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Conformational Changes in Sindbis Virus Envelope Proteins Accompanying Exposure to Low pH

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The attachment of high multiplicities of Sindbis virus to tissue-cultured cells followed by brief treatment at low pH has been shown to produce cell fusion (fusion from without). In this report, experiments to determine the effects of low pH on the physical and biological properties of Sindbis virus are described. Exposure of purified Sindbis virions to mildly acidic conditions resulted in a rapid and irreversible alteration in particle density and sedimentation characteristics, followed by a slower loss of infectivity. Infectivity was not restored by a return to neutral pH; rather, the loss of virus infectivity seemed to be initiated by exposure to low pH but continued at neutral pH. The formation of a virus-cell complex in which virions were attached to the cell surface protected the particles from low-pH inactivation, although low pH could still expose virus functions responsible for cell fusion. Low pH was found to induce a conformational change in the E_2 polypeptide of the intact virion. These results are discussed with respect to the process of Sindbis virus infection of tissue-cultured cells.

Recent years have witnessed a renewed interest in the initial stages of the interaction between viruses and host cells (for reviews, see Dimmock [4], Meager and Hughes [14], Lonberg-Holm and Philipson [10], Howe et al. [8], and Helenius et al. [6]). Experimental examination of virus-cell interactions which precede virus-directed protein and nucleic acid synthesis (traditionally referred to as attachment, penetration, and uncoating) have produced numerous conflicting and contrasting reports on this important biological process. Interpretations have yielded two general points of view, which may be summarized as supporting a penetration event that occurs (i) by direct interaction of the virus with the cell surface (3, 9, 16) or (ii) takes place in an intracellular location after endocytosis of the infecting virion (6, 13). The experiments leading to these models suffered from the common problem that in none of them could the investigator confidently state he was examining an event resulting in the infection of a cell by a virus. The virus preparations employed in these studies could not be shown to be 100% infectious. Indeed, particle-to-PFU ratios in the best of circumstances could not be shown to be better than 5:1, and, in most circumstances, this ratio was much higher. Thus, for any observation, whether of morphological or biochemical characteristics, it can be argued that the predominating contributor to the results observed was noninfectious material. Perhaps a more serious but less considered problem is the fact that experiments investigating the interaction of viruses with host cells have required enormous numbers of virus particles (infectious and noninfectious) per cell to generate a sample size producing particles identifiable by electron microscopy or allowing chemical and physical assays of alterations in the "infecting" viruses. Such high-multiplicity experiments make the unsubstantiated assumption that all virus particles presented to a cell are treated in the same way. Indeed, recent evidence suggests that cells are capable of dealing with limited numbers of virus particles through routes leading to an infection (19). Thus, many virus particles may engage in nonproductive cell interactions.

The differing views on the cellular location at which penetration by membrane-limited viruses may take place are unanimous on one point, that the membrane-bound virus reaches the cell cytoplasm by fusing its membrane with a cell membrane. This contention has received strong support from the recent observation that many viruses can cause the fusion of cell plasma membranes after exposure to mildly acidic pH (26, 28) (for a review, see Dimmock [4]). The fact that low pH can cause the expression of a fusion function in many enveloped viruses suggests that this pH-induced event may mimic an event occurring during the fusion of virus membranes with cell membranes during normal infection. In the investigations reported here, we

examined the effects of low pH on the infectivity and physical integrity of the alphavirus Sindbis in an attempt to relate the expression of this fusion function to the process of infection.

MATERIALS AND METHODS

Cells, medium, and viruses. BHK-21 cells were grown in Eagle minimal essential medium (5) supplemented with 10% fetal calf serum, 2 mM glutamine, and 10% tryptose phosphate broth, as described previously (15). Heat-resistant Sindbis virus (SVHR), which served as the wild type in these studies, was originally provided by Elmer Pfefferkorn (Dartmouth Medical College) and has been propagated in our lab for the past 10 years. Stocks of this virus were prepared as described previously (15). The virus concentration was determined by plaque assay, as described previously (15). Infectious center assays were carried out as described by Riedel and Brown (17).

