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Interaction of Sindbis Virus Glycoproteins During Morphogenesis

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In cells infected with the Sindbis temperature-sensitive mutants ts-23 and ts-10 (complementation group D), which contain a defect in the envelope glycoprotein E1, the precursor polypeptide PE2 is not cleaved to the envelope glycoprotein E2 at the nonpermissive temperature. This defect is phenotypically identical to the defect observed in the complementation group E mutant, ts-20. The lesion in ts-23 is reversible upon shift to permissive temperature, whereas that of ts-10 is not. Antiserum against whole virus, E1, or E2 also prevents the cleavage of PE2 in cells infected with wild-type Sindbis virus. Because the cleavage of PE2 is inhibited by the lesion in mutants that are genotypically distinct and by anti-E1 or -E2 serum, it appears that PE2 and E1 exist as a complex in the membrane of the infected cell.

Sindbis virus, the prototype of the alpha togavirus group, is the simplest of all enveloped animal viruses. The virion contains an icosahedral nucleocapsid composed of a single repeating subunit (C) and a single-stranded RNA genome of approximately 4.0×10^6 daltons (10, 24). The nucleocapsid is enveloped by a lipid bilayer from which project the spikes responsible for attachment and hemagglutinating activity (7, 8, 12, 13). These spikes are composed of two glycoproteins, E1 and E2 (22). Recently, a third glycoprotein, E3, was reported in virions of a closely related virus, Semliki forest virus (11). The virus structural proteins appear to be formed from a 130,000-dalton precursor by posttranslational cleavage (11, 15, 21).

Several temperature-sensitive (ts) Sindbis mutants from three RNA^+ complementation groups have been isolated (5). They are presumed to be defective in either virion structural proteins or the precursors from which these structural proteins are derived. Two of these groups, D and E, have lesions involving very late maturation functions (14, 19). We recently reported that ts-20, a member of complementation group E, fails to cleave a glycoprotein precursor, PE2, to the structural membrane glycoprotein E2 during morphogenesis at the nonpermisssive temperature (14). The present report describes the lesion in two members of complementation group D, ts-23 and ts-10. In cells infected with these mutants, PE2 fails to be cleaved to E2 at the nonpermissive temperature. This cleavage event also fails to occur when cells are treated with antiserum raised against the virion glycoprotein E1, indicating a close association between PE2 and E1 in the infected cell.

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MATERIALS AND METHODS

Virus and cells. The growth and quantitation of Sindbis virus in chicken embryo (CE) cells were described previously (14). The temperature-sensitive mutants were generously supplied by E. R. Pfefferkorn (Dartmouth Medical School) and were plaque purified as described previously (14).

Radioactive labeling. In experiments with temperature-sensitive mutants, monolayer cultures of CE cells were treated for 2 h with 2.5 or 5 μg of actinomycin D per ml in either leucine- or methionine-free medium. After virus adsorption (1 h at room temperature), cells were incubated at 29°C for 2 to 4 h. Prewarmed medium was then placed on one-half of the cultures, and incubation was continued at 41 or 29°C for 1 h. At this time, either 25 μCi of [^{3}H]leucine (Amersham/Searle; specific activity, 50 Ci/mmol) per ml or 3 μCi of [^{35}S]methionine (Amersham/Searle; specific activity, 380 Ci/mmol) per ml was added to the cultures. Incubation was continued for 1 to 2 h, and the cells were harvested by direct addition of 4% sodium dodecyl sulfate in 0.125 M Tris (pH 6.8). After the addition of 5% β-mercaptoethanol, the lysates were heated at 100°C for 2 min and subjected to polyacrylamide gel electrophoresis (PAGE).

If pulse-chase experiments were performed, the procedure was identical except that the isotopic label was added to the cells for 20 min. After this time, one set of cultures was harvested as described above. Three sets were washed extensively in warm Hanks balanced salt solution (45°C) containing 100 times

the concentration of methionine in Eagle medium, and then incubated for 5 min at 41°C in the methionine-containing Hanks solution. Two sets were then shifted to 29°C by the addition of 29°C Eagle medium containing 100 times the normal concentration of methionine. For one of these two sets of cultures, the above-mentioned medium contained 100 μg of cycloheximide per ml. A fourth culture was routinely chased at 41°C as a control.

In experiments in which cells were treated with antisera, the cultures were pretreated with actinomycin D, as described above, and infected with wild-type virus at a multiplicity of approximately 50. At 3 h after infection, antisera diluted 1:2 in medium without methionine were added to the cultures. [^{35}S]methionine was present from 5 to 7 h after infection. Cells were processed as described above.

