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Conformational Alteration of Sindbis Virion Glycoproteins Induced by Heat, Reducing Agents, or Low pH

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Sindbis virions undergo a conformational rearrangement after attachment to cells but prior to entry, as detected by exposure of epitopes on virus-cell complexes which are not accessible to their cognate monoclonal antibodies on native virions (D. C. Flynn, W. J. Meyer, and R. E. Johnston, J. Virol. 64:3643–3653, 1990). The rearrangement did not appear to require transit of virions through a low-pH environment, and the altered virions participated in a productive infection. This naturally occurring structural alteration could be mimicked, although not precisely duplicated, by any of three artificial treatments of purified virions in vitro: brief incubation at 51°C, treatment with 1 to 5 mM dithiothreitol, or incubation at pH 5.8 to 6.0. Infectivity was maintained after all three treatments, suggesting that Sindbis virions are metastable and can exist in at least two infectious conformations. The integrity of external, neutralizing epitopes was maintained on cell-associated virions and in the altered conformations induced by heat and dithiothreitol, whereas these epitopes were unreactive under low-pH conditions that induced an analogous exposure of previously inaccessible epitopes. The pH at which the conformational change was induced and the pH at which virions could mediate cell-cell fusion from without were coordinately shifted when these two parameters were determined for another strain of Sindbis virus. This coordinate shift in pH optima suggests that the conformational change in virion structure observed at the cell surface may be causally related to fusion.

Sindbis virions are composed of three proteins, a capsid protein and glycoproteins E1 and E2 (reviewed in reference 28). Multiple copies of the capsid protein form a T = 4 icosahedral nucleocapsid in conjunction with the single-stranded, positive-polarity RNA genome (5, 6, 22). The two glycoproteins, associated as heterodimeric units, are constituents of the lipoprotein envelope (26). The E1-E2 heterodimers are arranged in clusters of three on the surface of the virion, where they form a T = 4 icosahedral lattice (13, 35). This ordered structure apparently results from lateral interactions between the glycoproteins as well as interactions across the lipid bilayer between the glycoproteins and the icosahedral nucleocapsid.

The virus particle must perform two important but seemingly incompatible functions. While the enclosed genome is protected by the virion structure from a sometimes hostile external environment, disassembly of the virion must occur during infection of cells to allow viral gene expression. One of the earliest steps in the disassembly of enveloped virions is thought to involve a glycoprotein rearrangement prior to fusion of the envelope with a cellular membrane. Such a rearrangement could initiate fusion by the juxtaposition of a hydrophobic fusogenic domain on a virion glycoprotein with appropriate elements of the cellular membrane.

We have previously presented evidence that the initial step in the programmed disassembly of Sindbis virus occurs at the plasma membrane after attachment to cells but prior to internalization (10). At this stage of infection, a conformational change in the arrangement of the virion glycoproteins was detected by using monoclonal antibodies (MAbs) as probes. A panel of E1 and E2 MAbs, specific for epitopes not accessible on native Sindbis virions, were unable to bind to virus-cell complexes established at 4°C. However, a subset of these MAbs were capable of binding to virus-cell complexes soon after they were shifted from 4 to 37°C to promote penetration. These newly exposed internal epitopes were termed transitional epitopes.

The rearrangement was detected at the plasma membrane by radioimmunochemistry and by visualization of immunogold-labeled particles in the electron microscope (10). With a neutralizing MAb used as an internal standard, approximately 30% of the particles at the cell surface displayed transitional epitopes. Penetration of infectious virions was retarded in the presence of a MAb to a transitional epitope, suggesting that the rearranged particles are structural intermediates in a disassembly pathway leading to productive infection of tissue culture cells. The rearrangement was not dependent on new viral or cellular protein synthesis. Moreover, the rearrangement occurred normally in the presence of ammonium chloride and in cells that were temperature sensitive for endosomal acidification. This suggested that the conformational change detected on virus particles at the cell surface was not likely to be the result of a rearrangement triggered by low pH in endosomes followed by recycling to the plasma membrane. A more likely interpretation is that the conformational change in the glycoprotein spike was triggered by the interaction of virions with the cell plasma membrane.

The conformational change in Sindbis virions induced at the cell surface could be sufficient to catalyze the fusion of the viral envelope with the plasma membrane, leading to

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release of the nucleocapsid into the cytoplasm. Alternatively, the rearrangement we have observed may be the first in a series of successive changes. The cell surface conformational change could be preparatory to endocytosis followed by a second rearrangement, triggered by the low-pH environment of the endosome, and subsequent fusion with the endosomal vesicle membrane.

To study the structure of the rearranged virions in more detail, we have attempted to mimic the conformational change detected on virus-cell complexes by in vitro treatment of purified virions with several artificial dissociative agents, such as heat, dithiothreitol (DTT), and low pH. As in the studies with virus at the cell surface, MAbs specific for internal, external, and transitional epitopes on the E1 and E2 glycoproteins were used to probe treated virions for changes in structure. Virion viability and the patterns of epitope exposition on virions induced by the three in vitro treatments were compared with one another and with those observed on virus-cell complexes during the early stages of infection. In addition, we have examined the relationship between the conformational change induced at low pH and the ability of such virions to mediate cell-cell fusion.

MATERIALS AND METHODS

Virus growth and purification. A molecularly cloned strain of Sindbis virus, TR5000, was used in most of these studies. TR5000 (16a) contains the sequences of our laboratory strain, Sindbis AR339, from nucleotides 2713 to 11484 (including all the structural protein genes) and Toto1101 sequences (25) from nucleotides 1 to 2712 and 11485 to 11703. RNA transcripts were obtained by in vitro transcription of a cDNA clone (pTR5000) containing these Sindbis virus sequences downstream from an SP6 promoter. Virus stocks were obtained by introduction of transcripts into BHK cells (American Type Culture Collection) by transfection, whereupon virus replication was initiated (24). SB-HR is a heat-resistant Sindbis virus strain selected from an AR339 parent (4) and was obtained from the laboratory of D. T. Brown, University of Texas at Austin. Uninfected and infected cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% tryptose phosphate broth and 10% donor calf serum.

Freshly grown virus was purified by equilibrium density centrifugation in potassium tartrate gradients (14). The virus-containing bands were collected and concentrated by pelleting through a cushion of 20% sucrose in TNE (0.05 M Tris [pH 7.2], 0.1 M NaCl, 1.0 mM EDTA). The pellets were suspended in PBS-D (phosphate-buffered saline [PBS] lacking divalent cations) and stored at 4°C for up to 48 h before use.