Preparation and purification of radioactively labeled Sindbis virus. Subconfluent monolayers of BHK-21 cells were infected with 50 to 100 PFU of Sindbis virus per cell. At 4 h postinfection, the medium was removed and replaced with methionine-free medium containing 15 μCi of [^{35}S]methionine per ml. The medium was collected at 18 h postinfection, and radioactive virus was purified by sedimentation to equilibrium density on 15 to 35% (wt/wt) linear potassium tartrate gradients (22). Velocity sedimentation analysis of purified Sindbis virus was carried out in 5 to 15% (wt/wt) linear potassium tartrate gradients at 4°C for 1 h at 26,000 rpm in a Sorvall AH627 rotor.

Polyacrylamide gel electrophoresis. Analysis of [^{35}S]methionine-labeled Sindbis virus proteins before and after exposure to acid pH or trypsin treatment was carried out by electrophoresis of the proteins through sodium dodecyl sulfate gels containing 11% polyacrylamide as described previously (20). Sample buffer was prepared as described previously (20). Fluorography of the gels was performed by the method of Bonner and Laskey (2).

Treatment of virus with low-pH medium. The effects of low pH on infectivity were determined by diluting virus (in minimal medium) 1:100 in low-pH medium (12); controls were diluted in an identical medium at pH 7.2. Virus was held at the appropriate pH for a period of time and then diluted into phosphate-buffered saline (PBS) (pH 7.2) with 3% fetal calf serum for titration by standard procedures on BHK-21 monolayers (15). The effects of low pH on physical properties were determined by diluting virus 1:100 in medium at pH 5.3 or 7.2, and when a trypsin digest followed the low-pH treatment the pH was raised to 7.2 with NaOH before trypsin was added.

Treatment of virus with trypsin. Trypsin (3× crystallized, treated with L-[tosylamido 2-phenyl]ethyl chloromethyl ketone; Worthington Diagnostics) was prepared as a stock solution at a concentration of 1 mg/ml in PBS and diluted to the desired concentration into low-pH-treated or untreated virus preparations. Soybean trypsin inhibitor (Worthington) was prepared as a stock solution at a concentration of 4 mg/ml in PBS deficient in calcium and magnesium and diluted to a concentration exceeding by fourfold the concentration of trypsin in any given reaction mixture (1).

RESULTS

Sindbis virus-mediated fusion from without. As a prerequisite to the studies that follow, it was necessary for us to demonstrate the conditions that most efficiently produced fusion of BHK-21 monolayers by attachment of Sindbis virions. The procedures employed in this study are basically those described by White and Helenius (26) and Mann et al. (12). We found that fusion from without could only be obtained at multiplicities in excess of 1,000 PFU/cell (the particle-to-PFU ratio for our virus ranged from 6:1 to 10:1, as determined by direct particle counts and total protein assay [B. Riedel, unpublished data]). Fusion from without occurred efficiently at pHs between 5.1 and 6.4, but was optimal at pH 5.3. Therefore, the protocols employed in this investigation were established after finding that fusion from without required (i) high cell densities, (ii) high multiplicities, and (iii) brief exposure to low pH followed by return to neutral pH, resulting in a series of fusion events in which an entire monolayer (2×10^6 cells) was converted to virtually a single polycaryon (Fig. 1). Interestingly, fusion from without, mediated by Sindbis virus, only occurred after virus-cell complexes were returned to neutral pH.

Effects of low pH on Sindbis virus infectivity. The observations presented above and in other published reports (25, 26) that alphaviruses can induce cell fusion through interaction with the surfaces of closely associated cells prompted us to determine what effects, if any, the low-pH treatment involved in this interaction had on virus infectivity. Exposure to pH 5.3 was found to rapidly and irreversibly inactivate Sindbis virus (Fig. 2). The kinetic rate of inactivation was found to depend on the temperature, with very rapid loss of infectivity at higher temperatures and slower loss rates at lower temperatures. The return to normal pH did not restore any of the lost infectivity. Surprisingly, a return to normal pH after brief exposure to low pH did not interrupt the inactivation process (Fig. 2). Stated otherwise, low-pH treatment seemed to immediately produce an alteration in the virus particle that led to an inactivation process, which was itself not dependent on the pH.

