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Intracellular Distribution of Sindbis Virus Membrane Proteins in BHK-21 Cells Infected with Wild-Type Virus and Maturation-Defective Mutants

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The association of Sindbis virus proteins with cellular membranes during virus maturation was examined by utilizing a technique for fractionating the membranes of BHK-21 cells into three subcellular classes, which were enriched for rough endoplasmic reticulum, smooth endoplasmic reticulum, and plasma membrane. Pulse-chase experiments with wild-type (strain SVHR) virus-infected cells showed that virus envelope proteins were incorporated initially into membranes of the rough endoplasmic reticulum and subsequently migrated to the smooth and plasma membrane fractions. Large amounts of capsid protein were associated with the plasma membrane fraction even at the earliest times postpulse, and relatively little was found associated with the other membranes, suggesting a rapid and preferential association of nucleocapsids with the plasma membrane. We also examined the intracellular processing of the proteins of two temperature-sensitive Sindbis virus mutants in pulse-chase experiments at the nonpermissive temperature. Labeled virus proteins of mutant *ts*-20 (complementation group E) first appeared in the rough endoplasmic reticulum and were then transported to the smooth and plasma membrane fractions, as in wild-type (strain SVHR) virus-infected cells. In cells infected with *ts*-23 (complementation group D), the pulse-labeled virus proteins appeared initially in the rough membrane fraction and were transported to the smooth membrane fraction, but only limited amounts reached the plasma membrane. Thus, in *ts*-23-infected cells, the transport of the virus-encoded proteins from the smooth membranes seemed to be defective. In both *ts*-20- and *ts*-23-infected cells the envelope precursor polypeptide PE_2 was not processed to E_2, and no label was incorporated into free virus at the nonpermissive temperature.

The alphatogavirus Sindbis virus is a structurally uncomplicated virion composed of single-stranded infectious RNA encased in an icosahedral capsid, which in turn is enclosed in a membranous envelope (30, 39). The capsid contains RNA and multiple copies of a single protein, which has a molecular weight of 30,000 (30, 39). The envelope of the virion is composed of two virus-specified proteins (E_1 and E_2) (34), which are glycosylated by host cell enzymes (20) and are present in the virus in equimolar ratios to the capsid protein (34). The lipids are of host cell origin (32).

It has been demonstrated that a 130,000-molecular-weight polyprotein serves as the precursor for all of the structural proteins of Sindbis virus (15, 33, 37). The progressive processing of this polyprotein results in the production of the three primary viral structural components, which are incorporated into the mature virion through two pathways of processing and assembly. As the 130,000-molecular-weight polyprotein is synthesized, the portion of that protein destined to be incorporated into the viral core (polypeptide C) is specifically cleaved from the amino-terminal end (15, 33, 37). It is suspected that the proteolytic activity for this cleavage is located within the primary structure of the capsid protein itself (15, 37). Multiple copies of the 30,000-dalton capsid protein are combined with a single copy of virus progeny RNA in the cytoplasm of a cell to produce a morphologically identifiable nucleocapsid structure (1, 13). The remainder of the polyprotein contains structural components destined to become the two envelope proteins (E_1 and E_2) of the mature virion, which become integrated into the cell membrane by the association of the E_2 region of the polypeptide with intracellular membranes (8, 35, 42). Experimental evidence has suggested that the integration of this virus membrane polypeptide is initiated by an amino-terminal sequence which is not removed from the integrated polypeptide (7), as is the case for most signal se-

quences (5, 6). As the integration of this 100,000-molecular-weight polyprotein into cellular membranes occurs, the protein is cleaved to produce proteins PE_2 (the precursor of virus structural polypeptide E_2) and E_1. Polypeptides PE_2 and E_1 migrate to the cell plasma membrane by an unknown pathway; it is assumed that host-specified glycosylation of the envelope proteins takes place during intracellular transportation. Maturation of the envelope proteins into the membrane of the mature virion occurs after the nucleocapsid interacts with the inner surface of the plasma membrane, presumably through an attachment of the nucleocapsid with the carboxy-terminal end of the PE_2 polypeptide, which is a transmembranyl structure (8, 18, 42).

