Sindbis Virus Membrane Fusion Is Mediated by Reduction of Glycoprotein Disulfide Bridges at the Cell Surface

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We have examined the role of thiol-disulfide exchange reactions during the penetration of cells by Sindbis virus. The protein-protein associations that form the rigid icosahedral lattice of the Sindbis virus envelope have been shown to be stabilized by disulfide bridges, and reduction of these critical disulfide bridges during cell penetration may be the mechanism by which the rigid protein lattice is disrupted prior to fusion (R. Anthony and D. T. Brown, J. Virol. 65:1187-1194, 1991; R. Anthony, A. Paredes, and D. T. Brown, Virology 190:330-336, 1992). Reduction of disulfide bridges occurs at near neutral pHs via thiol-disulfide exchange reactions, and these reactions can be blocked by covalent modification of the thiol involved. In this study, the effects of the reducing agent 2-mercaptoethanol on Sindbis virus-mediated cell-cell fusion from without and the effects of the membrane-impermeable thiol-alkylating reagent 5,5'-dithiobis(2-nitrobenzoic acid) on Sindbis virus penetration were determined. The presence of exogenous reducing agent was found to induce fusion from without under conditions unfavorable to both typical Sindbis virus-mediated fusion from without and cysteine-mediated thiol-disulfide exchange reactions. In addition, the thiol-alkylating reagent was found to inhibit Sindbis virus entry when present during infection. These results are consistent with a model for Sindbis virus entry in which reduction of critical disulfide bridges at the cell surface disrupts the rigid protein-protein associations of the envelope, allowing membrane fusion and release of the viral genome into the cell.

Sindbis virus is a structurally well defined alphavirus composed of 240 copies each of the three virally encoded proteins E1, E2, and C. The positive-strand RNA genome is protected by a T=4 icosahedral nucleocapsid composed of the viral protein C (4, 5, 19). This nucleocapsid is in turn surrounded by a host-derived lipid envelope containing the viral glycoproteins E1 and E2. Sindbis virus is a unique enveloped virus in that its envelope proteins are rigidly organized as a T=4 icosahedral lattice (1, 14). In contrast to other viral envelopes, which are regarded as membranes with associated protein, the Sindbis virus envelope can be considered a rigid protein lattice with associated lipid. E1-E2 heterodimers trimerize to form the envelope capsomeres via E1-E1 associations which are stabilized by intramolecular disulfide bridges (1). Additional E1-E1 and E2-E2 interactions organize these capsomeres into a T=4 icosahedral lattice (see Fig. 7 in reference 1), which appears to be maintained primarily by disulfide bridge-stabilized E1-E1 associations (2). The unique rigid organization of the Sindbis virus envelope proteins makes it imperative that these protein-protein interactions be disrupted during entry.

The disulfide bridges which maintain the structural integrity of the envelope protein lattice are protected from exogenous reducing agents by the conformation of the capsomere (2). Conformational changes induced by extended treatment with dithiothreitol (DTT) or by very brief treatment at low pH expose these critical disulfide bridges to reduction by DTT. This reduction results in disassembly of the envelope and release of the nucleocapsid (2). In vitro disassembly of Sindbis virus following low-pH (5.3) treatment requires a return to neutral pH as reduction by DTT occurs via a thiol-disulfide exchange reaction which is highly pH dependent (26). In this two-step process, the in vitro disassembly of the Sindbis virus envelope by DTT parallels the pH requirements demonstrated for Sindbis virus-mediated fusion from without (FFWO); fusion induced by the HR strain of Sindbis virus requires an initial exposure to pH 5.3 followed by a return to neutral pH (9).