The effects of alterations in the glycosylation of Sindbis virus envelope proteins and of changes in the lipid composition of the virus membrane on the pH stability of virus infectivity were tested by comparing the inactivation kinetics of BHK-grown to *Aedes albopictus*-grown virus (Fig. 2). It was shown previously that the viruses produced from these two phylogenetically unrelated cell types have very different carbohydrate and lipid compositions (11, 23) that do not seem to significantly affect their biological

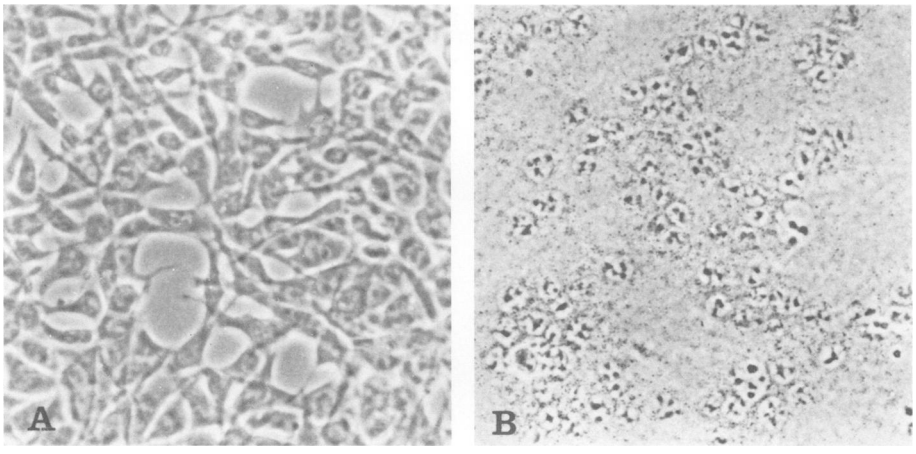

FIG. 1. Sindbis virus-induced fusion from without. BHK-21 monolayers were treated with cycloheximide and infected with 1,000 to 2,000 PFU of Sindbis virus per cell for 1 h at 0°C. (A) Control uninfected cells. (B) Cells infected as described and treated with pH-5.3 medium.

properties (23). Under the experimental conditions employed in this study, no difference in the inactivation rate of Sindbis virus produced in mosquito cells relative to that in BHK cells could be detected (Fig. 2), and thus major alterations in either the carbohydrate or lipid composition did not affect the pH stability of virus infectivity.

Effect of virus attachment to cells on the sensitivity of infectivity to low pH. Earlier studies by White et al. (27) showed that low-pH treatment of cell-associated Semliki Forest virus could bypass a block in infection produced by treating the cells with chloroquine. Although these experiments were carried out at high multiplicities, they implied that enough virus survived the low-pH treatment to infect the cells through fusion with the plasma membrane.

The following experiment was carried out to determine whether the attachment of Sindbis virus to the cell surface provided protection against the inactivating effects of low-pH treatment. Identical subconfluent monolayers of BHK-21 cells were infected with Sindbis virus for 1 h at 4°C. The cells were washed with cold (4°C) PBS and treated with cold pH-5.3 medium for 15 min. We showed above (Fig. 2) that this treatment is sufficient to inactivate over 99% of non-cell-associated virus. The control monolayer was treated similarly, but with cold pH-7.2 medium. The cultures were returned to normal medium and incubated at 37°C for 20 min. The monolayers were then trypsinized, and the cells were plated in an infectious center assay (17). These experiments were carried out at two very low multiplicities, ensuring that no cell interacted with more than a single PFU (Table 1; the

