A Conformational Change in Sindbis Virus Glycoproteins E1 and E2 Is Detected at the Plasma Membrane as a Consequence of Early Virus-Cell Interaction

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A conformational change in the structure of Sindbis (SB) virus was detected after virion attachment to baby hamster kidney cells but before internalization. The alteration was manifested as increased virion binding of certain glycoprotein E1 and E2 monoclonal antibodies (MAbs) that recognized transitional epitopes. These epitopes were inaccessible to MAb on native virions but became accessible to their cognate MAbs in the early stages of infection. Transit of virions through a low-pH compartment apparently was not required for the conformational change. Exposure of transitional epitopes was unaffected by treatment of BHK cells with NH_4Cl and occurred normally in Chinese hamster ovary cells temperature sensitive for endosomal acidification. However, the rearrangement was correlated with both the time course and temperature dependence of SB virus penetration, and the rearrangement occurred earlier with an SB virus mutant having an accelerated penetration phenotype. In addition, MAb to a transitional epitope, a probe specific for rearranged particles, retarded penetration of infectious virions. These results suggested that the SB virus E1/E2 glycoprotein spike undergoes a structural rearrangement as a consequence of virion interaction with the cell surface and that this altered virion form may be an important early intermediate in an entry pathway leading to productive infection.

Virus particles require a structure sufficiently stable to withstand the extracellular environment. Yet in order for viral genomes to replicate, the surrounding virion structures must be capable of programmed disassembly upon infection of a susceptible cell. Therefore, one would predict that as uncoating of the genome progresses, defined conformational changes in virion structure will occur, with each such rearrangement being triggered by a specific stimulus encountered during the interaction between virion and host cell. The results reported here suggest that in Sindbis (SB) virus infection, the earliest of these structural alterations may occur at the cell surface in response to interaction between SB virions and elements of the host cell plasma membrane.

SB virus is the prototype member of the *Alphavirus* genus. The SB virion has a single-stranded RNA genome, contained within an icosahedral nucleocapsid (47, 51, 52). The nucleocapsid is surrounded by a host-derived lipoprotein envelope acquired upon budding from the plasma membrane (1, 7, 33). Traversing the lipid envelope are two viral glycoproteins, E1 and E2 (44, 52). The carboxy-terminal portions of E1 and E2 are interior to the lipid bilayer and are presumed to interact with the icosahedral nucleocapsid (40, 58). E1 and E2 are closely associated in heterodimeric units (40), which are in turn assembled as trimers (24). The interaction between the glycoproteins and the underlying capsid produces the ordered icosahedral organization of the glycoprotein spikes on the virion exterior (19, 54).

SB virus attaches to tissue culture cells through an unidentified receptor(s). Successful interaction with the receptor(s) eventually leads to uncoating of the virion and release of the viral genome into the cytoplasm. The precise se-

quence of events leading to uncoating of the SB virus genome remains unclear. A widely accepted hypothesis suggests that alphaviruses penetrate cells via receptor-mediated endocytosis, followed by fusion of the virion envelope with the endosomal membrane and consequent release of the nucleocapsid into the cytoplasm (25, 30, 34). Other studies suggest that SB virus may penetrate cells by direct fusion of the viral envelope with the plasma membrane (8, 11). Both hypotheses postulate that fusion is preceded by a structural alteration in the virion, presumably in the glycoprotein spikes, which exposes a hydrophobic fusogenic peptide at the virion surface. The hypotheses differ with respect to the location and trigger for the proposed rearrangement.

In the endocytosis model, the low endosomal pH (5.0 to 6.0) (36, 53) may trigger a rearrangement in the glycoprotein spike and exposure of the putative fusogenic hydrophobic domain (56). Changes in alphavirus structure occurring after exposure to low pH have been detected as alterations in antigenic profile, in physical virion parameters, and in differential protease sensitivity of the glycoproteins (16, 28, 46). In the direct fusion model, it is suggested that a rearrangement could be induced by the interaction of the virion glycoprotein spike with a plasma membrane receptor(s), leading to exposure of the fusogenic glycoprotein domain.

In the experiments reported here, monoclonal antibodies (MAbs) were used to detect an early conformational transition in the glycoproteins of infecting SB virions. The transition was detected at the cell surface and occurred as a consequence of virus-cell interaction. Although these experiments have not determined whether the conformationally altered particles gain access to the cytoplasm by endocytosis or direct fusion, the results suggest that the phased disassembly process leading to successful uncoating and productive SB virus infection begins at the plasma membrane.

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MATERIALS AND METHODS

Cells, virus, and antibody. Baby hamster kidney (BHK-21) cells were obtained from the American Type Culture Collection in passage 53 and used through passage 65. Cells were grown in Eagle minimal essential medium (MEM; GIBCO Laboratories) supplemented with 10% donor calf serum (DCS; Hazelton), 10% tryptose phosphate broth (Difco Laboratories), 50 U of penicillin per ml, and 50 μ g of streptomycin (Sigma Chemical Co.) per ml. Chinese hamster ovary (CHO) cell lines WTb and B3853 were the kind gift of Calvin Roff and April Robbins and were grown as previously indicated (42).

SB virus strain AR339 was obtained initially from Henry Bose (University of Texas, Austin) and was grown in BHK cells. Purification of SB virus as well as the isolation and characterization of SB-RL, a rapidly penetrating SB virus mutant, were described previously (4, 37). Unless otherwise noted, virus was freshly grown in BHK cells and purified immediately before use. In similar virus preparations, particle/PFU ratios for AR339 and SB-RL were approximately 16:1 and 3:1, respectively.