MAbs. The MAbs used in this study were from four sources (Table 1). To generate the R500 series of MAbs, BALB/c mice were initially infected with SB-RL (SB-reduced latent period [2]) and then given a booster inoculation with sodium dodecyl sulfate-denatured virions. This immunization protocol favored selection of hybridomas secreting antibodies specific for linear internal or inaccessible epitopes. MAbs 31, 49, and 50 were the kind gift of Alan Schmaljohn and have been characterized previously (29, 30, 34). MAb 5806 was generated from a fusion by using as immunogen the nuclear inclusion body induced in plant cells infected with tobacco etch virus (TEV) (31). This antibody is unreactive with Sindbis virus by standard enzyme-linked immunosorbent assay (ELISA) and by the capture ELISA described below and was used in individual experiments as a negative control. Other MAbs were obtained from a fusion in which mice were immunized with whole SB-RL particles. Of this group, MAbs R2, R6, R8, R9, R10, and R15 have been described previously (20, 21), and MAb R12 is specific for a transitional epitope on native virus (10).

TABLE 1. Characterization of MAbs

MAb	Antibody binding class[a]	Glycoprotein specificity	Isotype	Neutralizing[b]
R6	Ext	E2	IgG2a	Yes
R8	Ext	E2	IgG3	Yes
R10	Ext	E2	IgG3	Yes
R15	Ext	E2	IgG2a	Yes
49	Ext	E2	IgG2a	Yes
50	Ext	E2	IgG2b	Yes
R502	Ext	E2	IgG2a	Yes
R2	Tr	E1	IgG2b	No
R4	Tr	E1	IgA	No
R9	Tr	E1	IgG2a	No
R12	Tr	E2	IgG1	No
R501	Tr	E2	IgG1	No
R503	Tr	E2	IgG2a	No
R505	Tr	E2	IgG2a	No
31	Tr	E1	IgG2b	No
R500	Int	E1	IgG1	No
R509	Int	E2	IgG1	No
R510	Int	E1	IgG2b	No
R512	Int	E2	IgG1	No
5806	Nonspecific	TEV	IgG1	

[a] Ext, MAbs recognizing external epitopes on native virus; Tr, MAbs recognizing transitional epitopes exposed on virions at the cell surface at 37°C but not on native virus; Int, MAbs recognizing internal epitopes exposed only on disrupted virions in a standard ELISA.

[b] Ability of MAbs to neutralize the infectivity of native virus in a plaque reduction neutralization assay.

Heating time course and capture ELISA. TR5000 was assayed for exposure of internal epitopes in a time course experiment at 51°C. Sixty micrograms of virus was placed in 1 ml of PBS–1% DCS (PBS with 1% donor calf serum) that had been preheated to 51°C. Aliquots containing 4 μg of virus were removed after incubation for appropriate times and brought to 1 ml in PBS with 3% ovalbumin for use in a capture assay. Individual MAbs were used as the capturing antibodies. The presence of captured virus was detected in each case with a horseradish peroxidase-conjugated MAb that was otherwise identical to the capturing antibody.

To assay heat inactivation of infectivity, the virus was diluted 1:5 in PBS–1% DCS (1.5 ml final volume) that had been preheated to 51°C. A 50-μl aliquot was removed immediately and placed on ice to serve as the 0-min-heated sample. The remainder of the virus solution was incubated at 51°C. At the indicated time points, 50-μl aliquots were removed and placed on ice. Each sample was serially diluted and titered in a plaque assay.

Plaque reduction neutralization test. A neutralization test was done with serial dilutions of MAb R2 or R505 and rabbit anti-mouse immunoglobulin G (IgG; Sigma) in the presence of guinea pig serum (Cedarlane; 5% final volume) as a source of complement. Identical samples of TR5000 (estimated titer of 2.3×10^6 PFU/ml) were either heated for 10 min at 51°C or incubated at room temperature. Both samples were diluted in PBS–1% DCS to a titer of 10^6 PFU/ml, and 50 μl of virus was incubated for 30 min at 37°C with each dilution of MAb R2 or R505 or a 1:100 dilution of MAb 5806 ascites fluid as a negative control. Rabbit anti-mouse IgG (10 μg) and 5 μl of complement were added to each sample, which was then

incubated for 30 min. A 1,000-fold dilution of the antibody-virus suspension was made to dilute antibodies and complement. A monolayer of BHK cells was infected for enumeration of residual infectivity by a plaque assay. The plaque reduction neutralization titer was the highest dilution of MAb capable of neutralizing 80% of the PFU measured in control incubations with a 1:100 dilution of MAb 5806, the TEV-specific MAb.

DTT treatment. Aliquots (32 μg) of purified TR5000 were brought to a 200-μl volume with PBS and DTT to create a range of DTT final concentrations. The samples were incubated for 1 h at 37°C. The volume was brought to 8 ml in PBS containing 3% ovalbumin to dilute residual DTT. The samples were analyzed for infectivity by plaque assay and for antigenic profile in a capture assay (0.2 μg of virus per well).

Treatment of virions at low pH. A range of pH buffers was generated with PBS containing 0.02 M 2-(*N*-morpholino) ethanesulfonic acid (MES) and 0.2% bovine calf serum. Purified TR5000 (8 μg per sample) was incubated at 37°C for 1 h in 500 μl of each buffer, a volume shown to remain at constant pH after addition of virus. The samples were neutralized to pH 7.0 with 2 M NaOH. A small sample was removed for dilution and titration by the plaque assay. The remainder was brought to 2 ml in PBS containing a final concentration of 3% ovalbumin for use in a capture assay (0.2 μg of virus per well).

Cell fusion assay. A procedure similar to that of White et al. (37) was followed for fusion from without. Twenty-four-well culture dishes, containing approximately 10^5 BHK cells per well, were placed at 4°C for 20 min. Culture fluids were aspirated, and Sindbis virus at a multiplicity of infection of 1,000 to 2,000 in MEM was added to each monolayer in a volume of 100 μl. The virus was allowed to adsorb in the cold for 1 h, at which time the cultures were moved to room temperature, and the inoculum was removed. One-half milliliter of 0.02 M MES-buffered MEM (without bicarbonate) at 37°C and at an appropriate pH (5.0 to 7.0 in 0.2-pH unit increments) was added to each well. After a 2-min incubation, the MES medium was removed, and fresh MES-buffered medium at the same pH was added. After an additional 2 min, the MES medium was replaced with MEM at neutral pH. The cultures were incubated at 37°C for 45 min, fixed with cold 10% neutral buffered Formalin, stained with Giemsa, and evaluated microscopically. The degree of fusion at a given pH was quantitated by observing each of 10 fields and counting the number of nuclei in cells containing at least 3 nuclei per cell. The pH of each MES-buffered medium was rechecked after removal from treated cell monolayers to ensure that the pH had remained constant.

