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Use of a λgt11 Expression Library To Localize a Neutralizing Antibody-Binding Site in Glycoprotein E2 of Sindbis Virus

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The Sindbis virus envelope contains two species of integral membrane glycoproteins, E1 and E2. These proteins form heterodimers, and three dimeric units assemble to form spikes incorporated into the viral surface which play an important role in the specific attachment of Sindbis virus to host cells. To map the neutralization epitopes on the surface of the virus, we constructed a λgt11 expression library with cDNA inserts 100 to 300 nucleotides long obtained from randomly primed synthesis on Sindbis virus genomic RNA. This library was screened with five different neutralizing monoclonal antibodies (MAbs) specific for E2 (MAbs 50, 51, 49, 18, and 23) and with one neutralizing MAb specific for E1 (MAb 33). When 10^6 λgt11 plaques were screened with each antibody, four positive clones that reacted with E2-specific MAb 23 were found. These four clones contained overlapping inserts from glycoprotein E2; the domain from residues 173 to 220 of glycoprotein E2 was present in all inserts, and we concluded that this region contains the neutralization epitope recognized by the antibody. No clones that reacted with the other antibodies examined were found, and we concluded that these antibodies probably recognize conformational epitopes not present in the λgt11 library. We suggest that the E2 domain from residues 173 to 220 is a major antigenic determinant of Sindbis virus and that this domain is important for virus attachment to cells.

Neutralizing antibodies bind to surface structures of a virion and interfere with the uptake and uncoating of a virus (13). In at least some cases, the antibody neutralizes by binding to the structure on the virus that interacts with a receptor on the cell surface, thus directly blocking the virus from interacting with its receptor, and antiidiotypic antibodies made against such antibodies may function as antireceptor antibodies (3, 6, 28). Various strategies have been used to map the neutralization epitopes of viruses. One is analysis of genetic variants that are resistant to a monoclonal antibody (MAb) (4, 5, 8, 11, 15, 21, 24, 27, 29). A second approach is to assay the interaction of the antibody with peptides; test polypeptides can be produced synthetically, by fragmentation of the protein, or by expression as fusion proteins in a convenient system (10).

Neutralization antibodies specific for both E1 and E2 of Sindbis virus have been isolated and characterized (2, 17, 19, 20, 22, 23). We previously sequenced variants resistant to one or more of six neutralizing MAbs specific for E2 and one neutralizing MAb specific for E1 to identify amino acid changes that led to escape from neutralization (24). All six E2-specific MAbs selected variants in a domain of E2 between residues 181 and 216, indicating that this region was of considerable importance for the antigenicity of E2. We have also shown (28) that anti-idiotypic antibodies to three of these E2-specific neutralizing antibodies function as antireceptor antibodies in chicken cells, suggesting that the neutralization epitopes defined by these antibodies form part of the virus antireceptor that attaches to the host cell receptor to initiate virus infection.

Mapping of amino acid changes that lead to loss of antibody binding has often correctly identified residues that form part of an epitope (reviewed in reference 9). Thus, it seemed likely that the E2 domain defined by the amino acid changes leading to escape from neutralization, which also lead to loss of the ability to bind the antibody, interacted directly with the neutralizing MAbs and, by extension, that this domain also forms part of the virus antireceptor. However, MAb-resistant variants in which single amino acid substitutions away from the actual antibody-binding site itself led to escape from neutralization have been described (5, 14). In such cases, changes in conformation of the antibody-binding domain were induced by substitutions outside the epitope. Thus, it is important to define, if possible, the antibody-binding domain by using a direct binding method. We reasoned that the bacterial expression vector λgt11, in which foreign epitopes are expressed as fusion proteins with β-galactosidase, might be suitable for this purpose, provided that an epitope is contiguous. Recombinant λgt11 cDNA libraries can be screened with antibodies to identify specific recombinant clones containing inserts leading to reactivity with the antibody (30).

Construction and screening of the bacteriophage library. Sindbis virus strain AR339, from A. Schmaljohn (24), was grown in monolayers of primary chicken embryo fibroblasts (16). Virus was purified, as previously described (1), and disrupted with 0.5% sodium dodecyl sulfate, and 49S genomic RNA was extracted with phenol-chloroform (7). After two ethanol precipitations, the RNA was suspended in distilled H_2O and stored at −70°C until use as a template for cDNA synthesis.