PAGE. Slab gels were treated by the method of Laemmli (16), using an apparatus (Hoeffer Scientific) similar to that described by Reid and Bieleski (18). Slab gels were dried by using a commercial gel dryer (Hoeffer Scientific), and the dried gel was exposed to sheets of Chronex 2 DC X-ray film (E. I. du Pont de Nemours & Co.) for 2 to 4 days. Autoradiograms were quantitated by using methods and equipment described by DeRosier and co-workers (9).

Complementation between Sindbis virus mutants. Mixed-infection experiments with pairs of temperature-sensitive mutants were carried out as described by Burge and Pfefferkorn (5). In some experiments, the virus in the inoculum was aggregated with $MgCl_2$ by using a modification of the Abel procedure (1), in which virus samples are mixed with an equal amount of 0.5 M $MgCl_2$ in phosphate-buffered saline and incubated at 37°C for 15 min. This results in aggregates of approximately 8 to 10 virions (3). In each experiment, both single- and double-mutant inocula were aggregated. In all experiments, three to four cultures were infected with each inoculum.

Antisera. Rabbit antisera were a generous gift of Joel Dalrymple (Walter Reed Hospital). Neutralization titers, reported as the reciprocal of the highest serum dilution capable of neutralizing 50% of an inoculum containing 50 to 150 PFU per plate, were: anti-Sindbis, 2,000; anti-E2, 20; anti-E1, <10; and anti-C, <10.

RESULTS

Proteins synthesized in cells infected with complementation group D mutants. In cells infected with the complementation group E mutant, ts-20, PE2 does not undergo cleavage to E2 at the restrictive temperature (14). ts-20-infected cells adsorb erythrocytes at the nonpermissive temperature (6), whereas those infected with ts-23 or ts-10 do not. The glycoprotein E1 has been demonstrated to be responsible for the hemagglutinating activity of the virion (8), and ts-23 is known to have a temperature-sensitive hemagglutinin (25). We examined the proteins synthesized in ts-23- and ts-10-infected cells by discontinuous PAGE (Fig. 1). The most prominent feature of these profiles is that, at the nonpermissive temperature, the glycoprotein precursor PE2 fails to undergo cleavage and the amount of E2 is reduced. These profiles are essentially indistinguishable from that of cells infected with the complementation group E mutant, ts-20, at the nonpermissive temperature (Fig. 1).

Our first concern was that the mutants we were using no longer demonstrated the same complementation patterns first reported. To test this possibility, complementation tests were done with ts-20, ts-10, and ts-23 (Table 1). Following the convention of Burge and Pfefferkorn (5), complementation indexes of two or less are considered negative, whereas those of three or more are considered positive. The results in Table 1 demonstrate that ts-10 and ts-23 belong to the same complementation group and ts-20 belongs to another.

Reversibility of the lesions in ts-10- and ts-23-infected cells. To determine whether the lesions in ts-23 and ts-10 were reversible, pulse-chase experiments were performed in conjunction with temperature shifts. CE cultures were infected at a multiplicity of approximately 10 to 50, and the cultures were incubated at the permissive temperature (29°C) for 5.5 h. At this time, the cells were warmed to the nonpermissive temperature (41°C) and, after incubation at 41°C for 30 min, label was added to the cultures. After a pulse period of 20 min, the cultures were chased at the permissive temperature in the presence or absence of cycloheximide. The profiles of proteins produced in cells infected with ts-23 are shown in Fig. 2. The appearance of the glycoprotein E2 during chase conditions suggested that precursor made at nonpermissive temperature can be chased into a polypeptide that corresponds to E2. This observation cannot be explained on the basis of leakiness of ts-23, since E2 does not appear under chase conditions at 41°C. The appearance of E2 does not represent de novo synthesis of E2 after a shift to the permissive temperature. Addition of 100× methionine completely inhibits incorporation of label into trichloroacetic acid-precipitable material within 5 min. In contrast, the PE2 synthesized at the nonpermissive temperature in cells infected by ts-10 fails to chase (Fig. 3).

To determine whether the precursor made by ts-10 and ts-23 could be chased into functional virions, temperature shift experiments were performed, and the appearance of PFU was monitored. A series of cultures was infected with ts-23 or ts-10 and incubated at the nonpermissive temperature. At 2, 4, and 6 h after