RESULTS

A panel of MAbs specific for Sindbis virus E1 and E2 glycoproteins was used to detect conformational alterations induced by three treatments: heat, DTT, and low pH. To reduce the possibility of structural damage due to freezing and thawing, the experiments detailed below were performed with freshly grown and purified virus. The virus used was TR5000, a virus derived from a full-length clone containing the structural genes of strain AR339 as well as Toto1101 sequences (see Materials and Methods) (25).

The characteristics of the diagnostic MAbs are listed in Table 1. These can be divided into three categories. The first group is capable of binding to external epitopes and can neutralize native virus, defining three neutralizing antigenic sites on the surface of Sindbis virions (7, 19a, 20, 21, 23, 27, 29, 30, 32–34). The remaining MAbs cannot bind to native virus because their cognate epitopes are either inaccessible or internal on native virus particles. A subset of these MAbs were identified as transitional epitope antibodies in that they could not bind to native virions or virus-cell complexes established at 4°C but did react with virus-cell complexes during the early stages of infection at 37°C (10).

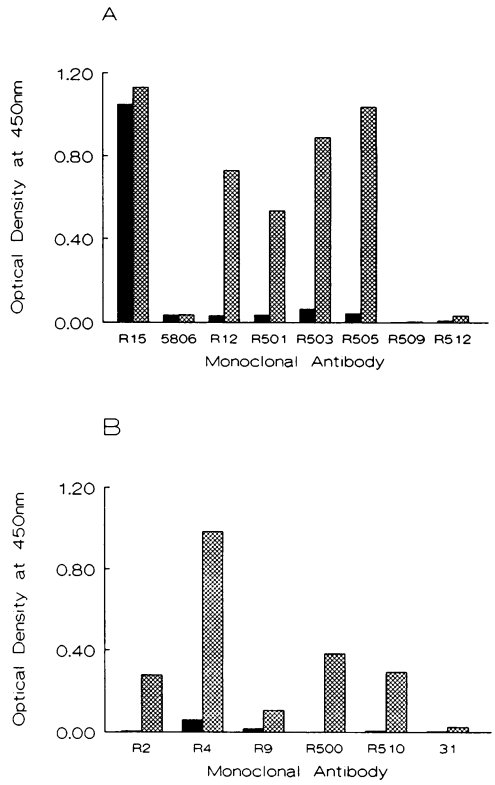

FIG. 1. Binding of MAbs to heated and unheated virus. Sindbis virus was incubated at room temperature or at 51°C for 10 min as described in Materials and Methods and then used in a capture assay to measure binding of external, transitional, internal, and nonspecific control MAbs to epitopes on the E1 and E2 glycoproteins. (A) R15 positive control binds to an external E2 epitope; all others are MAbs that bind to transitional or internal E2 epitopes. Solid bars, unheated TR5000. Hatched bars, heated TR5000. (B) E1-specific MAbs that bind to transitional or internal epitopes. Solid bars, unheated TR5000. Hatched bars, heated TR5000.

Exposure of normally inaccessible epitopes by heat treatment. MAbs specific for internal or transitional epitopes were tested for their ability to bind heated and unheated virus in a capture assay. In this experiment, parallel aliquots of TR5000 were either heated at 51°C for 10 min or left at room temperature. Figure 1 shows the data gathered for a representative sample of the 20 MAbs tested. The positive control neutralizing antibody, R15 (Fig. 1A), captured both heated and native virus, consistent with its specificity for an external site on the E2 glycoprotein that is not affected by incubation at 51°C. Antibody 5806, an MAb generated against the 58K nuclear inclusion protein of a plant virus, TEV, served as the negative control. All other antibodies are directed against inaccessible or internal epitopes on glycoprotein E2 (Fig. 1A) or E1 (Fig. 1B) of Sindbis virus. Of the 20 MAbs tested, at least 9 captured heated virus but not native virus, demonstrating a heat-induced rearrangement of the E1 and E2 glycoproteins of TR5000.

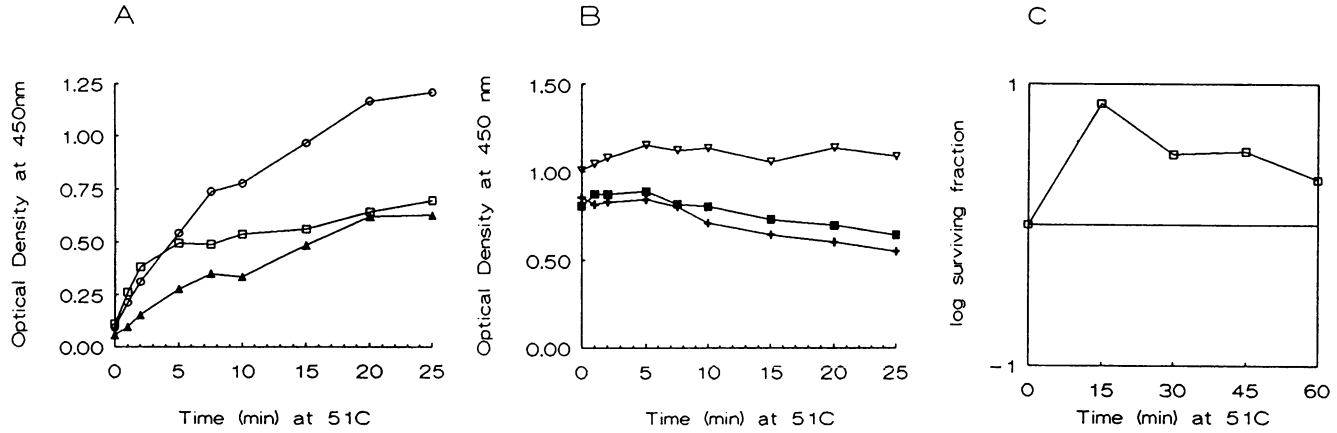

FIG. 2. Reactivity of transitional and external E1 and E2 epitopes as a function of time at 51°C. Sindbis virus was incubated at 51°C for the times indicated and assayed for MAb binding in a capture assay. (A) Transitional-epitope MAbs: E1 MAb R4, □; E2 MAb R12, ▲; E2 MAb R505, ○. (B) E2 external-epitope MAbs: R6, +; R10, ▽; 50, ■. (C) Sindbis virus infectivity as a function of time of incubation at 51°C.

