Structural Rearrangement of Infecting Sindbis Virions at the Cell Surface: Mapping of Newly Accessible Epitopes

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Sindbis virus glycoproteins E1 and E2 undergo a conformational alteration during early virus-cell interaction at the cell surface (D. Flynn, W. J. Meyer, J. M. MacKenzie, Jr., and R. E. Johnston, *J. Virol.* 64:3643-3653, 1990). Certain epitopes normally internal on native virus become accessible to monoclonal antibody (MAb) binding after attachment but before internalization of virus particles. These newly exposed epitopes, termed transitional epitopes, may be part of functionally important domains made accessible at the surface of the altered virus to facilitate entry into cells. Heating Sindbis virions at 51°C for a short time induced a similar, although not identical, exposition of transitional epitopes on the E1 and E2 glycoproteins (W. J. Meyer, S. Gidwitz, V. K. Ayers, R. J. Schoepp, and R. E. Johnston, *J. Virol.* 66:3504-3513, 1992). In the current report, we have identified several of the transitional epitopes that become exposed as a consequence of early virus-cell interactions. Transitional epitope MAbs that bound to rearranged, heated virions and virus-cell complexes were used in antibody competition binding assays on heated Sindbis virions to map the spatial relationships between native, external, neutralizing antigenic sites and newly exposed transitional epitopes. Because the heated, rearranged particles retained their infectivity, MAbs that bound to transitional epitopes also were used to isolate MAb neutralization escape mutants. Sequencing the glycoprotein genes of the escape mutants identified specific E1 and E2 loci where mutation prevented MAb binding to transitional epitopes. One of the transitional epitopes identified (E2 residues 200 to 202) lies in the E2 190-216 region, which harbors two major neutralization sites, E2a and E2b, and an N-linked glycosylation site at E2 196. The glycosylation signal was eliminated by site-directed mutagenesis of a full-length cDNA clone of the Sindbis virus genome. The absence of a carbohydrate moiety did not expose the transitional epitopes mapped to this locus, suggesting that on native virions, the inaccessibility of the E2 200-202 determinant was inherent in the structure of the glycoprotein spike.

The early stages of virus infection include a number of critical steps, each involving an interaction of the infecting virion with an element of the cell, culminating with the release of the viral genome in a form suitable for gene expression. Understanding the nature of these events will require identification and characterization of the virion structural intermediates generated during this disassembly process and the cellular elements which catalyze the conversion of one intermediate to the next. We report here the antigenic and topographic characterization of the initial structural intermediate in the disassembly pathway for the prototype alphavirus, Sindbis virus.

The genome of Sindbis virus is a positive-sense, single-stranded RNA and is contained within a T=4 nucleocapsid composed of capsid (C) protein subunits (9, 31, 32). The nucleocapsid is surrounded by a host cell-derived, lipid envelope containing two viral glycoproteins, E1 and E2 (19, 38). Within the envelope, E1 and E2 are associated as heterodimers which in turn are assembled in groups of three to form an icosahedral arrangement at the virus surface (15, 31, 36). The carboxy terminus of one (or both) of the glycoproteins, probably that of E2, is thought to associate with capsid protein subunits at the inner face of the envelope, accounting for the T=4 symmetry of the glycoprotein spikes on the exterior face. Lateral interactions between the glycoproteins serve to stabilize the structure (2).

In the infection of baby hamster kidney (BHK) cells, Sindbis virions appear to be converted to a first disassembly intermediate at the cell surface, presumably in response to their interaction with receptor or the microenvironment at the cell surface (8, 13). Additional structural changes may occur at the cell surface, leading to fusion with the plasma membrane and release of the nucleocapsid into the cytoplasm (1). Alternatively, the initial cell surface alterations may precede virion endocytosis, subsequent rearrangement(s) triggered by the low-pH environment of the endosome, fusion with the endosomal membrane, and release of the capsid (22, 50). The final event is uncoating, removal of the genome from the nucleocapsid, may be mediated by an interaction of the newly released capsid with cytoplasmic ribosomes (42, 49).

Changes in the conformation of Sindbis virus and other alphaviruses have been detected by probing suspected structural intermediates with monoclonal antibodies (MAbs) or proteases. Structural rearrangements in response to treatment at low pH or after endocytosis have been inferred by changes in the protease sensitivities of E1 and/or E2 (12, 22) or by changes in the reactivities of MAbs raised to low-pH-treated virions (37). Under conditions of hemagglutination or during morphogenesis, alterations in glycoprotein conformation were detected as changes in the constellation of accessible glycoprotein epitopes (40). Our laboratory has used MAb probes to demonstrate an early conformational alteration of Sindbis virions at the cell surface (13). A subset of MAbs specific for inaccessible epitopes on glycoproteins E1

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and E2 of native virus, and hence not capable of binding intact virus in solution, nevertheless binds to virus-BHK cell complexes at 37°C. Such newly exposed epitopes have been termed transitional epitopes. A step early in the interaction of the virus with the cell triggers the rearrangement in the absence of a low-pH involvement. The alteration occurs either before or coincident with penetration, and rearrangement of a rapidly penetrating mutant is accelerated. Treatment of virus-cell complexes with MABs to a transitional epitope retarded penetration of replication-competent virions, suggesting that the rearrangement produced an early intermediate in a disassembly pathway leading to productive infection.

A glycoprotein rearrangement very similar to that apparent at the cell surface can be artificially induced by heating Sindbis virions to 51°C for 5 to 10 min (28). The constellation of accessible native and transitional epitopes displayed on virions at the cell surface is closely approximated on heated particles. In this study, we have used these heated virions to determine the topographic relationships between several of the transitional epitopes as well as between the transitional epitopes and previously mapped neutralization sites on glycoprotein E2. The rearranged, heated virions retain infectivity and can be neutralized by the transitional epitope MABs (28). Therefore, we have isolated neutralization escape mutants which bound reduced amounts of the selecting transitional MAB. By sequencing the glycoprotein genes of such mutants, the presumptive epitopes for several of the transitional MABs have been mapped to specific regions of the primary amino acid sequence.

MATERIALS AND METHODS

Virus strains and cells. All virus strains were grown on BHK cells maintained in Eagle's minimal essential medium (GIBCO) containing 5% penicillin and streptomycin, 10% tryptose phosphate broth (Difco), and 10% donor calf serum. Our laboratory strain of Sindbis virus, AR339, was supplied originally by H. R. Bose, University of Texas, Austin. SB-RL (SB-reduced latent period) was derived from Sindbis virus AR339 as previously described (5). TR5000 is a recombinant virus obtained by transfection of BHK cells with transcripts generated *in vitro* from a plasmid containing Toto 1101 nucleotides 1 through 2712 and 11485 through the 3' poly(A) tract (34) and Sindbis virus AR339 sequences from nucleotides 2713 through 11484. The Sindbis virus AR339 nucleotides encode all of the structural proteins of TR5000. Plasmids pTR5196 and pTR5278 were derived by site-directed mutagenesis of TR5000 by the method of Kunkel et al. (23). The pTR5196 construct has a glutamine codon (CAG) substituted for the wild-type asparagine (AAC) at amino acid position 196 of the E2 glycoprotein. The pTR5278 construct has a tyrosine codon (UAC) substituted for the wild-type histidine (CAC) at E2 position 278.