A λgt11 library containing short inserts of Sindbis virus cDNA was constructed by a modification of the procedure of Young and Davis (30). cDNA synthesis was randomly primed with sonicated salmon testis DNA; [^{32}P]dCTP was included during cDNA synthesis to monitor the product. After treatment with the Klenow fragment of DNA polymerase I, methylation with *Eco*RI methyltransferase, and addition of *Eco*RI linkers (Collaborative Research), the modified cDNA was digested with an excess of *Eco*RI restriction enzyme. The digested cDNA was then fractionated on a Sephadex CL-6B column (Pharmacia), and Sindbis virus

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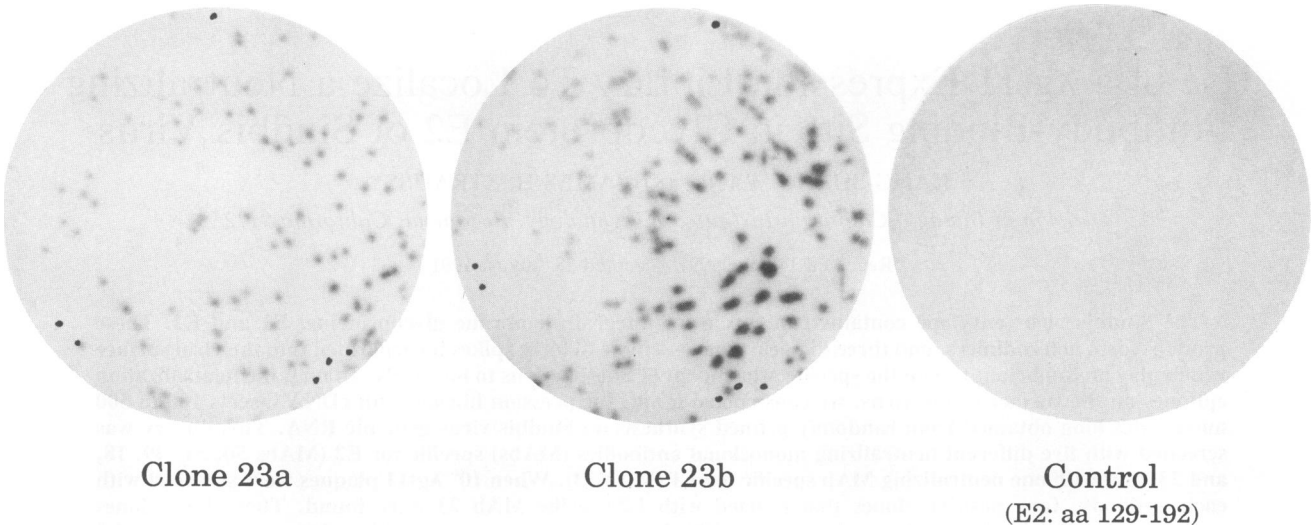

FIG. 1. Reactivities of phage clones λ23a and λ23b with MAb 23. Immunoreactive phage plaques were picked and rescreened until a uniformly reactive population was obtained. Illustrated are the final populations for two reactive clones and a nonreactive clone selected for other reasons. These phage stocks were plaqued on *E. coli* Y1090 in the presence of the inducer, and plaques were transferred to nitrocellulose filters as described in the text. The filter was incubated with MAb 23 and then with ^{125}I-conjugated protein G and autoradiographed. Comparison of the autoradiogram with the plaque population found on the petri plates from which the filters were obtained showed that all of the phage plaques in λ23a and λ23b reacted with the antibody. A λgt11 population expressing E2 residues 129 to 192 did not react, as shown at the right. aa, amino acids.

cDNA fragments 100 to 300 bp long were pooled and ligated to dephosphorylated λgt11 arms (Promega). After in vitro packaging into phage heads (Stratagene), the percentage of phage containing Sindbis virus cDNA inserts was found to be 90% by plating phage on *Escherichia coli* Y1090 in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Plaques were screened for reactivity with the various MAbs. Phage plaques were grown for 6 h at 42°C, nitrocellulose disks (Schleicher & Schuell) soaked in 10 mM isopropyl-β-D-thiogalactopyranoside were then placed on the top of the agar layer, and the plates were transferred to 37°C for 15 h. The filters then were lifted and washed successively in 10 mM Tris-Cl (pH 7.5) and 150 mM NaCl containing 5% nonfat milk. The filters were incubated overnight at 4°C with a MAb (10 μg/ml in phosphate-buffered saline containing 5% nonfat milk) and washed, ^{125}I-conjugated protein G (0.5 μCi/ml in 5% nonfat milk) was added, and the filters were incubated for at least 2 h at room temperature. After washing and drying, the filters were exposed overnight at −80°C to Kodak X-Omat film. Immunoreactive phage were picked and rescreened until a uniformly reactive population was obtained.