MAbs R2, R3, R4, R9, R12, and R15 were produced against intact, purified SB-RL virions by Olmsted et al. (37). MAbs in the R500 series were produced against sodium dodecyl sulfate-denatured SB-RL virions (W. J. Meyer and R. E. Johnston, unpublished data). MAbs 31, 35, and 38 (E1 specific) and 49 (E2 specific) were the kind gift of Alan Schmaljohn and Joel Dalrymple (45). MAbs K3 and R5806 were raised against tobacco etch virus (TEV) proteins (15, 49) and are specific for the TEV capsid protein and putative RNA polymerase, respectively. Antibodies were concentrated by ammonium sulfate precipitation, dialyzed against phosphate-buffered saline (PBS), and purified by affinity chromatography on a protein A-Sepharose column (17, 22).

Iodination of goat anti-mouse immunoglobulin G. Goat anti-mouse immunoglobulin G (GaM; Sigma) was purified by protein A-Sepharose column chromatography, concentrated by lyophilization, and resuspended in PBS to 10 mg/ml. Lactoperoxidase iodination of GaM was accomplished by mixing 50 μ l of 0.2 M PO_4 buffer (pH 7.2), 10 μ l of GaM (10 mg/ml), 25 μ l of Enzymobeads (1 mg/ml; Bio-Rad Laboratories), 25 μ l of 1% β -D-glucose (Sigma), and 10 μ l of Na^{125}I (100 mCi/ml; ICN Radiochemicals) for 45 min at 21°C. Iodinated antibodies were purified over a Bio-Gel P-6DG desalting column (Bio-Rad) equilibrated with PBS (PBS without Ca^{2+} or Mg^{2+}). ^{125}I -GaM was checked for its ability to bind mouse MAb, using a radioimmunoassay in 96-well enzyme-linked immunosorbent assay (ELISA) plates.

MAb binding at the cell surface. SB virus or SB-RL was grown in BHK cells and gradient purified immediately before the experiment. BHK cells (10^5 cells per 16-mm tissue culture well) were chilled to 4°C for 30 min. A 100- μ l sample of virus suspension (120 μ g of virion protein per ml in PBS with 1% DCS [PBS-1%]) was added to cells for 60 min at 4°C. Wells were washed with PBS-1% and blocked with PBS containing 10% DCS (PBS-10%) for 30 min at 4°C. A 100- μ l sample of purified MAb (20 μ g/ml in PBS-1%, pH 7.2) was added to each well for 30 min at 4°C. The treated cultures were shifted to 37°C. At intervals from 0 through 60 min after the shift to 37°C, duplicate cultures were washed with cold PBS-1% and blocked with PBS-10% or PBS with 10% nonimmune goat serum for 30 min at 4°C. Then 100 μ l of ^{125}I -GaM (approximately 10 μ g/ml in PBS-1%) was added to each well for 30 min at 4°C. Cells were washed five times with cold PBS and solubilized with 300 μ l of 1% sodium

dodecyl sulfate in H_2O . Duplicate samples were assayed in a gamma counter.

Similar experiments were performed on CHO cell lines WTb and the temperature-sensitive mutant B3853. In this case, however, establishment of the temperature-sensitive phenotype required incubation of the mutant for several hours at nonpermissive temperature (42). Each cell line (10^5 cells per 16-mm tissue culture well) was incubated for 4.5 h at either 4, 34, or 40°C before removal of the medium and addition of 100 μ l of PBS-1% containing 20 μ g of purified SB virus and 2 μ g of MAb. Incubation was continued at the given temperature, and binding of MAb was detected with ^{125}I -GaM as described above.

Penetration assay. Approximately 100 to 200 PFU of SB virus was allowed to attach to 10^6 BHK cells in 60-mm tissue culture plates at 4°C for 60 min. The virus-cell complexes then were shifted to 37°C. At intervals between 0 and 60 min, two plates were overlaid with agarose for plaque assay to quantitate the number of PFU that had attached. Two plates were trypsinized for 5 min at 37°C. The cells were removed from the tissue culture plate, centrifuged at $600 \times g$, and suspended in 25 ml of MEM. Therefore, only those PFU resistant to removal by trypsinization were quantitated as infectious centers. A 200- μ l sample of cell suspension was plated in each of 96 wells, and wells containing infectious centers, as judged by the appearance of cytopathic effect, were counted at 48 h. The number of infectious centers in the original suspension was calculated by using the Poisson distribution. The proportion of PFU that penetrated cells (became resistant to trypsin) was calculated by dividing the number of infectious centers by the number of PFU attached at each time point.

Preparation of immunogold beads. Gold beads approximately 30 nm in diameter were prepared as follows. Distilled and deionized water (247.5 ml) was brought to a boil in a 500-ml Erlenmeyer flask. Then 2.5 ml of 1% $\text{HAuCl}_4 \cdot 2\text{H}_2\text{O}$ (wt/vol in H_2O ; Sigma) was added, quickly followed by the addition of 3.9 ml of trisodium citrate dihydrate (Sigma). The solution was boiled for 30 min over low heat with stirring to produce a gold sol. The flask was cooled under running tap water and then placed on ice. To a 1.5-ml Eppendorf tube were added 950 μ l of gold sol, 20 μ l of 0.1 N NaOH, and 13 μ l of affinity-purified GaM (10 mg/ml). The mixture was vortexed briefly after each addition. The stability of the GaM-Au bead conjugates was measured by flocculation with NaCl. (The concentration of NaCl required to flocculate the Au sol was determined by the method of Muller and Baigent [35]). The GaM-Au beads were blocked by the addition of 20 μ l of cold water fish gelatin (45% solution in H_2O ; preheated to 45°C; Sigma), followed by slow mixing for 10 to 15 min at room temperature. GaM-Au beads were purified over a glycerol step gradient by the method of Birrell et al. (5). Purified GaM-Au beads were dialyzed against TBS (20 mM Tris hydrochloride pH 7.6, 0.15 M NaCl, 1 mM sodium azide). The ^{125}I -GaM and GaM-Au beads were derived from the same preparation of purified GaM.