It is unlikely that the exposure of these epitopes was due to a generalized degradation of viral particles for two reasons. First, 12 antibodies that bound to partially disrupted virus in the ELISA failed to efficiently capture heated virus (3 of these are shown in Fig. 1). Second, when treated under these conditions, the genomes of virions containing [^{3}H]uridine-labeled RNA remained insensitive to digestion with RNase (14).

Time course of epitope exposure at 51°C. MAbs that could bind to epitopes on heated, rearranged virions and to transitional epitopes on virus-cell complexes (10) were used in a time course capture assay to detect the initial time and degree of exposure of their cognate epitopes. TR5000 was heated at 51°C for the indicated times; aliquots were removed, and appropriate dilutions were used in the capture assay. TR5000 that had been heated for 20 to 25 min was captured by MAbs R4, R12, and R505 (Fig. 2A). The initial exposure of the epitopes bound by these three MAbs occurred as early as 1 min after heating began. The maximum rate of epitope exposure occurred between 0 and 7 min of heating for the R4, R12, and R505 epitopes. Unlike the other epitopes, the epitope represented by R2 binding had a very gradual rate of epitope exposition (data not shown).

MAbs that bind to external neutralizing domains were also used in the time course experiment to monitor the effect of heat on their cognate epitopes (Fig. 2B). MAbs R6 and 50 had only a slightly reduced capacity to bind TR5000 after incubation at 51°C for 25 min. MAb R10 showed no decrease in binding capacity through the same period. Thus, although previously inaccessible epitopes were exposed as a result of heating TR5000, external epitopes present on E2 remained intact. This finding suggests that the heat treatment induced a relatively subtle conformational alteration of TR5000 glycoproteins.

Infectivity of heated, rearranged particles. When TR5000 was heated at 51°C for 60 min, there was no decrease in titer, suggesting that rearranged TR5000 was infectious (Fig. 2C). To determine the viability of heated, rearranged virions, plaque reduction neutralization tests were performed with heated and unheated TR5000. R2, an E1 glycoprotein-specific, transitional-epitope MAb, was capable of binding heated but not unheated TR5000 (Fig. 1B). R2 ascites fluid had an 80% plaque reduction neutralization endpoint titer of >1:3,200 on heated TR5000, yet little or no reduction in the titer of unheated TR5000 was observed at any dilution tested (1:100 to 1:3,200; data not shown). Considering that reactivity with transitional-epitope antibodies defines the rearranged particles, then neutralization of infectivity by these same MAbs would only have been detected if the rearranged particles themselves were infectious.

Rearrangement of Sindbis virions induced by DTT. The E1 and E2 glycoproteins of Sindbis virus contain numerous cysteine residues that form disulfide bonds. The majority of these bonds are probably intramolecular, since E1 and E2 from virions can be separated from one another under nonreducing conditions (1, 38). Previously, Gidwitz et al. (14) determined that 15 mM DTT decreased the infectious titer of Sindbis virus. Therefore, TR5000 was incubated in a range of DTT concentrations from 0 to 10 mM to ascertain whether the structure of the virus could be altered without loss of titer. After a 1-h incubation at 37°C at a given DTT concentration, the samples were diluted in PBS containing 3% ovalbumin (Materials and Methods). This dilution was sufficient to reduce residual DTT to a concentration which did not affect the ability of MAb to bind virus in the subsequent capture assay.

DTT concentrations between 0.25 and 0.5 mM exposed epitopes normally inaccessible on the glycoproteins of native TR5000. The epitope bound by R4, an E1-specific MAb, was initially exposed between 0 and 0.25 mM DTT, and as the DTT concentration was increased through 10 mM DTT, the R4 epitope became more accessible to R4 binding in the capture assay (Fig. 3A). Epitopes bound by E1 MAbs R2, R9, and 31 were not detected at any concentration of DTT used in this experiment (Fig. 3A). E2 MAbs R12, R503, and R505 had similar binding profiles, with initial exposure of their cognate epitopes occurring between 0.25 and 0.5 mM DTT and maximum binding occurring at 5 mM DTT. Exposure of the R501 epitope was detected initially between 0.5 and 0.75 mM DTT, with maximum binding at 0.75 mM DTT (Fig. 3B).

External epitopes on native virus were also examined in this study (Fig. 3C). All external epitopes were affected at DTT concentrations above 1 mM. The least stable epitopes were those bound by R15 and R502. These epitopes were altered with as little as 0.5 mM DTT. At 10 mM DTT, the binding ability of all MAbs specific for external epitopes was