Mapping of the location of E2 sequences reactive with MAb 23. The λgt11 library containing randomly generated 100- to 300-bp Sindbis virus cDNA inserts in the *lacZ* gene was tested for reactivity with six MAbs by using ^{125}I-labeled protein G (Amersham) to detect the presence of the MAbs (all were immunoglobulins G) bound to immunoreactive phage clones on nitrocellulose filters. Four positive phage clones, designated λ23a, λ23b, λ23c, and λ23d, were identified when MAb 23 was used to screen 10^6 plaques. The reactivities of λ23a and λ23b with the antibody are shown in Fig. 1. Results obtained with a negative λgt11 clone that expresses a different region of E2 are also shown.

DNA was prepared from each of these four reactive phages (30). The inserts were removed with *Eco*RI, subcloned into vector M13mp18, and sequenced by the dideoxy-chain termination procedure (18). The four inserts contained overlapping sequences from the central region of glycoprotein E2 (Fig. 2). The insert in λ23a comprised E2 residues 155 to 258, that in λ23b comprised residues 173 to 251, that in λ23c comprised residues 145 to 223, and that in λ23d comprised residues 169 to 220. Thus, the domain from residues 173 to 220 is present in all four inserts and the neutralizing epitope recognized by MAb 23 must lie within this region.

During these experiments, additional λgt11 clones which failed to react with MAb 23 were sequenced. Some of these were found to express regions of E2. Noteworthy among these were clones expressing E2 residues 129 to 192 (Fig. 1), 135 to 187, 141 to 203, 147 to 183, and 153 to 191. Thus, the presence of residues between 204 and 220 are required (but are not necessarily sufficient) for reactivity with MAb 23.

Results obtained with other MAbs. We also attempted to identify fusion proteins immunoreactive with four other E2-specific neutralizing MAbs, namely, MAbs 18, 50, 51, and 49, as well as fusion proteins immunoreactive with MAb 33, which is specific for glycoprotein E1. In each case, 10^6 plaques were screened. No positive plaques could be identified with any of these antibodies. We conclude that these antibodies probably react with conformational epitopes not present in the λgt11 library, because these epitopes either are discontinuous or consist of conformations not assumed by the fusion proteins.

An antibody-binding domain in E2. Various approaches have been used to map the epitopes on proteins that react with MAbs. Only determination of the three-dimensional structure of the antigen-antibody complex can fully map the interactive residues; of the five such structures determined, the epitopes comprise 15 to 22 amino acids and are discontinuous, formed from different parts of the polypeptide chain (reviewed in reference 9). Furthermore, it is to be expected that even if an epitope is contiguous, its conformation in the native protein is important for its reactivity and short syn-