The reactivity of the GaM-Au beads was tested by mixing 200 μ l of GaM-Au with 500 μ l of PBS containing either 0.5 mg of purified SB virus or 0.5 mg of purified SB virus that had been reacted previously with 5 μ l of MAb R15 ascites fluid. These mixtures were then applied to a linear gradient (15 to 35% sucrose in TNE [20 mM Tris hydrochloride, pH 7.2, 0.15 M NaCl, 1 mM EDTA]) and centrifuged for 1.5 h at 24,000 rpm in a Sorvall AH629 rotor. A positive reaction was indicated by a reddish tint imparted to the viral band, an increased sedimentation velocity, and the presence of GaM-

Au beads on the virus itself, as visualized by negative-stain electron microscopy. By these criteria, the GaM-Au beads recognized only MABs and did not bind to virus alone.

Immunocytochemical labeling. SB-RL (multiplicity of infection of 10^4 PFU per cell) was allowed to attach to duplicate cultures of 5×10^5 BHK cells on 35-mm culture dishes at 4°C for 60 min. Plates were washed three times with PBS-1% at 4°C, and 0.2 ml of MAB R15, R12, or R509 was applied for 30 min at 4°C. One set of plates was shifted to 37°C, and another set remained at 4°C. After a 30-min incubation, excess MAB was removed and the plates were washed three times with PBS at 4°C. The cells were fixed with formaldehyde (3.7% in PBS) for 30 min at 4°C. Plates were washed with PBS-1% three times, and 0.2 ml of GaM-Au (optical density at 520 nm of 0.8) was applied to each plate. The plates were placed on an orbital shaker and gently rotated for 60 min at 22°C. The cells were washed three times with PBS. The treated cells were gently scraped from the plates with a rubber policeman, pelleted in a microcentrifuge for 5 min, and postfixed with 3.7% formaldehyde for 30 min at 4°C. The cells then were washed three times with cold PBS and fixed with 1% OsO₄ (in 0.1 M phosphate buffer, pH 7.2) for 60 min at 4°C, washed with PBS, and dehydrated at 4°C in 30, 50, 70, and 95% ethanol (15 min each at 4°C). The cells were dehydrated further by three consecutive 15-min incubations in 100% ethanol, followed by three changes in propylene oxide. Infiltration of the cell pellet was accomplished overnight at room temperature in a 1:1 mixture of propylene oxide and Epon LX-112 (Ladd). The cells then were placed in Epon LX-112 for 3 days at 60°C. Gold or silver thin sections were obtained on an LKB Nova ultramicrotome. Sections were placed on slotted grids (0.5% Formvar and carbon coated) and stained for 60 min with 2% uranyl acetate (in 50% ethanol-50% H₂O; Ladd) and poststained with Reynolds lead citrate for 5 min. Electron micrographs were taken on a JEOL 100s operating at 80 keV.

Effect of antibody binding to transitional epitopes. Freshly grown SB virus was partially purified on a potassium tartrate step gradient as described previously (21). The virus band at the interface was removed, diluted in NTE (0.1 M NaCl, 0.05 M Tris, pH 7.2, 1 mM EDTA), and centrifuged through a 20% sucrose cushion in an AH629 rotor (Sorvall) at 24,000 rpm for 2.5 h. The pellet was suspended in PBS-D.

The structural integrity of each purified SB virus preparation was monitored by using a capture ELISA. MABs R12, R15, and R5806 were nonspecifically attached to an ELISA plate, followed by addition of purified SB virus. The ability of each MAB to capture purified virions was detected by polyclonal, horseradish peroxidase-labeled, SB virus-specific hyperimmune mouse ascites fluid. MAB R15, which binds to an E2 surface epitope, served as a positive control. MAB R5806, specific for the putative RNA polymerase of TEV, served as a negative control. MAB R12 was specific for an E2 transitional epitope. Therefore, the inability of MAB R12 to bind SB virus in a capture ELISA was diagnostic for preservation of native structure.

The virus was diluted to approximately 10^3 PFU/ml in PBS-1%; 200 μ l of diluted virus was applied to 60-mm culture dishes containing BHK cells that had been cooled to 4°C. Triplicate cultures were washed once with 1 ml of cold PBS-1%, followed by the addition of 200 μ l of PBS-1% or one of the indicated MABs (1:500 dilution of ascites fluid). Where indicated, 5% guinea pig serum (Cederlane) was added as a source of complement to maximize the neutralizing activity of the MAB. The cultures were incubated for 45

min at 4°C to allow MAB to interact with virus-cell complexes. The cultures were shifted to 30°C for 45 min, a temperature and time that would permit penetration. The cultures were then washed with 1 ml of PBS-1%, followed by addition of 200 μ l of PBS-1% or an appropriate MAB(s) (1:500 dilution of ascites fluid with complement); incubation was continued to allow neutralization of virus particles remaining at the cell surface. After this incubation, the inoculum was removed and the plates were overlaid with 0.9% agarose in MEM for enumeration of plaques.

RESULTS

MAB binding to SB virus-BHK cell complexes. MABs to the SB virus glycoproteins may be classified experimentally as reactive with either external or internal epitopes. External epitopes are those present on freshly grown and purified (presumably native) virions that are accessible to MAB as shown by capture ELISA or by the ability of native virions to adsorb MAB in solution (38, 45, 46). External epitopes include at least three neutralizing antigenic sites on E2 (38, 41, 46, 50), while one neutralizing E1 antigenic site has been identified (9, 46). Conversely, internal epitopes are positioned on native virions such that binding of their defining MABs is effectively prevented.