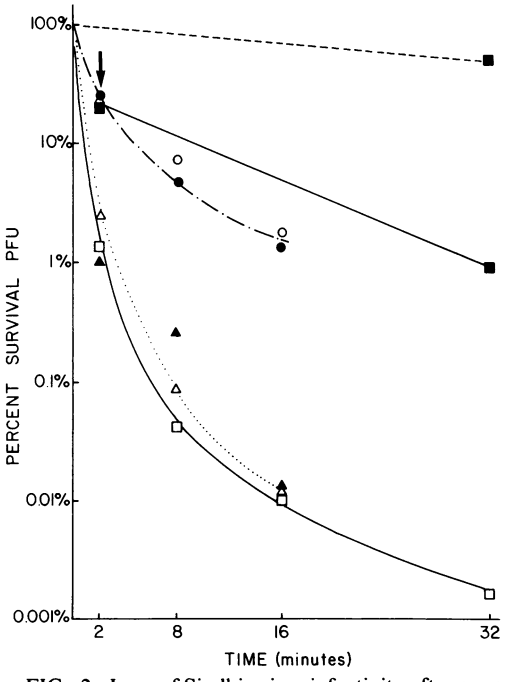

FIG. 2. Loss of Sindbis virus infectivity after exposure to pH 5.3. Sindbis virus was exposed to low pH and, at the time point indicated, diluted into PBS (pH 7.2) at 4°C (unless otherwise specified) and assayed as described in the text. The SVHR-infected cells and treatment conditions were as follows: BHK-grown virus, pH 7.2, 22°C (control) (■– – –■); BHK-grown virus, pH 5.3, 4°C (○); *A. albopictus*-grown virus, pH 5.3, 4°C (●); BHK-grown virus, pH 5.3, 22°C (△); *A. albopictus*-grown virus, pH 5.3, 22°C (▲); BHK-grown virus, pH 5.3, 37°C (□); and BHK-grown virus, pH 5.3 for 2 min at 4°C, then transferred to pH 7.2 (arrow) for an additional 30 min at 22°C (■——■).

TABLE 1. Protection of Sindbis virus from low-pH inactivation by association with (attachment to) BHK-21 cells

Expt conditions	Expt no.	No. of infectious centers produced[a] (% of total cells) at:	
		pH 7 (control)	pH 5.3
Virus adsorbed to cells (4°C, 1 h), exposed to pH 5.3 or 7.2 (15 min, 4°C), treated immediately with trypsin		0	0
Virus[b] adsorbed to cells (4°C, 1 h), exposed to pH 5.3 or 7.2 (15 min, 4°C), warmed to 37°C for 20 min, then treated with trypsin	1	58,000 (0.7)	84,000 (0.9)
	2	152 (0.0039)	134 (0.0045)

[a] Infectious center assay was carried out as described by Riedel and Brown (17).
[b] Multiplicity of infection was 0.01 for experiment 1 and 0.0001 for experiment 2.

ratio of infected to uninfected cells in the control experiments was less than 1:100 or less than 1:10,000). These experiments were, therefore, extremely sensitive assays of the effect of low pH on a single virus-cell complex destined to yield a productive infection. No difference in the number of infectious centers produced after treatment with low pH relative to the number in virus-cell complexes treated at neutral pH could be detected (Table 1). These results show that the Sindbis virus attached to cells is protected from the inactivating effect of acidic pH. When cells were treated with trypsin immediately after the low-pH treatment described above, no infectious centers were obtained in either the control or the low-pH experiment (Table 1). This latter result showed that the virus was still located on the cell surface after the low-pH treatment and had been neither internalized nor fused with the plasma membrane. Numerous attempts to demonstrate fusion from without by low pH at 4°C have failed, whereas identically treated monolayers fused readily at temperatures above 10°C (data not shown), further suggesting that fusion with the cell surface does not occur at low temperatures.

Conformational changes in Sindbis virus accompanying exposure to low pH. The rapid and irreversible loss of the infectivity of free virions after exposure to low pH seems to be a complex process occurring in more than one step (see above). Infectivity loss seems to be initiated after low-pH exposure, but proceeds in the absence of low-pH treatment (Fig. 2). This observation prompted us to determine whether alterations in the physical properties of the virus particles could be detected after low-pH treatment.

