Cleavage of a Viral Envelope Precursor During the Morphogenesis of Sindbis Virus

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In cells infected with a temperature-sensitive mutant of Sindbis virus, the cleavage of the precursor to one of the viral envelope proteins does not occur at the nonpermissive temperature. This precursor is found associated with the plasma membrane fraction obtained from the infected cell. Since this is the site at which the virus matures, this finding suggests that during Sindbis virus replication the precursor to the smaller proteins associates with the cell membrane and is then cleaved during the maturation of the virus.

The alpha togaviruses, of which Sindbis is the prototype, are chemically the simplest of all enveloped viruses. The virion contains a nucleocapsid composed of RNA and a single polypeptide species (25). Surrounding the nucleocapsid is a protein-free lipid bilayer (11, 13). External to this lipid bilayer is a protein shell composed of two envelope glycoproteins, E1 and E2 (19). Because of its relative chemical simplicity, Sindbis has been employed extensively as a model system to study viral envelope acquisition.

Recent evidence indicates that the viral structural proteins are formed by the cleavage of higher molecular weight precursor polypeptides (8, 17, 18, 20, 24). The capsid protein appears early and is presumed to be the first cleavage product (20). In the infected cell both capsid protein and E1 are present in significant quantities, but it is unclear whether E2 is found in the cell (19, 22). However, in whole cell extracts PE2, a precursor to E2, can be detected. This precursor is cleaved prior to or during the budding process (19).

Alpha togaviruses acquire their envelopes by budding through the plasma membrane (1, 3) and the membranes delimiting cytoplasmic vacuoles (1, 3, 9). This suggests that terminal events of maturation occur on or in association with these membranes. We have examined, plasma membrane fractions obtained from cells infected with a temperature-sensitive (ts) mutant of Sindbis virus, ts-20, in an attempt to characterize the nature and location of the terminal steps in Sindbis virus maturation.

MATERIALS AND METHODS

Virus and cells. The large-plaque variant of Sindbis virus was isolated from a stock culture of strain Ar 399 by single-plaque passages and is designated wild-type (WT). Ts-20 was generously supplied by E. R. Pfefferkorn. Ts mutants were plaque purified and grown at 29 C in monolayer cultures of chicken embryo (CE) cells in Eagle medium containing twice the original amino acid concentration.

Radioactive labeling and fractionation of infected cells. Monolayer cultures of CE cells were treated for 2 h prior to infection with 5 μg of actinomycin D per ml (Mann Research Laboratories) in leucine-free Eagle medium. After virus adsorption (1 h at room temperature) cultures were incubated for 4 h in the above medium at which time 25 μCi of [³H]leucine per ml (Amersham, specific activity 58 Ci/mmol) were added. The cultures were then incubated for an additional 3 to 4 h. The cells were harvested with a rubber policeman into ice-cold buffer (10^{-2} M Tris, 10^{-3} M EDTA, pH 7.0) and allowed to swell for 30 min. Homogenization was accomplished with 20 strokes of a glass Dounce homogenizer (Kontes). Nuclei and undisrupted cells were removed by sedimentation at $800 \times g$ for 10 min. These cytoplasmic extracts were either digested for polyacrylamide gel electrophoresis (PAGE), or, alternatively, membrane fractions were obtained by discontinuous gradient centrifugation as described previously (3, 4). To obtain soluble and pellet fractions, extracts were put into small tubes (0.8 ml) and centrifuged at $130,000 \times g$ for 1.5 h in a 50.1 rotor by using special adaptors (Beckman Instruments). Pellets and supernatant fluids were then digested for PAGE.

When pulse-chase experiments were done, cells were labeled in the following manner. Secondary cultures were infected with ts-20 or WT at a multiplicity of 10 and incubated at 29 C in leucine-free Eagle medium (Gibco) containing actinomycin D (5 μg/ml) for 5 h. At this time Hanks' Balanced Salt Solution (HBSS) at 42 C was added, and the cultures were incubated in a water bath held at 42 C. After 5 min, the HBSS was replaced with 5 ml of prewarmed leucine-free Eagle medium containing 300 μCi of [³H]leucine per ml. The cultures were incubated for 8

to 9 min at 42 C and the labeled medium was removed. The cultures were washed once with warm HBSS, and the HBSS (42 C) containing unlabeled leucine (5 mg/ml) was added. Incubation was continued for an additional 5 min. The HBSS was removed, and 10 ml of MEM (29 C) containing excess leucine was added to each culture, and incubation continued for 2 h at 29 C. The culture fluids were then harvested and the viruses were purified as described below. A culture was harvested and processed for PAGE as described above at the end of both the pulse and chase periods.