In these experiments, we have used the relative exposure of internal and external epitopes to monitor the structure of the SB virus glycoprotein spike during the early stages of virus-cell interaction. Freshly purified SB virions (estimated multiplicity of infection of 10^3 PFU per cell) were allowed to attach to BHK cells at 4°C. At 4°C, virus binds to the cell surface but does not penetrate. Individual MAB preparations were added to the virus-cell complexes, and MAB binding was monitored with ¹²⁵I-GaM (Fig. 1). At 4°C (time 0), an external neutralization epitope on glycoprotein E2 was detected with MAB 49, but very little binding was observed with MAB R12, which recognizes an internal epitope on E2, with MABs R2 and R510, which recognize internal epitopes on E1, or with MAB K3, which is specific for the capsid protein of TEV (15). However, when the temperature of the virus-cell complex was increased to 37°C in the presence of these MABs, binding of R12 (E2 specific) and R2 (E1 specific) to their cognate epitopes was evident. The degree to which the MAB R12 and R2 epitopes were exposed increased through 30 min and then remained stable through 60 min. If one considers MAB 49 as an internal standard, it is estimated that 20 to 30% of the attached virions bound MABs R12 and R2. We have used the term "transitional epitopes" to denote those internal epitopes which become accessible to MAB binding under these conditions. In a control experiment, incubation of SB virions in the absence of cells at 37°C did not induce any structural change detectable by MAB binding, nor was MAB R12 binding detected at the cell surface in the absence of SB virus (data not shown). Not all internal sites were detected, as typified by the inability of MAB R510 or the nonspecific TEV control antibody K3 to bind to virus-cell complexes under any of the above conditions. This experiment suggests that the structure or orientation of the SB virus glycoprotein spike was altered early in the infection process. The alteration required the formation of a virus-cell complex, was a function of temperature, and was detectable within the first 30 min at 37°C.

The exposure of the transitional epitopes was detected only when the MABs were present from the time of temperature shift to 37°C (Fig. 2). When virus-cell complexes established at 4°C were shifted to 37°C for 30 min, returned

FIG. 1. Exposure of transitional epitopes at the cell surface. BHK cells were infected with gradient-purified SB virus at 4°C, treated with MAb, and then shifted to 37°C in the presence of the MAb as detailed in Materials and Methods. MAb binding was quantitated with ^{125}I -GaM. Shown are results for MAbs 49 (Δ), R12 ($\circ$), R2 ($\square$), R510 ($\bullet$), and K3 ($\blacksquare$).

to 4°C, and then probed with transitional epitope MAbs, the altered conformation was not found. This result suggests that the rearranged glycoproteins reside in a relatively short-lived intermediate structure. In the experiment depicted in Fig. 1, in which the transitional epitope MAbs were present at the time of the rearrangement, the antibodies bound to the rearranged glycoproteins and apparently trapped them at the cell surface.

Sixteen MAbs that react with internal epitopes have been analyzed for their reactivity with SB virus-BHK cell complexes at 4°C or after shift to 37°C in the presence of MAb (Table 1). The MAbs showed two predominant patterns of reactivity. MAbs R509 and R512 were typical of nine antibodies reactive with internal epitopes. They reacted poorly with virus-cell complexes either after attachment at 4°C or after incubation at 37°C. This result suggests that the transition detected in the previous experiment (Fig. 1) was not the result of generalized virus degradation. A second pattern (seven MAbs) was typical of the transitional epitopes defined by MAbs R12 and R2 (Fig. 1). For these epitopes, a low level of binding was detected at 4°C, whereas a substantial increase in binding was evident after incubation of virus-cell complexes for 30 min at 37°C. Four MAbs (R3, R4, 35, and 38) gave anomalous results. MAbs 35 and 38, nominally internally reactive antibodies, gave a significant signal after virus attachment at 4°C followed by additional antibody binding at 37°C. Taken together, the observed changes in MAb reactivity suggest that the SB virus glycoprotein spike is subject to at least one conformational transition during the early stages of virus-cell interaction.

Morphology of the conformationally altered glycoprotein

FIG. 2. Detection of transitional epitopes with MAb present before or after the shift to 37°C. BHK cells were infected with gradient-purified SB virus at 4°C and treated with MAb R12 (bars A and C) or PBS (bar B). Cell cultures were then shifted to 37°C for 30 min (bars B and C) or held at 4°C (bar A). The cultures at 37°C (bars B and C) were then returned to 4°C. For cultures depicted in bar B, MAb R12 was added for 30 min, followed by addition of ^{125}I -GaM to all of the samples.

spike. Immunogold labeling was used to determine whether the altered glycoprotein spikes, detected immunochemically at the cell surface, were contained within intact virus particles or were glycoprotein forms associated with the plasma membrane (Fig. 3; Table 2). Instead of detecting bound MAb with ^{125}I -GaM as described above, these experiments used GaM adsorbed onto electron-opaque, colloidal gold beads (GaM-Au), thus allowing visualization of the complex in the electron microscope. Gold beads 30 nm in diameter were used to facilitate morphometric analysis. Three MAbs were

TABLE 1. Characterization of internally reactive MAbs

MAb	Protein specificity	Type of epitope ^a
R2	E1	TR
R9	E1	TR
R12	E2	TR
31	E1	TR
R501	E2	TR
R503	E2	TR
R505	E2	TR
R504	?	INT
R506	?	INT
R507	?	INT
R508	?	INT
R509	E2	INT
R510	E1	INT
R511	?	INT
R512	E2	INT
R513	?	INT

^a MAbs were classified as specific for transitional epitopes (TR; similar to R12 [Fig. 1]) or as internal epitopes (INT; similar to R510 [Fig. 1]).

