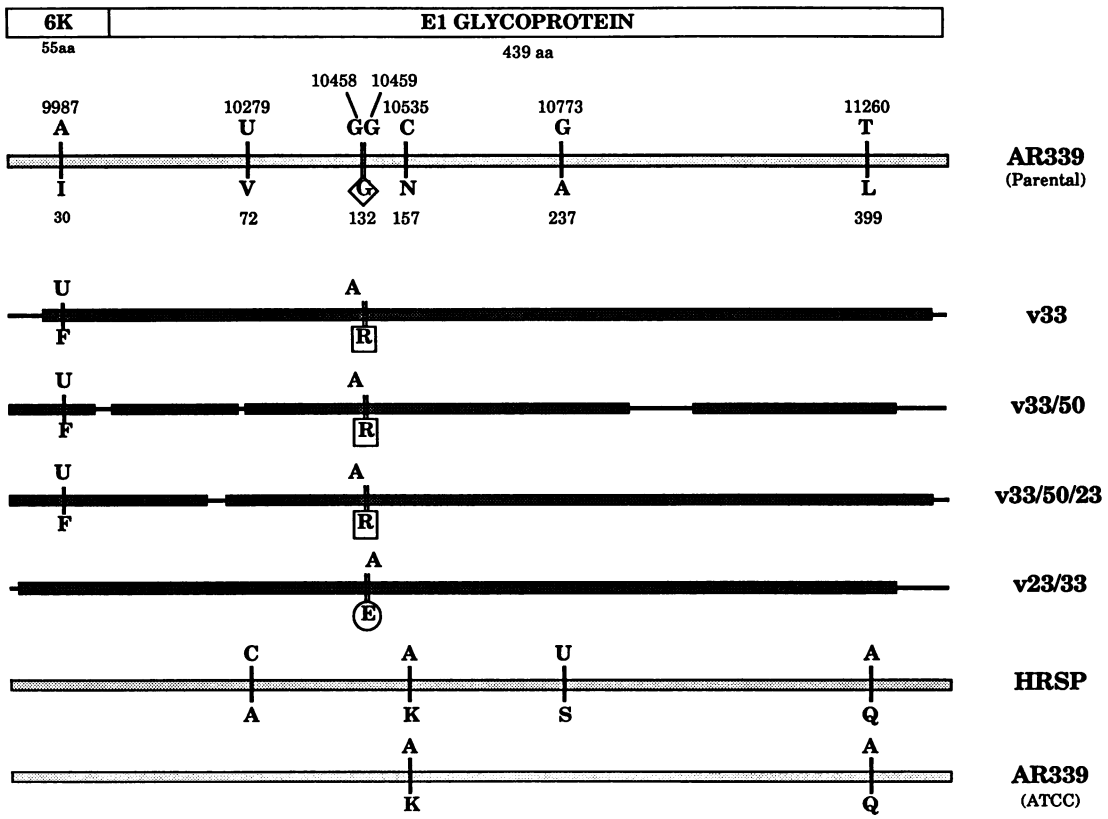

FIG. 2. Schematic representation of the 6K protein and E1 glycoprotein genes for various strains of Sindbis virus showing the regions sequenced and the nucleotide (and resultant amino acid [aa]) changes found. For the AR339 parent, obtained from J. Dalrymple, nucleotides numbered from the 5′ terminus of the genome (41) are shown above the line for each position where a change occurs in any of the strains shown below; below the line are shown the encoded amino acids, given in the single-letter amino acid code, numbered from the beginning of the 6K and E1 proteins, respectively. Changes at the boxed Gly confer resistance to neutralization by MAb 33. Below are schematics of various antibody escape variants, where the stippled boxes show the regions sequenced. Only nucleotides or amino acids different from AR339 are shown; if no nucleotide or amino acid is indicated, the sequence is identical to AR339 in the first line. At the bottom are the schematics for HRSP (41) and ATCC AR339 strain (16), showing the nucleotide and amino acid differences from the AR339 strain obtained from Dalrymple.

of the E2 changes in HRSP distinguishing it from AR339 (positions 3, 172, and 216) are also required for resistance to MAb 49.