MABs. MABs against Sindbis virus were produced from two separate hybridoma fusions. In the first fusion, mice were immunized and boosted with native SB-RL. Isolation and characterization of MABs R6, R8, R10, and R15 have been described previously (29). MABs R2, R4, and R12 were also from the first fusion and are specific for inaccessible epitopes on the E1 and E2 glycoproteins of native Sindbis virus (13). Antibody specificity was determined by radioimmunoprecipitation as described previously (29) or with the following modifications. Infected cells were labeled with 20 μ Ci of [³⁵S]methionine per ml for 10 min at 4.5 h postinfection. After overnight incubation of the labeled lysates with

MAB, 2.5 μ l of goat anti-mouse immunoglobulins (Ig) (Sigma) was added, and the mixture was incubated for 4 h at 4°C before addition of Pansorbin (Calbiochem). Immunoprecipitates were washed in lysis buffer containing 1 M NaCl. In some experiments, lysates were prepared in sodium dodecyl sulfate (SDS)-containing buffer either with or without 1 M NaCl. In other experiments, Affi-Gel 10 (Bio-Rad) or cyanogen bromide-activated Sepharose (Pharmacia), conjugated with goat anti-mouse Ig, was used to precipitate protein-antibody complexes. The manufacturers' suggested conditions were used in both cases.

The R500 series of antibodies was generated from a second fusion. BALB/c mice were injected intraperitoneally and subcutaneously with 25 μ g of SB-RL in Freund's complete adjuvant. Four days later, the mice were infected with 10⁸ PFU of purified SB-RL by subcutaneous inoculation. After 4 months, one mouse was selected and boosted intravenously with 100 μ g of SB-RL that had been boiled for 5 min in 0.1% SDS. The next day, the mouse was injected intraperitoneally with 100 μ g of SB-RL that had been boiled in 0.36% SDS. Two days later, the mouse was sacrificed, the spleen was removed, and the fusion was completed. Glycoprotein E1 or E2 specificity of the antibodies was determined either by Western immunoblot analysis (45) or by nucleotide sequence analysis of MAB-selected neutralization escape mutants.

MABs 31, 49, and 50 were gifts of Alan Schmaljohn and have been characterized previously (39). Isotyping of all MABs listed above was performed either by using a mouse immunoglobulin subtype identification kit (Boehringer Mannheim) or by immunodiffusion.

Capture and CAB assays. The capture assay was designed to maintain virion structure in as near a native state as possible. MABs were bound nonspecifically to an enzyme-linked immunosorbent assay (ELISA) plate, and then virus was added. The ability of the capturing antibody to bind the virus was detected with horseradish peroxidase (HRP)-labeled anti-Sindbis virus hyperimmune ascites fluid (HMAF). The competitive antibody binding (CAB) assay was a modified version of the capture assay designed to evaluate the spatial relationships between determinants of the virus and has been described previously (30). Briefly, the competing MAB was adsorbed to the surface of a microtiter plate to capture freshly purified, native or heated virus. Additional competing MAB was added to bind the remainder of the available cognate epitopes. HRP-labeled test MAB was added. The degree to which binding of the HRP-labeled MAB was inhibited was determined by comparison with binding of HRP-labeled test MAB to capture virus that received no additional competing MAB, measuring optical density (OD) values. Heated virus was prepared for use in the capture or CAB assay by diluting freshly grown and gradient purified SB-RL to a volume of 1 to 1.5 ml in phosphate-buffered saline (PBS) with 1% donor calf serum (PBS-1%). The sample was heated for 5 or 10 min at 51°C to expose transitional epitopes. After heating, the virus was further diluted to a working volume with PBS-3% ovalbumin and used in the capture or CAB assay.

ELISA. Virus was placed in the wells of a 96-well microtiter plate. The plate was placed at 37°C overnight to allow the virus solution to evaporate. This procedure disrupts the virus structure so that inaccessible epitopes become exposed. However, external epitopes remain intact and accessible to antibody binding. Details of the assay have been described previously (30).

Selection of MAb neutralization escape mutants. All MAb neutralization escape mutants were selected from stocks of SB-RL. Escape mutant 12-1 was isolated by heating an infected-cell supernatant containing SB-RL (approximately 8×10^8 PFU/ml) at 51°C for 5 min to expose transitional epitopes. The stock was diluted to 2.5×10^5 PFU in 0.25 ml of PBS-1%. A 100- μ l aliquot of MAb R12 ascites fluid and 50 μ l of guinea pig serum (as a source of complement; Cedarlane) were added to the heated virus stock. The mixture was incubated at 37°C for 1 h. After incubation, 20 μ l of goat anti-mouse IgG (Sigma), 50 μ l of complement, and 30 μ l of PBS-1% were added to the virus-MAb R12 mixture to enhance virus neutralization. The solution was incubated for 1 h at 37°C, and then a 60-mm-diameter tissue culture plate of BHK-21 cells was infected with 200 μ l of the mixture. Progeny virus-containing culture supernatant was harvested following the appearance of cytopathic effect. This procedure was repeated three more times to selectively amplify the variant population, using 10 μ l of the culture supernatant as inoculum for the ensuing round of enrichment. After amplification, the supernatants were titrated in a plaque assay, and isolated plaques were picked. The agarose plugs in PBS-1% were seeded directly onto BHK cells in 24-well tissue culture plates. The supernatants from wells showing cytopathic effect were assayed for virus capable of escaping R12 binding in a capture assay. These candidate mutants were grown and purified. The virus suspensions were evaporated to dryness in a microtiter plate, and the lack of reactivity with R12 was reconfirmed by ELISA. One of the isolates (RLmc12-1) was grown again in the presence of MAb R12 and plaque purified to ensure its genetic purity.

Neutralization escape mutants were isolated with MAbs R15, R501, R502, R503, R505, R2, and R4, using the same basic protocol as that used for RLmc12-1 except that the starting stock of SB-RL was diluted to 10^5 PFU in 200 μ l of PBS-1% and MAb concentrations were optimized for the ability to neutralize in the presence of a rabbit anti-mouse IgG (Sigma). Optimal final dilutions of neutralizing MAb varied from 1:90 to 1:4,500. MAb and virus were allowed to incubate for 1 h at 37°C before the addition of 50 μ l of rabbit anti-mouse IgG. The mixture was incubated at 37°C for 15 min, 50 μ l of complement was added, and the mixture was further incubated for 45 min at 37°C. Antibody R2 and R4 escape mutants appeared to be less common in the population of virus stocks, so dilutions of the collected supernatants were not made after their passage under antibody selection. Amplification of all escape mutants was accomplished by repeating this procedure four times.

