Envelopment of Sindbis Virus: Synthesis and Organization of Proteins in Cells Infected with Wild Type and Maturation-Defective Mutants

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The synthesis and organization of Sindbis virus structural proteins was investigated in BHK cells infected with wild-type virus (SVHR) or temperature-sensitive (ts) mutants defective in maturation. Cells infected with ts-23 or ts-20 (complementation groups D and E) were similar in the polypeptides synthesized at the nonpermissive temperature and differed from SVHR-infected cells in that the envelope protein E2 was not cleaved from the PE2 precursor. Data from experiments utilizing pulse-chase procedures or protein synthesis inhibitors indicated that although infectious virions were released from cells infected with these mutants in shift-down experiments, the particles were produced almost exclusively from proteins synthesized after the return to permissive temperature. This suggests that a stable complex may be formed among the structural proteins before budding. A membrane fraction isolated from cells infected with either ts mutants or SVHR contained the PE2, E1, and C polypeptides, whereas E2 was restricted to fractions obtained from SVHR-infected cells. Although equivalent amounts of virus-specific protein were synthesized in cells infected with either mutant and the cells contained qualitatively the same proteins in the isolated membranes, cells infected with ts-23 did not have virus-specific proteins exposed on their surface that could be detected by ferritin-conjugated antibody-labeling procedures or lactoperoxidase-mediated iodination. In contrast, ts-20-infected cells had significant amounts of viral protein, mainly E1, that could be detected on the plasma membrane by either procedure. Iodine was incorporated into E1 and E2 on the surface of SVHR-infected cells in the same relative amounts as seen in iodinated virions. PE2, however, although present in membranes, could not be iodinated on the surface of infected cells under any of the conditions used in this study. We also monitored the relative efficiency with which these viral proteins could be removed from intact cells by dilute solutions of nonionic detergents. The results indicated that E2 was most efficiently removed, followed by E1. PE2 (the precursor to E2) and C remained associated with the cell and could be subsequently isolated in the membrane fraction.

Sindbis virus (an alpha togavirus) is a structurally uncomplicated virion composed of single-stranded RNA enclosed in an icosahedral capsid which is in turn enclosed in a membranous envelope (5, 21). The capsid of the virion contains the viral RNA and multiple copies of a protein having a molecular weight of 30,000 (21, 23, 26). The envelope of the virion is composed of two virus-specified proteins that are glycosylated by host cell enzymes and are present in the virion in equimolar ratios with the capsid protein (11, 13, 20, 21, 26). The lipids are of host cell origin (20). Structurally the viral envelope

is a membrane bilayer (14). X-ray diffraction and freeze-fracture studies have suggested that this bilayer contains no protein elements penetrating into the hydrophobic interior (5, 14). Enzymatic digestion of the surface glycoproteins of togaviruses by thermolysin has indicated that small hydrophobic tails of the viral glycoproteins anchor these proteins into the membrane (33). Studies based on protein cross-linking agents have produced conflicting results with regard to the extent to which these "tails" penetrate the membrane (12; J. S. Manning, D. E. Felice, and R. R. Traut, *Abstr. Annu. Meet. Am. Soc. Microbiol.* 1976, S279, p. 251). However, electron spin resonance studies

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have suggested that this hydrophobic anchor penetrates less than halfway across the envelope bilayer (27). The envelope of the virion is acquired in the terminal stages of virus morphogenesis by a process referred to as budding. The viral capsid is assembled around the RNA in the cytoplasm of the infected cell and then attaches to the inner surface of the host cell plasma membrane after that membrane is first modified by the insertion of virus-specified glycoproteins (1, 4, 5). The envelopment of the viral capsid takes place as it is progressively wrapped in the modified plasma membrane while moving from the cytoplasm to a position physically outside the cell. The release of the virion from the cell surface occurs as the viral envelope is brought into contact with itself at the base of the developing particle, and a process similar to membrane fusion severs the continuity of the viral envelope and host plasma membrane (5). Although the process of virus budding is well described in morphological terms, many aspects of this process remain to be described biochemically and structurally. (i) What is the nature of the virus-induced alteration that exists on the inner surface of the modified cell plasma membrane which is recognized by the nucleocapsid and to which the nucleocapsid attaches? (ii) What mechanism ensures that the viral envelope proteins are incorporated into the viral envelope in an equimolar ratio to the capsid protein? (iii) What process provides the energy for the sequential envelopment of the viral capsid?

Two models have been proposed to answer these questions. The first suggests that the viral glycoproteins are transmembranyl structures in both the modified cell plasma membrane and the mature virion (12). A sequential association of the capsid protein with the ends of the glycoproteins protruding through the inner surface of the plasma membrane accounts, in this model, for the stoichiometric relationship of the glycoproteins to the core protein and would drive the budding process to completion. A second proposal is based on data suggesting that the glycoproteins in the mature virion are peripheral, i.e., not transmembranyl, structures, and assumes that this peripheral status also exists in the modified plasma membrane (27). This latter model suggests that a reduced fluidity found in the lipids of the viral membrane results from the presence of the viral glycoproteins and suggests that such a reduced fluidity exists in the host plasma membrane after the insertion of the virus glycoproteins. In this hypothesis, budding would occur through the association of the virus capsids with local-

ized areas of the plasma membrane containing the viral glycoproteins and having this increased viscosity. It was suggested that this association alone could be sufficient to initiate the budding process.

Both of these models assume that the viral glycoproteins have the same configuration in the plasma membrane before budding as they have in the mature virion. However, the modified plasma membrane contains the viral glycoprotein E1 and a precursor to the second glycoprotein, PE2 (3, 15, 21, 25, 28). The PE2 polypeptide is cleaved to form the E2 viral glycoprotein in the very terminal stages of budding. The cleavage process appears to be essential to the budding process, since PE2 is never found in mature virions and mutations that block the budding process produce no E2 polypeptide. However, attachment of nucleocapsids to the inner surface of the plasma membrane is not dependent on prior cleavage of PE2 (4, 15). This change in the structure of one of the two envelope proteins during the budding process suggests that a topological change may occur in the cell plasma membrane as it is converted from plasma membrane to viral envelope. Such a structural change has already been suggested morphologically. Freeze-etching studies of the Sindbis virus maturation process have revealed that intramembranyl particles disappear from the interior of viral envelopes as budding progresses, resulting in the release of a mature virion that is free of intramembranyl structure (5).