The envelopment of the virus in the modified cell plasma membrane occurs as polypeptide PE_2 is cleaved to E_2, with the loss of a 10,000-molecular-weight fragment from the amino-terminal end of the protein (33, 35). We have proposed that this cleavage results in a topological rearrangement of polypeptide PE_2 in the membrane, during which this protein loses its transmembrane configuration and assumes a more peripheral position in the developing viral envelope (38). Polypeptide E_1 seems to possess the same configuration in the cell membrane that it has in the envelope of the mature virion (38). The interaction of PE_2 with capsid protein, which is coupled to the cleavage of PE_2 to E_2 and the resulting topological rearrangement, proceeds such that the viral nucleocapsid is moved to a position outside the confines of the host cell; i.e., it is enveloped and released. The transient association of the icosahedral capsid with virus glycoproteins in the cell plasma membrane generates an icosahedral protein lattice on the surface of the mature virion. The threefold arrays in this icosahedron are stable in the absence of the nucleocapsid (41).

Although very little is known regarding the intracellular pathway followed by Sindbis virus polypeptides from the time of synthesis until their appearance in mature viruses, a number of points seem clear. The surfaces of infected cells contain virus antigens detectable by a variety of procedures within 2 h after infection (4). Treatment of cells with tunicamycin (an inhibitor of glycosylation) prevents the detection of virus proteins on cell surfaces (19, 28); similar results occur after infection of cells with certain temperature-sensitive mutants of Sindbis virus at the nonpermissive temperature (3, 14, 38). A large body of experimental evidence has suggested that PE_2 is cleaved to E_2 at the plasma membrane during the final stages of envelopment and that PE_2, E_1, and E_2 may be present on the surfaces of infected cells (38). Indeed, E_1 and E_2 can be detected by direct radioiodination of infected cell surfaces by the lactoperoxidase procedure (36, 38). However, the presence of PE_2 on cell surfaces has never been demonstrated directly by any procedure.

We employed a technique of cell fractionation which separated cellular membranes into enriched rough endoplasmic reticulum, smooth endoplasmic reticulum, and plasma membrane fractions. This technique allowed the determination of processing, transportation, and intracellular distribution of virus-encoded structural proteins in cells infected with either wild-type virus or temperature-sensitive mutants defective in maturation. The results of these experiments provided information on the processing and intracellular migration of virus proteins before their incorporation into mature virions.

MATERIALS AND METHODS

Cells, viruses, and media. BHK-21 cells were grown as monolayers in Eagle minimal essential medium (16) supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.), 10% tryptose phosphate broth, and 2 mM glutamine, as described previously (31). The heat-resistant strain of Sindbis virus (strain SVHR), which served as wild type, and Sindbis temperature-sensitive mutants were provided by Elmer Pfefferkorn, Dartmouth Medical College, Hanover, N.H. Virus stocks were prepared and titrated in BHK cells as described previously (31).

Infection of cells, radioactive labeling, and processing of labeled cells. To insure maximal breakage of cells during subsequent homogenization (see below), BHK cells were kept in the logarithmic phase of growth by repeated passages before confluency for at least 48 h before infection. Subconfluent monolayers in 100-mm petri plates were pretreated at 37°C with Eagle minimal essential medium containing 4 μg of actinomycin D (Calbiochem) per ml for 1 h. The cells were infected with 50 to 100 PFU of wild-type (strain SVHR) virus or temperature-sensitive mutant per cell, as described previously (31, 38). The cells were incubated at the desired temperature for 4 h, washed with warm phosphate-buffered saline, and incubated for an additional 1 h in Eagle minimal essential medium minus methionine (supplemented with 1% fetal calf serum). In experiments involving tunicamycin (Miles Laboratories, Inc., Elkhart, Ind.), the drug was added to the cells at 2 h postinfection at a concentration of 1 μg/ml. At the end of the incubation period, [^{35}S]methionine (40 μCi/ml; 1,100 Ci/mmol; Amersham/Searle) was added to the infected cells, and incubation was continued at the appropriate temperature. After a 7-min pulse with [^{35}S]methionine, the monolayers were washed with phosphate-buffered saline and transferred into warm medium containing 50 μg of cycloheximide (Sigma Chemical Co., St. Louis, Mo.) per ml and 2 mM cold methionine and incubated at the desired temperature for different chase periods. Cells which were only pulse-labeled were washed, placed on ice, and fractionated immediately after the pulse. The chase medium was collected

and examined for the presence of labeled virus by density gradient centrifugation (see below). At the end of the chase, the monolayers were placed on ice and washed four times with cold calcium- and magnesium-free phosphate-buffered saline and once with E-T buffer (0.001 M Tris, 0.01 M EDTA, pH 8.0), and 5.0 ml (final volume) of E-T buffer was added to each plate. At this point the cells became detached from the plate, and they were held in suspension for 10 min, during which the cells were allowed to swell. Then the cells were ruptured by 25 strokes of a Dounce homogenizer. Cell breakage was monitored carefully with a phase-contrast light microscope. Nuclei were removed from the cell homogenate by centrifugation at 1,500 rpm for 4 min.