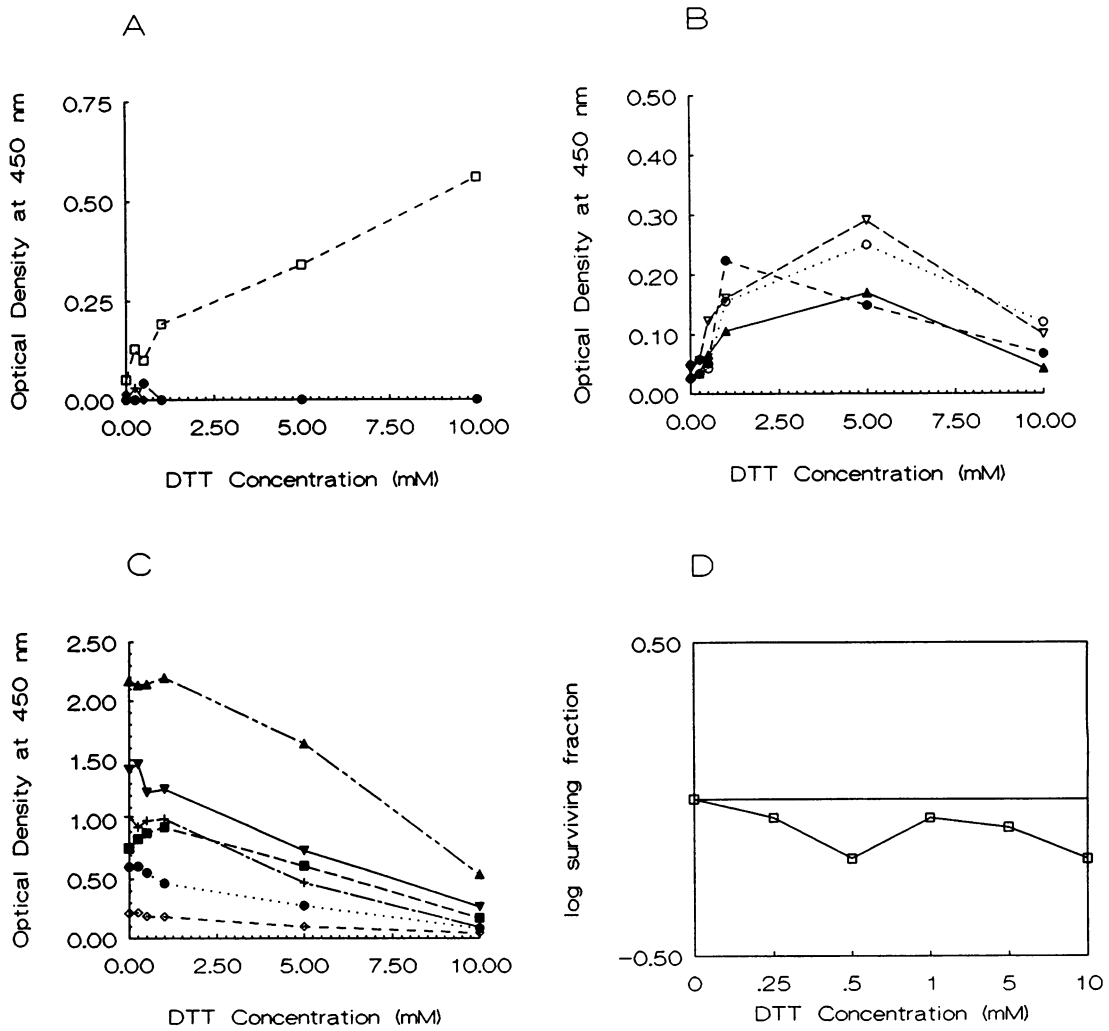

FIG. 3. Reactivity of transitional and external E1 and E2 epitopes as a function of DTT concentration. Sindbis virus was incubated for 1 h at 37°C at the DTT concentration indicated, the samples were diluted 1:40, and the treated virus was analyzed in a capture assay. (A) E1 transitional-epitope MAbs: R2, +; R4, □; R9, ●; 31, ◆. (B) E2 transitional-epitope MAbs: R12, ▲; R501, ●; R503, ▽; R505, ○. (C) E2 external-epitope MAbs: R15, ▼; 49, ◇; 50, ■; R502, ●; R6, +; R8, ▲. (D) Sindbis virus infectivity as a function of DTT concentration.

decreased by 75 to 80%; the infectious titer of TR5000 was decreased by approximately 20% (Fig. 3D).

Structural rearrangement of Sindbis virions at acidic pH. Alphaviruses mediate cell-cell fusion in tissue culture after brief exposure of virus-cell complexes to acid pH, followed by return to neutrality (17, 37). The fusion is presumably facilitated by a pH-induced conformational alteration in the structure of the virus. This rearrangement may expose a hydrophobic domain(s) present on one or both of the glycoproteins that is required for cell membrane fusion. Acid pH-induced rearrangement of alphavirus E1 and E2 glycoproteins has been demonstrated previously by using alteration in protease sensitivity (9, 16, 30) or formation of new oligomeric arrangements among the glycoproteins (36) as indicators of conformational change. To explore further the nature of a pH-induced structural alteration of Sindbis virus in the context of the rearrangement described above, we exposed TR5000 to a range of pH conditions in vitro and used MAbs specific for internal, transitional, and external epitopes in a capture assay to monitor the change in structure of the virus. TR5000 was incubated for 1 h at 37°C at the indicated pH in PBS containing 0.02 M MES and 0.2% bovine calf serum. Following incubation, the samples were returned to neutral pH and used in the capture assay.

In contrast to heat-treated virus, in which the structure of the external epitopes was conserved in the rearranged particles, the reactivity of these epitopes was substantially reduced after incubation at pH 5.8 to 6.0 (Fig. 4A). Concomitant with the decline in external epitope reactivity, transitional epitopes became accessible as the result of pH treatment (Fig. 4B and C). Maximal exposure of transitional epitopes, as determined by antibody binding, occurred at a pH of 5.8 to 6.0 (R4, R12, R501, R503, and R505). Exposure of the epitopes to which MAbs R2, R9, and 31 bind was not detected in this assay at any pH tested (Fig. 4B). The virus maintained viability at tested pH values higher than 6.0 (Fig. 4D), and the infectivity of virions treated at pH 6.0 could be neutralized by a transitional-epitope MAb (R505; data not shown).

The rearrangement induced by low pH differed from that observed in virus-cell complexes (10) or after incubation of virions at 51°C or in the presence of DTT. Also exhibiting