Polyacrylamide gel electrophoresis. A digestion solution of 10% mercaptoethanol, 2% sodium dodecyl sulfate (SDS), 0.01% bromophenyl blue, and 10% glycerol in 0.75 M Tris, pH 6.8, (14) was diluted 1:1 with samples to be electrophoresed. Immediately prior to electrophoresis samples were boiled for at least 1 min.

SDS phosphate gels (10%) were prepared by the technique of Ornstein (16), as modified by Hay et al. (12). Electrophoresis was performed in glass tubes (0.7 by 20 cm) on vertical 14-cm separating columns at 8 mA/gel for 7.5 to 8 h. After electrophoresis, the gels were frozen at −70 C and sliced into 1-mm disks with a transverse gel slicer (Diversified Scientific Instruments, Inc.). Gel slices were placed in scintillation vials containing 0.5 ml of deionized water and were allowed to swell overnight. Bray's scintillation fluid (10 ml) was added to each vial, and samples were counted in a Nuclear Chicago Mark 1 liquid scintillation counter.

Discontinuous gels (14 cm) were formed in 20-cm glass tubes essentially as described by Laemmli (14). Stacking gels were at least three times the volume of the sample. Discontinuous gels were run at 5 V/cm for 12 to 16 h. The gels were fractionated and radioactivity was monitored as above.

^{14}C-labeled Sindbis virions were digested and co-electrophoresed with all ^{3}H-labeled samples. The location of these polypeptides in the gels are indicated by bold arrows. The location of PE2 is marked by a light arrow.

Virus purification. Virus was purified by the method of Martin (15). Fifty percent potassium tartrate and 30% glycerol solutions in 10^{-3} M EDTA, 10^{-2} M Tris, 10^{-1} M NaCl (pH 7.0) were adjusted with buffer to refractive indices of 1.3974 and 1.3694, respectively. A linear 25-ml gradient was formed in an SW27 tube (Beckman Instruments) and clarified (10,000 × g for 15 min) medium containing isotopically labeled virus was layered on the gradient. Centrifugation was for 15 h at 82,500 × g. Fractions were monitored for radioactivity and infectivity. Those fractions containing peaks of radioactivity and infectivity were pooled and dialyzed against two changes of phosphate-buffered saline (PBS). The dialyzed samples were then lyophilized, digested, and subjected to PAGE.

RESULTS

Nature of the defect in ts-20. Ts-20 is an RNA$^+$ mutant of Sindbis virus, which was originally isolated and described by Burge and Pfefferkorn (5, 6). In cells infected with ts-20 and incubated at nonpermissive temperature, nucleocapsids are assembled and the cultures hemadsorb (7). Studies conducted prior to the identification of the second envelope protein in Sindbis virions indicated that proteins synthesized in ts-20 infected cells at nonpermissive temperature were similar to those synthesized in wild-type infected cells (18). Since RNA synthesis and nucleocapsid assembly proceeded at nonpermissive temperature (18), it seemed probable to us that the defect of ts-20 was in one of the two envelope proteins recently resolved by Schlesinger et al. (19). To test this possibility, the proteins synthesized in cells infected with WT and with ts-20 at permissive and nonpermissive temperatures were compared by using discontinuous Tris SDS gels which resolve these two viral envelope proteins (19). CE cultures were infected at a multiplicity of 50 and were incubated at the appropriate temperature for 5 h in leucine-free medium containing actinomycin D (5 µg/ml). Medium containing [^{3}H]leucine (25 µCi/ml) was then added and the cultures were incubated for an additional 3 h. At this time the cultures were washed extensively with cold Eagle medium (4 C). The cells were homogenized, and cytoplasmic extracts prepared as described in Materials and Methods were analyzed by PAGE. The polypeptides synthesized at each temperature in WT and ts-20 infected cells are shown in Fig. 1. The protein profile obtained from cells infected with ts-20 at the nonpermissive temperature differed from that obtained at the permissive temperature and from that of cells infected with WT in two major respects. Relative to E1, an increased amount of the PE2 was present, and a polypeptide with the electrophoretic mobility of E2 was not detected. This suggested that the lesion in ts-20 results in the failure of PE2 to be cleaved to E2 at the nonpermissive temperature.