Virus was exposed to pH 5.3 for 20 min and returned to neutral pH conditions. This treatment was sufficient to inactivate 99% of the virus population (see above). The virus was then subjected to either velocity sedimentation (Fig. 3B) or equilibrium density sedimentation (Fig. 3A) as described above. Virus exposed to low pH was found to band more lightly and sediment more slowly than untreated virus. The fact that most of the virus exposed to low pH sedimented more slowly than untreated virus did (Fig. 3B) showed that the change of density (Fig. 3A) was probably not the result of aggregation of particles, but rather resulted from the conformational change in the virus structure. The small amount of labeled material sedimenting faster than untreated virus may be virus aggregates. These aggregates cannot be much larger than dimers, as their sedimentation constant was not much greater than that of untreated virus. No larger material pelleted in these experiments. No aggregates of this type were seen when low-pH-treated virus was examined in the electron microscope, and their formation may have resulted from the high concentrations of virus employed in these centrifugation studies.

The conversion of virus conformation from the normal to the low-pH configuration was found to be rapid and complete. Intermediate forms between the two types were not detected. Attempts to stabilize partially inactivated populations of virus by glutaraldehyde fixation failed to produce virus populations that would band in both positions in the gradient (bimodal distribu-

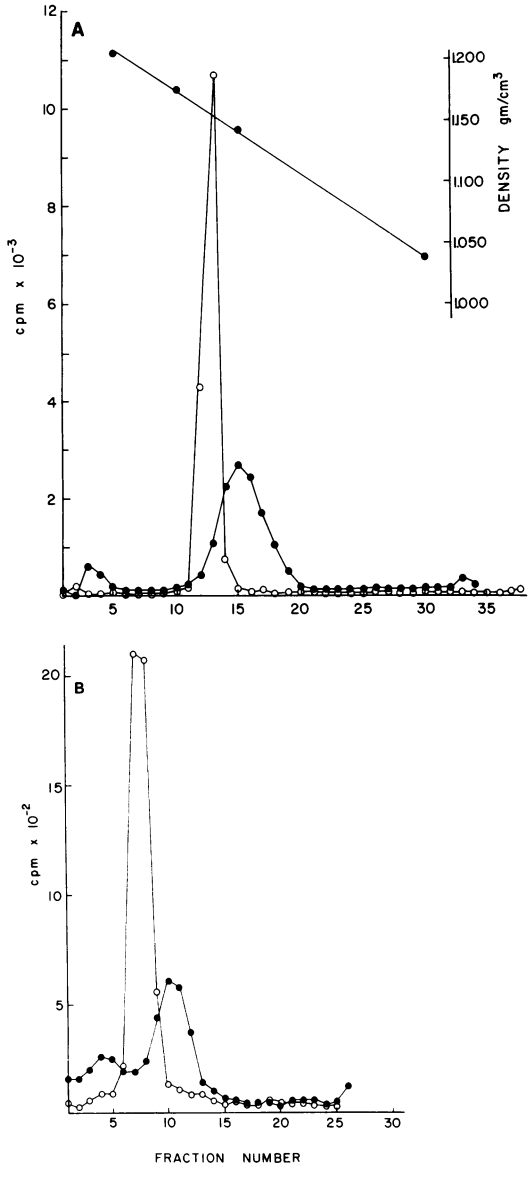

FIG. 3. Effect of low-pH treatment on Sindbis virus density and sedimentation characteristics. [^{35}S]methionine-labeled Sindbis virus was treated at pH 5.3, returned to neutral pH, and subjected to either density (A) or velocity (B) sedimentation as described in the text (equal amounts of label were applied to the gradients).

tion) or at intermediate densities (data not shown). Even when these experiments were carried out after a brief (2-min) exposure to low pH, all virus was found to band with the characteristics of low-pH-treated virus, as shown in Fig. 3A. These collective results support the notion that low-pH treatment induces a very rapid conformational change in Sindbis virus and that the loss of virus infectivity (described above) follows this change at a somewhat slower rate.

Conformational changes in Sindbis virus glycoprotein after low-pH treatment. The change in sedimentation and density characteristics of the virions after low-pH treatment (Fig. 3) suggests that some change in virus composition or conformation accompanied exposure to low pH. A comparison of the protein content of low-pH-treated virus to untreated virus (taken from the appropriate fractions shown in Fig. 3) revealed that these two preparations contained polypeptides E_1, E_2, and C (capsid protein) in equivalent amounts (Fig. 4, lanes B and C). In addition, the fact that no methionine-labeled material was found at the top of the gradients suggested that low-pH treatment does not result in the loss of protein from virus particles.