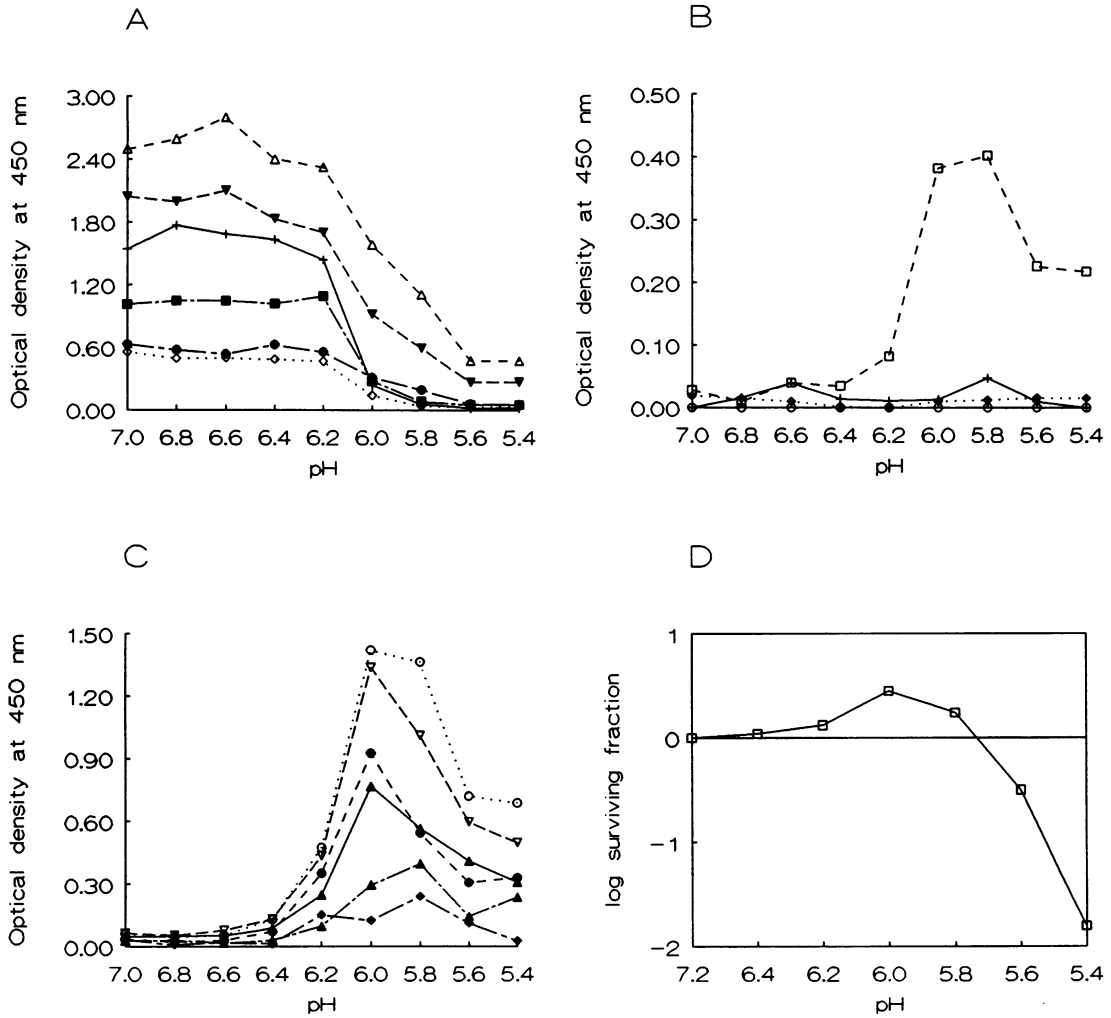

FIG. 4. Reactivity of transitional and external E1 and E2 epitopes as a function of pH. Sindbis virus was incubated at the indicated pH for 1 h at 37°C, returned to pH 7.0, and used in a capture assay. (A) E2 external-epitope MAbs: R6, +; R8, △; R15, ▼; 49, ◇; 50, ■; R502, ●. (B) E1 transitional-epitope MAbs: R2, +; R4, □; R9, ○; 31, ◆. (C) E2 transitional-epitope MAbs: R12, —▲—; R501, ●; R503, ▽; R505, ○; E2 internal-epitope MAb R509, ———▲———; TEV-specific control MAb 5806, ◆. (D) Sindbis virus infectivity as a function of pH.

detectable binding at pH 5.8 to 6.0 were MAb R509, an internal-epitope MAb which did not bind virions under any of the other conditions examined, and MAb 5806, the plant virus-specific MAb. The fact that the negative control MAb 5806 captured acid-treated TR5000 likely reflects nonspecific interactions between MAb 5806 and denatured protein. At lower pHs, the reactivity of all the MAbs and virus infectivity were decreased markedly. In conjunction with the loss of external epitopes, these data suggest that the exposure of transitional epitopes in response to pH 5.8 to 6.0 may have resulted from a more dramatic alteration in virion structure than that induced by the other artificial treatments or by interaction of virions with cell plasma membranes.

Correlation of structural rearrangement and cell-cell fusion from without. Incubation of virus-cell complexes at acidic pH followed by a return to neutral pH induces cell-cell fusion from without (17, 37). The pH at which fusion occurs varies with different Sindbis virus strains, and specific mutations affecting fusion have been identified (3). For two Sindbis virus strains, TR5000 and SB-HR, the pH dependence of both the structural rearrangement and cell-cell fusion were determined (Fig. 5). Maximal exposure of the transitional epitopes reactive with MAbs 503 and 505 was shifted from pH 6.0 for TR5000 to pH 5.6 for SB-HR. A similar shift occurred with respect to the loss of reactivity with an external-epitope antibody, MAb R6 (Fig. 5A). This result suggests that the loss of reactivity with external MAbs and the increase in reactivity with transitional-epitope MAbs are a consequence of the same conformational change. Maximal cell-cell fusion catalyzed by TR5000 occurred at pH 5.6. Like the pH dependence of the conformational alteration, the pH of maximal fusion induced by SB-HR was shifted 0.4 pH units, to pH 5.2 (Fig. 5B).

DISCUSSION

The structure of the Sindbis virion spike is altered during early interactions with susceptible cells (10), and MAbs specific for transitional epitopes exposed on the altered particles at the cell surface act to retard the formation of infectious centers. We infer that the altered virions are intermediates in the normal process of infection and that the

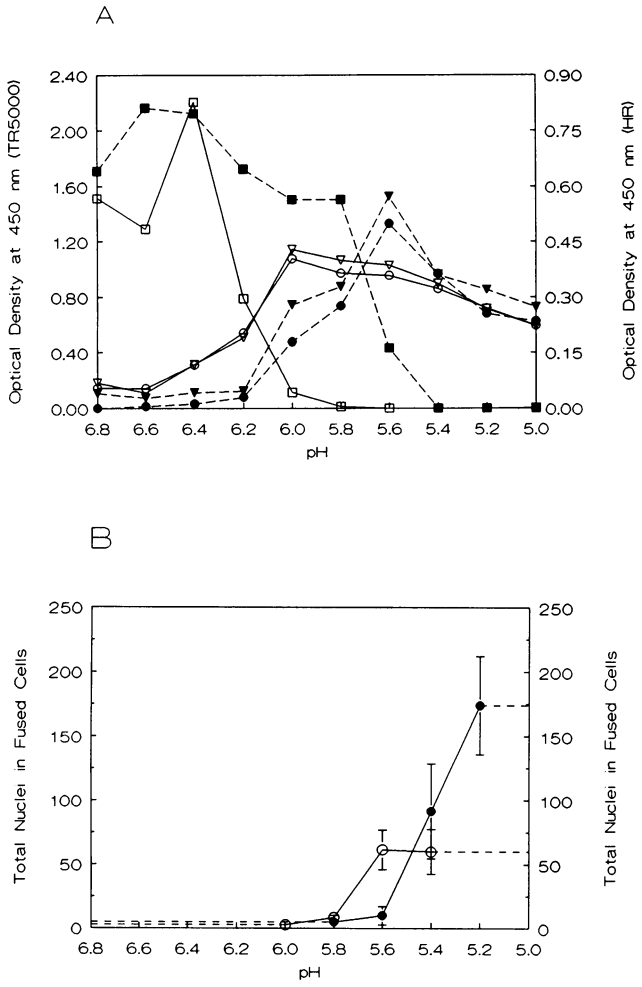

FIG. 5. Comparison of low-pH-treated TR5000 and SB-HR reactivity with transitional-epitope MAbs and cell-cell fusion. (A) Purified TR5000 or SB-HR virions were treated at the indicated pH and assayed for reactivity with external- and transitional-epitope MAbs by capture ELISA. TR5000, open symbols and solid lines; SB-HR, solid symbols and dotted lines. MAbs: R6, □/■; R503, ▽/▼; R505, ○/●. (B) Degree of cell-cell fusion mediated by purified TR5000 or SB-HR was determined as a function of pH as described in Materials and Methods. TR5000, open circles; SB-HR, solid circles. Dotted lines indicate estimates based on visual observation. Error bars indicate one standard deviation.

observed conformational change detected at the cell surface represents the first step in the entry, disassembly, and uncoating of the Sindbis virus genome. In the present study, we have attempted to mimic the rearrangement found at the cell surface by treatment of isolated virions with heat, DTT, or decreased pH.