Fractionation of cell homogenates. Cellular membrane fractions were prepared by using a modification of the techniques of Hunt and Summers (21, 22) and Morrison and McQuain (29). The postnuclear homogenate (see above) was sedimented through 20% (wt/wt) sucrose onto a 60% (wt/wt) sucrose cushion in a Sorvall OTD-627 rotor at 22,000 rpm for 16 h at 5°C. The membranous material banding at the interface between the 60 and 20% sucrose layers was collected, made 50% (wt/wt) with respect to sucrose, placed in the bottom of a Sorvall polyallomer centrifuge tube, and overlaid sequentially with 7 ml of 44% (wt/wt) sucrose, 9 ml of 39% (wt/wt) sucrose, 9 ml of 34% (wt/wt) sucrose, 6 ml of 29% (wt/wt) sucrose, and 1 ml of E-T buffer (all sucrose solutions were made in E-T buffer). The samples were centrifuged to equilibrium for 18 h at 22,000 rpm and at 5°C. Fractions (1.4 ml) of each gradient were collected from the bottom of the centrifuge tube, and the relative concentration of membranes in each fraction was determined by measuring the optical density (Fig. 1). A portion of each fraction was counted with a Packard liquid scintillation counter, and the resulting profiles were plotted as percentages in each fraction of the total counts per minute on the gradient. Fractions corresponding to each of the peaks were pooled, diluted with E-T buffer, and pelleted by centrifugation for 2 h at 26,500 rpm in a Sorvall OTD-627 rotor. The pelleted membranes were dissolved in a sample buffer (see below) in preparation for electrophoresis on sodium dodecyl sulfate-polyacrylamide gels.

The location of the plasma membrane fraction in the discontinuous sucrose gradient and the extent of contamination of other fractions by plasma membranes were determined as follows. BHK cell monolayers were infected with wild-type Sindbis virus and incubated at 37°C for 5 h. The monolayers were washed and incubated at 4°C with anti-SVHR serum for 1 h. After two washes with cold phosphate-buffered saline, the cells were further incubated at 4°C with ^{125}I-labeled staphylococcal protein A (Sigma Chemical Co.) for 30 min. After extensive washing with cold phosphate-buffered saline, the cells were homogenized, and the membrane fractions were isolated as described above. The results of this fractionation are shown in Fig. 1. From this experiment it was determined that most of the plasma membrane-associated label (ca. 80%) corresponded to the low-density peak of radioactivity. Control experiments indicated little binding of labeled protein A to similarly treated uninfected cells and very low levels of binding to cells infected with mutant *ts*-23 at the nonpermissive temperature (Table 1) (3, 14, 38).

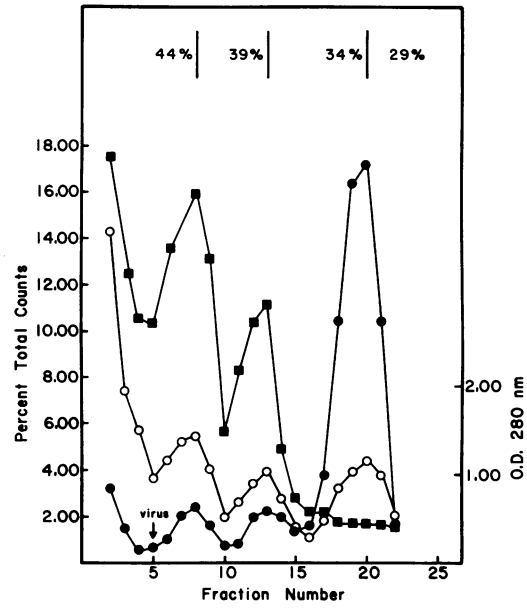

FIG. 1. *Fractionation of membranes of strain SVHR-infected BHK-21 cells labeled for either plasma membranes or rough endoplasmic reticulum membranes. The details of the fractionation are described in the text. Sucrose solution interfaces and concentrations are indicated at the top of the figure. Symbols: ■, [^{3}H]uridine-labeled cells; ●, ^{125}I-labeled cells; ○, optical density (O.D.) at 280 nm of gradient fractions.*