The following report describes our investigations into the orientation of the viral envelope proteins before and during the budding process through the use of temperature-sensitive (ts) mutants. Mutants in complementation groups D and E are particularly well suited for such a study since they produce intact nucleocapsids and the viral glycoproteins PE2 and E1 at a nonpermissive temperature (3, 4, 8, 15, 23; this study). However, budding does not occur in cells infected with either group of mutants. It has also been demonstrated that these two groups differ in the distribution of the virus-specified proteins in the cells infected at a nonpermissive temperature. Burge and Pfefferkorn demonstrated that the surface of cells infected with a group E mutant contained virus-specified hemadsorbing capability and therefore was modified by the insertion of virus protein. ts mutants of group D produced no such alteration in the cell surface (6). Brown and Smith have further demonstrated that in group E mutant-infected cells the virus nucleocapsids specifically attach to the inner surface of the host cell

plasma membrane. Group D mutants, on the other hand, have no capsids attached to the plasma membrane even though large numbers of these structures can be seen in the cell cytoplasm (4). It would thus appear that the group D mutants are defective in some stage of virus development that is earlier in the infection cycle than occurs with the group E mutant. The group E mutant would appear to be defective in a very terminal stage of budding, since the plasma membrane contains virus-specified polypeptides and capsids are attached to the inner surface of the plasma membrane, but budding does not occur. These mutants would seem to be ideal tools for a study of the process of development of the Sindbis virus envelope.

MATERIALS AND METHODS

Cells, virus, and media. Sindbis virus (heat resistant; SVHR) and Sindbis ts mutants were a gift of Elmer Pfefferkorn (Dartmouth Medical School, Hanover, N.H.). BHK-21 cells were obtained from Peter Faulkner (Queens University, Kingston, Ontario, Canada) and were grown in Eagle minimal essential medium (11) supplemented with 10% fetal calf serum. Virus stocks were prepared after plaque purification by passage at low multiplicity in BHK-21 cells in media containing 1% fetal calf serum. ts mutants were produced at 28°C; SVHR was produced at 37°C. All virus titrations were carried out using BHK-21 cells. After adsorption of the virus dilution at room temperature for 1 h, the monolayers were overlaid with 1% agarose in Eagle medium containing 2% fetal calf serum. The monolayers were stained with neutral red, and the plaques were counted after 3 days of incubation. Stocks of Sindbis virus ts mutants used in this study had the following titrations at 28°C (permissive temperature) and 39.5°C (nonpermissive temperature), respectively: ts-23 (complementation group D), 8.2×10^8 and less than 10^5 ; and ts-20 (complementation group E), 4.0×10^9 and less than 10^5 . In a complementation test (6), ts-20 complemented ts-23 at a level of 30. Other characteristics of these mutants are presented in a previous publication (22).

Infection of cells and radioactive labeling procedures. To minimize the host cell protein synthesis and maximize the labeling of virus-specified polypeptides, the following infection procedure was used. Cells were pretreated at 37°C with medium containing 2 to 4 μ g of actinomycin D per ml for 1 h. The monolayers were washed twice with cold PBS (phosphate-buffered saline) and infected with 50 to 100 PFU of the appropriate mutant or wild-type (SVHR) virus per ml at 4°C for 1 h. The flasks were then transferred to 39.5°C for an additional hour. After the infection period, fresh medium containing actinomycin D was added to the flasks, which were placed at the desired temperature for the period of time specified in the text. At the end of this incubation period, the cells were washed four times with warm PBS and labeled for the specified time period with tritiated leucine (60 to 100 μ Ci/ml) in leucine-

free Eagle medium. In shift-down experiments, cells were maintained at the nonpermissive temperature in nonradioactive media for an additional 1 h before being placed at 28°C.

Iodination of surface proteins. The lactoperoxidase iodination procedure, which is specific for substrate amino acids of proteins exposed on the cell surface (18), was performed on intact monolayers at 39.5°C as follows: cultures infected with ts mutants or SVHR were washed extensively with PBS and covered with 1.5 ml of PBS containing 250 μ Ci of carrier-free iodine-125 (Amersham) and 50 μ g of lactoperoxidase (Sigma Chemical Co., St. Louis, Mo.). The reaction was initiated and maintained by the addition and mixing of 20 μ l of 0.1% H_2O_2 every 2 min for 12 min. The cultures were then allowed to stand at 39.5°C for an additional 8 min. They were then extensively washed with PBS and processed as described below for precipitation of viral proteins or directly for gel electrophoresis.

Preparation of rabbit antisera. Sindbis virus that had been purified through three consecutive equilibrium gradients and one velocity gradient in potassium tartrate was dialyzed against PBS and mixed with an equal volume of Freund complete adjuvant. An aliquot of this preparation (containing 100 μ g of viral protein) was injected subcutaneously, and 10 days later this procedure was repeated. After 3 weeks an additional injection of Sindbis virus in PBS was given intravenously. A 1:10,000 dilution of the resulting antiserum inactivated 99% of virus infectivity in 15 min at room temperature.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Polypeptide analysis was carried out in 14-cm vertical slab gels, using a discontinuous system modified from Laemmli (17). DATD (*N,N'*-dialyltartardiamide; Eastman, Rochester, N.Y.) was used as the cross-linker and was found to give better resolution of the viral glycoproteins. However, the sieving capacity of DATD-cross-linked gels is not as great as in gels cross-linked with *N,N'*-methylene bisacrylamide, and hence the stacking and separating gels were formed at 5 and 14%, respectively. The ratio of acrylamide to DATD was 30:1.6. The sample buffer contained 2% sodium dodecyl sulfate, 5% mercaptoethanol, 10% glycerol, 0.005 M phosphate buffer (pH 7.0), and 5 μ M phenylmethylsulfonyl fluoride as a protease inhibitor. Samples were dissolved directly in buffer preheated to 100°C and dissociated at 100°C for 2 min. Electrophoresis was carried out using constant voltage (100 V) for 7 h. The gels were then fixed and stained with Coomassie brilliant blue. Individual tracts were frozen and sliced, and the gel slices were dissolved in 0.5% sodium periodate, evaporated onto filters (Whatman GF/A), and counted in toluene-based scintillation fluid.