Escape mutant RLmc12-19 was isolated by a different approach. Virus was diluted and heated as described above, and 50 μ l of R12 ascites fluid was added. After incubation at 37°C for 1 h, 150 μ l of a gold-labeled goat anti-mouse IgG preparation (13) was added, and the mixture was incubated for 1 h at 37°C. After incubation, the solution was centrifuged at 13,000 rpm for 20 min in a Fisher microcentrifuge. The supernatants were collected and used to infect monolayers of BHK cells in 60-mm-diameter dishes. This process was repeated three more times to amplify the mutant population, using the supernatants from plates displaying cytopathic effect. Plaque-purified isolates of the escape mutants were obtained and screened for lack of R12 reactivity by the method described above.

Sequencing the mutants. Sequencing of the glycoprotein genes of the variants was done by oligonucleotide-primed dideoxynucleotide chain termination (51) directly on purified

viral or intracellular RNA, using primers and protocols as detailed by Davis et al. (11).

Intracellular RNA was isolated by the following method. BHK cells were infected at a multiplicity of infection of 2 to 10. Six hours postinfection, the cells were washed twice with a hypotonic buffer (3.3 mM NaCl, 0.5 mM MgCl₂, 3.3 mM Tris-HCl [pH 8.5]). The cells were collected in 1 ml of the buffer by using a rubber policeman and allowed to swell for 10 min before the addition of 0.45% Nonidet P-40. The mixture was vortexed and then centrifuged at $800 \times g$ to eliminate nuclei. RNA was extracted from the supernatants by using phenol-chloroform-isoamyl alcohol. The RNA was precipitated overnight at -20°C in 2.5 volumes of ethanol and 0.025 volume of 4 M NaCl.

In vitro mutagenesis. TR5196 and TR5278 were products of site-directed mutagenesis performed by a modification of the method of Kunkel et al. (23) in which Klenow fragment (2,250 U; Promega) was used in place of T4 DNA polymerase. An M13 DNA template containing SB-RL E2 sequences was used for oligonucleotide-primed mutagenesis. Restriction endonuclease digestions of the replicative forms of the plasmids containing the mutagenized E2 genes and of pTR5000 were performed, and an E2 fragment (*Stu*I nucleotide 8571 to *Bss*HII nucleotide 9804) of pTR5000 was replaced by the mutagenized homologous sequences. The plasmids were used to transform *Escherichia coli* JM101, and transformants were picked and grown. Plasmid DNA samples were screened by restriction enzyme digestion, the appropriate plasmid DNA was cut with *Xho*I, and full-length Sindbis virus transcripts were produced in vitro from an SP6 transcriptional promoter. The specific infectivities of the transcripts were shown to be equivalent to that of wild-type pTR5000 transcripts. Transfection of BHK cells was accomplished with Lipofectin (Bethesda Research Laboratories) as recommended by the manufacturer. TR5196 and TR5278 were recovered from the transfection supernatants. The genomic RNAs of TR5196 and TR5278 were sequenced to confirm the base changes at E2 codons 196 and 278, respectively.

RESULTS

Accessibility of Sindbis virus glycoprotein epitopes. The MAbs used in this study, their isotypes, E1 or E2 glycoprotein specificity, and epitope accessibility, based on previously published results (13, 28), are summarized in Table 1. Accessible epitopes are present on the surface of the glycoprotein spike and allow binding of MAb to native virus. MAb binding to inaccessible epitopes is inhibited either because of steric hindrance at the surface of the glycoprotein as a result of the size of the MAb molecule or because the tertiary structure of the glycoprotein places the epitope beneath the surface of native virions.

The accessibility of an epitope was determined by the binding pattern of the MAb in two assays. In the first assay, Sindbis virus was evaporated in wells of an ELISA microtiter plate, resulting in partial disruption of virions and exposure of previously inaccessible epitopes, while accessible epitopes remained intact. Individual MAbs were tested for the ability to bind disrupted virus as detected by HRP-labeled goat anti-mouse Ig. In the second assay, MAb was bound nonspecifically to an ELISA plate, and purified native SB-RL was added. Virion capture was detected by HRP-labeled anti-Sindbis virus HMAF. If a MAb bound virus in both assays, the epitope was considered accessible on the surface of native virions. If the MAb bound to disrupted

TABLE 1. Accessibility of Sindbis virus glycoprotein epitopes

MAb	Glycoprotein specificity	Isotype	Epitope accessibility		
			Native virions ^a	Cell surface virions ^b	Heated virions ^c
R509	E2	IgG1	—	—	—
R512	E2	IgG1	—	—	—
R3	E1	IgA	—	ND ^d	+
R4	E1	IgA	—	±	+
R500	E1	IgG1	—	—	+
R510	E1	IgG2b	—	—	+
31 ^e	E1	IgG2b	—	+	—
R9	E1	IgG2a	—	+	±
R2	E1	IgG2b	—	+	+
R12	E2	IgG1	—	+	+
R501	E2	IgG1	—	+	+
R503	E2	IgG2a	—	+	+
R505	E2	IgG2a	—	+	+
R6	E2	IgG2a	+	+	+
R8	E2	IgG3	+	+	+
R10	E2	IgG3	+	+	+
R15	E2	IgG2a	+	+	+
R502	E2	IgG2a	+	+	+
49 ^e	E2	IgG2a	+	+	+
50 ^e	E2	IgG2b	+	+	+

^a MAb reactivity with use of freshly grown and gradient-purified Sindbis virus as antigen in a capture ELISA (13, 28).

^b MAB reactivity of Sindbis virus-BHK cell complexes established at 4°C and then shifted to 37°C for 30 min in the presence of the MAb (13).

^c MAB reactivity in a capture ELISA using as antigen freshly grown and gradient-purified Sindbis virus which had been heated to 51°C for 10 min (28).

^d ND, not determined.

^e Gift of Alan Schmaljohn (39, 40, 44).

virus in ELISA but not to native virus in the capture assay, the epitope was considered inaccessible on native virions.

Epitopes inaccessible on native virions may be further subdivided into transitional epitopes, those which become accessible to their cognate MAb at the cell surface, and internal epitopes which remain inaccessible to MAb at the cell surface in virus-cell complexes (13). Heating native Sindbis virions to 51°C mimicked the exposure of transi-

tional epitopes at the cell surface in that most of the transitional epitope MAbs bound to heated virus (28).

Topographical mapping of E1 and E2 antigenic sites on heated virions. CAB assays have been used previously to examine the spatial relationships between neutralizing antigenic determinants present on the E2 glycoprotein of native Sindbis virus and SB-RL (11, 30). In the present study, we wished to explore the spatial relationships among neutralizing antigenic determinants and the epitopes newly accessible on freshly purified Sindbis virions that had been heated for 10 min at 51°C. Therefore, heated virions were used in a CAB assay which sought to maintain virion structure as much as possible (see Materials and Methods). The assay judged the spatial proximity of two epitopes by noting the ability of an unlabeled antibody to inhibit the binding of an HRP-labeled test antibody.