Consideration of the reactivities with MAb 49 of the variants selected for resistance to MAb 50 are illuminating in this regard. v50 (Lys-190 to Met) is only partially resistant to MAb 49 (Table 2). v50/23, which also has the change Lys-216 to Glu, rendering the variant resistant to MAb 23, is, however, completely resistant to MAb 49. Similarly, v23/50 is resistant to MAb 49 and has Glu-216 (as well as a change of Glu-181 to Val). Glu-216 is also present in HRSP. This suggests that Glu-216 is required to obtain resistance to MAb 49 in a single step. Note that the results with v33/50 show that Glu-216 is not essential for resistance to MAb 49, as this variant has Lys-216. This variant does have two changes in E2, however, Lys-190 to Asn and Asp-205 to Gly, consistent with the hypothesis that at least two changes in the AR339 E2 sequence are required to obtain a viable virus resistant to MAb 49.

DISCUSSION

The results with the panel of MAb-resistant variants reported here indicate that domains in both E1 and E2 near glycosylation sites are important for virus neutralization. We assume that these domains interact directly with the neutralizing antibodies, although other techniques are required before this is established with certainty. A 50-amino-acid domain in E2 is illustrated in Fig. 6. Charged residues are highlighted, and the highly charged nature of this domain (25% Asp, Glu, Lys, Arg) is clear. Also clear is the importance of charged residues in the interaction with antibodies, since all but one of the changes observed affect the net charge. The carbohydrate attachment site at position 196 is indicated. In all alphaviruses sequenced to date there are carbohydrate chains attached to E2, but in only five (Sindbis, western equine encephalitis, Venezuelan equine encephalitis, Semliki Forest, and Ross River viruses) is there a site near residue 200. Furthermore, this domain is quite divergent among the various alphaviruses. There are, however, four conserved Cys residues (Sindbis virus E2 positions 201, 203, 220, and 226) in this domain, suggesting that there is a defined structure in this region even if the primary sequence is variable. The charged nature of this domain and the fact that in five viruses it contains a carbohydrate attachment site indicates that it is exposed on the surface of the protein, where it can interact with the solvent, with antibodies, and possibly with cellular receptors.