Immune selection of cell-associated virus polypeptides. Direct or indirect precipitation of viral proteins from cell extracts was performed essentially as described by Kaluza (16), modified such that 1% bovine serum albumin was included in the lysis buffer. Indirect precipitates were formed by using sheep anti-rabbit globulin (GIBCO, Grand Island, N.Y.). Equivalence zone concentrations were

determined by checkerboard titrations. Alternatively, viral proteins were selected by affinity chromatography in which unfractionated rabbit antisera proteins or ammonium sulfate-precipitated immunoglobulins were covalently linked to a Bio-Gel A support (Affi-Gel 10), purchased from Bio-Rad Laboratories, Richmond, Calif. A 4-ml amount of rabbit antisera was mixed with 25 ml of 0.1 M phosphate buffer (pH 6.8) and 1 g of Affi-Gel 10 and shaken at 4°C overnight. Unreacted groups were saturated by incubation of the adsorbent with 1 M glycine in 0.1 M phosphate buffer (pH 6.8) for 5 h at 4°C. Columns with a 3-ml bed volume were prepared and equilibrated with lysis buffer. Coupling of purified immunoglobulins did not increase the amount of virus-specific proteins bound to the column in these experiments. A 0.5- to 1.0-ml amount of the infected cell lysate, prepared as described above, was allowed to enter the column bed and to react for 5 h at 4°C. The column was then washed extensively with lysis buffer to remove the unadsorbed proteins and finally with lysis buffer lacking bovine serum albumin. The viral proteins were eluted in 3.5 M potassium thiocyanate in 0.5% Nonidet P-40 (NP-40). The eluted proteins were then dialyzed and lyophilized, and the NP-40 was subsequently removed by ethanol extraction. The final precipitate was processed for electrophoresis as described above. Selection of viral proteins by affinity chromatography gives results equivalent to immunoprecipitation, but has the advantage that equivalence zones need not be determined and viral proteins are obtained free from large amounts of immunoglobulins that remain coupled to the column.

Preparation of cell membranes. Tritium-labeled infected cells were washed four times with cold PBS and scraped from the monolayer into ice-cold 0.001 M Tris, pH 7.0 (5% sucrose and 0.001 M magnesium acetate). The cells were disrupted by homogenization in a Dounce homogenizer with a tight-fitting pestle. The cell homogenate was clarified at 2,000 rpm for 5 min to remove nuclei, and the membranes remaining in the supernatant were sedimented through 20% (wt/wt) sucrose onto a 60% (wt/wt) sucrose cushion in a Beckman SW27 rotor at 20,000 rpm overnight. The membranes were then made 70% (wt/wt) in sucrose concentration by the addition of sucrose. This solution was overlaid with 3 ml of 60% (wt/wt) sucrose followed by 30% sucrose and was centrifuged overnight at 30,000 rpm in the Beckman SW41 rotor. The membranes were recovered at the 60 to 30% interface.

Treatment of infected cells with NP-40. Cells infected with wild type (SVHR), ts-23, or ts-20 as described above were labeled with tritiated leucine between 4.5 and 6 h of incubation at 39.5°C. The cells were then washed six times with ice-cold PBS, and the monolayers were covered with a minimal volume (about 5 ml) of cold 0.01% NP-40 in PBS (obtained from Shell Chemical Co.) and maintained at 4°C with gentle agitation for 30 min. The NP-40 solution was then removed from the cells, and the monolayer was washed six more times with cold PBS. The NP-40 supernatant was treated by centrifugation at 2,000 rpm to remove any cells released

from the monolayer, and the supernatant was lyophilized. The lyophilized material was resuspended in absolute ethanol to remove the NP-40 and stored overnight at -20°C. The ethanol solution was centrifuged at 12,000 rpm for 30 min at 0°C in a Sorvall SS-34 rotor, the ethanol was decanted, and the pellet was dried under vacuum. The pellet was then resuspended in ice-cold 10% trichloroacetic acid and centrifuged at 12,000 rpm as described above. The supernatant was removed, and the ethanol step described above was repeated. After the second lyophilization, the pellet was dissolved directly in electrophoresis buffer and electrophoresed as described above.

Electron microscopy. The presence of Sindbis virus antigens exposed on the surface of wild-type- and ts mutant-infected cells was determined by electron microscopy of infected cells after treatment with ferritin-conjugated antiviral antiserum. The procedures of embedding and thin sectioning have been described previously (5). Anti-Sindbis serum was prepared as described above and was adsorbed three times with uninfected BHK cells and diluted 1:10 in PBS. Ferritin-conjugated anti-rabbit serum prepared in goats was purchased from the Cappel Laboratories (Downingtown, Pa.). The indirect labeling procedure was used. Infected as well as uninfected cells were treated at 39.5°C with the anti-Sindbis serum for 30 min (conditions that allowed capping of viral membrane proteins) or at 4°C after fixation in 1% glutaraldehyde (to prevent capping of virus antigens). After treatment with the primary antiserum, the cells were washed exhaustively with cold PBS and the ferritin conjugate was applied for 30 min at 4°C. The cells were then washed with PBS and embedded for ultrathin sectioning. The ultrathin sections were examined in a Siemens 101 electron microscope.

RESULTS

Viral protein synthesis in cells infected with ts mutants. Cells infected with ts mutants 23 or 20 at the nonpermissive temperature were labeled with tritiated leucine and analyzed by polyacrylamide gel electrophoresis as described above. The protein profiles of these genetically distinct mutants are shown in Fig. 1 and are compared with those of wild-type (SVHR)-infected cells incubated and labeled under similar conditions. The proteins synthesized in cells infected with ts-23 and -20 were identical to one another in their migration pattern and differed from SVHR in that both mutants produced little or none of the E2 polypeptide. These observations are consistent with the results of Jones et al. (15) and Brache and Schlesinger (3) and extend their observations to the group D mutant, ts-23. The results presented in Fig. 1 show that although the mutants are capable of complementing one another (6, 22), they are similar in terms of the proteins they synthesize at the nonpermissive temperature.

FIG. 1. Virus proteins synthesized in BHK-21 cells infected with Sindbis virus wild type (SVHR) and *ts* mutants. Cells were infected and labeled as described in the text between 5.5 and 7 h of incubation at 39.5°C. The positions of ¹⁴C-labeled marker proteins added to the sample before electrophoresis are indicated by arrows in this and the following gel electropherograms. (A) SVHR; (B) *ts*-20; (C) *ts*-23.