The results of the CAB assay using Sindbis virus are summarized in Table 2. The data suggest that at least three newly accessible determinants reside on the E1 glycoprotein at the surface of heated Sindbis virus. Reciprocal competition was evident for epitopes bound by R3 and R4, and likewise for R500 and R510. These two sets of epitopes defined two determinants on the E1 glycoprotein. The third E1 site was defined by MAb R2, which did not compete strongly or reciprocally for binding with any of the other E1 or E2 specific MAbs.

The transitional epitope MAbs R12, R501, R503, and R505 exhibited strong reciprocal competition for binding on heated Sindbis virus, which suggested that they recognize the same determinant on the E2 glycoprotein. The antigenic site defined by these four MAbs has been designated the E2d determinant. The E2 MAbs which bind to accessible epitopes on native virus define three immunodominant E2 neutralizing antigenic sites: E2a (MAbs 49 and 50), E2b (MAbs R8, R10, R15, and R502), and E2c (MAb R6). Each of these sites is independently mutable in that neutralization escape mutations selected with an E2a MAb retains reactivity with E2b and E2c MAbs, for example. However, the E2a and E2b sites are in close physical proximity to each other, as judged from reciprocal competition (11, 30) (Table 2). Little competition was evident between the E2d-specific

TABLE 2. Competitive binding of MAbs on captured Sindbis virions

Capturing/ competing MAb	% Inhibition of HRP-conjugated MAb binding ^a												
	Glycoprotein E1 MAb					Glycoprotein E2 MAb							
	R2	R3	R4	R500	R510	R6	R10	R502	49	R12	R501	R503	R505
R2	10	6	4	7	9	3	1	4	2	2	3	0	1
R3	7	9	9	7	7	0	0	4	7	5	5	2	0
R4	4	10	10	5	10	0	0	3	3	3	1	2	3
R500	7	6	7	9	8	3	4	6	4	5	5	6	5
R510	4	2	5	8	10	1	2	2	7	5	5	2	0
R6	5	5	3	3	6	10	5	4	5	6	3	2	3
R10	2	4	2	6	8	8	9	9	10	5	3	3	3
R502	2	5	4	7	7	7	9	10	9	7	6	6	5
49	9	4	4	7	8	8	9	9	10	8	8	5	6
R12	4	3	1	5	6	1	1	3	5	10	10	10	10
R501	4	0	9	7	0	1	2	4	2	10	10	10	10
R503	2	3	0	8	9	3	2	5	5	10	10	10	10
R505	1	0	0	8	8	5	4	5	5	10	10	10	10

^a Purified heated Sindbis virions were captured by the indicated MAb, excess capturing/competing MAb was added, and then the HRP-conjugated test MAb was added. Percent inhibition of binding of conjugated MAb was calculated as $[1 - (\text{OD with competing MAb} / \text{OD without competing MAb})] \times 100$. Values shown are percent inhibition divided by 10 and rounded to the nearest integer.

TABLE 3. E1 and E2 neutralization escape mutants selected by using transitional and internal epitope MABs

Virus isolate	Codon position	Codon change (parent to mutant)	Amino acid change (parent to mutant)
RLmc12-1	E2 200	GAG to CAG	Glu to Gln
RLmc12-19	E2 200	GAG to GUG	Glu to Val
RLmc501-12	E2 200	GAG to CAG	Glu to Gln
RLmc501-2A	E2 200	GAG to GAA	Glu to Lys
RLmc501-4	E2 202	AAG to AUG	Lys to Met
RLmc503-7A	E2 202	AAG to AUG	Lys to Met
RLmc505-1A	E2 200	GAG to GAA	Glu to Lys
RLmc505-1C	E2 200	GAG to CAG	Glu to Gln
	E2 374	GCU to UCU	Ala to Ser
RLmc505-12F	E2 202	AAG to AUG	Lys to Met
RLmc2-7B	E1 381	AAA to AAU	Lys to Asn
RLmc2-11A	E1 250	CCA to CCG	No Change
	E1 361	CCA to CUA	Pro to Leu
RLmc4-1D	E1 300	AAA to ACA	Lys to Thr
RLmc4-6F	E1 300	AAA to GAA	Lys to Glu
RLmc4-7H	E1 300	AAA to AUA	Lys to Ile

antibodies and the E2a-, E2b-, or E2c-specific MABs on heated Sindbis virus particles.

These data, in conjunction with those previously obtained in parallel experiments with native virus, suggest that the rearrangement induced by heating the virions was subtle. The spatial relationship between the three neutralizing antigenic sites, E2a, E2b, and E2c, on heated virions was virtually identical to that observed on native Sindbis virus particles (11, 30).

Selection of MAB neutralization escape mutants. To map transitional epitopes to specific amino acid sequences on E1 and E2, we took advantage of the observation that heated virions retained infectivity, that they displayed a subset of the transitional epitopes, and that their infectivity could be neutralized by the cognate transitional epitope MABs. This allowed the selection of MAB escape mutants, the genomes of which could be sequenced to map putative amino acid constituents of the transitional epitopes. SB-RL was used in these selections, because this strain grew to higher titers than did its parent, AR339, and because neutralization with certain MABs was more efficient with SB-RL (29).

Two approaches employing a second antibody to enhance neutralization were used to isolate the escape mutants. In both cases, the primary MAB was reacted with heated SB-RL virions to establish virus-MAB complexes. One approach used the addition of gold-labeled goat anti-mouse IgG as a second antibody, followed by centrifugation to pellet heated virus-MAB complexes (see Materials and Methods). Another, more traditional approach used either a rabbit anti-mouse IgG or goat anti-mouse IgG as second antibody and complement to aid neutralization of the heated virus-MAB complexes. Multiple growth cycles in BHK cells under MAB pressure were used in all cases to select against wild-type particles that might have outgrown a mutant during the amplification of the escape mutant population. Plaque isolates were grown on BHK cells, heated, and screened in both a capture and a conventional ELISA for lack of binding to the selecting MAB. Mutants having significantly reduced binding activity were selected for sequencing. None of the mutants lost the ability to bind MABs to the three E2 neutralizing antigenic sites.

The E1 and E2 glycoprotein genes of the escape mutants

FIG. 1. Single-step escape mutants in the major E2 neutralization domain. The schematic diagram shows the structural features of the region including E2a and E2b neutralization sites, the glycosylation site at E2 Asn-196, and the E2d transitional epitope at amino acid positions 200 and 202. Letters at the bottom represent the E2 amino acid sequence of Sindbis virus AR339. The symbols represent each of the three immunodominant E2 neutralization sites defined by reciprocal MAB competition and patterns of reactivity of neutralization escape mutants (○, E2a; □, E2b; ◇, E2 transitional). The letters within the symbols are amino acid substitutions identified among single-step neutralization escape mutants. Mutants E2 Ile-216, E2 Pro-214, and E2 Met-190 were reported by Strauss et al. (44). The remaining mutants were reported by Davis et al. (11) and in this report.

were sequenced, and Table 3 shows a compilation of the data for representative isolates. All of the E2 variants, whether selected with R12, R501, R503, or R505, had mutations at either E2 amino acid position 200 or E2 amino acid position 202. Also, each of the mutations, regardless of the selecting MAB, significantly reduced binding to all four of these MABs. At E2 codon 200, a single base change could theoretically give seven amino acid substitutions for the wild-type glutamic acid. Three of these, glutamine, lysine, and valine, were represented among the mutants. The double mutant RLmc505-1C had amino acid substitutions at positions 200 and 374. The change at 374 was seemingly conservative (serine substituted for wild-type alanine) and occurred in the E2 transmembrane region (17, 33). At E2 amino acid 202, only methionine substitutions for the wild-type lysine were recorded among the several variants sequenced.