The location of the fraction containing membranes of the rough endoplasmic reticulum was determined as follows. BHK-21 cells were labeled with tritiated uridine for 17 h. These cells were washed and placed in media enriched with 1,000× nonradioactive uridine for an additional 2 h. The cells were infected with strain SVHR as described above, and they were maintained in media containing 1,000× cold uridine for 5 h. This procedure should have resulted in labeling of stable rRNA. The subsequent chase period, combined with Sindbis virus-induced host cell shutoff (39), should have eliminated membrane-associated host cell messengers. The cells were disrupted, and the membrane fractions were isolated as described above. The distribution of trichloroacetic acid-insoluble radioactivity in this gradient is shown in Fig. 1. The distribution of label in the three membrane fractions was 62% in heavy membranes, 32% in medium-density membranes, and 6% in low-density membranes. This result corresponded to the results obtained when SVHR-infected cells were labeled with [^{35}S]methionine for a short pulse period (see Fig. 2). We concluded that the dense membrane fraction contained the site of synthesis of the virus membrane proteins, the rough endoplasmic reticulum. By exclusion, we assumed that the medium-density fraction contained the mem-

TABLE 1. *Immunological detection of Sindbis virus proteins on the surfaces of BHK-21 cells infected with wild-type virus (strain SVHR) and temperature-sensitive mutants at 39.5°C*[a]

Expt	^{125}I cpm at the following times postinfection:[b]				
	1 h	2 h	3 h	4 h	5 h
SVHR	7,737	9,674	16,135	24,027	33,752
ts-20	4,083	6,740	8,324	17,805	31,733
ts-23	3,637	3,940	4,197	7,013	10,803
Uninfected cells	NT[c]	NT	NT	NT	1,989
SVHR + protein A[d]	NT	NT	NT	NT	1,770

[a] Cells were infected and labeled with anti-Sindbis virus serum and ^{125}I-labeled staphylococcal protein A as described in the text.

[b] Data were derived from monolayers containing equal numbers of cells treated with equal quantities of antibody and staphylococcal protein A. Antibody prepared as described in the text was preabsorbed against uninfected BHK-21 cells.

[c] NT, Not tested.

[d] SVHR-infected cells were treated with ^{125}I-labeled staphylococcal protein A only as a control for nonspecific binding.

branes of the smooth endoplasmic reticulum and the Golgi membranes. For the sake of clarity, we refer to these enriched fractions as the membrane classes which they primarily represent.

Mature virions did not account for the label in the three classes of membranes, as demonstrated by two control experiments. In one experiment, radioactive viruses were purified on a membrane isolation gradient by the procedure described above. Mature viruses were recovered quantitatively at a position (Fig. 1, arrow) well below the peak containing the membranes of the rough endoplasmic reticulum. In another control experiment, purified radioactive viruses were mixed with nonradioactive virus-infected cells, and the mixture was subjected to the protocol of washing, swelling, and homogenization described above. Less than 2% of the virus-added counts were recovered with the membrane fractions of infected cells (data not shown), indicating that any virus that matured in the course of these experiments was completely removed from the cell population before disruption.

The above-described protocol was chosen after experimental evaluation of a variety of published procedures (2, 10, 25). This protocol was found to be superior for the fractionation of the cellular membranes of Sindbis virus-infected cells for the following reasons: (i) clumping of membranes and aggregation of organelles were reduced; (ii) the need for scraping cells from monolayers, a process which results in the physical disruption of large numbers of cells, was eliminated; (iii) superior separation of cellular membrane classes resulted; and (iv) higher yields of cell membranes could be produced without the need to add unlabeled carrier cells.

Purification of progeny virus from chase medium. The chase medium containing the radioactive viruses produced from proteins labeled during the pulse was subjected to centrifugation at 2,000 rpm for 10 min and then dialyzed overnight against phosphate-buffered saline at 5°C. The dialyzed supernatant was layered onto a 20 to 35% (wt/wt) linear potassium tartrate gradient, which was centrifuged overnight in a Sorvall OTD-627 rotor at 21,000 rpm and 5°C. Fractions (1.4 ml) were collected from the bottom of each gradient, and radioactivity was determined by counting a portion of each fraction with a Packard Tri-Carb liquid scintillation counter.