Detection of viral polypeptides in the membranes of cells infected with *ts* mutants. The difference in the alteration of the cell membranes after infection at the nonpermissive temperature with *ts*-20 or *ts*-23 was investigated by analysis of the virus-specified proteins present in the membranes of *ts* mutant- and

wild-type-infected cells. Figure 2 shows the proteins present in the fractionated membranes prepared as described in Materials and Methods. As was found in whole-cell extracts, the membranes of *ts*-20- and *ts*-23-infected cells were similar in that they lacked E2, which was readily detected in the membranes of wild-type-infected cells. However, the mutants differed from one another in that the membranes from *ts*-23-infected cells contained much less capsid protein that did the membranes of either *ts*-20- or wild-type-infected cells. This result agrees with the ultrastructural data (4), which showed

FIG. 2. Virus proteins recovered in membranes of BHK-21 cells infected with Sindbis virus wild type (SVHR) and *ts* mutants. Cells were infected and labeled between 5 and 6 h of incubation at 39.5°C as described in the text. Cell membranes were isolated as described in the text and were electrophoresed after the addition of ¹⁴C-labeled marker as in Fig. 1. (A) SVHR; (B) *ts*-20; (C) *ts*-23.

that nucleocapsids could attach to the inner surface of plasma membranes of cells infected with ts-20 but that this attachment did not occur in ts-23-infected cells. Group D mutant ts-23 therefore appears to be defective in the association of nucleocapsids with cell plasma membranes. That the group D mutant has any viral protein in the membrane fraction would seem surprising in view of the data indicating that cells infected with this mutant lack hemadsorbing capability (8) and, as will be shown below, have no virus-specified polypeptides exposed on the cell surface that are detectable antigenically or by radioiodination. The explanation for this apparent contradiction may reside in the fact that the material analyzed in this study represents a collection of cell membranes and not solely plasma membranes, and thus the virus-specified polypeptides detected in ts-23-infected cell membranes may be in fact restricted to interior membranes. Attempts to clearly separate plasma membranes from internal membranes using established procedures (2) were not successful.

Nonreversible nature of ts-23 and ts-20 defects. It was demonstrated previously that the mutants comprising the complementation groups D and E were capable of producing virus after a shift to the permissive temperature (4, 8, 15). To determine whether virus polypeptides synthesized at the nonpermissive temperature could be chased into mature virions after return to the permissive temperature, we followed the fate of virus proteins that had been produced at 39.5°C after a shift to 28°C (Fig. 3, only ts-20 shown). The gel profiles of cells infected with either mutant remained essentially unchanged after a shift to the permissive temperature even when these cultures were maintained at the permissive temperature for 8 h before analysis. Under identical conditions with SVHR-infected cultures, the PE2 polypeptide was greatly decreased during the chase period as was originally demonstrated by Schlesinger and Schlesinger (24), and in an 8-h chase nearly 70% of the total radioactivity incorporated during a 1-h pulse was recovered in virus (data not shown). Although cultures infected with either ts mutant produced titers of infectious virus comparable to those of SVHR-infected cultures after a shift to the permissive temperature, radioactivity incorporated at the nonpermissive temperature was very inefficiently chased into these virions (Table 1). That none of the three virus structural polypeptides appeared in mature virions after a shift to the permissive temperature in the ts-23-infected cell system is particularly interesting, since electron microscopy (4) and

FIG. 3. Virus proteins in BHK-21 cells infected with ts-20 and labeled with [^3H]leucine at 39.5°C between 5.5 and 7 h after infection. Cells were washed, received fresh medium, and were incubated at 39.5°C for 30 min before incubation at 28°C for an additional 2 h before electrophoresis. Symbols: (●) ^3H -labeled purified virus; (○) ^{14}C -labeled purified virus.

TABLE 1. Transfer of radioactivity incorporated into virus protein at 39.5°C to mature virions after shifting infected cultures to 28°C for 8 h^a

Virus	Yield (PFU $\times 10^{10}$)	cpm ($\times 10^{-3}$) in purified virus
SVHR	6.5	972
ts-23	7.4	4
ts-20	7.2	34

^a BHK-21 cells were infected and labeled between 4.5 and 6 h of incubation at 39.5°C as described in the text. Cells were washed and maintained in complete medium at 39.5°C for an additional hour before transfer to 28°C.

the gel electrophoresis study presented above suggested that the virus capsids were not associated with cell membranes in ts-23-infected cells and thus could be free to be converted into mature virions. In the case of ts-20, where sufficient radioactivity was obtained in the virus produced after a shift to the permissive temperature to be analyzed by gel electrophoresis, the profile shown in Fig. 4 was obtained. Although the virus produced after the shift to the permissive temperature contained all three structural proteins, the ratio of radioactivity in the E1 and E2 bands suggested that the E1 polypeptide was more readily incorporated into virions than E2 (obtained from PE2 produced at the nonpermissive temperature).

To further examine the role of new protein synthesis in the production of virions by the mutants in shift-down experiments, we monitored the production of PFU by the mutants

after a shift to the permissive temperature in the presence of cycloheximide. Production of virus under these conditions was reduced by three orders of magnitude by the presence of cycloheximide (Table 2). These data combined with those presented above indicate that virus production after a shift to the permissive temperature is almost completely dependent upon the synthesis of new viral proteins, and among the limited amount of protein produced at non-permissive temperature by ts-20 that is incorporated into virions after shift-down, E2 chases with the lowest efficiency.

Presence of virus-specified polypeptides on the surface of cells infected with ts mutants. We have used two procedures to detect virus proteins on the surface of mutant- and wild-type-infected cells: (i) direct observation by electron microscopy after labeling the surface of the infected cells with ferritin-conjugated antiserum, and (ii) direct labeling of the surface proteins with iodine-125, using the lactoperoxidase-labeling procedure. The ferritin-antibody studies allowed us to determine the relationship of antigens on the outer cell surface to capsid structures associated with the inner sur-

TABLE 2. Effect of cycloheximide on the growth of Sindbis virus RNA⁺ ts mutants after shift to permissive temperature^a

Mutant	Complemen- tation group ^b	Yield (PFU/ml) of virus after 2 h at:		
		39.5°C	28°C	28°C with cy- cloheximide
ts-2 ^c	C	8.0×10^4	9.2×10^5	1.2×10^5
ts-23	D	3.2×10^4	1.0×10^7	9.6×10^3
ts-20	E	2.0×10^4	4.6×10^7	1.7×10^4

^a BHK-21 cells infected for 5 h with 20 PFU of ts mutant virus per cell were washed and treated with either warm (39.5°C) medium or warm cycloheximide (complete medium, 50 μ g/ml) for 30 min. The cells were either maintained at 39.5°C or shifted to 28°C for 2 h.