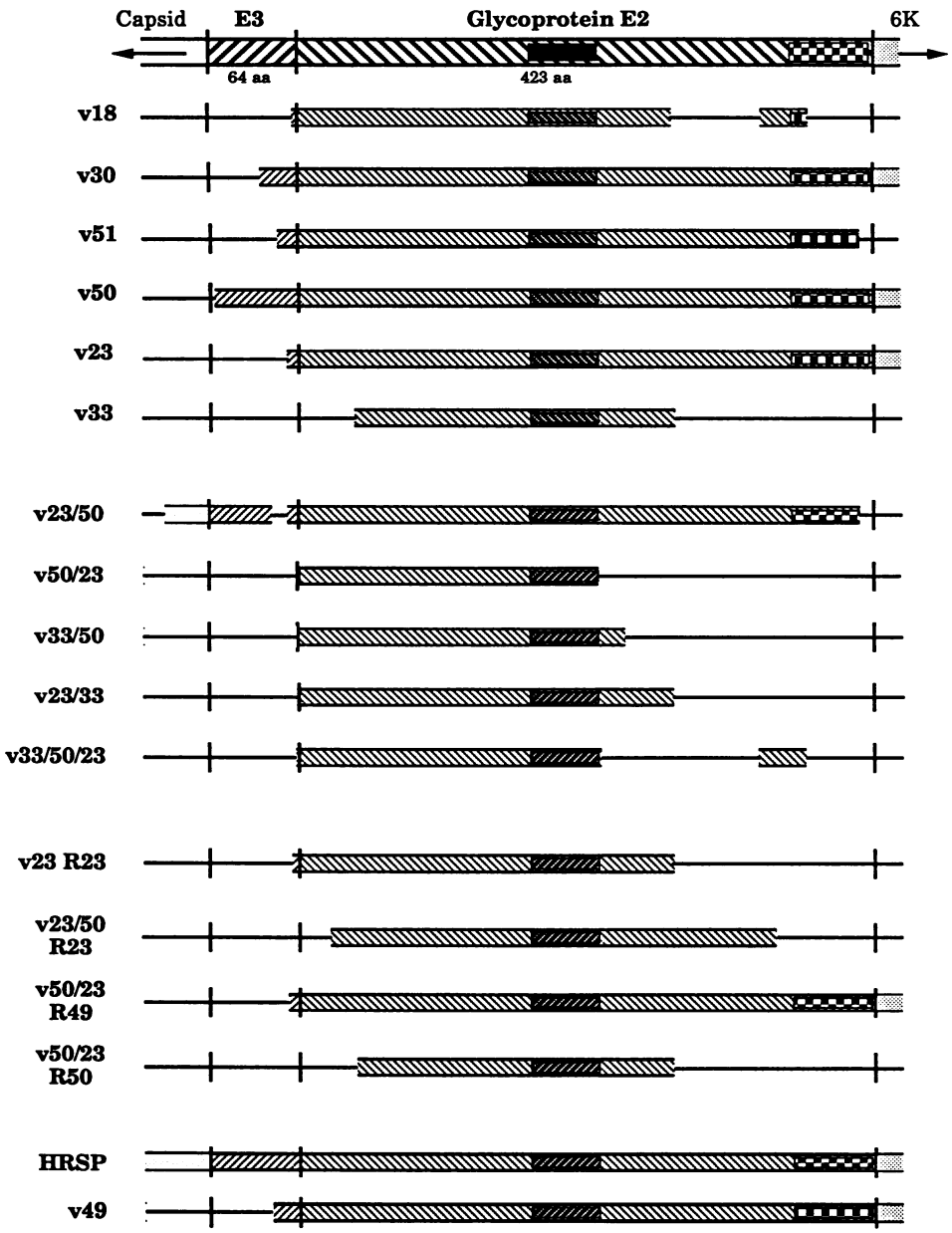

FIG. 3. Schematic of the region encoding glycoproteins E3 and E2 to illustrate the exact regions sequenced in each case. The domains shown as single lines were not sequenced. The heavily shaded box within E2 is the variable region in which all of the changes conferring resistance to neutralization were found; the checkerboard box is the hydrophobic membrane spanning domain. Abbreviation: aa, amino acid.

The variability of this region is also illustrated by the fact that in a stretch of 36 amino acids, changes in six different residues, all but one involving a change in charge, have been found that confer resistance to one or another of the MAbs examined (Fig. 6). Furthermore, four other amino acids in the 50-residue domain illustrated in Fig. 6 have been found to vary in different strains of Sindbis virus, as shown; three of these also involve changes in charge. Thus the sequence in this domain is flexible in that many changes are tolerated, including many changes in charge.

Even though two neutralization epitopes, A and B, can be defined from the pattern of cross-reaction of resistant variants, it is clear that these epitopes are overlapping and interrelated. First, competition experiments showed that all A- and B-specific MAbs tested compete for binding (Fig. 1). Second, changes in residues 184 (MAb 30) and 216 (MAbs 51, 23, and 18) render the virus resistant to B-epitope MAbs, which overlap the changes in residues 181, 190, or 205 (MAb 50) and 214 (MAb 49) that are involved in resistance to A-epitope MAbs. Third, it is clear that residue 216 modulates the reaction of the virus with the A-epitope MAb, MAb 49, as well as being a dominant residue for interaction with B-epitope MAbs.

The importance of residue 216 in the antigenicity of Sindbis virus has also been found by Davis et al. (5), who reported results with MAb 23 similar to that reported here. In addition, they obtained one MAb (MAb 8) whose phenotype is the inverse of MAb 23 in that MAb 8 requires Glu-216

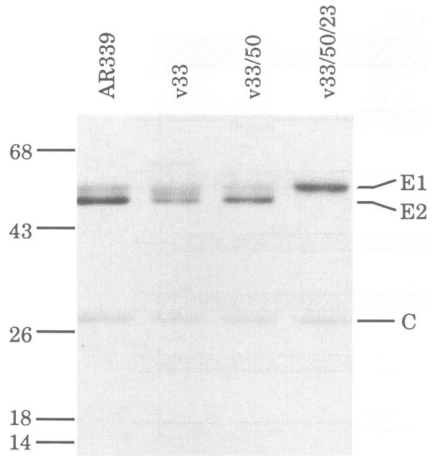

FIG. 4. Virion proteins of strain AR339, v33/50/23, and other variants analyzed by polyacrylamide gel electrophoresis (PAGE). Purified virions were dissolved in Laemmli sample buffer under reducing conditions and loaded directly onto 12.5% polyacrylamide gels (13) cross-linked with *N,N'*-diallyltartardiamide (DATD) (34). Molecular weights of marker proteins (in thousands) are indicated at the left; the locations of E2, E1, and the capsid protein from AR339 (C) are indicated at the right. Note that glycoprotein E2 of v33/50/23 has a decreased mobility and comigrates with E1.

for reactivity; variants containing Lys-216 or neutral amino acids at residue 216 were resistant to neutralization by MAb 8.