To determine if pulse-chase experiments were feasible, temperature-shift experiments were performed. Replicate CE cultures were infected with ts-20 and incubated at 41 C. At 2, 4, and 6 h after infection, the cultures were shifted to the permissive temperature, and the growth of the virus was monitored. As indicated in Fig. 2, the length of time at nonpermissive temperature did not affect the slopes of the virus growth curves. This experiment suggests that: (i) the lesion in ts-20 does not appear to affect any early viral functions, since the growth curve at the permissive temperature after 2 h at 41 C was identical to the 29 C control, and (ii) the capacity of the virus to replicate was not inter-
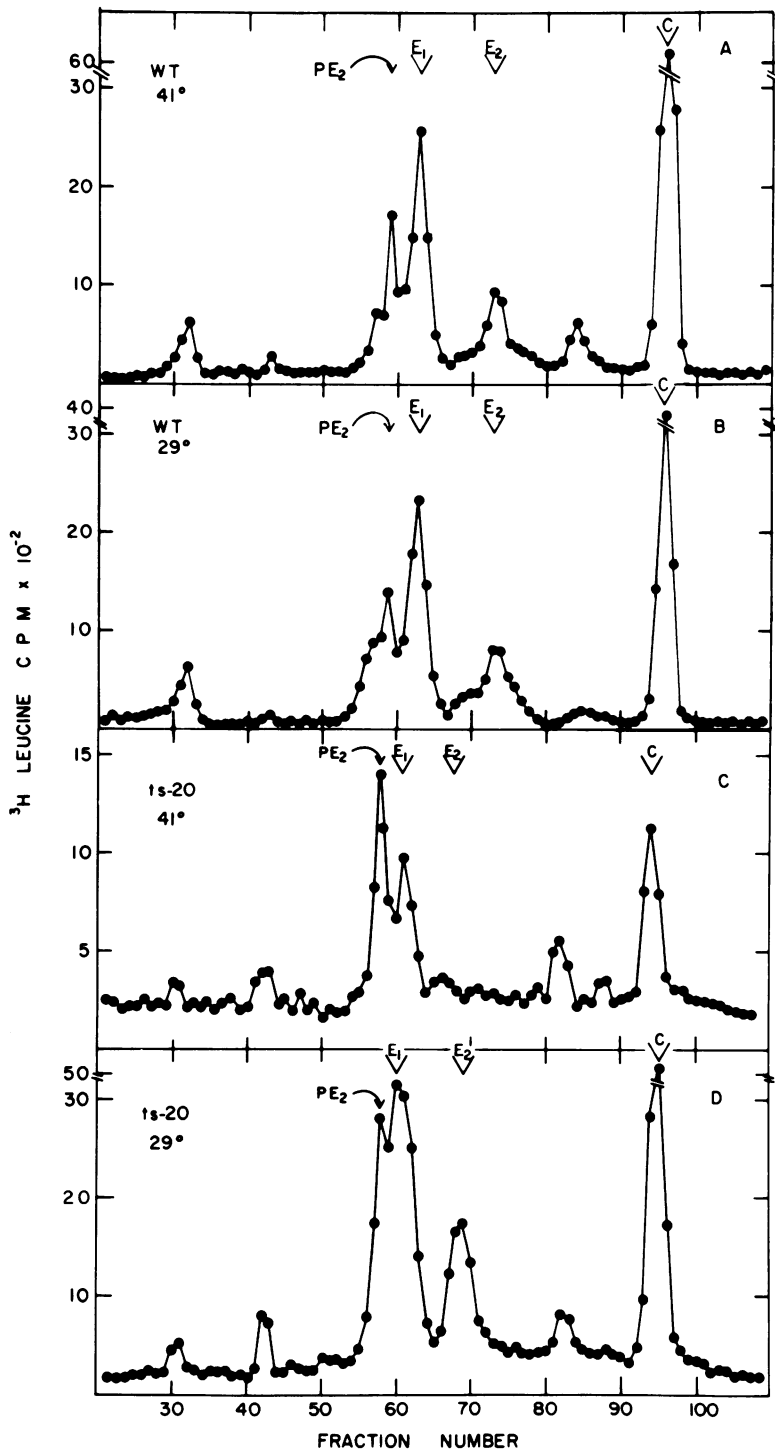
FIG. 1. *Electrophoresis on discontinuous-SDS gels of polypeptides from Sindbis virus-infected cells. Actinomycin D pretreated CE cultures were infected with either wild type (WT) or ts-20 and incubated at the appropriate temperature for 5 h in the continued presence of actinomycin D. [³H]leucine (25 µCi/ml) was then added to the medium and incubation was continued for an additional 3 h. The cells were then harvested, and cytoplasmic extracts were prepared and subjected to electrophoresis. Migration was from left to right. A, polypeptides from cells infected with WT at 41 C; B, polypeptides from cells infected with WT at 29 C; C, polypeptides from cells infected with ts-20 at 41 C; D, polypeptides from cells infected with ts-20 at 29 C.*
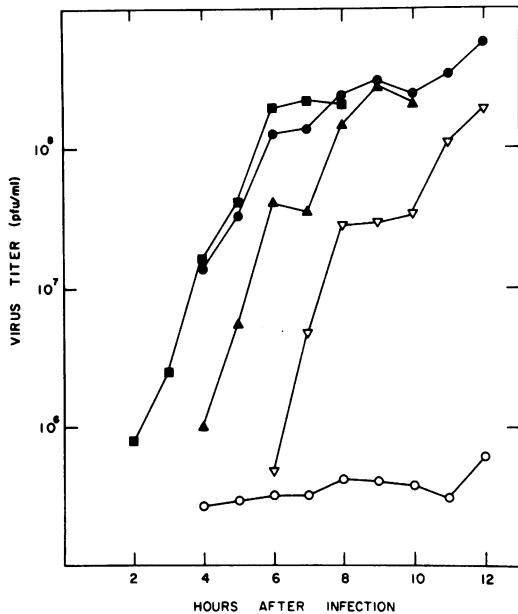
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FIG. 2. *Effect of time at nonpermissive temperature on the ability of ts-20 to replicate. Replicate cultures were infected with ts-20 and incubated at 41 C. A control culture was infected and incubated at 29 C. At various times after infection, cultures were shifted to 29 C and the growth of the virus was monitored. Symbols: 29 C throughout infection, ●; 41 C throughout infection, ○; shifted at 2 h, ■; 4 h, ▲; 6 h, ▽.*