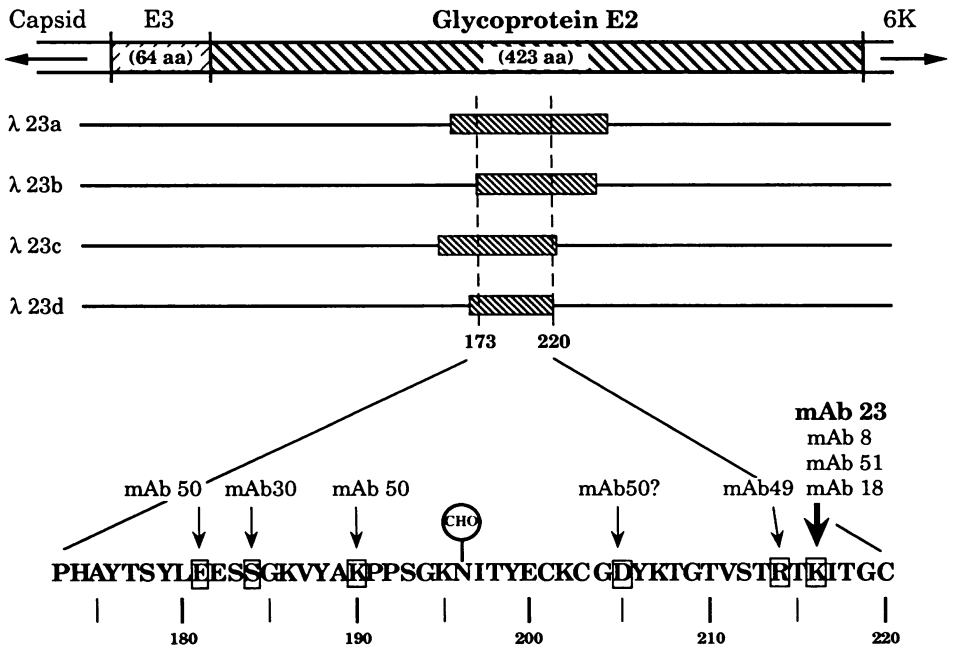

FIG. 2. Schematic representation of an antigenically important domain of Sindbis virus glycoprotein E2. The genome region encoding glycoprotein E2 is represented. The relative locations of the inserts in four λgt11 clones reactive with MAb 23 are mapped. The overlap region in these four clones between residues 173 and 220 of E2 is expanded below, with a number of key features indicated. Residues altered in variants resistant to MAbs are boxed (24), and a carbohydrate attachment site (CHO) is indicated. aa, amino acids.

thetic peptides might not react with the antibody. Previous attempts to map the binding site for MAb 23 and other anti-Sindbis virus MAbs by using synthetic peptides representing residues 175 to 194 and 206 to 220 of Sindbis virus E2 were not successful (18a). We have explored the use of the λgt11 system as a way to identify regions of a virus protein that react with neutralizing antibodies. If an epitope is substantially contained within a contiguous region of the polypeptide chain, and the polypeptide domain containing the epitope is capable of folding into a reactive conformation as part of the fusion protein, some fusion proteins expressed in phage plaques might react with the MAb. We found that one neutralizing MAb tested, MAb 23, reacted with fusion proteins in λgt11 plaques.

From the sequence of the inserts in the four clones immunoreactive with MAb 23, it is clear that this antibody can react with a single contiguous region of Sindbis virus glycoprotein E2 and that the neutralization epitope must be contained substantially, if not completely, within the 48 residues between amino acids 173 and 220. This result is consistent with the results from mapping of antibody escape variants resistant to MAb 23 (Fig. 2). Sequencing of three independent variants resistant to MAb 23 and two independent revertants selected to be sensitive again to MAb 23, as well as of other variants, has shown that residue 216 is important for reactivity with MAb 23 (24). Viruses with Lys-216 were fully sensitive to MAb 23, viruses with Val-216 or Ile-216 demonstrated reduced sensitivity to MAb 23, and viruses with Glu-216 were resistant to MAb 23. From the results obtained here, it appears likely, therefore, that residue 216 interacts directly with MAb 23, forming part of the epitope.

Although the remaining antibodies tested failed to react with the λgt11 library, it seems likely that E2-specific MAbs 50, 51, 49, and 18 also bind to epitopes at least partially encompassed within this same domain. Variants selected to be resistant to these MAbs were all found to have amino acid changes responsible for the escape from neutralization within the domain from residues 181 to 216 (Fig. 2) (24). Furthermore, MAb 23 and these other MAbs all react with closely spaced or overlapping epitopes as defined by competition assays or by the pattern of cross-reactivity of different variants resistant to the various antibodies (4, 20, 24). Interestingly, residue 216 appears to form a component not only of the epitope for MAb 23 but also of the epitopes of MAbs 51, 18, and 8 (Fig. 2). The epitope for MAb 23 appears to be contiguous and located entirely within this region. Those for the other MAbs tested, including 51 and 18, are either discontinuous, composed in part of residues within this region and in part of residues in other regions, or continuous, in which case the MAbs must react with conformations not present in the fusion library. The latter explanation seems likely for at least MAbs 49 and 50, because these MAbs react with sodium-dodecyl sulfate-denatured and reduced E2 in a Western blot (immunoblot), as is also the case for MAb 23 (18a).

The results are consistent with the hypothesis that the E2 domain between 173 and 220 forms a major antibody-binding region important for neutralization of virus infectivity. This domain is illustrated in Fig. 2, with the locations of antibody escape variants shown and the region selected by MAb 23 indicated. This domain is hydrophilic, containing 25% charged residues, and has a glycosylation site at Asn-196 and thus is almost certainly exposed on the surface of the glycoprotein spike (25).

We have previously found that an anti-idiotypic antibody to MAb 23, as well as anti-idiotypic antibodies to MAbs 49 and 50, functions as an antireceptor antibody in chicken cells (28). This suggests that the E2 domain defined by the fusion protein reactive with MAb 23 and by the antibody escape

variants forms part of the antireceptor on the virus spike that binds to the cellular receptor. This hypothesis is supported by the observation that two strains of Sindbis virus that differ only in having Gly or Arg at residue 172 of E2 differ in both their ability to bind to neuroblastoma cells in culture (26) and in their neurovirulence for mice (12).

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