Mapping of neutralization escape variants of other alphaviruses has also shown the importance of this E2 domain in neutralization. Vrati et al. (45) found that in Ross River virus three MAbs selected variants at positions 216, 232 to 234, and 246 to 251. Four of six amino substitutions found involved a change of charge. Using four MAbs, Johnson et al. (11) found that in Venezuelan equine encephalitis virus, there were changes at residues 182, 183, 199, and 207, two of which involved changes of charge.

The E2 neutralization epitopes identified by the MAbs used here, together with the E1 neutralization epitope identified by MAb 33, are clearly immunogenic and important for virus neutralization. The triple variant v33/50/23 is neutralized only 10% as efficiently as the wild-type virus by anti-wild-type Sindbis virus antisera from either rabbits or mice (33). This phenomenon is reciprocal: sera from mice infected with v33/50/23 neutralize the variant much more

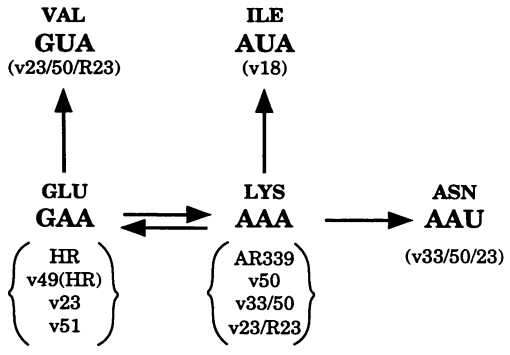

FIG. 5. Codons and encoded amino acids at E2 residue 216 that have been found in antigenic variants and their revertants.

efficiently than wild-type AR339 Sindbis virus (31a). The remaining 10% of neutralizing activity that reacts with both virus strains is presumably reactive with other epitopes.

One such additional neutralization epitope in E2 has been identified by Pence et al. (22). These authors mapped variants of Sindbis virus resistant to epitope C MAbs and showed that changes at residue 62, 96, or 159 were involved (changes at 62 and 159 also involved changes in charge). This neutralization epitope is thus distributed over disparate parts of E2, but appears distinct from the domain defined by E2 residues 170 to 220 studied here. Using a different approach, Stanley et al. (38) found that 10 of 12 MAbs in a panel specific for E2 would discriminate between two strains of Sindbis virus that differed in their neurovirulence for mice. These two strains were later found to differ at only two positions in E2, residues 55 and 209 (16), and both changes (His-55 to Gln and Gly-209 to Arg) involve charge alterations. The epitopes with which these MAbs react have not been mapped, and it is unclear whether residues 55 and 209 represent contact residues for the MAbs studied by Stanley et al. (38) or whether these two changes might induce conformational changes within E2 or the E1-E2 heterodimer that lead to differences in reactivity with multiple antibodies responding to different epitopes.

The domain of E2 identified by these studies as an important antigenic site is very likely to be involved in binding of the virus to susceptible cells and in the virulence of the virus. An anti-idiotypic antibody to MAb 49 appears to function as a high-affinity antireceptor antibody in chicken cells, and anti-idiotypic antibodies to MAbs 23 and 50 appear to be low-affinity antireceptor antibodies (46). This suggests that this domain plays a major role during binding of the virus to receptors on susceptible cells. Furthermore, two strains of Sindbis virus that differ only by having Gly or Arg at position 172 of E2 differ in their neurovirulence for mice (16) and in their ability to bind to cultured neuroblastoma cells (44), consistent with the hypothesis that this domain is important for binding to cellular receptors and suggesting that differences in neurovirulence may arise from differences in receptor affinities.