The observation that some enveloped viruses demonstrate low-pH-induced FFWO is believed to support the hypothesis that these viruses enter cells via an acidic, endosomal route (16). For Sindbis virus, such coupling of low-pH-induced FFWO and entry from an acidic endosome has been put in question by several observations. Agents which block endocytosis do not block penetration of cells by Sindbis virus (3, 6). In addition, a temperature-sensitive mutant CHO cell line defective in the acidification of endosomes undergoes FFWO at the normal acidic threshold pH and yet is efficiently infected with Sindbis virus at temperatures which prevent endosome acidification to this pH (10). Furthermore, Sindbis virus-mediated FFWO does not occur at the low-pH threshold required to induce fusion but instead requires a return to a neutral-pH environment (9). Finally, different cell lines show different pH optima for fusion, including one at pH 4.6, well below the acidification capability of the cells; these cells are also efficiently infected by Sindbis virus (8). The above observations suggest that the low-pH threshold required during FFWO is not required during natural entry, and thus penetration of cells is not restricted to the acidic environment of the endosome.

The observation that DTT can induce Sindbis virus envelope disassembly in vitro suggests that cleavage or rearrangement of the intramolecular disulfide bridges stabilizing the critical E1-E1 associations may be required to disrupt the envelope protein lattice during entry (2). Thiol-disulfide exchange reactions are biologically important for reducing and/or rearranging disulfide bridges in proteins. They require neutral pH conditions and can be blocked by covalent modification of the thiol involved (26). An envelope disas-

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semiby mechanism including such a reaction could explain the two-step nature of the FFWO phenomenon, as the low-pH step would induce conformational changes in the virions, allowing critical reductive events to occur upon a return to neutral pH. During the natural route of cell penetration, conformational changes in the envelope proteins induced by binding to the receptor at the plasma membrane (13) could trigger the thiol-disulfide exchange reactions, bypassing the need for low-pH-induced conformational changes. Such a reductive event could provide a mechanism for the disruption of the protein-protein associations of the envelope and allow direct penetration of cells at the plasma membrane. In an attempt to investigate this possibility, the virus-mediated phenomenon of FFWO has been reexamined and the effect of the membrane-impermeable thiol-alkylating reagent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) on virus penetration has been studied.

MATERIALS AND METHODS

Cells, virus, and media. BHK-21 cells were cultured at 37°C in Eagle’s minimal essential medium (MEM) supplemented with 10% fetal calf serum (GIBCO), 5% tryptose phosphate broth, and 2 mM L-glutamine. Heat-resistant Sindbis virus (SVHR), originally provided by E. Pfefferkorn (Dartmouth Medical College), was passaged at low multiplicity and titrated on BHK-21 cells as described previously (21).

Sindbis virus-mediated FFWO. Variations of Sindbis virus-mediated FFWO described previously were performed (9, 18). Briefly, FFWO at low pH was induced by adsorbing 1,000 FFU of SVHR per cell to BHK monolayers at 4°C for 60 min; the viral inoculum with then replaced with 37°C fusion medium, consisting of Eagle’s MEM without bicarbonate, 10 mM MES (2(N-morpholino)ethanesulfonic acid), and 10 mM HEPES (N-2-hydroxyethylpipерazinе-N'-2-ethanesulfonic acid) adjusted to pH 5.3 with and without various concentrations of 2-mercaptoethanol (2-ME). Fusion was assayed with a phase-contrast microscope after 5 h of incubation at 37°C and scored as described previously (−, no fusion; +, less than 25% of cells fused; ++, 25 to 50% of cells fused; ++++, 50 to 95% of cells fused; ++++, more than 95% of cells fused); the degree of fusion was calculated as 1 (number of cells/number of nuclei) (18). Two-pH-step FFWO was induced as described above except that various multiplicities of SVHR were used, the fusion medium contained 0.05 mM 2-ME, and the virus-cell complexes were returned to growth medium after 5 min of incubation in low-pH fusion medium. The monolayers were photographed under phase-contrast illumination after 1 h of incubation at 37°C and scored as described above (18).

Drugs. DTNB (Pierce) was solubilized in serum-free MEM. DTNB concentrations as high as 5 mM did not affect cell viability, as assayed by incorporation of [3H]uridine (ICN Biochemicals) into trichloroacetic acid (TCA)-precipitable cellular RNA, trypan blue exclusion, or neutral red uptake (data not shown). Direct effects of DTNB on Sindbis virus infectivity were determined by incubating the reagent with the virus for various periods of time, quenching the incubation by serial dilution into phosphate-buffered saline containing 3% fetal calf serum (GIBCO), and titrating on BHK cells in a standard plaque assay as described previously (21).