fered with by long periods of incubation at 41 C. These results indicated that pulse-chase experiments could be conducted to determine the fate of PE2 made at 41 C.

Fate of labeled precursor made at 41 C. To determine whether PE2 made at 41 C was subsequently cleaved and incorporated into virions when shifted to permissive temperature, pulse-chase experiments were performed (18). Replicate cultures were infected with ts-20 or WT Sindbis, and incubated for 4 h at 29 C. At this time, the cultures were rapidly warmed to 42 C (see Materials and Methods) and held at this temperature for 5 min. A pulse-chase labeling procedure combined with a temperature shift was then performed, and labeled virus was purified as described in Materials and Methods. Figure 3 (A and B) represents discontinuous PAGE profiles of the polypeptides of WT and ts-20 virions released after pulse labeling at 41 C and chase at 29 C. The specific activity of the WT and ts-20 harvested after the chase were very similar (4 × 10⁻⁵ to 8 × 10⁻⁵ counts per min per PFU), indicating the effectiveness of the pulse and subsequent chase. The profiles

of ts-20 and WT do not differ significantly, indicating that PE2 made at 41 C is cleaved and incorporated into virions after shift to the permissive temperature. Figure 4 (A and B) represents discontinuous gel profiles of the ts-20 infected cultures harvested after the pulse (A) and subsequent chase (B). The amounts of label in PE2 and E1 decrease at approximately the same rate during the chase period. Although E2 was not detected after the pulse, a small amount could be detected after a chase period of 2 h. Thus, label which appeared in PE2 made at 41 C was subsequently chased to E2 in whole virions.