We showed previously that Sindbis virus infectivity is insensitive to trypsin (1). We now tested the relative sensitivity of low-pH-treated and normal virus to protein cleavage by this enzyme. These experiments were carried out as described above to determine whether the

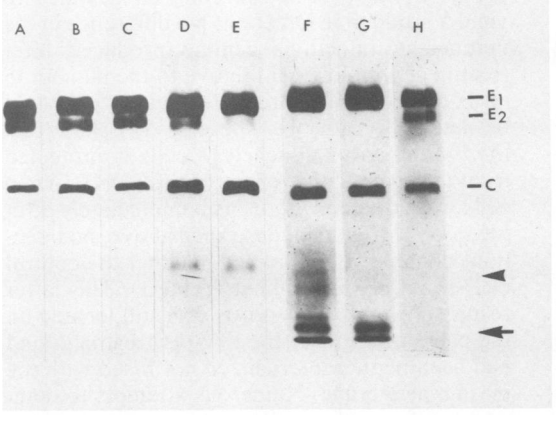

FIG. 4. Effect of low pH on the trypsin sensitivity of Sindbis virus proteins. Sodium dodecyl sulfate-polyacrylamide gel of [^{35}S]methionine-labeled SVHR with and without low-pH treatment (30 min at 37°C) followed by trypsin treatment. Lanes: (A and H) marker SVHR; (B) SVHR treated with pH-5.3 medium, followed by a 90-min treatment with a mixture of trypsin (10 μg/ml) and soybean trypsin inhibitor (40 μg/ml) at 20°C; (C) SVHR treated with pH-7.2 medium, followed by trypsin treatment (10 μg/ml) for 80 min at 20°C. (D through G) SVHR treated with low-pH medium followed by trypsin at 10 μg/ml for 10 min at 20°C (D), 10 μg/ml for 40 min at 20°C (E), 50 μg/ml for 10 min at 20°C (F), or 100 μg/ml for 90 min at 37°C (G). Arrowhead, Intermediate-molecular-weight, possibly glycosylated, peptides. Arrow, Low-molecular-weight peptides.

changes described above in physical properties were accompanied by the exposure of a trypsin-sensitive site in the envelope glycoproteins. Sindbis virus was exposed to low pH at 37°C for 30 min and returned to neutral pH. These and control viruses were then treated with trypsin for various periods of time at either 20 or 37°C. The preparation was then prepared for polyacrylamide gel electrophoresis as described above.

Virus treated with trypsin without previous exposure to low pH showed no sensitivity to the enzyme (Fig. 4, lane C). We showed previously that an identical trypsin treatment does not result in a reduction in infectivity (1). Virus exposed to low pH and then treated with trypsin mixed with soybean trypsin inhibitor also produced no demonstrable changes in the protein composition of the virion (Fig. 4, lane B). When virus was exposed to low pH and then treated with trypsin, a loss of E_2 protein occurred (lanes D through G). The loss of E_2 from the virion was found to be progressive with time (lanes D and E) and concentration and temperature (lanes F and G) and was accompanied by the appearance of low-molecular-weight peptides. Changes in pH had no apparent effect on the trypsin sensitivity of virus proteins E_1 or C regardless of the duration of incubation in the enzyme. Five methionine-containing peptides were produced by trypsin cleavage of the E_2 polypeptide (lane F). Two of these peptides appeared when milder (shorter times, reduced trypsin concentration, or lower temperature) trypsin treatments were employed (lanes D, E, and F, arrowhead). These peptides had a molecular weight in the range of 15,000 and banded in a diffuse pattern, suggesting that they may be derived from glycosylated domains of the E_2 protein. Extended digestion with trypsin resulted in the production of three lower-molecular-weight peptides (lanes F and G, arrow), the heaviest of these being reduced with extended digestion. These low-molecular-weight proteins banded in a sharp pattern, indicating that they may not be glycosylated.

We are presently establishing the regions of the E_2 protein from which the above-described tryptic peptides are produced.