Penetration assays. Viral RNA synthesis was assayed by determining incorporation of [3H]uridine (ICN Biochemicals) into TCA-precipitable material in BHK cells treated with 4 μg of dactinomycin per ml for 90 min prior to infection and maintained in dactinomycin throughout the experiment. Monolayers of equal numbers of BHK cells were treated with 1 mM DTNB for 30 min at 37°C at three different times with respect to a 1-h adsorption of 5 PFU of SVHR per cell at 4°C. After adsorption, all of the monolayers were washed with serum-free MEM and incubated at 37°C to allow penetration of the adsorbed virus. The 30-min DTNB treatments were done either immediately prior to adsorption, during the period of penetration at 37°C, or immediately after this penetration period. After the DTNB treatments, the monolayers were washed. [3H]uridine (10 μCi/ml) was added 90 min after the period of penetration, and at 5 h, the monolayers were lysed with sodium dodecyl sulfate and precipitated with 10% ice-cold TCA, and radioactivity was counted. Penetration was also assayed by plaque formation on BHK monolayers that had been exposed to 1 mM DTNB for 60 min under the treatment conditions described above. Plaque formation was determined as described previously (21).

RESULTS

FFWO at low pH. The rate of Sindbis virus-mediated FFWO is dependent upon the pH to which the cells are returned after exposure to low pH. Fusion proceeds faster at higher pHs and does not occur at the low-pH threshold of 5.3 required to establish conditions for fusion upon a return to neutrality (9). Thiol-disulfide exchange reactions are similarly pH dependent in that the reaction rates are directly affected by pH, occurring efficiently only in neutral to alkaline environments. If reduction of critical viral disulfide bridges is necessary to disrupt the rigid envelope protein lattice prior to membrane fusion, it would be blocked at low pH.

To examine a role for such a reductive event in Sindbis virus-mediated FFWO of BHK cells, fusion was attempted at low pH in the presence of the reducing agent 2-ME as described in Materials and Methods (Fig. 1). Unlike DTT and cysteine, 2-ME is an effective reducing agent at pH 5.0 and above (26). Treatment with a wide range of 2-ME concentrations was found to induce FFWO at pH 5.3, although this range of concentrations varied between indi-
vidual experiments. 2-ME-induced fusion at low pH is highly sensitive to a number of factors, including the passage number of the cells and the degree of confluency of the monolayers, which are not as critical to the process of fusion after a return to neutral pH. Treatment of monolayers with 2-ME in the absence of virus and incubation of virus-cell complexes at pH 5.3 in the absence of 2-ME do not induce fusion. The extremely low concentrations of reducing agent necessary to induce FFWO suggest that the critical disulfide bridges being reduced are highly strained. High concentrations of 2-ME do not promote fusion, possibly because the viral envelope is destroyed before the fusion event can occur, in a manner analogous to the complete destruction of the viral envelope during in vitro DTTR reduction of low-pH-treated virus (2). The observation that Sindbis virus-mediated FFWO occurs at low pH in the presence of low concentrations of 2-ME is consistent with the hypothesis that reduction of critical viral disulfide bridges may be important for virus-cell fusion. In addition, it may explain the FFWO requirement for a return to a neutral-pH environment.

Effect of 2-ME on the multiplicity requirement for FFWO. The observation that 2-ME induces fusion at low pH implies that the efficiency of Sindbis virus-induced FFWO may depend upon a reductive event. Figure 2 shows the effect of 2-ME on the multiplicity requirement for typical Sindbis virus-mediated FFWO. This reducing agent, when present during the brief exposure of the virus-cell complexes to low pH during FFWO, increases the efficiency of fusion after a return to neutrality. Optimal fusion in the 2-ME-treated monolayers is obtained at multiplicities as low as 100 PFU/cell, whereas multiplicities of 500 to 1,000 PFU/cell are typically required in the absence of 2-ME treatment (9, 18). The high-multiplicity requirement for FFWO must in part reflect the frequency with which attaching virions form multiple cell contacts, a steric requirement for the cell-virus-cell fusion event. If this fusion phenomenon is dependent upon a thiol-disulfide exchange reaction, the high-multiplicity requirement may also reflect the frequency with which the critical viral disulfide bridges associate with appropriate thiol donors. An increase in the availability of thiolates due to the exogenous reducing agent 2-ME would reduce the number of virions required to produce maximal cell-cell fusion. Such a model is supported by the decrease in multiplicity required for maximal fusion after brief exposure to 2-ME.