Location of virion proteins within the cell. In alpha togavirus-infected cells, virion structural proteins are not found in the soluble fraction of cell extracts (2, 10). Since PE2 is a precursor to one of the envelope proteins, it was reasonable to assume that it might be membrane associated. The possibility existed, however, that the failure of cleavage of PE2 in ts-20 infected cells at the nonpermissive temperature was a result of the failure of PE2 to become membrane associated. To test this possibility, cells were infected at a multiplicity of 50 with ts-20, incubated at 41 C, and labeled from 4 to 8 h after infection with [³H]leucine (25 μCi/ml). At the end of the labeling period, the cells were homogenized and pellet and soluble fractions obtained as described in Materials and Methods. The supernatant fluids and the pellets were then digested for PAGE and analyzed on continuous phosphate gels which do not resolve the two envelope proteins. The polypeptide profiles of the soluble and pellet fractions of the ts-20 infected cell extracts are shown in Fig. 5 A and B. It is evident that at the nonpermissive temperature both PE2 and the virion structural proteins are located within the pellet fraction and not in the soluble portion of the cell extract. The soluble fraction contained less than 10% of the counts found in the pellet.

Detection of precursor associated with a membrane fraction. Since virion proteins were not found in the soluble fraction of the ts-20 infected cells and both PE2 and E1 are located in the 130,000 × g pellet, it seemed probable that they were membrane associated. Sindbis virus is known to mature at the plasma membrane of the infected cells. We, therefore, were interested in determining whether PE2 could be detected in association with the plasma membrane fraction obtained from ts-20 infected cells. Cultures were infected with ts-20 and labeled with 25 μCi of [³H]leucine per ml from 4 to 8 h after infection at permissive and nonpermissive temperature. The cells were homoge-

nized as before and centrifuged at 4,000 × g for 15 min. The supernatant fluids were made 45% with respect to sucrose and layered in a discontinuous sucrose gradient and centrifuged as previously described (3). The gradient was fractionated, and those fractions enriched in 5′ nucleotidase activity (plasma membrane enzyme marker) were pelleted, digested, and analyzed by SDS-phosphate (15) and discontinuous acrylamide gel electrophoresis (13). As indicated in Fig. 6, the precursor PE2 was associated with plasma membrane fractions of cells infected with ts-20 at both nonpermissive and permissive temperatures. At the nonpermissive temperature, the relative amounts of PE2 and E2 corresponded to the level found in whole cell extracts.

Previous studies in this laboratory have indicated that Sindbis virion structural proteins are associated with the plasma membranes of infected cells (3). The above study extends these observations and demonstrates that a precursor to one of the envelope proteins is also associated with the plasma membrane of infected cells.