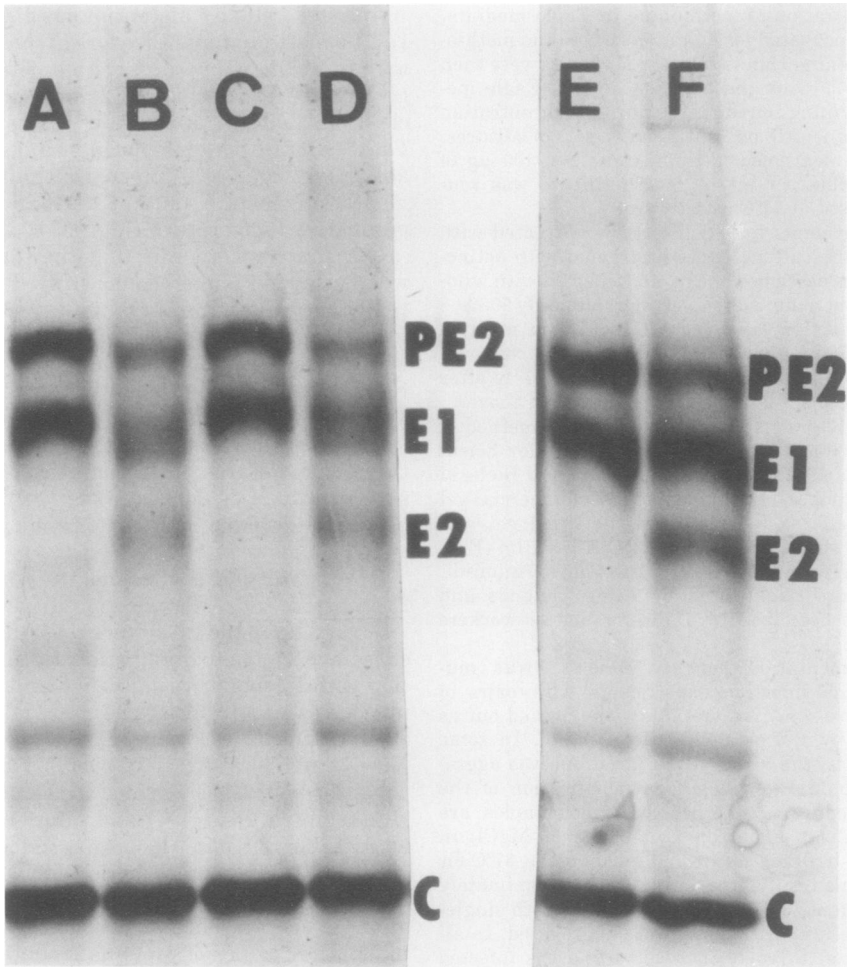


FIG. 1. *Polypeptides synthesized in cells infected with ts-23, ts-10, or ts-20 at the permissive and nonpermissive temperature. CE cells were infected with the appropriate ts mutant at a multiplicity of 10 to 50 PFU per cell. At 3 h after infection, a portion of the cultures was shifted to 41°C. Medium containing 25 μCi of [³H]leucine per ml was present from 4 to 6 h after infection. Cells were harvested, and polypeptides were analyzed by PAGE. A, ts-10 at 41°C; B, ts-10 at 29°C; C, ts-23 at 41°C; D, ts-23 at 29°C; E, ts-20 at 41°C; F, ts-20 at 29°C.*

infection, two cultures, one of which had been treated with cycloheximide for the preceding 20 min, were shifted to the permissive temperature, and the subsequent release of PFU was monitored. Replication at the nonpermissive temperature was used as the control. Figure 4 depicts the growth of ts-23 under the above-mentioned conditions. In untreated cultures, virus immediately appears in the culture fluids after the shift-down to permissive temperature. When cycloheximide is added to the culture fluids before the shift, an immediate rise in titer is also seen. This rise is as much as 50 to 100 times the control titers at 12 h after infection for the 4-, 6-, and 8-h shift times. As expected, the 2-h shift time does not show an increase in titer, since viral structural protein synthesis has not begun at 2 h after infection. Therefore, some PE2 made at the nonpermissive temperature must be cleaved and incorporated into infectious virions in ts-23-infected cells after a shift to the permissive temperature. These results are consistent with the above-mentioned finding that PE2 labeled at the nonpermissive temperature can be chased into E2 after a shift to the permissive temperature.

The results of a similar experiment with ts-10

TABLE 1. *Complementation between temperature-sensitive mutants of Sindbis virus*[a]

Single infection, 41°C		Double infection, 41°C		Complementation index[b]
Mutant	Titer	Mutants	Titer	
ts-10	3.0×10^4	ts-10/ts-20	3.6×10^6	10.3
	1.0×10^2		1.8×10^4	16
ts-23	7.0×10^4	ts-10/ts-23	1.3×10^5	1.3
	5.0×10^1		1.5×10^2	1.0
ts-20	3.2×10^5	ts-20/ts-23	1.6×10^6	4.1
	1.0×10^3		8.0×10^3	7.6

[a] Results from two separate experiments are presented. The upper figures represent an experiment in which the virus was not aggregated; the lower figures represent an experiment in which the virus was aggregated.