^b As established by Burge and Pfefferkorn (7).

^c ts-2 included as an example of a mutant that is reported not to produce virus after a shift to the permissive temperature (7).

face of the cell plasma membrane, and the radioiodination procedure provided the opportunity to determine which virus-specified polypeptides were exposed on the surface of the infected cells.

Cells infected with either wild type or the ts mutants were examined in the electron microscope after labeling with ferritin-conjugated antibody, using the indirect procedure described in Materials and Methods. The cells were labeled under conditions that either allowed or prevented the capping or clustering of the virus polypeptides exposed on the cell surface (31). Under conditions that permitted capping, SVHR-infected cells were observed to bind a large amount of antibody in discrete patches on the cell surface (Fig. 5a). The patches were of three basic morphological types: (i) patches associated with areas of the cell surface actively producing virions (Fig. 5a, arrow 1); (ii) antibody associated with areas not actively producing virions but with viral capsids associated with the inner surface of the plasma membrane opposite the ferritin (Fig. 5a, arrow 2); and (iii) antibody located in a cluster on the cell surface with no capsids associated with the membrane (Fig. 5a, arrow 3). When cells were examined in the absence of capping (Fig. 5b), the antibody was found to be more uniformly distributed on the cell surface, and the clustering of virus structures (capsids and partially mature virions) seen in Fig. 5a was not readily detected. The observation that the clustering of viral antigens exposed on the surface of the infected cell could result in a clustering of the nucleocapsids attached to the inner surface of the plasma membrane suggests that there is some interaction between the viral

FIG. 4. Proteins of Sindbis virus ts-20 released from cells after a shift to the permissive temperature. Infected BHK cells were labeled at 39.5°C between 5 and 6 h of incubation at 39.5°C. The culture was washed, fresh medium was added, and the culture was incubated for 30 min at 39.5°C and then placed at 28°C overnight. The virus produced was purified by two sequential centrifugations to equilibrium density in potassium tartrate. The entire virus band was electrophoresed after concentration by lyophilization.

antigens exposed on the outer surface of the plasma membrane and the nucleocapsids.

Large amounts of antibody were also bound to the surface of cells infected at the nonpermissive temperature with the mutant ts-20 (Fig. 6). In the absence of capping, the antibody was distributed randomly over the cell surface (Fig. 6a) and nucleocapsids were seen associated at various points on the inner surface of the plasma membrane. Capping of viral antigens on the cell surface by antiviral serum resulted in some clustering of the nucleocapsids on the inner surface of the plasma membrane as was seen with the wild type. In Fig. 6b such a cluster of nucleocapsids is seen in an area where the cell membrane has been torn away from the cell cytoplasm, revealing the contact between the virus capsid and the inner surface of the plasma membrane.

Experiments with ferritin labeling of cells infected at the nonpermissive temperature with mutant ts-23 produced negative results; no antibody could be detected on the surface of the infected cells (data not shown).

To determine which virus-specific protein(s) was responsible for the ferritin binding described above, infected cells were labeled with iodine-125 by the lactoperoxidase procedures described in Materials and Methods. Cells were iodinated after infection with the appropriate mutant or wild-type virus after 8 h of incubation at the nonpermissive temperature. After the iodination procedure was completed, the cells were extensively washed and the virus proteins were selected from the infected cells by the immunological procedures described in Materials and Methods. The immunoprecipitation was found to be essential, since many host-specific surface proteins were iodinated by this procedure, and their presence in the gels was found to obscure to some extent the bands of virus polypeptide (Fig. 7). The amount of virus-specific iodinated protein from the mutant-infected, wild-type-infected, and uninfected cells is shown in Table 3. The amount of radioactivity that was recognized by the antiviral antibody used in this study with ts-23-infected cells was nearly identical to that of the mock-infected cells. Therefore, although large amounts of virus-specific proteins could be detected on the surface of SVHR- and ts-20-infected cells, no significant difference was seen between ts-23-infected and uninfected cells, despite the fact that equivalent amounts of structural proteins were synthesized in cells infected by the two mutants (Fig. 1).

An analysis of the immunoprecipitate of wild-type- and ts-20-infected cells by gel elec-

trophoresis is shown in Fig. 8. SVHR-infected cells possessed a large amount of virus polypeptide E2, which was exposed to iodination by lactoperoxidase. Virus polypeptide E1 was also labeled by this procedure. PE2, the precursor of the E2 polypeptide, was not labeled even though isolated cell membranes from wild-type-infected cells contained as much PE2 as E2 (Fig. 2), suggesting that only after cleavage are the E2 sequences in PE2 accessible to iodination. The relative amounts of iodine incorporated into E1 and E2 seen in the wild-type-infected cells were identical to the profile of iodinated purified virus (data not shown; 28).

E1 and E2 were also iodinated on the surface of cells infected with ts-20 and incubated at the nonpermissive temperature (Fig. 7 and 8). The amount of E2 labeled relative to E1 was much lower than in the wild-type-infected cells; the E1 and E2 peaks contained equivalent amounts of radioactivity. The E2 labeled on ts-20-infected cells presumably reflects a leakiness of the mutant, which is accentuated by a high efficiency of labeling of the E2 glycoprotein and the fact that any E2 that is formed accumulates throughout the period of incubation and is not removed from the cells by budding, as is the case in a wild-type infection. The leakiness was at such a low level that it was not detected in the pulse-labeling procedures used to obtain the data presented in Fig. 1 and 2. As was the case in the preceding experiment with cells infected with wild-type virus, PE2 could not be detected as an iodinated species on the surface of ts-20-infected cells, even though the pulse-labeling experiment (Fig. 2) showed that the membranes of cells infected with this mutant contain as much PE2 as E1. It thus appears that, as in wild-type-infected cells, PE2 is present in the plasma membrane in a form that is not accessible to lactoperoxidase iodination. The argument that the antibody used in this study might not be able to bind to PE2 is negated by the data presented in Fig. 8D, showing the immunoprecipitation of PE2 and the other membrane proteins in the expected ratios ($PE2 + E2 = E1$) (24, 28), and by the recent observation of Brache and Schlesinger (3) that specific antibodies against E2 can block the development of virus in wild-type-infected cells and prevent the cleavage of PE2 to E2. The absence of an iodinated capsid peak in these experiments suggests that little disruption of the cells occurred during the iodination procedure, and thus proteins detected by this procedure were in fact externally located.