Heat-induced alterations of Sindbis virus structure. Virion conformation, the arrangement of the two glycoproteins in particular, was analyzed following short-term incubation at 51°C. Neither virus infectivity nor the reactivity of MAbs directed to external epitopes were decreased under the conditions used. However, a structural alteration in the virion was evident, as many of the same transitional E1 and E2 epitopes identified by Flynn et al. (10) became accessible to their cognate MAbs. The conformational change was relatively subtle, in that reactivity with external E2 MAbs did not degrade during a 25-min incubation at 51°C and the relative positions of these epitopes remained constant, as measured by competition binding assays (19a). These results suggest that intact Sindbis virions can exist in at least two stable conformational states, one represented by native particles, and a second induced by mild heat treatment. The neutralization of infectivity, mediated by MAbs to transitional epitopes exposed only on virions in the alternative conformational state, strongly suggested that such virions retained infectivity.

Identical MAb reactivity patterns were observed on heat-treated virions and on virus-cell complexes for 15 of the 20 MAbs screened on both. For the remaining five MAbs, the reactivity patterns were distinctly different (Table 2). Therefore, the heat-induced conformation was similar, although not identical, to the naturally occurring conformation that virions assume shortly after interaction with the cell surface.

Brief incubation of Sindbis virions at 51°C consistently resulted in a 2- to 10-fold stimulation in the number of PFU detected. The stimulation could be explained by disruption of virus aggregates, although Sindbis virus prepared by an identical protocol was not aggregated, as determined from UV inactivation kinetics (11). Alternatively, the excess infectivity could have been derived from activation of particles which are uninfectious under the conditions of a standard plaque assay. Rather than being defective, a significant fraction of such uninfectious particles may be too stable and thus fail to undergo a requisite rearrangement at the cell surface prior to entry. Incubation at 51°C may artificially trigger a rearrangement similar to that which normally oc-

TABLE 2. Comparison of transitional- and internal-epitope exposure induced by heat, low pH, and DTT with epitopes exposed at the cell surface

Epitope exposure	Reactivity[a]												
	Anti-E1 antibodies						Anti-E2 antibodies						Anti-TEV antibody 5806[c]
	R2	R4	R9	31	R500[b]	R510[b]	R12	R501	R503	R505	R509[b]	R512[b]	
Heat	+	+	±	−	+	+	+	+	+	+	−	−	−
DTT	−	+	−	−	ND[d]	ND	±	+	+	+	−	−	−
pH	−	+	−	−	ND	ND	+	+	+	+	+	−	+
Cell surface	+	±	+	+	−	−	+	+	+	+	−	−	−

[a] Reactivity of treated virions was detected in a capture ELISA. MAb binding at the cell surface was detected with ^{125}I-goat anti-mouse IgG; includes data from Flynn et al. (10). Symbols: +, optical density with treated virus >0.15 and at least three times greater than that of untreated virus; ±, optical density with treated virus ≥0.10 but ≤0.15 and at least three times greater than that of untreated virus; −, optical density with treated virus <0.10.

[b] MAbs R500, R509, R510, and R512 are specific for internal epitopes.

[c] MAb 5806 is specific for the putative TEV polymerase and does not bind to native Sindbis virions or to disrupted Sindbis virions in ELISA.

[d] ND, not determined.

curs at the cell surface, allowing these virus particles to successfully initiate an infection.

DTT-induced conformational changes. Alterations in virion structure induced by treatment with DTT were presumed to result from reduction of disulfide bridges. Incubation of virions in 5 mM DTT altered the pattern of reactivity with the diagnostic MAbs with only a slight decrease in infectivity. This indicated that DTT-treated virions assumed a conformation similar to, but again not identical with, the transitional intermediate structure found at the cell surface and the virion conformation induced by heating at 51°C (Table 2). A critical disulfide bridge(s) may hold virions in their native conformation at 37°C, whereas reduction of this bond(s) may favor the assumption of an alternative, but still infectious, conformation. Heating to 51°C or interaction with an element of the plasma membrane may be sufficient to convert the native structure to similar alternative conformations while leaving the critical disulfide bond(s) intact. Another possibility is that interaction with the plasma membrane during the earliest stages of infection mediates the conformational rearrangement by reduction of a critical disulfide bond(s).

There appear to be no free sulfhydryl groups in native virions which are accessible to reagents such as iodoacetamide (14). After treatment of native virions with 5 mM DTT, both glycoproteins could be labeled with iodo[^{14}C]acetamide (14). At lower concentrations of DTT, E1 was preferentially labeled. Therefore, it is unclear whether it was a disulfide bridge(s) on E1 and/or E2 which was reduced by DTT in order to allow the virion glycoproteins to assume an alternative conformation.

Conformational changes induced in Sindbis virus at low pH. We have confirmed and extended the observations of others that incubation in low-pH buffers results in an altered virion structure, as determined by sensitivity of the glycoproteins to protease digestion (9, 16) and formation of alternative oligomeric configurations (36). A group of transitional epitopes inaccessible on native TR5000 virions became available for MAb binding, with a maximum at pH 5.8 to 6.0. The rearrangement evident following low-pH treatment had several features in common with the conformational changes induced by heat or DTT. Reactivity with MAb probes showed that the virion conformation induced by a 1-h incubation at low pH was similar to but did not precisely mimic that found on virion intermediates at the cell surface (Table 2). Also similar to the heat-induced conformational change, a small but reproducible increase in infectious titer was observed coincident with maximal exposure of transitional epitopes at pH 5.8 to 6.0, and the infectivity of treated virions could be neutralized by MAb R505, a transitional-epitope MAb.