[b] Titer of double infection at 41°C divided by the sum of the single-infection titers.

are presented in Fig. 5. Like ts-23, in the absence of cycloheximide, virus is released into the medium after a shift-down to permissive temperature. However, the addition of cycloheximide to the culture medium before the shift completely inhibits this release, indicating that in ts-10-infected cells PE2 made at nonpermissive temperature is not cleaved to a functional envelope protein upon a shift to the permissive temperature. These results are consistent with our inability to chase labeled PE2 made at the nonpermissive temperature in ts-10-infected cells upon a shift to 29°C.

Effect of anti-Sindbis serum on the cleavage of PE2. The cleavage of PE2 to E2 is considered to be one of the terminal steps in the assembly and release of Sindbis virus (14, 20). This cleavage event occurs at the surface membrane of the infected cell (14). Cells infected with temperature-sensitive mutants that fail to cleave PE2 do not release virions. We were interested, therefore, in determining whether substances that inhibit virus release might also interfere with the cleavage of PE2. Antiviral serum is known to inhibit the release of certain enveloped viruses (23). Therefore, cells infected with wild-type Sindbis were exposed to normal rabbit serum and anti-Sindbis virus serum from 3 to 5 h after infection. The cells were then labeled for 2 h with [^{35}S]methionine, harvested, and processed for PAGE. The polypeptides were separated by PAGE, gels were autoradiographed, and the density of each band was determined as described in Materials and Methods. The areas under the peaks corresponding to PE2, E1, E2, and C were determined, and the ratios in control and anti-Sindbis-treated cultures were compared (Table 2). There is a relative accumulation of PE2 with

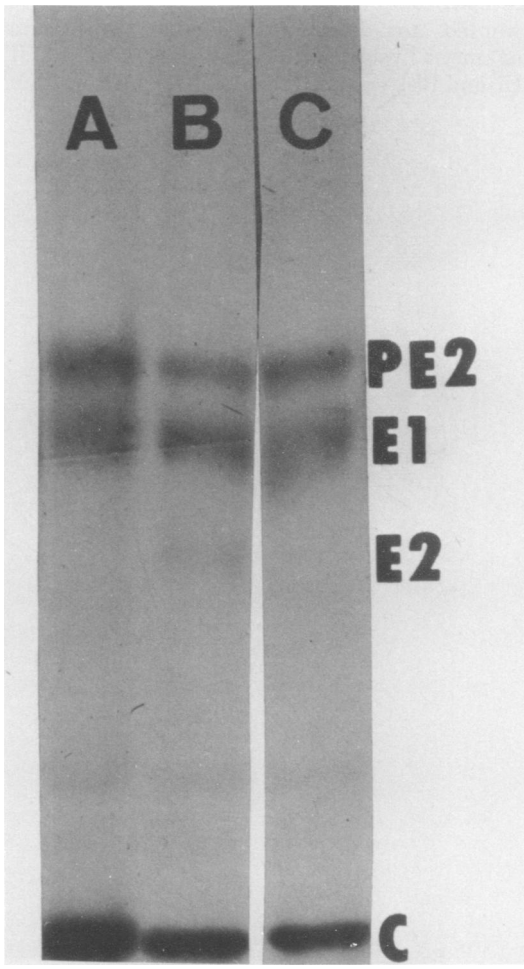


FIG. 2. *Fate of proteins labeled at 41°C in ts-23-infected cells after a shift to 29°C. CE cultures were infected with ts-23 and shifted to 41°C at 3 h after infection. After 1 h at 41°C, 9 μCi of [^{35}S]methionine was added to each culture, and incubation was continued for 20 min. At the end of the pulse period, one culture was harvested immediately, one was chased at 41°C, and another was chased at 29°C in the presence of cycloheximide, as described in the text. After a 1-h chase period, cultures were harvested and proteins were analyzed as described in the text. A, Pulse at 41°C; B, chase at 29°C plus cycloheximide; C, chase at 41°C.*

respect to E2 in the antibody-treated cells, indicating that the cleavage of PE2 is partially inhibited in cells treated with anti-Sindbis serum.