DISCUSSION

We have demonstrated that in ts-20 infected cells at the nonpermissive temperature the precursor protein PE2 is not cleaved to the envelope glycoprotein E2. The substantial increase in the amount of precursor at nonpermissive temperature has facilitated the localization of PE2 in the infected cell. The observations that PE2 is a direct precursor to E2 (20) and is associated with the plasma membrane suggest that its cleavage to E2 is one of the final events in the maturation of Sindbis virus. Since E1 and E2 are present in mature virions in the same ratio, the cleavage of PE2 to E2 may coincide with the budding of the virion. Consistent with this hypothesis, Schlesinger and co-workers were unable to find significant quantities of E2 in cells infected with wild-type Sindbis virus (20). Sefton et al. (22), on the
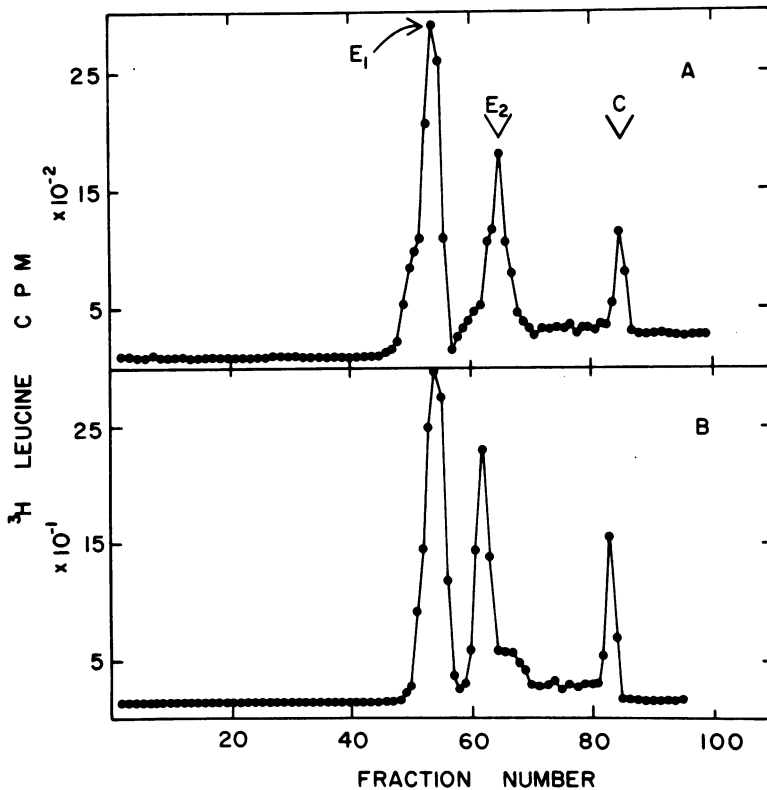

FIG. 3. *Discontinuous Tris-SDS polyacrylamide gels of polypeptides labeled at 41 C and subsequently chased into virions. Cultures were infected with WT or ts-20 and incubated for 4 h at 29 C. The cultures were then shifted to 41 C and pulse labeled. The virus produced in the subsequent chase was purified and subjected to electrophoresis. Migration was from left to right. A, WT; B, ts-20.*
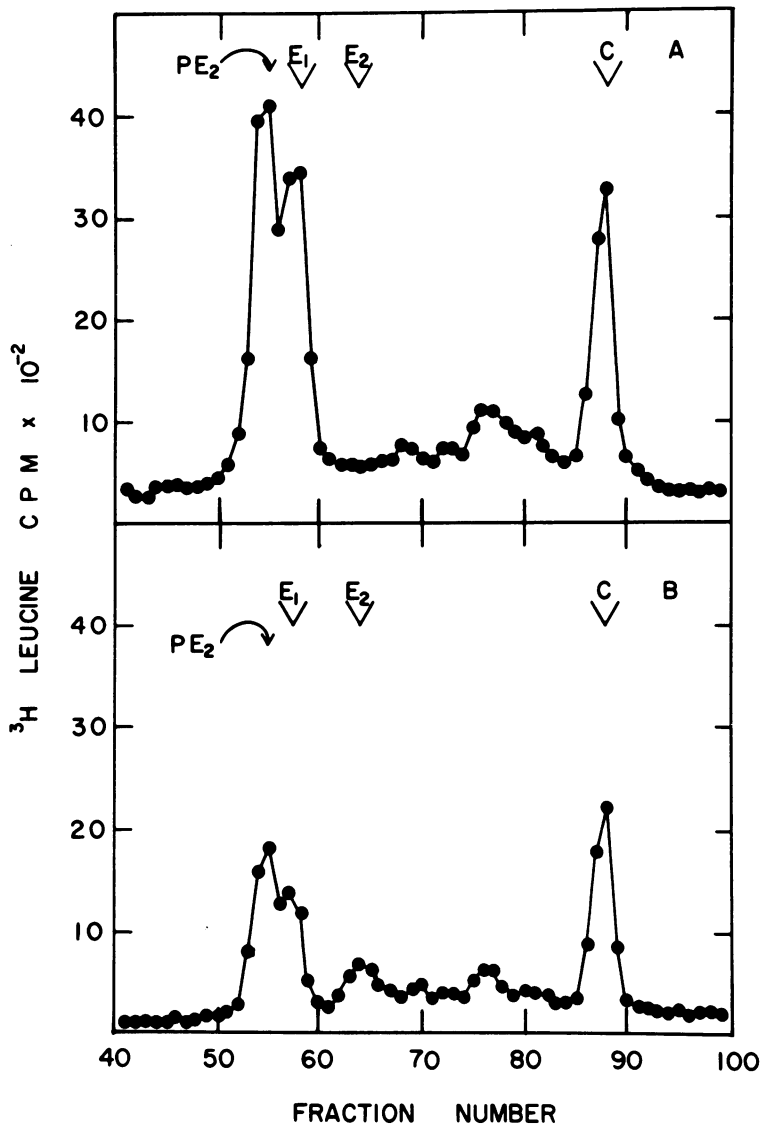
FIG. 4. *Discontinuous Tris-SDS polyacrylamide gel profiles of proteins synthesized in ts-20 infected cells during pulse at 41 C and subsequent chase at 29 C. Cultures were infected with ts-20 and labeled. One culture was harvested immediately after the pulse, and another immediately after the chase. Cytoplasmic extracts were prepared and subjected to electrophoresis. A, polypeptides synthesized during pulse at 41 C; B, polypeptides after a 2-h chase at 29 C.*