Other characteristics of Sindbis virus after exposure to low pH. In addition to the physical and biological alterations described above, we made three other observations on the characteristics of low-pH-treated, purified virions.

(i) The attachment of virus to cell monolayers, as determined by binding of [^{35}S]methionine-containing virus (18) is not affected by low-pH treatment (data not shown). (ii) If virus exposed to low pH and returned to neutral pH is attached to cells in large numbers, these attached virus particles, in contrast to untreated virus particles, cannot induce fusion from without when the cell-virus complex is exposed to low pH as described in the legend to Fig. 1. (iii) Electron microscopy of Sindbis virions exposed to low pH and cross-linked with glutaraldehyde or of virions stained for electron microscopy in pH-5.3 phosphotungstic acid revealed no obvious morphological differences compared with particles stained at neutral pH.

DISCUSSION

We confirmed in this report the observations of White and Helenius (26) that large numbers of cell-associated alphaviruses can mediate cell fusion at low pH. We extended these observations to further show that exposure to low pH produces a rapid conformational change in the virus particle. This conformational change does not affect the ability of the virus to become cell associated, but does eliminate its ability to induce cell fusion after attachment. The conformational changes in the virion produced by low pH are followed by rapid inactivation of the virus infectivity that, once initiated, can continue at neutral pH. Viruses attached to cells do not lose infectivity when exposed to low-pH conditions. It is not clear what low-pH-induced changes occur in the virion that cause the loss of infectivity. The conformational changes in the virion induced by low pH are related, at least in part, to an irreversible reorganization of the E_2 protein. Infectivity loss, however, may result from conformational changes in the virus interior that were not detectable in the experiments described above. A conformational change in the isolated hemagglutinin of influenza virus has also been shown at the pH at which this virion causes membrane fusion (21).

The conformational changes in Sindbis virus induced by low-pH treatment may mimic events occurring normally during the infection of a cell with Sindbis virus. After collision with cell surfaces, a tight association between the cell membrane and the virus is formed. This attachment to the cell surface may position the virus glycoproteins against the cell membrane in a configuration favoring a conformational change in the glycoproteins (similar to that induced by low pH), exposing the fusion function in the E_1-E_2 complex. This change would normally occur only in that region of the virus in contact with the host cell. This conformational change would be followed by the initiation of fusion of the virus membrane with the cell membrane (penetration). Subsequent conformational changes in the nucleocapsid complex may be induced at this point, initiating the process of disassembling the ribonucleoprotein complex (uncoating). A similar conformational change in the virus nu-

cleocapsid structure may follow the reorganization of the glycoprotein seen after low-pH treatment; however, in free virions the conformational change results in loss of infectivity. The mature Sindbis virion may be a metastable structure that can be induced to pass through a sequence of irreversible conformational changes by either interaction with the cell surface or exposure to low pH. Our data suggest that if one of these stages is induced by artificial conditions, such as low pH, before the step normally preceeding it, loss of infectivity may result.

In our initial experiments establishing the conditions for fusion from without of Sindbis virus-BHK-21 cell complexes (see above), we found that cell fusion only occurred when the cell-virus complexes were exposed to low pH and returned to neutral pH. Fusion could not be detected as long as the cells were maintained in low-pH medium (up to 1 h). Other published protocols for inducing cell fusion with alphaviruses also indicate that the virus-cell complex was exposed briefly to low pH and then returned to neutral pH (this was also true for experiments investigating fusion from within [12]). Thus, low pH seems to set up conditions for fusion of the virus membrane with the cell membrane; however, the fusion event itself requires neutral pH. It is difficult, at present, to reconcile this observation with existing models for the infection of tissue cultures by membrane-limited viruses that implicate the low pH of the lysosome as essential for the fusion event resulting in infection (7, 13, 24).

That immature (newly synthesized, cell-associated) virus proteins can also induce fusion events after exposure to low pH is shown in an accompanying paper (12). The results presented there show that E_2 production is not essential for expression of the fusion function. Thus, if a domain of polypeptide E_2 is responsible for the fusion event, the precursor of this protein (PE_2) is also capable of undergoing a conformational change to expose this domain on the cell surface.

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