The work reported here has also identified a domain in E1 near a glycosylation site as being important for neutralization and presumably forming a neutralization epitope. Neutralizing antibodies that interact with E1 are comparatively rare. Anti-E1 MAbs isolated by Schmaljohn et al. (32, 33) were mapped to five spatially distinct epitopes by competitive binding studies. Only one epitope (defined by MAb 33) led to virus neutralization, and we show here that the change from Gly-132 to Arg or Glu renders variants resistant to this MAb. These variants grow poorly compared with the parental virus, at least under the conditions used here to prepare viral RNA, suggesting these changes are not neutral. The functional unit in the virus glycoprotein spike is the E1-E2 heterodimer (8, 25, 50), and the antireceptor on the virus that binds to the cellular receptor may be composed of domains derived from both E1 and E2. The competition studies reported here suggest that the E1-specific neutralization domain defined by MAb 33 (presumably near residue 132) and the E2-specific neutralization domain defined by MAbs 49, 50, 23, 18, 30, and 51 (presumably the domain from 170 to 220) are spatially close in the E1-E2 dimer and may form a heterodimeric domain that binds to cellular receptors to initiate infection. It would be of interest to examine the neurovirulence of MAb-resistant mutants, in particular the v33 variants, and to determine the effect of the mutations on virus binding to various cell types.

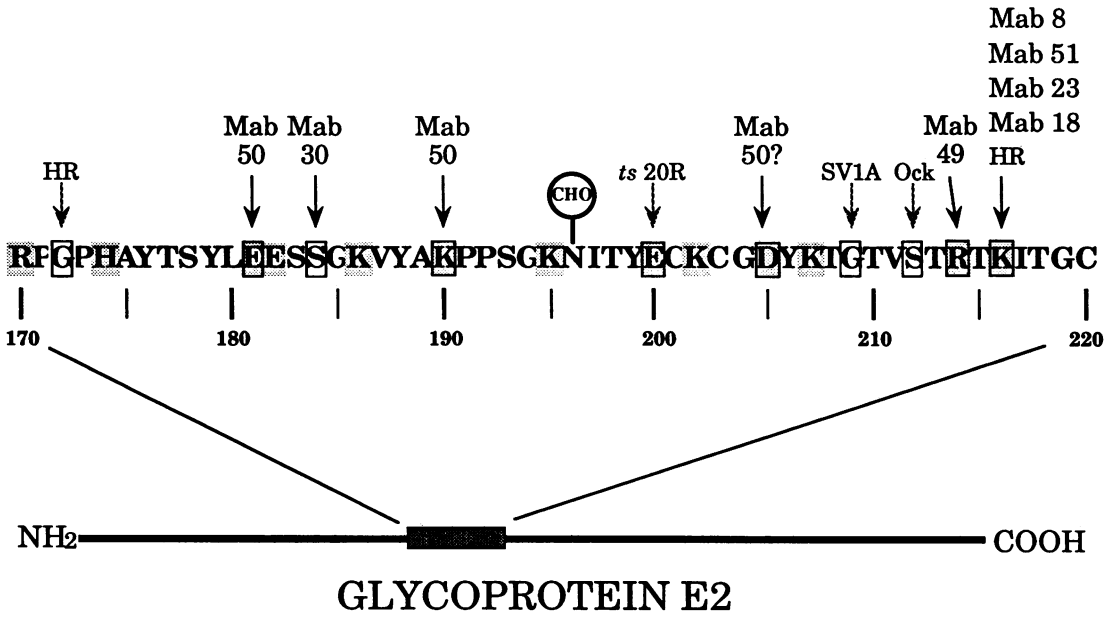

FIG. 6. Schematic diagram of glycoprotein E2, and an expanded view of the region containing the neutralization determinants. The amino acid sequence of AR339 is shown, with charged residues shaded. The location of the attachment site for a complex carbohydrate chain at residue 196 is indicated by a circle on a stem. Boxed residues are those changed in either HR (Gly-172 to Arg; Lys-216 to Glu) (41), the ATCC strain of AR339 (Gly-209 to Arg) (16), *ts*20R (Glu-200 to Lys) (14), the Ockelbo (Ock) strain of Sindbis virus (Ser-212 to Thr, Lys-216 to Glu) (36), or antibody escape variants (Table 3). MAb 8 variants were characterized by Davis et al. (5).

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