Inhibition of PE2 cleavage by anti-E1 serum. Since treatment of infected cells with anti-Sindbis results in the inhibition of PE2 cleavage similar to that observed with mutants

in both complementation groups D and E, we wanted to determine whether antiserum against E1 would inhibit the cleavage of PE2. If E1 and PE2 exist as a complex in the cell, then antiserum directed against E1 might be expected to prevent the cleavage of PE2. Six replicate cultures were infected with ts-23 at a multiplicity of 10 and incubated at the permissive temperature for 6 h. At this time the cultures were washed extensively with prewarmed methionine-free medium and shifted to the nonpermissive temperature. After 20 min, the cultures were pulse-labeled for 10 min with 25 μCi of [^{35}S]methionine per ml. The cells from one culture were harvested immediately, and the remaining five cultures were washed with prewarmed medium containing 100 times the usual concentration of methionine. They were incubated at the nonpermissive temperature in the above-mentioned medium containing 100 μg of cycloheximide per ml. After 10 min the cultures were exposed to the appropriate serum diluted in medium containing 100× methionine and cycloheximide. After a 20-min incubation period at the nonpermissive temperature, four cultures were shifted to the permissive temperature for 45 min. One culture containing normal serum was held at the nonpermissive temperature. At the end of the chase period, the cultures were harvested and subjected to PAGE (Fig. 6).

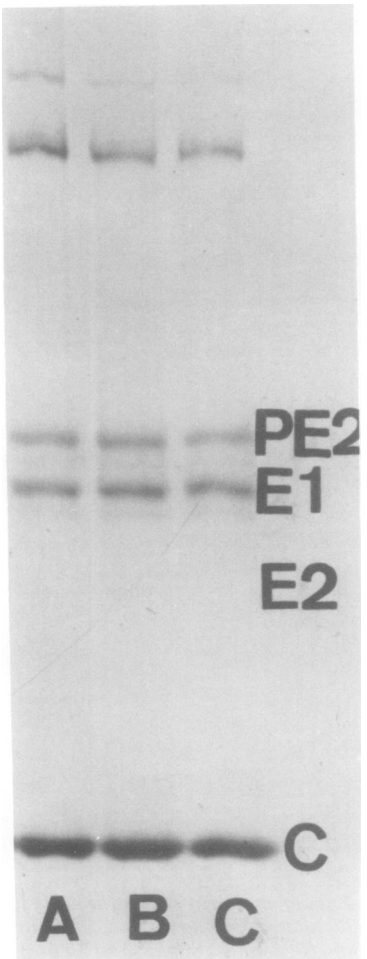


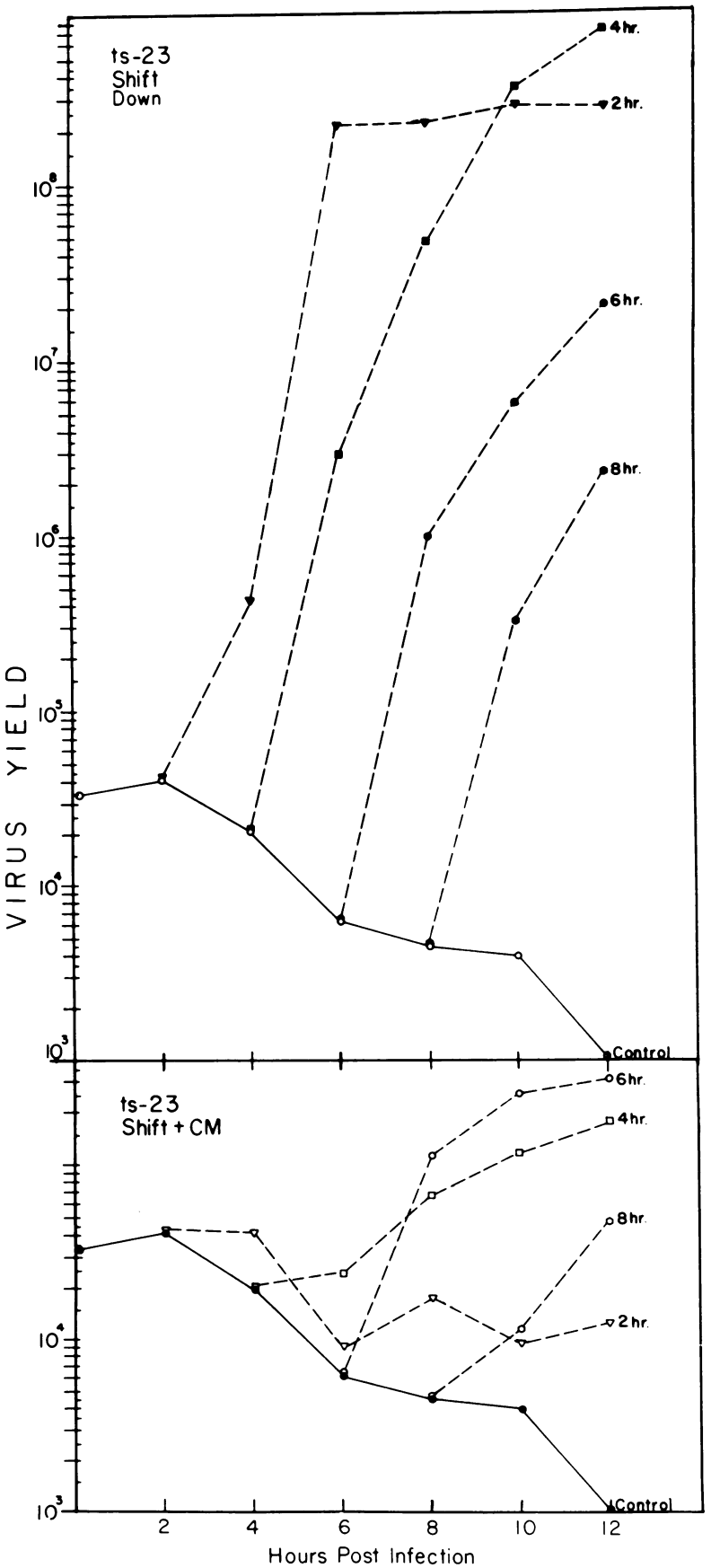
FIG. 3. *Fate of proteins labeled at 41°C in ts-10-infected cells after a shift to 29°C. CE cultures were infected with ts-10 and shifted to 41°C at 3 h after infection. After 1 h at 41°C, 9 μCi of [^{35}S]methionine was added to each culture, and incubation was continued for 20 min. At the end of the pulse period, one culture was harvested immediately, one was chased at 41°C, and another was chased at 29°C in the presence of cycloheximide as described in the text. After a 1-h chase period, cultures were harvested and proteins were analyzed as described in the text. A, Pulse at 41°C; B, chase at 29°C plus cycloheximide; C, chase at 41°C.*