FIG. 3. Immunocytochemistry of virus-BHK cell complexes at 4 and 37°C. SB-RL-BHK cell complexes were treated with MAb at 4°C and either held at 4°C or shifted to 37°C in the presence of the MAb. Binding of MAb was detected with GaM complexed with colloidal gold beads. (A) MAb R15, 4°C; (B) MAb R12, 4°C; (C) MAb R12, 37°C; (D) MAb R509, 37°C.

used on the basis of the results of previous experiments. MAb R15 is a neutralizing antibody in the same class as MAb 49 (Fig. 1). It reacted with an external epitope on E2 both at 4°C and after 37°C incubation and served both as a positive

control and as an internal standard. MAb R509 was used as a negative control antibody that did not bind detectably to virus-cell complexes under either condition. The test antibody was R12, which did not bind to attached virions but did bind to virus-cell complexes after incubation at 37°C.

After attachment at 4°C, significant GaM-Au labeling of virions was observed only when the virus-cell complexes were incubated with MAb R15 (Fig. 3A). Seventy-seven percent of the GaM-Au beads seen were associated with morphologically identifiable virions at the cell surface (Table 2). The density of GaM-Au labeling was much lower in samples incubated with MAb R12 (Figure 3B) or R509, and none of the observed gold beads were associated with virions.

After shifting of the virus-cell complex to 37°C in the presence of MAb R15, 82% of the GaM-Au beads seen at the cell surface were associated with virus particles (Table 2). Under these conditions, the density of GaM-Au labeling was higher than at 4°C. When the virus-cell complex was shifted to 37°C in the presence of MAb R12, the density of gold labeling increased dramatically compared with R12 at 4°C. All of the increase was due to association of GaM-Au with virus particles. Seventy-two percent of the GaM-Au beads

TABLE 2. Immunogold labeling of cell-associated viral antigens^a

Condition	Au particles/100 μm^2		
	Not virion associated	Virion associated	% Virion associated
4°C, R15	11.8 (55)	38.7 (180)	77
4°C, R12	9.6 (38)	0.0 (0)	0
4°C, R509	4.6 (22)	0.0 (0)	0
37°C, R15	13.6 (56)	60.9 (251)	82
37°C, R12	7.2 (33)	18.7 (86)	72
37°C, R509	8.4 (40)	0.2 (1)	2

^a The length of cell perimeter examined for each condition was estimated in the electron microscope with a grid bar at $\times 1,000$. Surface area was calculated by multiplying the perimeter distance by 100 nm (the estimated thickness of each section), and the number of Au particles per 100 μm^2 is presented. The surface area scanned for the various conditions ranged from 395 to 482 μm^2 . Numbers in parenthesis are the total number of Au beads observed associated with virions or the number lying greater than one virion diameter from a particle.

FIG. 4. Effect of cycloheximide and NH₄Cl on transitional epitope exposure. BHK cell monolayers were treated with either cycloheximide (20 µg/ml) or NH₄Cl (10 mM) for 30 or 60 min, respectively. The cultures were then infected as described for the experiment in Fig. 1. MAb binding to virus-cell complexes at 4°C or after 30 min of incubation at 37°C was quantitated with an iodinated second antibody. Background binding (in controls with PBS-1% instead of MAb) was subtracted from the values shown. Length of 37°C incubation: 0 min (■) or 30 min (□).

observed in the MAb R12 samples were associated with virus particles, compared with 0% at 4°C (Table 2; Fig. 3C). Virtually no labeling of virions was observed with R509, a MAb that recognizes an internal E2 epitope which did not become accessible upon incubation of virus-cell complexes at 37°C. These data suggested that the conformational transition detected with MAb R12 occurred on the glycoprotein spikes of morphologically intact virus particles as opposed to a membrane-associated form of the glycoprotein. Figure 3D illustrates a typical field showing cell-associated GaM-Au labeling in samples treated with MAb R509 at 37°C.

The proportion of virions exhibiting the R12 epitope may be inferred from the density of labeling with MAb R12 compared with MAb R15. Since MAb R15 reacts with an external epitope presumably exposed on every virion, the density of labeling with this MAb represents the maximum expected. The labeling density with MAb R12 was approximately 30% that of R15, suggesting that 30% of the particles had rearranged.

The transition at 37°C occurs in the presence of protein synthesis inhibitors, in the presence of a lysosomotropic agent, and in CHO cells defective for endosomal acidification. The cellular physiological requirements for the observed conformational alteration were investigated. Pretreatment of cells with cycloheximide had no effect on the exposure of transitional epitopes, suggesting that neither early virus translation products nor new cellular protein synthesis was required (Fig. 4). Under the conditions used, cycloheximide inhibited host cell protein synthesis by 85% (data not shown).

The conformational transition that we have detected at the cell surface could have resulted from internalization of virions by receptor-mediated endocytosis, rearrangement induced within a low-pH endosomal compartment, and recycling of endosomes containing bound virus to the plasma membrane. To determine whether transit through a low-pH compartment was required for the rearrangement, BHK

FIG. 5. Transitional epitope exposure after infection of CHO cells defective in endosomal acidification. Wild-type CHO cells (WTB) or mutants temperature sensitive for endosomal acidification (B3853) were incubated at 4, 34, or 40°C before addition of a mixture of SB virus and MAb. Incubation was continued at the given temperature for 45 min, the cultures were placed at 4°C, and MAb binding was quantitated with ¹²⁵I-GaM. Shown are results for incubation at 4°C (■), 34°C (■), and 40°C (□). Values shown were reduced by background binding evident in uninfected controls receiving MAb R12. Binding of MAb 49 ranged from 4,600 to 5,600 cpm under the various conditions of cell type and temperature.

cells were treated for 60 min with medium containing 10 mM NH₄Cl. This NH₄Cl concentration is thought to inhibit alphavirus penetration by approximately 75% (26, 29) and increases the endosomal pH to approximately 6.5 (36). The temperature was lowered to 4°C, the cultures were infected with SB virus in the presence of NH₄Cl, and after 1 h the infected, NH₄Cl-treated cells were shifted to 37°C. The rearrangement detected by MAb R12 at the cell surface was unaffected by NH₄Cl treatment (Fig. 4). However, the yield of progeny virus from NH₄Cl-treated cultures was only 10% that of untreated controls, consistent with previous reports (25, 26).