The mutants isolated by using E1-specific MAB R4 all had amino acid substitutions at position 300. The wild-type lysine was changed to a threonine, glutamic acid, or isoleucine at this position. R2 escape mutants were isolated with coding changes at either E1 361 or E1 381, which substituted leucine and asparagine for wild-type proline and lysine, respectively. Since the R2 escape mutations map 20 residues apart on the primary sequence, this epitope may be conformational. Several attempts were made to isolate escape mutants with MABs R500 and R510. Although these antibodies strongly neutralized heated virions, no escape mutants were recovered. The E1 glycoprotein contains several regions which are highly conserved among the alphaviruses. If MABs R500 and R510 recognize epitopes which consist of such conserved domains, then it is possible that any mutation which would allow escape from MAB neutralization also would be lethal.

Role of the complex N-linked oligosaccharide at E2 amino acid 196 in the accessibility of the E2d determinant. Two predicted N-linked glycosylation sites, Asn-X-Thr, are utilized on the E2 glycoprotein of Sindbis at amino acid positions 196 and 318 (20, 27, 35). The proximity of the complex carbohydrate moiety at residue 196 to the E2 200-202 region raised the possibility that antibody binding to the E2d determinant on native virus was occluded by the bulky oligosaccharide (Fig. 1). To test this hypothesis,

TABLE 4. Accessibility of transitional epitopes on TR5000 and TR5196

MAb	Epitope accessibility					
	Standard ELISA ^a		Capture ELISA ^b			
	TR5000	TR5196	TR5000	Heated TR5000	TR5196	Heated TR5196
49	100	100	100	88	100	31
R12	93	93	32	420 (13)	21	90 (4)
R501	103	104	20	300 (15)	6	48 (8)
R503	97	104	8	284 (36)	6	60 (10)
R505	99	99	16	284 (18)	13	54 (4)

^a TR5000 and TR5196 were assayed under standard ELISA conditions (Materials and Methods), which partially disrupt virion structure and expose transitional and internal epitopes. The test MAb was added, and then HRP-labeled goat anti-mouse IgG was added. OD values (0.04) obtained with a nonspecific MAb (MAb 5806, specific for the tobacco etch virus 58K protein [43]) were deducted from the OD values obtained with the test MAbs (ranging from 0.67 to 0.81) and then were normalized by setting the MAb 49 value to 100%.

^b In the capture assay, the indicated MAb was adsorbed to an ELISA plate, and then either fresh native virus or virus that had been heated for 5 min at 51°C was added. Captured virions were detected with HRP-labeled anti-Sindbis virus HMAF. OD values (ranging from 0.01 to 0.07) obtained with the nonspecific control MAb 5806 were deducted from the OD values obtained with the test MAbs (ranging from 0.03 to 1.10) and then were normalized by setting the MAb 49 value to 100%. Fold increase over the level for unheated, native virions is given in parentheses.

site-directed mutagenesis was used to substitute a glutamine codon (CAG) for the wild-type asparagine codon (AAC) at residue 196. TR5196 was gradient purified, and the migration pattern of its viral proteins was compared by SDS-polyacrylamide gel electrophoresis with the parental TR5000 pattern. Without the carbohydrate moiety, PE2 (the precursor of E2) and E2 proteins of TR5196 migrated faster than the corresponding proteins of TR5000 (data not shown).

TR5196 was compared with TR5000 in an ELISA and in a capture assay (Table 4). In these experiments, MAb 49, specific for an external neutralization epitope, E2a, functioned as the positive control capable of binding TR5196 or TR5000 virions, and MAb 5806, specific for the putative RNA polymerase of tobacco etch virus (43), served as the

negative control. The ELISA data indicated that neither the carbohydrate moiety nor the amino acid (glutamine) at position 196 was part of the E2d antigenic site, since disrupted TR5196 and TR5000 bound equivalent amounts of each MAb. In the capture assay, the two virus strains showed similar binding patterns. MAbs R12, R501, R503, and R505, which were specific for E2d transitional epitopes, did not efficiently capture native TR5196 or TR5000. Upon heating of the two virus strains at 51°C, the E2d epitopes became exposed, as reflected by the increased amount of TR5196 or TR5000 captured. These data indicate that the oligosaccharide present at E2 196 did not contribute to the inaccessibility of the E2d determinant on native virus.

Although neither the amino acid at position 196 nor the oligosaccharide appeared to be within the E2d determinant, it was noted that compared with heated TR5000, less heated TR5196 was captured by MAbs R12, R501, R503, and R505 (Table 4). This result may reflect a structural difference between the two strains as a result of the replacement of the wild-type residue at E2 196 or the lack of a carbohydrate moiety at this position.

E2b MAb escape mutations. Another feature of the E2 glycoprotein in close proximity to the transitional E2d site at E2 200 to 202 is the E2b neutralization site (Fig. 1). Davis et al. (11) mapped a major determinant of the E2b neutralizing antigenic site to E2 residue 216 by analyzing the sequences and MAb reactivity patterns of several laboratory strains of Sindbis virus as well as MAb R8 neutralization escape mutants. Similar results were obtained by Strauss et al. (44), who examined escape mutants selected with single or multiple MAbs and revertants of such mutants which had regained E2b reactivity. To determine the extent of the E2b site, additional escape mutants have been selected with two other MAbs, R15 and R502, which were tentatively mapped to the E2b site on the basis of competition for binding with other E2b MAbs (data not shown).

Table 5 shows the reactivities of previously isolated R8 (E2b)-selected neutralization escape mutants with MAbs R15 and R502. MAbs R15 and R502 both bound to an R8 mutant (RLmc8-1) having a Glu-to-Val change at E2 216, but binding of R502 was somewhat reduced with an E2 Gly-216

TABLE 5. Reactivities of R8-, R15-, and R502-selected variants with E2a, E2b, and E2c MAbs in ELISA^a

Virus isolate	MAb dilution	ELISA result ^b with MAb:						
		49 (E2a)	50 (E2a)	R8 (E2b)	R10 (E2b)	R6 (E2c)	R15 (E2b)	R502 (E2b)
SB-RL (Gly-209, Thr-213, Glu-216, His-278)		100	100	100	100	100	100	100
RLmc8-1 (Val-216) ^c	10 ⁻²	174	128	5	ND	114	88	84
RLmc8-3 (Gly-216)	10 ⁻²	138	107	7	ND	85	77	41
RLmc8-c (Asn-213)	10 ⁻²	104	76	11	ND	81	100	34
	10 ⁻³	70	106	8	0	163	34	8
RLmc15-2 (Glu-209)	10 ⁻³	87	84	6	1	108	79	13
	10 ⁻⁴	ND	ND	ND	ND	ND	29	ND
RLmc502-a (Arg-209)	10 ⁻²	118	106	87	141	112	50	14
RLmc502-b (Arg-209, Asn-213)	10 ⁻²	3	90	6	0	126	52	9
	10 ⁻³	0	46	5	0	80	ND	0
RLmc502-c (Asn-213, Tyr-278)	10 ⁻²	4	91	6	1	114	53	14
	10 ⁻³	0	23	0	ND	85	ND	0
TR5278 (Tyr-278) ^d	10 ⁻³	94	95	89	ND	104	100	105

^a Gradient-purified viruses were adsorbed to microtiter wells by evaporation, and 50 µl of ascites fluids containing MAb, diluted as indicated in PBS, was added. Bound primary MAb was detected with HRP-labeled goat anti-mouse IgG.