As indicated in lane C, antiserum raised against E1 inhibits the cleavage of PE2. In contrast, in cultures exposed to normal serum (lane D), cleavage of PE2 to E2 occurs after the shift to the permissive temperature. In this experiment, the cleavage of PE2 was partially inhibited by antiserum directed against E2 (not shown) or antiserum prepared against disrupted virions (lane E). Since E2 and PE2 are known to be antigenically cross-reactive, the observed inhibition of PE2 cleavage by E2 is not surprising (17). The profile of the chase with anti-whole virus is similar to the one used to formulate Table 2. We do not know why the inhibition of cleavage with anti-serum with activity against both E1 and E2 or anti-E2 is not as complete as it is with anti-E1.

DISCUSSION

The terminal maturation events in Sindbis virus morphogenesis occur at the plasma membrane of the infected cell (14). The glycoprotein PE2 has been found in association with this membrane fraction (14) and its cleavage to E2 is one of the final events to occur during mor-

FIG. 4. *Interaction of Sindbis virus glycoproteins during morphogenesis. Cultures were infected with ts-23 and incubated at the nonpermissive temperature. At 2, 4, and 6 h after infection, two cultures, one of which had been treated with cycloheximide (100 μg/ml) for the preceding 20 min, were shifted to the permissive temperature, and PFU release was monitored. Replication at the nonpermissive temperature served as a control for leakiness.*


ts-23
Shift
Down
4hr.
2hr.
6hr.
8hr.
Control
ts-23
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VIRUS YIELD
Hours Post Infection
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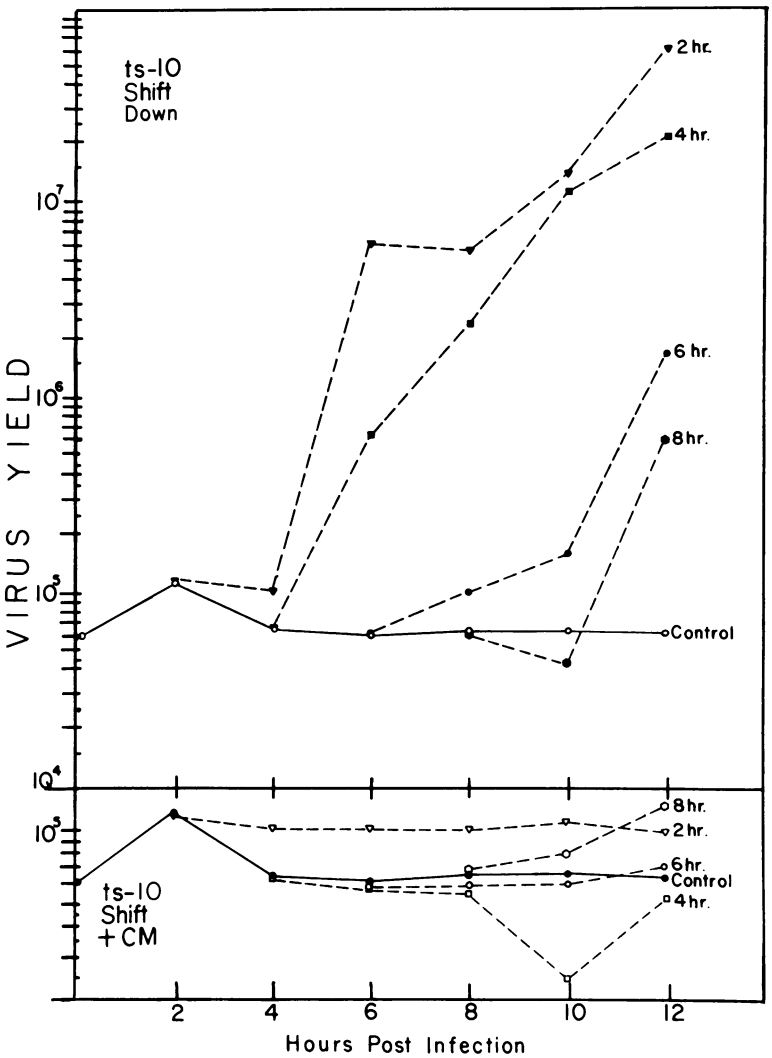