The putative role of a low-pH compartment also was investigated in a CHO cell mutant with a temperature-sensitive defect in endosomal acidification (42). The conformational change detected on BHK cells by MAb R12 also was detected to the same extent on both mutant and parent CHO cells at both permissive and nonpermissive temperatures (Fig. 5). These data in conjunction with the NH₄Cl experiments and morphological studies suggest that the glycoprotein transition occurred at the cell surface and that it was unlikely that prior transit of virions through a low-pH compartment was required.

Correlation of penetration with exposure of transitional epitopes. The biological significance of the observed alteration in glycoprotein structure was explored in several experiments. In an initial experiment, we compared the time course for detection of transitional epitopes at the cell surface to the time course for penetration of SB virus (Fig. 6). In this experiment, the extent of penetration was defined as infectious centers resistant to removal by trypsinization. The detection of the structural rearrangement, as indicated by an increase in ¹²⁵I-GaM labeling, occurred with a time

FIG. 6. Comparison of transitional epitope exposure and penetration. Exposure of the R12 epitope was assessed as for Fig. 1. Penetration was measured as the appearance of trypsin-resistant SB virus infectious centers as described in Materials and Methods. Background binding was deducted from the plotted values as for Fig. 5. Symbols: ●, ¹²⁵I cpm; ○, infectious centers.

course similar to or narrowly preceding the detection of trypsin-resistant infectious centers.

The rate of SB virus penetration is dependent on temperature. The effect of temperature on penetration and on the expression of transitional epitopes was examined in the experiment depicted in Fig. 7. The percentage of PFU that penetrated BHK cells in 60 min increased dramatically with increasing temperature. The amount of MAb R12 binding at the cell surface increased with temperature in an analogous manner. In a separate experiment, very little penetration was detected at 24°C, and we were unable to detect the conformational change under these same conditions. In every case, there was no change in the binding of a neutralizing MAb (MAb 49), indicating that the number of virus particles associated with the cell surface remained the same for each temperature (data not shown).

If the glycoprotein conformational change occurred immediately before or concomitant with penetration, then virus mutants with altered penetration kinetics might display altered kinetics of transitional epitope exposure. SB-RL is a mutant of SB virus that is characterized by a more rapid rate of penetration (4, 37). The accelerated penetration phenotype results from a single point mutation in glycoprotein E2 that substitutes arginine for serine at amino acid position 114 (13, 39). When equivalent amounts of SB-RL and SB virus were attached to BHK cells at 4°C and then shifted to 37°C, the MAb R12 epitope of SB-RL was detected earlier and at higher levels than observed with SB virus (Fig. 8). Equivalent binding of MAb R12 to SB virus and SB-RL was observed in standard ELISA in which virions are disrupted before exposure to antibody (R. A. Olmsted and R. E. Johnston, unpublished data). The implication of this result is that the arginine substitution in SB-RL facilitates the glycoprotein rearrangement at the cell surface, leading to the apparent increase in penetration efficiency.

Effect of a transitional epitope MAb on penetration. The

FIG. 7. Temperature dependence of penetration and exposure of transitional epitopes. Penetration and exposure of the R12 epitope were compared 60 min after the shift to the indicated temperatures as for Fig. 6. Symbols: □, ¹²⁵I cpm; ■, infectious centers (percentage of attached PFU).

close correlative association of the structural transition with virion penetration and the detection of the transition at the cell surface suggested that conformationally altered virions were early intermediates in the entry process. Alternatively, the rearrangement may have occurred exclusively on noninfectious particles which predominate in most alphavirus

FIG. 8. Exposure of transitional epitopes on SB virus and SB-RL. The time courses for exposure of the R12 epitope on SB virus (●) and SB-RL (○) were compared. Background binding was subtracted as for Fig. 5. No binding above background was detected for MAb K3 or R510; MAb 49 binding ranged from 10,234 to 10,569 cpm and from 10,480 to 11,434 cpm for SB virus and SB-RL, respectively.

		30°C		45 min		60 min		Overlay	
		60 min		45 min					
4°C									
Infect								PFU ^a	% Neut.
	PBS				PBS			237±9	0
	R15+C'				PBS			29±8	88
	PBS				R15+C'			225±35 ^b	5
	PBS				R15+R12+C'			224±45	5
	R12				PBS			282±47	0
	R12				R15+C'			162±13 ^b	32

FIG. 9. Penetration of infectious SB virions in the presence of a transitional epitope antibody. Infected cultures were incubated as described in Materials and Methods at the temperatures and for the times indicated above. Treatments with MAb or diluent (PBS-1%) were at the end of the attachment period at 4°C and after a penetration period at 30°C. a, Average of three cultures. b, comparison of these two values by Student's *t* test yields *P* = 0.05. Analogous values for *P* in two other independent experiments of this type were *P* < 0.02 and *P* < 0.005.

populations and therefore might not be related to an entry pathway leading to productive viral infection. To distinguish between these two possibilities, the biological effect of antibody binding to transitional epitopes on rearranged virions was measured. In initial experiments, the ability of MAb R12 to prevent infection was tested. SB virus was allowed to attach to BHK cells for 60 min at 4°C. The virus-cell complexes were treated with MAb R12 or MAb R12 and complement. In the presence of the MAb, the cultures were shifted to 37°C for 60 min. The cultures were then overlaid with agarose for the formation of plaques. PBS and MAb R509 were used instead of MAb R12 in control experiments. Under these conditions, MAb R12 had no consistent effect on the establishment of infectious centers (data not shown).