other hand, have reported that whole cell homogenates of Sindbis virus-infected cells contained significant amounts of E2. The results obtained in our laboratory with long-term labeling generally have been in agreement with those of Sefton et al. (22).

We are not certain whether the E2 present in Sindbis-infected cells represents that which has not yet been incorporated into virions, virions that remain bound to the cell surface, or complete virions which have budded into cytoplasmic vacuoles and are physically inside the cell but topologically outside of it.

Although we have demonstrated that PE2 is associated with the plasma membrane of the infected cell, it is not exposed on the outer surface of the cell (22). Cells infected with ts-20 hemadsorb normally at the nonpermissive temperature, whereas those infected with ts-10 and ts-23, which are in a separate complementation
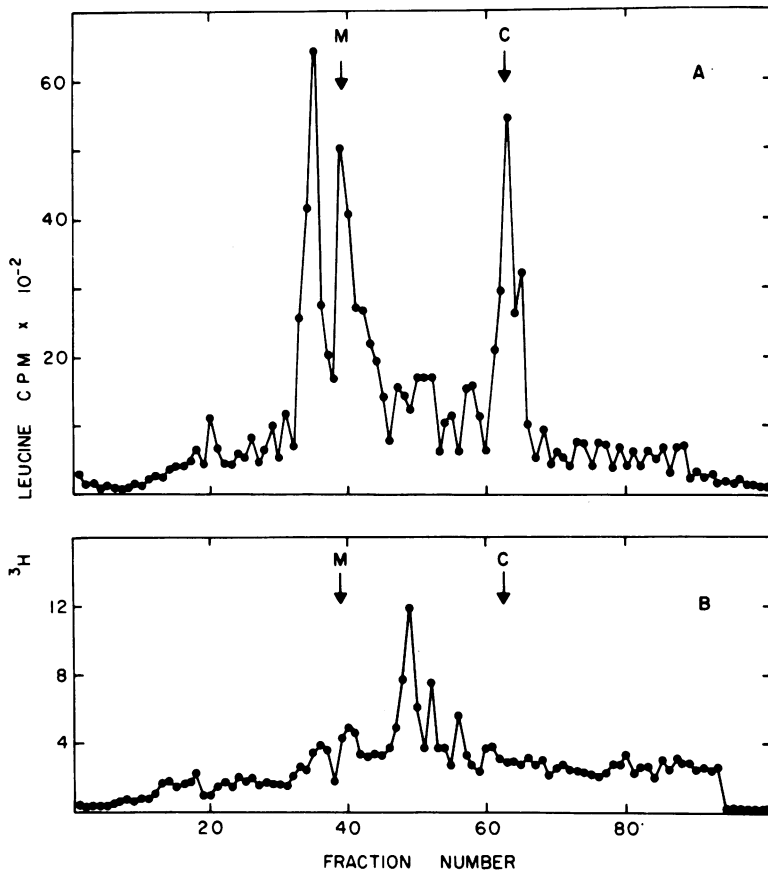
FIG. 5. *Continuous phosphate-SDS polyacrylamide gel profiles of pellet and soluble fractions from cells infected with ts-20 at nonpermissive temperature. Cultures were infected with ts-20 and incubated at 41 C. The cultures were labeled as in Fig. 1. Extracts were prepared and subjected to high-speed centrifugation. A, pellet fraction; B, soluble fraction. Migration is from left to right, and locations of virion structural proteins are indicated by the arrows.*