FIG. 5. *Interaction of Sindbis virus glycoproteins during morphogenesis. Cultures were infected with ts-10 and incubated at the nonpermissive temperature. At 2, 4, and 6 h after infection, two cultures, one of which had been treated with cycloheximide (100 μg/ml) for the preceding 20 min, were shifted to the permissive temperature, and PFU release was monitored. Replication at the nonpermissive temperature served as a control for leakiness.*

phogenesis (14, 20). Previously, we demonstrated that in cells infected with ts-20 this cleavage did not occur at the nonpermissive temperature (14). ts-20 represents one of the three RNA^+ complementation groups (group E) in the Sindbis virus system (5). ts-23 and ts-10 represent a second RNA^+ complementation group (group D), which is similar to group E in that the temperature-sensitive lesions affect late events in maturation (19). Most of the available evidence indicated that the lesion in ts-23 affects the envelope glycoprotein E1 (8, 25). We were somewhat surprised, therefore, when the protein profiles of the cells infected with ts-23, ts-10, and ts-20 indicated that in each case PE2 was not cleaved at the restrictive temperature. There are several plausible ways to explain how genotypically distinct ts mutants could have similar defects in a structural precursor protein. One is that the E1 protein itself acts either directly or in combination with another protein to effect the cleavage of PE2. In this way, a lesion in either of the envelope glycoproteins could result in the failure of PE2