We next examined the possibility that although R12 did not prevent penetration, binding of this MAb to an intermediate particle might nevertheless retard its penetration (Fig. 9). In a control experiment, MAb R15, a neutralizing E2-specific antibody, was added to virus-cell complexes after attachment at 4°C but before penetration was stimulated by shift to 30°C. Eighty-eight percent of the attached PFU were neutralized under these conditions. However, when MAb R15 was added after the penetration period at 30°C, only 5% of the PFU remained susceptible to neutralization. No additional neutralization was observed when cultures were treated with both MAbs R15 and R12 after the penetration period at 30°C. These control experiments established that MAb R15 neutralized attached but unpenetrated virions and that penetration of infectious virions was essentially complete (95%) at the end of the 30°C incubation. In contrast to R15, MAb R12 added to virus-cell complexes before the shift to 30°C did not neutralize attached virions, although prior experiments indicated that MAb R12 bound to rearranged virions under such conditions. When parallel MAb R12-treated cultures were probed with MAb R15 after the penetration period at 30°C, 32% of the attached infectious virions remained susceptible to neutralization, compared with 5% in the absence of MAb R12. These data suggest that when the glycoprotein rearrangement occurred, MAb R12 bound to the conformationally altered intermediate particles and retarded their penetration, leaving a higher proportion of them susceptible to MAb R15 neutralization at the end of the 30°C incubation. Since MAb R12 was specific for the rearranged particles and since the retardation of penetration was measured in a biological assay for infectivity, it is probable that

the altered virions were intermediates in a normal entry pathway leading to productive infection.

DISCUSSION

One of the paradoxical features of virus structure is that virions must remain stable under a variety of adverse environmental conditions yet be capable of uncoating during the initial stages of infection. The earliest steps in the disassembly cascade may occur extracellularly, at the plasma membrane, or in association with intracellular compartments such as endosomes. In nature, reoviruses enter susceptible hosts orally, eventually reaching the intestine and infecting intestinal epithelial cells. The initial stage of reovirus disassembly occurs extracellularly in the proteolytic environment of the intestine (6). In the intestine, reovirions are converted into infectious intermediate subviral particles upon removal of the sigma 3 protein and generation of the μ lc cleavage product, delta (6). The sigma 1 attachment protein of the infectious intermediate subviral particles is extended from the virion surface (20), a conformation that presumably facilitates cell attachment or entry. Influenza virus may undergo an analogous proteolytic activation in the respiratory tract (3).

A significant alteration in picornavirus structure occurs early in infection of intact cells (12, 31, 32). The alteration probably results as a consequence of attachment at the plasma membrane, since it is also observed after incubation of virions with membrane preparations bearing the appropriate receptor (14, 23). Picornavirus particles eluted from the cell surface after attachment are characterized by an altered protease susceptibility and antigenic profile, lower sedimentation rate, loss of VP4, and exposure of the amino terminus of VP1 (12, 18, 31, 32). In the native structure, VP4 is not exposed at the virion surface but is positioned directly below each of the vertices (27, 43). The absence of VP4 in eluted particles implies a major structural rearrangement that allows release of VP4 from its interior position. VP4 is myristylated at its amino terminus (10). This modification may assist in anchoring attached virions to the plasma membrane and cause VP4 to remain associated with cells after virion elution. In addition, Fricks and Hogle (18) have shown that the altered virions expose the amino terminus of VP1, which is lipophilic, suggesting its potential role in an interaction between an altered virion and cellular membranes in subsequent entry stages.

MAbs have been used to document intracellular conformational changes of influenza virion structure during the early stages of infection. It has been proposed that entry of influenza virus is by receptor-mediated endocytosis, followed by a low-pH-induced conformational change in the hemagglutinin glycoprotein (HA) structure (57). In the altered HA structure, the hydrophobic region at the amino terminus of HA2 is exposed, suggesting a conformation that could potentiate fusion of the influenza virus and endosomal membranes. Such a rearrangement in response to an acidic environment has been demonstrated in solution by using the bromelain-solubilized influenza virus HA (48). Low pH also induces structural changes in native influenza virions, as detected by increased or decreased binding of MAbs directed against the four major antigenic regions of HA (55, 59). A MAb raised against influenza virions treated at low pH does not react with native virions, cytoplasmic HA, or cell membrane-associated HA (2). The MAb does bind to influenza virions contained within endosomes, suggesting that the low endosomal pH induces a structural rearrange-

ment in virions similar to that induced in soluble bromelain-solubilized HA at low pH.

The structural rearrangement of the SB virus glycoprotein spike described in this report may be one of the first steps in the alphavirus disassembly cascade. Using MAbs reactive with epitopes not normally accessible on the surface of native virions, we have detected an altered plasma membrane-associated virion intermediate on which these transitional epitopes were exposed. Four issues regarding this structural rearrangement will be discussed: its biological relevance, the cellular signals that induce it, the mechanism by which it occurs, and the nature of the altered virion structure.

Biological significance of the structural rearrangement. Several arguments support the hypothesis that the rearranged particle we have observed represents an intermediate structure in the process of productive infection rather than the fate of noninfectious particles in the virion population. First, the rearrangement probably did not result from non-specific degradation of virus particles. There was no evidence of degradation in the electron microscopy study, and only a subset of previously inaccessible glycoprotein epitopes became accessible to their cognate MAbs when virus-cell complexes were incubated at 37°C. Approximately 30% of total virions had rearranged (Fig. 1; Table 2), including at least 30% of the infectious virus in the population (Fig. 9). Second, the rearrangement was correlated with both the time course and temperature dependence of viral penetration, as measured in a biological assay. Third, the structural rearrangement and penetration were linked genetically. A mutant having an accelerated penetration phenotype, SB-RL, also showed an accelerated time course for the rearrangement.