Role of thiol-disulfide exchange reactions in Sindbis virus penetration of BHK cells. The data presented above suggest that the reduction of disulfide bridges in the rigid envelope protein lattice of Sindbis virus may be required for membrane fusion during entry. It has been demonstrated that cell surfaces have a reductive capability mediated by thiol-disulfide exchange reactions (11). Such plasma membrane-associated activity has been implicated in the reduction of the disulfide bridge connecting the A and B chains of diphtheria toxin (31). This hypothesis has received dramatic support from experiments in which it has been demonstrated that chemical reagents such as DTNB which block thiol groups on the cell surface prevent the cleavage of the diphtheria toxin disulfide bridge and render sensitive cells resistant to the toxin (23). DTNB is a membrane-impermeable sulfhydryl-blocking reagent that covalently modifies sulfhydryls via a thiol-disulfide exchange reaction. As it does not directly affect Sindbis virus infectivity (Fig. 3) or BHK cell viability (as described in Materials and Methods), it is an excellent reagent for testing the hypothesis that such reactions occur at the plasma membrane during Sindbis virus penetration.

The effect of this reagent on viral penetration at low multiplicities of infection was assayed by [3H]uridine incorporation into viral RNA under three treatment conditions as described in Materials and Methods (Table 1). In the presence of dactinomycin, BHK cells were treated for 30 min with 1 mM DTNB either prior to adsorption of the virus at 4°C, during penetration of the virus at 37°C, or immediately after the penetration period. In each case, viral RNA was assayed at 5 h after the penetration period by measuring the incorporation of [3H]uridine into TCA-precipitable material. DTNB treatment of cells prior to infection has a slight inhibitory effect on viral RNA synthesis, while treatment during virus penetration inhibits RNA production to approximately 40% of control levels. The decrease in RNA synthesis seen with treatment of the cells prior to infection likely results from a failure to remove all of the drug prior to infection. Treatment with DTNB after an initial period of penetration has a limited inhibitory effect; however, the depression in RNA synthesis after this treatment may be due to the fact that Sindbis virus infection increases the permeability of cells, allowing some membrane-impermeable reagents direct access to the cytoplasm, where they can have secondary effects (10, 12).
TABLE 1. Effect of DTNB on penetration of cells by Sindbis virus

<table>
<thead>
<tr>
<th>Determination</th>
<th>Treatment time</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral RNA synthesis&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Before penetration</td>
<td>82 ± 11</td>
</tr>
<tr>
<td></td>
<td>During penetration</td>
<td>40 ± 10</td>
</tr>
<tr>
<td></td>
<td>After penetration</td>
<td>81 ± 13</td>
</tr>
<tr>
<td>Plaque formation&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Before penetration</td>
<td>80 ± 11</td>
</tr>
<tr>
<td></td>
<td>During penetration</td>
<td>46 ± 10</td>
</tr>
<tr>
<td></td>
<td>After penetration</td>
<td>110 ± 10</td>
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</tbody>
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<sup>a</sup> Virus was adsorbed to equal numbers of BHK cells for 1 h at 4°C, replaced in warm MEM, and incubated at 37°C to allow penetration. Cells were treated for 30 min (A) or 60 min (B) with 1 mM DTNB at the indicated times. The monolayers were then washed to remove the drug prior to further incubation at 37°C in DTNB-free medium. Results are the averages of five independent experiments ± standard deviation.

<sup>b</sup> Total viral RNA level was determined 5 h after the cells were warmed to 37°C to initiate penetration.