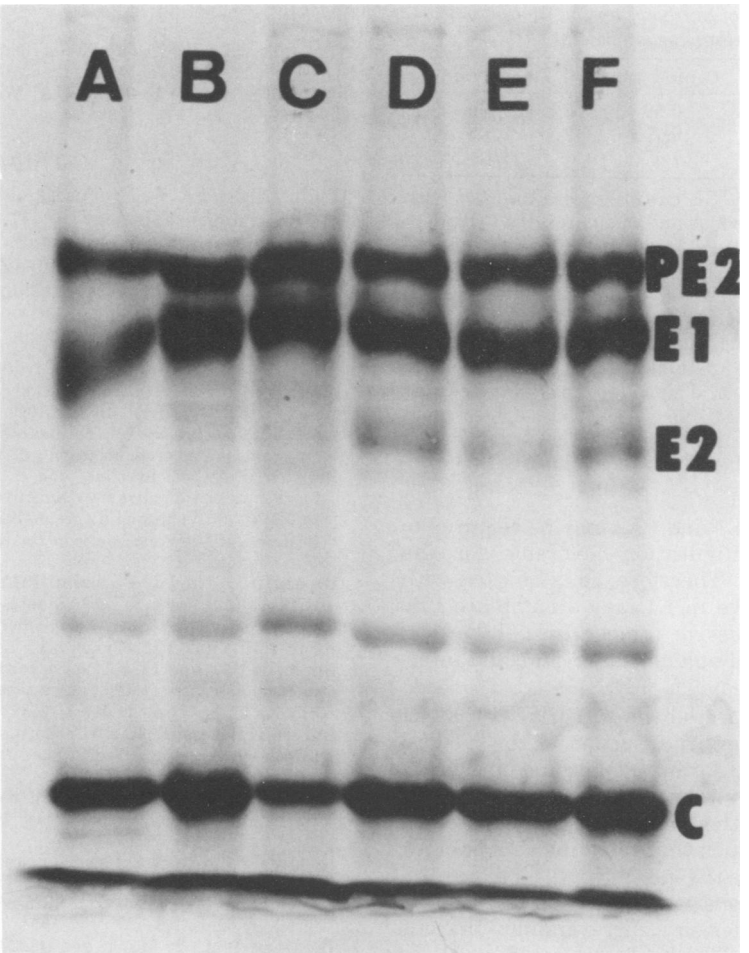

FIG. 6. *Inhibition of PE2 cleavage by antiserum. Six replicate cultures were infected with ts-23 at a multiplicity of infection of 10 and incubated at the permissive temperature for 6 h. At this time, the cultures were washed extensively with prewarmed, methionine-free medium and shifted to the nonpermissive temperature. After 20 min, the cultures were pulse-labeled for 10 min with 25 μCi of [^{35}S]methionine per ml. The cells from one culture were harvested immediately, and the remaining five cultures were washed with prewarmed medium containing 100 times the usual concentration of methionine. They were incubated at the nonpermissive temperature in the 100× methionine medium containing 100 μg of cycloheximide per ml. After a 20-min incubation period, the appropriate antiserum was added to each monolayer, and incubation at the nonpermissive temperature was continued for 20 min. Four cultures were shifted to the permissive temperature for 45 min. One culture containing normal serum was held at the nonpermissive temperature. At the end of the chase period, the cultures were harvested and subjected to PAGE. A, Profile of the polypeptides synthesized during the pulse period at the nonpermissive temperature. B, Chase with normal serum at 41°C. C, Chase at 29°C in the presence of anti-E1 serum. D, Chase at 29°C in the presence of normal serum. E, Chase at 29°C in the presence of antiserum prepared against disrupted Sindbis virus. F, Chase at 29°C in 100× methionine-containing medium in the absence of serum.*

to cleave. We view this as unlikely; the conversion of PE2 does not occur in disrupted Sindbis-infected cells (unpublished data). A second explanation is that the glycoprotein E1 is necessary for the insertion of PE2 into the appropriate host cell membrane fraction. However, PE2 is present in plasma membrane fractions isolated from cells infected with mutants of both complementation groups at the nonpermissive temperature (unpublished data). A third possibility is that E1 and PE2 exist as a complex and that a lesion in either would affect the cleavage event. This possibility was suggested by Schlesinger and Schlesinger (20). An inter-

TABLE 2. *Effect of antibody on the production of Sindbus virus protein*[a]

Proteins compared	Control ratio	Treated ratio
C/E1	0.59	0.59
PE2/E1	0.79	1.32
PE2/E2	1.77	3.13

[a] Two secondary CE cultures were infected with wild-type Sindbis virus and incubated at 37°C for 6 h in methionine-free medium containing 5 μg of actinomycin D per ml. At 4 h after infection, rabbit anti-Sindbis serum was added to one culture (treated) and normal rabbit serum was added to the other (control). After incubation for an additional 2 h at 37°C, 5 μCi of [^{35}S]methionine per ml was added to each plate for 1.5 h. The cells were harvested and digested, and the polypeptides were subjected to electrophoresis. Autoradiograms were scanned, and the areas under the peaks were compared.

action between E1 and PE2 may be required to establish a configuration favorable for the cleavage of PE2. Therefore, cells infected with mutants defective in E1 may accumulate PE2 at the nonpermissive temperature. Likewise, antisera directed against E1 or E2 could interfere with the cleavage of PE2.

An equally reasonable alternative is that E1 influences the proper insertion of the E1-PE2 complex into the surface membrane of the cell. In cells infected with ts-10, the defect in E1 could result in the complex being oriented in the membrane such that refolding cannot occur after a shift to the permissive temperature. This model is consistent with the observation that the ts-10 lesion is irreversible. In cells infected with ts-23, the defect could result in the insertion of the complex such that PE2 cannot be cleaved at the nonpermissive but can be refolded upon a shift to the permissive temperature. It is difficult to define exactly why an E1 defect could prevent PE2 cleavage according to this model. However, the model is consistent with the observation that nucleocapsids fail to bind to membranes of ts-23-infected cells at the nonpermissive temperature (4), suggesting either the absence or improper orientation of binding sites for the assembled cores.

The last two models presented require a close association of PE2 with E1 in the infected cell membrane. We are currently conducting experiments with glycoprotein cross-linkers on infected cell membranes to determine whether such an association exists.

ACKNOWLEDGMENTS

We are especially indebted to Joel Dalrymple for providing the antiserum against the isolated, purified Sindbis virus glycoproteins. We are also grateful to Elmer Pfefferkorn for the generous gift of the temperature-sensitive mutants.

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ADDENDUM IN PROOF

While this work was in press, M. Bracha and M. J. Schlesinger (Virology 74:441-449, 1976) reported essentially identical results to those described here, with the exception of the data concerning the reversibility of the lesion in ts-20.

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