The rearranged particle was detected only when transitional epitope MAbs were present continuously as virus-cell complexes were shifted from 4 to 37°C. In experiments in which virus-cell complexes were shifted to 37°C in the absence of MAb and subsequently probed with the appropriate transitional epitope MAbs, binding of antibody was not detected. This finding suggests that the conformationally altered particle was a relatively short-lived intermediate that was trapped at the cell surface by the transitional epitope antibodies soon after the rearrangement had occurred. Consistent with this view is the finding that in the presence of transitional epitope antibodies, the internalization of infectious virions was retarded. These results also provide strong evidence that the observed conformational intermediate was generated during the normal course of productive viral infection. Had the rearrangement been limited to noninfectious virus particles, no effect of MAb specific for rearranged virions would have been observed in the biological assay of penetration.

Possible cellular triggers for the rearrangement. The rearrangement did not occur on SB virions in the absence of cells, suggesting strongly that it was triggered in response to a cellular signal or interaction between virions and a cellular component. Simple attachment of virions at 4°C was not sufficient to induce the virion rearrangement described in this report. De novo protein synthesis was not required.

The cell surface is the most likely location for the virus-cell interaction which triggers the transition that we have observed. In support of this argument, rearrangement was detected at the cell surface with iodinated antibodies added to intact cells, and transitional epitope MAbs, which bound to conformationally altered particles but not native virions, retarded internalization of infectious virus. Alternatively,

virions could have entered the receptor-mediated pathway, these rearrangements could have been triggered by the reduced-pH environment within the endosomes, and the particles could have been recycled rapidly to the cell surface to be detected in our assays. This alternative seems less likely. Neither treatment of BHK cells with NH_4Cl nor CHO cell mutations that limit the acidification of endosomes had any measurable effect on the rearrangement we observed. In addition, the constellation of epitopes displayed on the rearranged virions described here differed qualitatively from that displayed by virions treated in suspension at low pH (W. J. Meyer, S. Gidwitz, and R. E. Johnston, unpublished data). Although these experiments do not indicate a requirement for endosomal recycling in the rearrangement, they also do not formally exclude this possibility. However, the most probable interpretation is that formation of the structural intermediates occurred before internalization of virions and was induced at the cell surface in response to interaction with an element(s) of the plasma membrane.

Our data do not allow definitive conclusions regarding the fate of the transitional intermediates subsequent to their formation, except that the genomes contained in such particles eventually participated in a productive infection. If a fusogenic peptide is exposed on the intermediate particles at the exterior face of the plasma membrane, then the rearranged glycoproteins potentially could mediate direct fusion at the cell surface. Alternatively, if exposure of a fusogenic domain requires the low-pH environment of an endosome, then the structural intermediate that we have identified may represent only one of several early conformational changes, leading to the eventual exposure of a fusogenic domain after internalization by receptor-mediated endocytosis. Therefore, two of the critical issues remaining to be resolved are as follows: (i) what constitutes a fusogenic domain in alphaviruses, and (ii) is this domain exposed only in endosomes, or is it included among the epitopes newly exposed by structural rearrangements detected at the cell surface?

Potential transition mechanisms. The mechanism by which such a rearrangement could occur is unknown. The MAb probes that detected the transition most likely reacted with those glycoprotein spikes above the plane of the virus-cell interface rather than with those in direct association with the plasma membrane, where steric hindrance would have prevented antibody binding. Therefore, the spikes on which the rearrangement was detected probably were not those in direct contact with a putative triggering element. This could be explained if an initial rearrangement of the glycoproteins in direct contact with the membrane was propagated around the virion surface through interactions between the spikes in the plane of the envelope or was propagated through interactions of the spike glycoproteins with the capsid structure. Alternatively, attachment to receptor and rearrangement could have occurred on a given spike or group of spikes, followed by transient disassociation of the virion from the surface and reestablishment of attachment at a different group of glycoprotein spikes on the same virion.

Nature of the intermediate particle. In native SB virions, the E1 and E2 glycoproteins are closely associated with one another (40). Trimers, each consisting of three E1-E2 heterodimer units, form groupings around five- and sixfold axes of symmetry in a $T = 4$ icosahedral arrangement visible by electron microscopy of negatively stained virions (19, 54). Within this structure, transitional E1 and E2 epitopes are inaccessible to their cognate MAbs. The occlusion of such epitopes may result from the formation of E1-E2 heterodimers, from interaction of heterodimers to form the

trimeric structures, or from higher-order associations between trimers. The transitional epitopes in the intermediate particle became accessible to MAb, presumably by a reorientation of one or more of these protein-protein interactions or by a change in the folding of individual glycoprotein polypeptides. The available data favor a relatively small change in the native virion leading to the intermediate structure. There were no obvious structural differences observed by electron microscopy of native and intermediate particles in thin section, and only a subset of inaccessible epitopes became accessible during transition to the intermediate particle. A subtle change in the association between trimers or the interactions between individual E1-E2 heterodimeric units forming the trimers could account for the simultaneous exposure of new epitopes on both E1 and E2. In such a model, it would not be necessary to invoke either a partial denaturation of the individual glycoproteins or a dissociation of the relatively stable E1-E2 heterodimer. However, it is clear that the information presently available is insufficient to formulate a more specific model or to rule out other models for the structure of the intermediate particle.

Regardless of the precise nature of the intermediate particle, the data presented in this paper suggest that (i) after attachment to BHK cells, SB virions undergo a reorganization of their glycoprotein spikes, (ii) the rearrangement is detected at the plasma membrane, without any apparent requirement for prior exposure to a low pH environment, and (iii) the structure resulting from the rearrangement is an early intermediate in a disassembly pathway leading to productive infection. It is our goal to determine the steps in this pathway subsequent to the formation of the intermediate and to identify the constituents of its characteristic transitional epitopes.

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