^b ELISA results were normalized by comparing the OD values obtained for the variants with that for the SB-RL control. The results are presented as a percentage of the SB-RL control level. ND, not determined.

^c Variants were isolated by selection with the indicated MAb as described in Materials and Methods.

^d Site-directed mutant in the pTR5000 clone as described in Materials and Methods.

mutant (RLmc8-3). Whereas E2 216 is the major determinant of the reactivity of E2b MAbs R8, R10 (11), 18, and 23 (44), it appeared that this residue was at the periphery of the R15 and R502 epitopes. In another R8-selected mutant (RLmc8-c), E2 Thr-213 was replaced with Asn, which was used as a site for N-linked glycosylation (11). This mutation and/or its attendant carbohydrate moiety strongly affected binding to R8, R10, R15, and R502. Little or no effect on E2a MAb reactivity (MAbs 49 and 50) was observed with either the E2 216 or E2 213 mutation. In the case of the E2 213 mutation, this was somewhat surprising, since a MAb 49 escape mutant had been mapped previously to E2 214 (44).

Also shown in Table 5 are the E2 mutations found in the genomes of neutralization escape mutants selected with R15 and R502 as well as the MAb reactivities of these mutants. These MAbs selected for single mutants at E2 209: Gly to Glu (RLmc15-2) or Arg (RLmc15-4 and RLmc502-a). The E2 Glu-209 substitution reduced binding of R8, R10, R15, and R502, whereas the E2 Arg-209 substitution preferentially affected R15 and R502 binding. These data in conjunction with the reactivity patterns of R15 and R502 with R8-selected mutants suggested that the E2b neutralization site contains at least two overlapping epitopes which span E2 residues 209 to 216.

Two double mutants also were among those selected by R502, one with Gly to Arg at E2 209 and Thr to Asn at E2 213 and the other with Thr to Asn at E2 213 and His to Tyr at E2 278. As expected of mutants containing the N-linked glycosylation site at E2 213, these double mutations caused reduced binding of E2b MAbs R8, R10, R15, and R502. Unlike the single mutants at E2 213, however, the double mutants also affected the binding of the E2a MAbs, abrogating binding of MAb 49 and reducing binding of MAb 50. Neither E2 Arg-209 nor E2 Tyr-278, as a single mutation (in escape mutant RLmc502-a and site-directed mutant TR5278, respectively), affected binding of either of the E2a MAbs.

DISCUSSION

Virus structure must be stable in the extracellular environment to prevent the degradation of its genetic material, yet the virus must uncoat to replicate. The shift from a stable to a disassembled form occurs early in infection. Disassembly of some viruses begins before the particle traverses the plasma membrane and can involve a series of structural intermediates rather than a single uncoating event. Conformational alterations in the structure of viral proteins during the early events of infection have been documented for T2 and T4 bacteriophage (3, 41) and several animal viruses such as reovirus (6, 16), influenza virus (4), and picornaviruses (10, 14, 24, 25). In the case of poliovirus, conformational rearrangements in virions which occur at the plasma membrane (14) can be reproduced *in vitro* by interaction with a soluble form of the poliovirus receptor (21).

We have demonstrated that a conformational rearrangement of the Sindbis virus glycoprotein spike can be detected as newly accessible E1 and E2 transitional epitopes at the plasma membrane after attachment but prior to entry of virions (13). In this study, we have mapped some of these transitional epitopes by using heated virions as a model for the structural intermediate detected at the cell surface. Although the heat-induced rearrangement of Sindbis virus is not identical to that observed on virus-cell complexes, the patterns of epitope exposition are similar (28). Compared with other means of artificially inducing a structural rearrangement, such as treatment with dithiothreitol or low pH,

the heated virions more faithfully reproduce the constellation of accessible epitopes found on the authentic cell surface intermediate. The heated virions retained their infectivity and could be neutralized by transitional epitope MAbs, making it possible to map the transitional epitopes by selection of MAb escape mutants and sequencing their glycoprotein genes.

The transitional epitope bound by MAb R2 appeared to be conformational, because it mapped to two amino acids (E1 positions 361 and 381) separated by 20 amino acids on the linear peptide sequence. Both amino acid changes lie adjacent to short alphavirus conserved amino acid regions (five and seven amino acids, respectively). These residues may be located near the base of the glycoprotein spike, as they are close to the transmembrane region. Hydrophobicity analysis indicated that E1 361 and 381 reside in hydrophilic regions (data not shown), suggesting that this epitope would be an exposed domain on isolated E1 glycoprotein molecules. Therefore, in the context of the native glycoprotein spike, this domain may be occluded by the association of E1 and E2 into heterodimers (36), by the association of heterodimers into trimers (15, 31, 36, 46), or by other E1 interactions involved in stabilization of the virion surface architecture (2).

The epitope recognized by MAb R4 is inaccessible on native virions, is inconsistently exposed at the cell surface, but is accessible on heated virions. MAb R4 has an IgA isotype so that the structural requirements for accessibility of the R4 epitope may be considerably different than would be the case if accessibility of the same epitope could be assayed with an IgG isotype MAb. The population of MAb R4 neutralization escape mutants contained three different amino acid substitutions at E1 300. This residue is in the vicinity of E1 313, at which mutation alters the pH requirements for cell-cell fusion by Sindbis virus (7).

Competition binding assays on heated virus suggested that all of the identified E2 transitional epitopes resided within a single antigenic site which we have termed E2d. Of nine independently isolated mutants selected by the four E2 transitional epitope MAbs, all contained mutations at either E2 200 or E2 202. This region of the E2 glycoprotein (Fig. 1) contains an N-linked glycosylation site (35), two of the Sindbis virus immunodominant neutralizing antigenic sites (E2a and E2b) (11, 44), and a putative virion site for binding a Sindbis virus receptor on chicken embryo fibroblasts (CEF) (48).

Removal of the N-linked carbohydrate at E2 196 by site-directed mutagenesis did not relieve the inaccessibility of the E2d site on native virions, nor did it alter the exposure of the E2d site on heated particles. This result suggests that the arrangement of the spike rather than glycosylation is the primary determinant of E2d accessibility.