Release of virus protein from the membrane of cells infected with wild-type and ts mutant

virus by treatment with NP-40. The results of the iodination study described above suggested that PE2 and its cleavage product, E2, might be exposed to differing degrees on the surface of infected cells. Furthermore, the observation that capsid protein remained associated with membranes during purification by centrifugation from wild-type- and ts-20-infected cells suggested that the nucleocapsids are tightly bound to the membrane (see also reference 4). The observation that the capsids could be clustered on the inner surface of the plasma membrane as the exposed viral antigens were capped by antiviral antiserum suggested that the nucleocapsids were attached to at least one of the viral membrane proteins that were exposed on both the inner and outer surface of the cell (a transmembranyl protein). We have therefore attempted to selectively remove virus proteins from the surface of infected cells in the hope that any transmembranyl polypeptides would remain anchored in the cell membrane while other, more peripheral proteins would be released. We have used the procedure described by Pearlstein and Seaver (19) to test this possibility. These authors demonstrated the selective release of a membrane protein from the surface of transformed cells under conditions that did not result in lysis of the cells by the use of low concentrations of the nonionic detergent NP-40 at 4°C.

Cells infected with SVHR, ts-20, or ts-23 were labeled and treated with NP-40 as described in Materials and Methods. The monolayers were then covered with fresh medium and placed at 28°C, or their membranes were isolated and analyzed by gel electrophoresis. The infected cells survived the NP-40 treatment remarkably well. Few cells were released from the monolayer, and if the cultures were placed at 28°C virus was produced from each of the cultures in normal amounts (Table 4).

The virus-specified polypeptides released from infected cells by the NP-40 treatment are shown in Fig. 9, 10, and 11, together with the profiles of those polypeptides that remained associated with the membranes of the infected cells. These profiles can be compared with the protein composition of untreated membranes shown in Fig. 2. NP-40 quantitatively removed

E2 from the membranes of wild-type-infected cells, and this polypeptide was recovered in the NP-40 solution (Fig. 9). The NP-40 solution also contained some PE2, E1, and capsid protein, although in lesser amounts than E2. The presence of C suggested some cell lysis, although PE2 and C were least efficiently released from the monolayer by this procedure. A decrease in the amount of E1 in the membranes accompanied by an increased amount of this protein in the NP-40 wash solution (relative to PE2) suggests that this protein is also removed from the membrane to some extent by detergent treatment.

Treatment of ts-20-infected cells with NP-40 also resulted in the preferential loss of E1 from the cell membranes into the wash solution (Fig. 10). Removal of viral proteins by NP-40 from ts-23-infected cells was less efficient and did not result in the preferential removal of any viral protein from the cell membranes (Fig. 11).

These experiments suggest that the virus polypeptides PE2 and C are most tightly associated with the cell membrane, whereas the cleavage product of PE2, E2, is most loosely bound. The E1 polypeptide is more tightly bound than E2, but is more readily removed by detergent treatment than either PE2 or C.

DISCUSSION

This paper confirms and expands upon previously published observations on the organization of Sindbis glycoproteins in cell membranes and the nature of the defects in Sindbis virus temperature-sensitive mutants defective in budding. Wild-type Sindbis produces large amounts of the three virus structural proteins and PE2, as shown previously (3, 15, 21, 25, 24, 27, 28). These viral proteins can also be detected in the purified membrane preparations of infected cells, but PE2 is not found in mature virions. The purified membranes of cells infected with wild-type virus also contain significant amounts of the capsid protein C. The copurification of this protein with the cell membranes implied that it is tightly bound to the modified membrane, most likely in the form of partially budded structures. Labeling the surface of cells infected with wild-type virus by the indirect ferritin-conjugated antibody procedure

FIG. 5. Labeling of BHK-21 cells infected with Sindbis wild-type virus with ferritin-conjugated antiserum. Cells were labeled and prepared for electron microscopy as described in the text at 7 h after infection. (a) Labeled at room temperature to allow clustering of viral antigens. Arrows: 1, Ferritin-labeled area where mature virions are being produced; 2, ferritin-labeled area where budding is not occurring but cores are attached to the inner surface of plasma membrane opposite bound ferritin; 3, ferritin-labeled areas of cell surface having no visible virus structures. (b) Labeled at 4°C after treatment with glutaraldehyde to prevent clustering of viral antigens. Arrows indicate mature virions on cell surface. Magnification, $\times 30,000$.

FIG. 6. Labeling of BHK-21 cells infected with Sindbis virus ts-20 at the nonpermissive temperature. Cells were treated as described in Fig. 5 and the text. (a) Labeled at 4°C after treatment with glutaraldehyde. Arrows indicate nucleocapsids associated with inner surface of plasma membrane. (b) A cluster of virus membrane-associated capsids produced by labeling at room temperature. The association of the capsids with the modified membrane is revealed in the region where the membrane is pulled away from the cell cytoplasm (arrow). Magnification, $\times 83,000$.

FIG. 7. Total proteins labeled on the surface of BHK-21 cells infected with Sindbis virus ts-20 at 39.5°C for 9 h. Surface proteins were labeled with ^{125}I by the lactoperoxidase procedure as described in the text. Position of virus polypeptides was determined by ^{14}C -labeled marker mixed with the sample before electrophoresis. Symbols: (●) ts-20-infected BHK cells; (○) uninfected BHK cells.

TABLE 3. Sindbis virus protein exposed to enzymatic iodination on the surface of cells infected with wild type, ts-23, and ts-20^a

Cells infected with:	Total ^{125}I bound to monolayer (cpm $\times 10^{-3}$)	Amt of ^{125}I bound to virus proteins as determined by:	
		Immunoprecipitation	Affinity chromatography ^b
SVHR	1 127.1	180.3 (16.0) ^c	84.7 (7.5)
ts-23	1,397.0	ND ^d	2.4 (0.17)
ts-20	1,136.2	23.9 (2.1)	24.1 (2.1)
Mock infected	1,086.4	2.1 (0.18)	2.3 (0.2)

^a Cells infected as described in the text were iodinated after 8 h of incubation at 39.5°C.