<sup>c</sup> Monolayers were overlaid with agarose and stained with neutral red 48 h after infection.

The effect of DTNB on Sindbis virus penetration was also determined by assaying plaque formation (Table 1). Pretreatment of the cells with DTNB results in a slight inhibition of plaque formation, while treatment during the period of infection inhibits plaque formation to approximately 46% of untreated-control levels. Again, the decrease in plaque formation seen when monolayers were treated with DTNB prior to infection probably results from an inability to remove all of the reagent prior to infection. Treatment with DTNB postinfection has no inhibitory effect on plaque formation, which is consistent with the above conclusion that the depression in RNA levels with DTNB treatment after infection is not due to a direct effect on penetration of the virus. Although paradoxical, the slightly higher number of plaques found in cell cultures treated after infection is highly reproducible, and at this time no explanation is available.

The interesting observation that Sindbis virus penetration is most effectively inhibited by DTNB when the reagent is present during the period of infection suggests that targeted thiol groups are unmasked during entry through a cooperative interaction between the virus and its receptor. The cooperative and rapid nature of this interaction may create circumstances in which DTNB cannot efficiently compete with a viral disulfide bridge (Fig. 4) undergoing a thiol-disulfide exchange reaction. Such reactions have rapid reaction rates, about 10<sup>8</sup> s<sup>-1</sup> at pH 8.0 (7), and an inefficiency in alkylating the critical thiols would result in an inability to completely block Sindbis virus penetration.

DISCUSSION

Fusion of enveloped viruses with cellular membranes during penetration is protein mediated, as are intracellular and intercellular fusion events (30). The exposure of a fusion domain in the envelope protein E1 has been implicated in the fusion of alphaviruses with cellular membranes (17). In addition, the observations presented above, together with those published previously by our laboratory (1, 2), suggest that the disulfide bridge-stabilized E1-E1 associations of the envelope must be disrupted for membrane fusion to occur. The most likely mechanism for disassembly of the Sindbis virus envelope protein-protein interactions during penetration is the reduction of these stabilizing disulfide bridges via thiol-disulfide exchange reactions.

A thiol-disulfide exchange reaction occurs by the nucleophilic attack of an ionized thiol on a disulfide bridge and is highly dependent on environmental pH, the pK<sub>a</sub> of the thiol, and steric hindrance. Cysteine-mediated thiol-disulfide exchange reactions thus require neutral to alkaline pHs (11, 26). FFWO mediated by the HR strain of Sindbis virus requires exposure to pH 5.3 with a subsequent return to pHs greater than 6 to induce fusion (9). The rate of fusion increases as the pH to which the virus-cell complexes are returned increases, and fusion does not occur at the low-pH threshold required for fusion. Likewise, rapid disassembly of the viral envelope by DTT also requires brief exposure to pH 5.3 followed by a return to neutral pH (2). These observations implicate a definite role for a neutral pH

FIG. 4. Model depicting conformational changes in E1 during Sindbis virus membrane penetration and low-pH-induced membrane fusion. (A) The E1 glycoprotein in the mature virion has both functional and structural domains, which are involved in membrane fusion and envelope integrity, respectively. (B) Conformational changes induced by the receptor-virus interaction or by exposure to low pH unmask critical disulfide bridges, favoring a subsequent reshuffling of disulfide bridges. (C) Reduction of critical disulfide bridges responsible for maintaining the protein-protein associations of the envelope disrupts the rigid protein icosahedral lattice, allowing subsequent fusion with a cellular membrane. Solid boxes, E1-E1 associations; hatched boxes, fusion peptide.
environment and in this way reflect the requirements for a thiol-disulfide exchange reaction described above. This is consistent with a model for virus-cell fusion in which thiol-disulfide exchange reactions are critical to the fusion process.