Analysis of E2a and E2b escape mutants indicates that these two sites are mutationally independent; E2b MAbs bind E2a escape mutants and vice versa. However, the locations of the mutations involved (E2a, 190 and 214; E2b, 209, 213, and 216) indicate that the two sites overlap on the primary amino acid sequence of the glycoprotein. Strong reciprocal competition between E2a and E2b MAbs, observed in CAB assays on intact virions, supports this interpretation and suggests that the two sites are in very close proximity in the native structure (11, 30). Surprisingly, the configuration of the E2a and E2b sites was not detectably changed on heated particles, while the E2d site, which mapped between E2a and E2b, became accessible to MAb. Also unexpected was the low level of reciprocal competition between the E2d MAbs and MAbs to the E2a and E2b sites.

One interpretation of these results is that the mutations at E2 200 to 202 allosterically affect E2d MAb binding, with the actual E2d site being located elsewhere on E2. While this possibility cannot be excluded, it seems unlikely that all of the escape mutations would fall in an allosterically active site but none would be in the actual epitope of nine different mutants selected with four different MAbs. We favor a second interpretation, that E2a and E2b MAbs bind on the same face of the three-dimensional E2 glycoprotein, one that is accessible to antibody in the native virion. The E2d site, although between E2a and E2b on the primary sequence, may lie on another face of the glycoprotein which is accessible to antibody only after structural rearrangement of the spike.

A putative Sindbis virus receptor was identified on CEF by using an anti-idiotypic antibody raised against E2a MAb 49 (48). The inferences are that the MAb 49 epitope constitutes the virion site which interacts with the CEF receptor and that E2 transitional epitopes are adjacent to it. However, BHK cells and CEF apparently do not share the same receptor (47, 48), so it remains unclear what relevance the CEF receptor binding site has to rearrangements induced by binding to the BHK receptor. It will be interesting to examine virion rearrangements induced by binding to CEF and to determine the ability of the E2d mutants to infect CEF.

The functional role of domains exposed following conformational rearrangement of infecting virions at the plasma membrane is not known, nor is it known whether the transitional epitopes identified in this report are themselves constituents of functional domains. The transitional epitopes may represent markers of the rearrangement, while putative function resides in other newly exposed domains not reactive with the available panel of MAbs. We hypothesize that the initial attachment step itself, and/or the microenvironment at the cell surface (8, 26), triggers the conformational rearrangement and that domains newly accessible after the rearrangement function in a second-stage interaction with an element(s) of the plasma membrane. Such a two-stage attachment sequence is compatible with the classical concept of an initial reversible binding of virus to cell followed by an irreversible tight binding. The second-stage interaction could catalyze direct fusion of the viral envelope with the plasma membrane either with or without subsequent rearrangements induced by the reductive environment at the cell surface (1). Alternatively, the postulated second-stage interaction could initiate receptor-mediated endocytosis. In the latter case, additional conformational changes could be induced by the low-pH environment of the endosome, leading to fusion with the endosomal membrane. Although our results do not distinguish between these models for subsequent steps in the disassembly pathway, they strongly suggest that the first step in the disassembly of Sindbis virus occurs at the cell surface and is characterized by the exposure of specific, previously inaccessible glycoprotein domains.

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REFERENCES

1. Abell, B. A., and D. T. Brown. 1993. Sindbis virus membrane fusion is mediated by reduction of glycoprotein disulfide bridges at the cell surface. *J. Virol.* **67**:5496-5501.
2. Anthony, R. P., and D. T. Brown. 1991. Protein-protein interactions in an alphavirus membrane. *J. Virol.* **65**:1187-1194.
3. Arscott, P. G., and E. B. Goldberg. 1976. Cooperative action of the T4 tail fibers and baseplate in triggering conformational change and in determining host range. *Virology* **69**:15-22.
4. Barbey-Morel, C. L., T. N. Oeltmann, K. M. Edwards, and P. F. Wright. 1987. Role of respiratory tract proteases in infectivity of influenza A virus. *J. Infect. Dis.* **155**:666-672.
5. Baric, R. S., D. W. Trent, and R. E. Johnston. 1981. A Sindbis virus variant with a cell determined latent period. *Virology* **110**:237-242.
6. Bodkin, D. K., M. L. Nibert, and B. N. Fields. 1989. Proteolytic digestion of reovirus in the intestinal lumens of neonatal mice. *J. Virol.* **63**:4676-4681.
7. Boggs, W. M., C. S. Hahn, E. G. Strauss, J. H. Strauss, and D. E. Griffin. 1989. Low pH-dependent Sindbis virus-induced fusion of BHK cells: differences between strains correlate with amino acid changes in the E1 glycoprotein. *Virology* **169**:485-488.
8. Brown, D. T., and J. Edwards. 1992. Structural changes in alphaviruses accompanying the process of membrane penetration. *Semin. Virol.* **3**:519-527.
9. Choi, H.-K., L. Tong, W. Minor, P. Duma, U. Boege, M. G. Rossmann, and G. Wengler. 1991. Structure of Sindbis virus core protein reveals a chymotrypsin-like serine proteinase and the organization of the virion. *Nature (London)* **354**:37-43.
10. Crowell, R. L., and L. Philipson. 1971. Specific alterations of coxsackie B3 eluted from HeLa cells. *J. Virol.* **8**:509-515.
11. Davis, N. L., D. F. Pence, W. J. Meyer, A. L. Schmaljohn, and R. E. Johnston. 1987. Alternative forms of a strain specific neutralizing antigenic site on the Sindbis virus E2 glycoprotein. *Virology* **161**:101-108.
12. Edwards, J., E. Mann, and D. T. Brown. 1983. Conformational changes in Sindbis virus envelope glycoproteins accompanying exposure to low pH. *J. Virol.* **45**:1090-1097.
13. Flynn, D. C., W. J. Meyer, J. M. Mackenzie, Jr., and R. E. Johnston. 1990. A conformational change in Sindbis virus glycoproteins E1 and E2 is detected at the plasma membrane as a consequence of early virus-cell interaction. *J. Virol.* **64**:3643-3653.
14. Fricks, C. E., and J. M. Hogle. 1990. Cell-induced conformational change in poliovirus: externalization of the amino terminus of VP1 is responsible for liposome binding. *J. Virol.* **64**:1934-1945.
15. Fuller, S. 1987. The T=4 envelope of Sindbis virus is organized by interactions with a complementary T=3 capsid. *Cell* **48**:923-934.
16. Furlong, D. B., M. L. Nibert, and B. N. Fields. 1988. Sigma 1 protein of mammalian reoviruses extends from the surfaces of viral particles. *J. Virol.* **62**:246-256.
17. Garoff, H., and H. Soderlund. 1978. The amphiphilic membrane glycoproteins of Semliki Forest virus are attached to the lipid bilayer by their COOH-terminal ends. *J. Mol. Biol.* **124**:535-549.
18. Helenius, A., J. Kartenbeck, K. Simons, and E. Fries. 1980. On the entry of Semliki Forest virus into BHK-21 cells. *J. Cell Biol.* **84**:404-420.
19. Hirschberg, C. B., and P. W. Robbins. 1974. The glycolipids and phospholipids of Sindbis virus and their relation to the lipids of the host cell plasma membrane. *Virology* **61**:602-608.
20. Hsieh, P., M. R. Rosner, and P. W. Robbins. 1983. Host-dependent variation of asparagine-linked oligosaccharides at individual glycosylation sites of Sindbis virus glycoproteins. *J. Biol. Chem.* **258**:2548-2554.
21. Kaplan, G., M. S. Freistadt, and V. R. Racaniello. 1990. Neutralization of poliovirus by cell receptors expressed in insect cells. *J. Virol.* **64**:4697-4702.
22. Kielian, M., and A. Helenius. 1985. pH-induced alterations in the fusogenic spike protein of Semliki Forest virus. *J. Cell Biol.*