^b As described in the text.

^c Numbers in parentheses are percentages.

^d ND, Not determined.

revealed three morphologically distinct structures. Under conditions that allowed for capping of virus antigens on the surface of the infected cell, clusters of antibody could be found where budding was obviously taking place. Clusters of antibody were also seen in regions where no virus budding occurred, and virus capsids were not associated with the inner surface of the membrane. Antibody could also be detected on the outer surface of the infected cell, with a cluster of virus capsids on the inner surface of the membrane opposite the externally located antibody. In this latter instance there was no indication of budding; rather it appeared as though nucleocapsids had attached to the membrane, but the budding process

either had not yet been initiated or was inhibited by the antibody. These three areas were not intermixed, suggesting that budding may take place more or less synchronously in localized regions on the cell surface. Brown et al. (5)

FIG. 8. Virus-specific proteins iodinated by lactoperoxidase and selected by direct immunoprecipitation (A) or by affinity chromatography (B and C). (A and B) BHK cells infected with SVHR and incubated at 39°C for 8 h before iodination. (C) BHK cells infected with ts-20 and incubated at 39.5°C for 8 h before iodination. (D) Immunoprecipitation of virus-specific proteins from BHK-21 cells infected with SVHR and incubated in the presence of [^3H]leucine (50 $\mu\text{Ci}/\text{ml}$) from 5.5 to 10 h after infection.

TABLE 4. Virus produced from SVHR-, ts-20-, and ts-23-infected cells after treatment with NP-40^a

Virus	Titer (PFU/ml) produced from:	
	NP-40-treated cells ^b	PBS-treated cells ^b
SVHR	1.0×10^9	2.2×10^9
ts-20	3.4×10^9	8.6×10^9
ts-23	1.6×10^8	3.2×10^8

^a NP-40 treatment was as described in the text.^b Titered in BHK-21 cells at 28°C.

have provided additional evidence for such localized simultaneous release of virus by using freeze-fracture techniques. These authors found that budding took place in distinct areas on the infected-cell surface. However, in the absence of capping, the cell surface was found to be more or less uniformly labeled by the ferritin conjugate, suggesting that even though the budding process occurred in patches, the plasma membrane was uniformly modified by the insertion of virus-specified protein. Radiiodination of wild-type-infected cells by the lactoperoxidase procedure confirmed the observation of Sefton et al. that E2 labeled very efficiently, E1 less efficiently, and PE2 not at all (28). The techniques used in our study differ from those of Sefton et al. in that virus-specific proteins were separated from cell proteins after the iodination procedure, thus allowing a better opportunity to detect the virus-specified proteins without their being obscured by co-electrophoresing host membrane proteins. In spite of this precaution and the fact that all of the amino acid sequences present in E2 are also present in PE2 (24), PE2 could not be detected as an iodinated species. PE2 is, however, present in the membranes as was shown by an analysis of the isolated membranes (Fig. 2). We conclude that although present in the cell membrane, its substrate amino acids for the iodination are not available to the lactoperoxidase enzyme. As budding takes place, the substrate amino acids are exposed as PE2 is cleaved to E2. This implies a structural rearrangement of the E2 portion of the PE2 polypeptide during cleavage and the accompanying budding process. E1 was not altered in its labeling characteristics and may not have undergone significant reorganization.

Cells infected with the group E mutant, ts-20, were found not to produce mature virions that could be detected by plaque assay or physical assay at the nonpermissive temperature. Cells infected with this mutant at the nonpermissive temperature contained large amounts

FIG. 9. Release of wild-type Sindbis virus (SVHR) proteins from infected BHK-21 cell membranes by treatment with NP-40. Cells were infected, labeled, and treated with detergent as described in the text. (A) Virus proteins removed from the cells and recovered in the NP-40 washing solution. (B) Virus proteins remaining associated with cell membranes after treatment with NP-40. Membranes were prepared as described in the text.

FIG. 10. Release of Sindbis virus *ts-20* proteins from infected BHK-21 cell membranes by treatment with NP-40. (A and B) As in Fig. 9.

of PE2, E1, and C but no detectable E2 (3, 15; this study). Membranes purified from *ts-20*-infected cells that had been maintained at the nonpermissive temperature contained PE2 and E1 in roughly the same proportions as found in the intact cell and large amounts of C.

This is consistent with the observation of Brown and Smith (4) that cells infected with *ts-20* have, at the nonpermissive temperature, large numbers of virus capsids associated with the inner surface of the plasma membrane. Although no budding could be detected, the attachment of the nucleocapsid to the inner surface of the plasma membrane was very stable

since the structures could be copurified in equilibrium density gradients. We have also found that the outer surface of the plasma membrane of *ts-20*-infected cells can bind anti-Sindbis antibody in areas opposite regions where capsids are attached. Enzymatic radioiodination indicates large amounts of glycoprotein E1 relative to E2 in this system, and although we have found the membranes to contain as much (mole for mole) PE2 as E1, none of the PE2 can be iodinated. This suggests that it is configurationally unavailable to the iodination process in this mutant-infected system, as it is in wild-type-infected cells. The failure to label PE2 in this static system argues against the criticism that can be directed against the data obtained from wild-type-infected cells, specifically that PE2 cannot be detected by iodination because it is rapidly converted to E2 once exposed on the cell surface. The association of the nucleocapsids with the inner surface of the *ts-20*-infected cell plasma membranes suggests that these

FIG. 11. Release of Sindbis virus *ts-23* proteins from infected BHK-21 cell membranes by treatment with NP-40. (A and B) As in Fig. 9.

structures are attached to some virus-specific material. The clumping of the capsids under the plasma membrane by the externally attached antibody molecules suggests some interaction between the virus proteins exposed on the cell surface and the virus-specified structure located on the inner surface of the membrane to which the capsids attach. This fact further suggests that the association of the nucleocapsid to the modified membrane is not in itself sufficient to initiate budding or result in the cleavage of PE2.

ts-23-infected cells were found to be similar in their virus-specified proteins to ts-20-infected cells at the nonpermissive temperature. An analysis of the membranes purified from ts-23-infected cells revealed the presence of some PE2 and E1, but very little capsid protein (Fig. 2). The small amount of capsid protein in the membrane fraction of ts-23-infected cells agrees with the ultrastructural observation that capsids do not associate with the inner surface of the plasma membrane in this system (4). Virus protein could not be detected on the surface of cells infected with ts-23 at the nonpermissive temperature either by the indirect ferritin-labeling procedure or by enzymatic radioiodination. The proteins associated with the membranes shown in Fig. 2 either are associated with the plasma membrane but not exposed externally or are associated with internal membranes that copurify with the plasma membrane in the procedures used here and are thus not accessible to either of the surface-labeling procedures used in this study.