The process of low-pH-mediated FFWO differs from the process of infection in that it requires high multiplicities of virus and is protein receptor independent (15, 18); however, both processes must supply a mechanism for the disruption of the envelope protein lattice prior to fusion. Our data suggest that both events are similarly dependent upon a thiol-disulfide exchange reaction. Addition of very low concentrations of the reducing agent 2-ME (which, unlike DTT and biological thiols, reduces efficiently at pH 5.3) promotes cell fusion in an acidic environment, suggesting that the chemical reduction of critical disulfide bridges can induce fusion in an otherwise unfavorable reductive environment. The extremely low concentrations of 2-ME needed to promote this fusion imply that the critical disulfide bridges being reduced are in very strained conformations. Low-pH-induced conformational changes in the envelope proteins could be responsible for exposing these strained critical disulfide bridges and may explain the very rapid nature of in vitro DTT-mediated virus disassembly following low-pH exposure (2).

The process of Sindbis virus penetration of host cells is receptor dependent, and several possible receptors have been identified (24, 27, 29). The interaction of the virus with a cell surface receptor at neutral pH induces conformational changes in the viral glycoproteins which precede penetration (13). The studies presented here also suggest that a cooperative interaction between the receptor and the virus induces conformational changes which allow the reduction of critical disulfide bridges within the envelope proteins by thiol-disulfide exchange reactions. The progression from the receptor-induced conformational changes to the disruption of the envelope protein lattice and fusion likely proceeds very rapidly. The fact that the stabilizing disulfide bridges and critical thiols are inaccessible to molecules such as DTT and DTNB while in the native state, combined with the extremely rapid rate at which the reductive event occurs after the receptor-induced conformational changes, may explain the inefficiency of DTNB in blocking this process. Thiols mediating the reductive events may reside either in the receptor protein or within the virus itself. In the latter case, a reshuffling of disulfide bridges following attachment would result in the required disruption of the envelope protein lattice. In such a model (Fig. 4), the interaction of the virus with an appropriate receptor alters the conformation of E1, favoring a reshuffling of disulfide bridges via thiol-disulfide exchange reactions. This reshuffling of disulfide bridges leads to disruption of the rigid protein-protein associations in the envelope, allowing subsequent fusion of the viral envelope with the plasma membrane.

Sturman et al. (25) have suggested that reshuffling of disulfide bridges is involved in the conformational changes occurring in coronavirus proteins during cell penetration. In collaboration with Shinji Makino (University of Texas at Austin), we have obtained preliminary evidence that mouse hepatitis virus is also inhibited by DTNB when the reagent is present during infection (unpublished observation). It is possible that such rearrangements of disulfide bridges are required for penetration by a variety of enveloped viruses.

A critical reductive event in Sindbis virus penetration could occur in an unidentified neutral-pH compartment within the cell, as is thought to be the case for ricin toxin (28). Like diphtheria toxin, ricin is a heterodimeric toxin with a disulfide bridge connecting the A and B chains that must be reduced for penetration into the cell cytoplasm. However, unlike that of diphtheria toxin, the ricin disulfide bridge is reduced not at the cell surface but in some internal compartment at neutral pH, possibly the trans-Golgi network (28). DTNB has no effect on the cytotoxicity of ricin while inhibiting the cytotoxicity of diphtheria toxin (23). Thus, if a critical reductive event occurs after internalization of Sindbis virus by endocytosis, DTNB would not be expected to inhibit entry.

Our data are in agreement with the studies of Flynn et al. (13), who identified the exposure of transitional epitopes in Sindbis virus glycoproteins during attachment and penetration. Antibodies to these epitopes were able to prevent virus infection when present during the process of infection. Flynn et al. (13) found that these antibodies were capable of blocking penetration of cells by 30% of the added virus, leading them to conclude, as we have concluded, that the rearrangements leading to infection occur very rapidly. Such rapid protein rearrangements would dramatically limit the ability of antibodies or DTNB to arrest the virus particles during entry.

The identification of conformational changes induced in viral structural proteins by interaction with receptors has resulted in a reappraisal of the mechanism of cell penetration by polioviruses (see reviews in references 20 and 22). An emerging view is that the conformational changes required for virus penetration, which were previously thought to be induced by exposure to acidic endosomal pH, may occur at the cell surface as virus-receptor interactions take place. These conformational changes may lead to the direct penetration of polioviruses at the cell surface, as we also propose here for the alphaviruses.

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