- 101:2284–2291.
23. Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**:367–382.
24. Lonberg-Holm, K., L. B. Gossler, and J. C. Kauer. 1975. Early alteration of poliovirus in infected cells and its specific inhibition. *J. Gen. Virol.* **27**:329–342.
25. Lonberg-Holm, K., and B. D. Korant. 1972. Early interaction of rhinoviruses with host cells. *J. Virol.* **9**:29–40.
26. Mandel, R., H. J.-P. Ryser, F. Ghani, M. Wu, and D. Peak. 1993. Inhibition of a reductive function of the plasma membrane by bacitracin and antibodies against protein disulfide-isomerase. *Proc. Natl. Acad. Sci. USA* **90**:4112–4116.
27. Mayne, J. T., J. R. Bell, E. G. Strauss, and J. H. Strauss. 1985. Pattern of glycosylation of Sindbis virus envelope proteins synthesized in hamster and chicken cells. *Virology* **142**:121–133.
28. Meyer, W. J., S. Gidwitz, V. K. Ayers, R. J. Schoepp, and R. E. Johnston. 1992. Conformational alteration of Sindbis virion glycoproteins induced by heat, reducing agents, or low pH. *J. Virol.* **66**:3504–3513.
29. Olmsted, R. A., R. S. Baric, B. A. Sawyer, and R. E. Johnston. 1984. Sindbis virus mutants selected for rapid growth in cell culture display attenuated virulence in animals. *Science* **225**:424–427.
30. Olmsted, R. A., W. J. Meyer, and R. E. Johnston. 1986. Characterization of Sindbis virus epitopes important for penetration in cell culture and pathogenesis in animals. *Virology* **148**:245–254.
31. Paredes, A. M., D. T. Brown, W. Chiu, E. Marietta, R. E. Johnston, R. J. Schoepp, and B. V. V. Prasad. Structure of Sindbis virus at 28Å resolution as determined by three-dimensional cryo-electron microscopy. *Proc. Natl. Acad. Sci. USA*, in press.
32. Paredes, A. M., M. N. Simon, and D. T. Brown. 1992. The mass of the Sindbis virus nucleocapsid suggests it has T=4 icosahedral symmetry. *Virology* **187**:329–332.
33. Rice, C. M., J. R. Bell, M. W. Hunkapiller, E. G. Strauss, and J. H. Strauss. 1982. Isolation and characterization of the hydrophobic COOH-terminal domains of the Sindbis virion glycoproteins. *J. Mol. Biol.* **154**:355–378.
34. Rice, C. M., R. Levis, J. H. Strauss, and H. V. Huang. 1987. Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. *J. Virol.* **61**:3809–3819.
35. Rice, C. M., and J. H. Strauss. 1981. Nucleotide sequence of the 26 S mRNA of Sindbis virus and deduced sequence of the encoded virus structural proteins. *Proc. Natl. Acad. Sci. USA* **78**:2062–2066.
36. Rice, C. M., and J. H. Strauss. 1982. Association of Sindbis virion glycoproteins and their precursors. *J. Mol. Biol.* **154**:325–348.
37. Salminen, A., J. M. Wahlberg, M. Lobigs, P. Liljeström, and H. Garoff. 1992. Membrane fusion process of Semliki forest virus II. Cleavage-dependent reorganization of the spike protein complex controls virus entry. *J. Cell Biol.* **116**:349–357.
38. Schlesinger, M. J., S. Schlesinger, and B. W. Burge. 1972. Identification of a second glycoprotein in Sindbis virus. *Virology* **47**:539–541.
39. Schmaljohn, A. L., E. D. Johnson, J. M. Dalrymple, and G. A. Cole. 1982. Non-neutralizing monoclonal antibodies can prevent lethal alphavirus encephalitis. *Nature (London)* **297**:70–72.
40. Schmaljohn, A. L., K. M. Kokubun, and G. A. Cole. 1983. Protective monoclonal antibodies define maturational and pH-dependent changes in Sindbis virus E1 glycoprotein. *Virology* **130**:144–154.
41. Simon, L. D., and T. F. Anderson. 1967. The infection of *Escherichia coli* by T2 and T4 bacteriophages as seen in the electron microscope. II. Structure and function of the baseplate. *Virology* **32**:298–305.
42. Singh, I., and A. Helenius. 1992. Role of ribosomes in Semliki Forest virus uncoating. *J. Virol.* **66**:7049–7058.
43. Slade, D. E., R. E. Johnston, and W. G. Dougherty. 1989. Generation and characterization of monoclonal antibodies reactive with the 49-kDa proteinase of tobacco etch virus. *Virology* **173**:499–508.
44. Strauss, E. G., D. S. Stec, A. L. Schmaljohn, and J. H. Strauss. 1991. Identification of antigenically important domains in the glycoproteins of Sindbis virus by analysis of antibody escape variants. *J. Virol.* **65**:4654–4664.
45. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354.
46. Von Bonsdorff, C.-H., and S. C. Harrison. 1975. Sindbis virus glycoproteins form a regular icosahedral lattice. *J. Virol.* **16**:141–145.
47. Wang, K. S., R. J. Kuhn, E. G. Strauss, S. Ou, and J. H. Strauss. 1992. High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. *J. Virol.* **66**:4992–5001.
48. Wang, K. S., A. L. Schmaljohn, R. J. Kuhn, and J. H. Strauss. 1991. Anti-idiotypic antibodies as probes for the Sindbis virus receptor. *Virology* **181**:694–702.
49. Wengler, G., D. Würkner, and G. Wengler. 1992. Identification of a sequence element in the alphavirus core protein which mediates interaction of cores with ribosomes and the disassembly of cores. *Virology* **191**:880–888.
50. White, J., J. Kartenbeck, and A. Helenius. 1980. Fusion of Semliki Forest virus with the plasma membrane can be induced by low pH. *J. Cell Biol.* **87**:264–272.
51. Zimmermann, D., and P. Kaesberg. 1978. 3'-terminal nucleotide sequence of encephalomyocarditis virus RNA determined by reverse transcriptase and chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **75**:4257–4261.