It thus seems that the budding process is initiated by the association of a completed nucleocapsid with the inner surface of a plasma membrane containing (i) PE2 in a configuration not accessible to enzymatic radioiodination and (ii) E1, which is exposed in the membrane to radioiodination much as it is in mature virions. As budding of the capsid occurs, PE2 is cleaved to yield E2, which is altered in such a way that it is now accessible to radioiodination by the lactoperoxidase procedure, as it is in mature virions. E1 seems to undergo the transition from host plasma membrane to mature virion without obvious topological alteration.

The pulse-chase experiments and the cycloheximide data demonstrated that the production of virus after a shift to the permissive temperature was almost completely dependent upon synthesis of new structural polypeptides. In neither of the mutant systems was it possible to efficiently chase the structural proteins, even though some are presumed to be nondefective. This could be taken as evidence that there is a complex formed among all three proteins

(PE2, E1, and C) such that a defect in one renders the entire complex nonfunctional. The failure to chase any of the ts-23-specified peptides may indicate that this complex is formed before the appearance of the proteins at the plasma membrane. The reduced amount of E2 relative to E1 and C chased into ts-20 virions (Fig. 4) may indicate that such a complex can occasionally be rescued by rearrangement with a nondefective protein, in this case PE2 synthesized after return to the permissive temperature. This would imply that the original ts lesion in this mutant resides in PE2. This also suggests that complementation of RNA⁺ mutants may be a function of the degree to which these complexes can rearrange. The generally low complementation indexes found in Sindbis mutants (6, 22) and the low efficiency with which proteins synthesized at the nonpermissive temperature are rescued in shift-down experiments indicate that these complexes are very stable. Additional evidence for such a complex has been reported previously (3, 20, 28).

The nature of the attachment of the capsid to the PE2-E1 complex is not directly resolved in this study. The inability to detect PE2 on the surface of infected cells and the finding that PE2 and C are most difficult to release from the cell membrane suggest that this attachment may be mediated through PE2. The finding that the PE2 cleavage product, E2, is most readily removed from the membrane by detergent treatment suggests that this initial PE2-C association is lost after the cleavage of PE2 during budding. Using experimental conditions similar to those we have used, it has been demonstrated that a detergent-stable association of the capsid with the membrane proteins does not exist in mature virions (30). A lack of association of the capsid with the membrane proteins is also indicated by several studies using unrelated techniques (5, 14, 27; Manning et al., *Abstr. Annu. Meet. Am. Soc. Microbiol.* 1976, S279, p. 251). We have attempted to determine which protein(s) is responsible for the binding of nucleocapsids by purification of such a complex from ts-20-infected cells. Although preliminary data have been obtained which indicate that PE2 can be more efficiently recovered than E1 bound to nucleocapsids, this complex appears to be generally unstable to the procedures used to dissolve the membrane.

The initial association between the capsid and the PE2 polypeptide postulated here and the subsequent disruption of this interaction suggest a model for the envelopment of Sindbis virus and provide plausible answers to the questions posed above. We suggest that a complex of PE2 and E1 is formed in cell membranes

soon after the synthesis of these polypeptides. Within this complex PE2 may be organized as a transmembranal protein, whereas E1 is more peripherally located. (E1 is readily iodinated and more efficiently removed from the membrane by detergent than PE2.) The nucleocapsids attach to PE2 at a site exposed on the inner surface of the plasma membrane after the PE2-E1 complex is prepared for that attachment by some maturation process that is defective in ts-23. The complex of these three proteins would be the starting point for the budding reaction. Subsequently the cleavage of PE2 occurs, breaking the initial association of C with the membrane proteins and allowing the E2 sequences to assume a more peripheral position in the developing envelope, thereby exposing its substrate amino acids to iodination by lactoperoxidase. It is in this step that ts-20 is presumed to be defective. In infection by wild-type virus, the sequential association (mole for mole) of the C and PE2 proteins and the subsequent cleavage of the latter may provide the energy to wrap the nucleocapsid in the developing envelope until the membrane fusion process described previously (5) releases the mature virion from the cell surface. Through such a sequence it would be possible for the capsid to identify and associate with the proteins that are to become part of its envelope and to incorporate them in 1:1 molar ratios with the C protein. The proposed topological rearrangement of the E2 protein during the budding process would produce a mature virion in which the envelope proteins do not cross the hydrophobic interior of the envelope membrane bilayer.

The origin of the proteolytic activity responsible for the cleavage of PE2 is not yet known. However, a proteolytic activity associated with the capsid protein has been suggested previously (9, 23, 29), and its proximity to the PE2 polypeptide in this model make it a possible source for the cleaving activity.

The sequence of maturational events described above could provide for two other viral functions. First, the initial attachment of the virion to the host cell (apparently mediated by the E2 polypeptide; 10) is followed by fusion of the viral envelope with the cell plasma membrane (B. M. Riedel and D. T. Brown, unpublished observation). This fusion activity is non-essential and might be detrimental in the infected-cell plasma membrane during virus maturation. It is possible that the PE2 polypeptide is organized such that it cannot carry out this function (cell fusion is not observed in monolayers infected with ts-20; unpublished observation). Hemadsorption, however (apparently mediated by the E1 polypeptide; 10), functions

in both ts-20-infected cells and in mature virions, supporting the idea that E1 does not undergo topological change during maturation. Second, the transient interaction of the capsid with the membrane proteins during morphogenesis may result in a structural alteration in the capsid as it is incorporated into a mature virion. Such a morphological rearrangement of the capsid could account for its stability in the cytoplasm of the cell in which it is assembled and instability (uncoating) in the cytoplasm of a subsequently infected host cell.

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