Notwithstanding these similarities, several observations suggest that low-pH-induced alterations in glycoprotein structure were fundamentally different from the rearrangement induced by heat and the conformational change at the surface of infected cells (10). Concomitant with maximal exposure of the transitional epitopes at pH 5.8 to 6.0, the reactivity of external glycoprotein epitopes decreased dramatically. In addition, particles incubated at pH 5.8 to 6.0 were reactive with MAb R509, which had been shown previously to react only with virions disrupted in a standard ELISA, but did not react with virions at the cell surface, heat-treated virions, or particles treated with DTT. Virions incubated at low pH also reacted with MAb 5806, a nonspecific control MAb which was raised against the putative TEV polymerase. MAb 5806 had no detectable cross-reactivity with TR5000 in a capture ELISA or in a standard ELISA in which virions were disrupted. Therefore, the alteration induced by acidic pH (5.8 to 6.0) appeared to be more extensive and disruptive than that caused by either of the other artificial treatments or than the rearrangement which occurs at the cell surface. A further decrease in pH to 5.6 was characterized by progressive loss of reactivity with all of the MAbs and a 90% reduction in infectious titer relative to its maximum at pH 6.0.

Cell-cell fusion mediated by low pH. One possible route for alphavirus entry involves transit through a low-pH compartment. The virus may reach such a compartment as a consequence of entry by receptor-mediated endocytosis and acidification of the resulting endosome (16, 18, 19). A conformational change in glycoprotein structure, induced by the low-pH environment of the endosome, is thought to expose a hydrophobic domain on the virus which promotes fusion of the viral envelope with the endosomal membrane. Cited in support of this hypothesis is the finding that a brief incubation of virus-cell complexes at low pH is followed by cell-cell fusion, presumably mediated by the simultaneous fusion of individual virus envelopes with the plasma membranes of more than one cell. We found that the conformational change in low-pH-treated Sindbis virions, detected by exposure of transitional epitopes, and the ability of such virions to mediate cell-cell fusion were coordinately affected by genetic differences between virus strains. This coordinate shift in pH optima suggests that the conformational change in virion structure may be causally related to fusion. Because the conformational change observed at the cell surface (10) is similar in some respects to that associated with low-pH treatment, one must consider the possibility that the cell surface conformational change may promote the fusion of the viral envelope with the plasma membrane. The ability of low-pH treatment to initiate cell-cell fusion after establishment of Sindbis virus-cell complexes at 4°C may result from an artificial, synchronous induction of a glycoprotein rearrangement similar to that which occurs naturally at the surface of the infected cell.

Maximal cell-cell fusion was observed for each virus strain at a slightly more acidic pH than that required for the glycoprotein rearrangement detected by the transitional-epitope MAbs. This may reflect the fact that the assays for rearrangement and fusion differ in several important respects, and the rearrangement and fusion may actually occur coordinately, as suggested by the experiment with SB-HR. Alternatively, fusion may require additional changes in the virion structure at the lower pH. If this is the case, then such additional alterations appear to involve the frank denaturation of the virion, as demonstrated by dramatic loss of antigenicity and infectivity.

Cycling of virions through endosomes and return to the cell surface. The detection of transitional epitopes on approximately 30% of the virions at the cell surface suggested that the interaction of the envelope spike with elements of the plasma membrane was sufficient to trigger the observed rearrangement (10). The rearrangement was detected with immunological reagents which cannot penetrate the cell, excluding the possibility that the observed rearrangement was strictly intracellular. However, the present finding, that a 1-h incubation of virions at pH 5.8 to 6.0 induced a similar rearrangement, suggests an alternative possibility. During normal infection, a substantial proportion of virions could be internalized in endosomes, undergo a conformational change in response to the low-pH environment, and then rapidly cycle back to the cell surface, where they would have been

detected by our immunological probes. This would also be consistent with the observation that cell-cell fusion mediated by Sindbis virus requires incubation of virus-cell complexes at low pH and then a return to neutrality (8, 37). However, we have found no evidence that low pH was involved in the conformational rearrangement detected at the cell surface. First, the rearrangement was unaffected by the presence of 10 mM NH_4Cl and occurred normally at the restrictive temperature in a Chinese hamster ovary (CHO) cell line that is temperature sensitive for endosomal acidification (10). While neither of these conditions is thought to raise endosomal pH to neutrality, the pH dependence of the conformational change (Fig. 4 and 5) would predict that even at endosomal pHs as low as 6.2, we should have observed a significant diminution in the extent of the rearrangement if low pH were the triggering event. This was not observed. Second, the data presented here clearly show that exposure of Sindbis virions to pH levels commonly found in endosomes (pH 6.0 and below) resulted in the profound denaturation of external epitopes. Yet such epitopes remained intact and functional in neutralization experiments, in which a transitional-epitope MAb retarded penetration of infectious virions (10). Therefore, the data accumulated to date indicate that the conformational change detected on Sindbis virions at the cell surface does not require transit through a low-pH compartment.

The glycoprotein rearrangement, which appears to be triggered by the interaction of Sindbis virions with the cell surface, may be a prelude to fusion of the virion envelope with the plasma membrane. This would predict that the putative fusogenic domain was among the epitopes exposed on the transitional intermediate particle. An analogous structural intermediate, characterized by an exposed hydrophobic domain, has been identified after interaction of poliovirus with the cell surface (12) or after interaction of virions with partially purified poliovirus receptor in vitro (15). Alternatively, the Sindbis virus conformational change detected at the cell surface may reveal a secondary ligand required for induction of receptor-mediated endocytosis and thus lead to subsequent structural alterations within the low-pH endosomal environment. Regardless of the events which follow the glycoprotein rearrangement, we believe that the conformational alteration we observed at the plasma membrane represents the first step in the pathway leading to entry, uncoating, and initiation of productive infection by Sindbis virus.

In summary, the results presented in this article strongly suggest that Sindbis virions are metastable and can retain infectivity while their glycoprotein spikes assume at least two distinct conformations. The native structure could be converted to an alternative one by a variety of artificial conditions, although none of the artificially induced rearrangements of virion architecture was precisely equivalent to the naturally occurring conformational change observed after interaction of virions with susceptible cells. Nevertheless, these in vitro treatments resulted in infectious virions which displayed transitional epitopes and which could be neutralized by MAbs to these epitopes. We are using this property to map the relative positions of the transitional epitopes on rearranged virions, to select transitional MAb neutralization escape mutants, and, by sequencing these mutants, to infer which specific new glycoprotein domains become exposed on virions during the early stages of virus-cell interaction.

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