group and have defective envelope proteins, do not (7). Since E1 is the only envelope glycoprotein present in significant quantity on the surface of the cell at nonpermissive temperature, it is likely that this protein is responsible for hemadsorption and is the one which is defective in ts-10 and ts-23 infected cells.

Ts-20 may be analogous to a cleavage-failure, temperature-sensitive mutant of Semliki forest virus recently described by Simons et al. (22). However, because a different electrophoresis system was used, it is difficult to correlate the precursor and product in their gel profiles with PE2 and E2.

We have previously demonstrated (3) that, during the maturation of Sindbis virus, the virion envelope glycoproteins are selectively associated with the plasma membranes of in-

fected cells. However, our present results indicate that the Sindbis virus glycoprotein is not initially associated with the cell membrane in the same form that it assumes in the mature virion. Instead, it can be detected on the membrane in the form of the precursor PE2, which undergoes a cleavage event to yield E2. It is possible that E1 is also associated with the membrane in the form of a precursor molecule. Schlesinger (21) has proposed that the high molecular weight polypeptide B1 is the direct precursor to PE2 and E1. We have preliminary evidence which suggests that the plasma membrane of the infected cell may contain virus-specific polypeptides of molecular weight greater than PE2. We are currently attempting to determine if a precursor which contains both of the envelope polypeptides is associated with
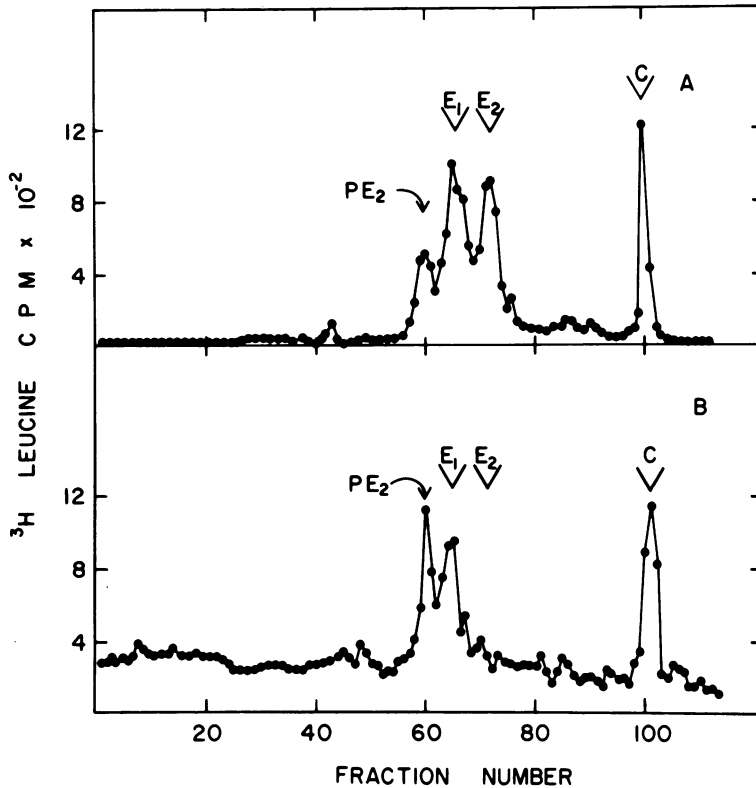
FIG. 6. *Discontinuous Tris-SDS polyacrylamide gel profiles of membrane fractions from ts-20 infected cells. Cells were infected and labeled as in Fig. 1. After harvesting, extracts were centrifuged on discontinuous sucrose gradients and those fractions enriched for plasma membranes were digested and subjected to PAGE. A, 29 C; B, 41 C.*

the plasma membrane, and, if so, what effect a defect in E1 would have on the subsequent cleavage of such a polypeptide.

ACKNOWLEDGMENTS

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