Preparation of rabbit antiserum. An antiserum to strain SVHR virus was prepared in a New Zealand white rabbit by repeated biweekly subcutaneous injections of gradient-purified SVHR.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. ^{35}S-labeled polypeptides were resolved in 11% polyacrylamide slab gels (14 by 12 by 0.15 cm) (26). The samples were prepared for electrophoresis by boiling for 3 min in a sample buffer containing 1% sodium dodecyl sulfate, 1.5% dithiothreitol, and 0.24 M Tris (pH 6.8), and they were then subjected to electrophoresis for 3.5 h at a constant power of 4.0 W/gel slab. The slabs were fixed in 20% trichloroacetic acid and impregnated with diphenyloxazole by the method of Bonner and Laskey (9). The gels were dried and then subjected to autoradiography with Kodak X-OMAT RP X-ray film.

RESULTS

Intracellular transport of Sindbis virus glycoproteins. Pulse-chase experiments were used to examine the migration of newly synthesized Sindbis virus membrane proteins through the three classes of cellular membranes. Wild-type Sindbis virus-infected cells were labeled at 5 h postinfection as described above for a pulse period of 7 min at 37°C, as experiments demonstrated extensive modification of infected cell surfaces at this time (Table 1). The cells were transferred into chase medium containing cycloheximide and 10 times the normal amount of cold methionine and were fractionated either immediately after the pulse or after different chase periods. The pulse or chase medium was collected and examined for the presence of labeled virus particles by density gradient centrifugation, as described above (Fig. 2 and 3). An analysis of the protein contents of the different membrane fractions by polyacrylamide gel electrophoresis was carried out to obtain informa-

tion on the state of processing of the virus polypeptides in the different classes of membranes (Fig. 4).

These data show that in the pulse-labeling period most of the virus membrane proteins were associated with the rough endoplasmic reticulum membrane fraction. Some label was also associated with the smooth endoplasmic reticulum and plasma membrane fractions. This pulse profile was identical to that obtained when cells were prelabeled with [^{3}H]uridine, as described above (Fig. 1). This similarity suggests that incomplete separation of the intracellular membranes may have accounted for the appearance of some pulse-labeled virus membrane proteins in the medium-density fractions. With increasing chase times, the amount of labeled virus protein associated with the rough endoplasmic reticulum fraction progressively decreased. At the same time, the amount of label in the smooth endoplasmic reticulum and plasma membranes progressively increased, and all three fractions were labeled approximately equally after a 20-min chase (Fig. 2).

It was not possible in any of these experiments to demonstrate clearly a sequential transfer of label from rough endoplasmic reticulum to the lower-density membranes. Rather, label seemed to be transferred simultaneously into both smooth endoplasmic reticulum and plasma membrane fractions. This may have resulted in part from rapid and preferential association of capsid protein with the plasma membrane (see below). Extended chase periods of 120 to 240 min resulted in a reduction in the amount of label in the smooth endoplasmic reticulum fraction compared with the plasma membrane fraction (data not shown). This observation suggests either a transfer of label from the smooth membranes which was only detectable at these times or a selective degradation of the proteins in these membranes. The progressive incorporation of the chased protein label into the plasma membrane fraction of the cells was accompanied by a rapid transfer of the label into mature viruses (Fig. 3).

Although no label could be detected in mature viruses at the end of the 7-min pulse period (Fig. 3), label was found in the viruses at the end of a 10-min chase. Thus, label could be incorporated into mature viruses within 17 min of its addition to the cells. An analysis of the composition of

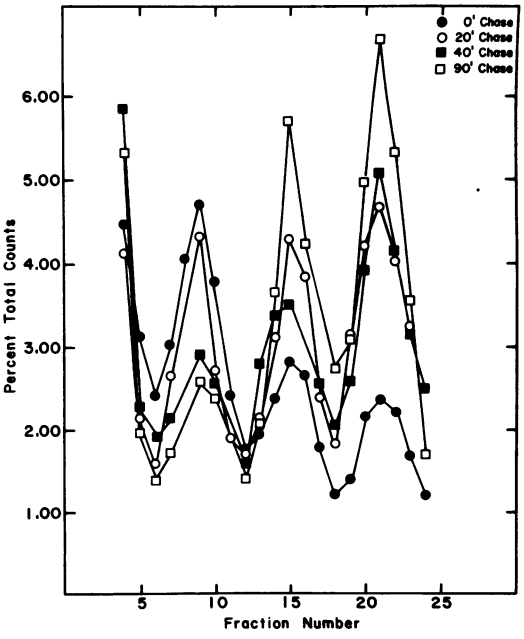

FIG. 2. *Amounts of protein label ([^{35}S]methionine) associated with cellular membrane fractions after pulse-chase labeling of SVHR-infected BHK-21 cells. Cells were labeled and fractionated as described in the text, and peak fractions corresponded to those shown in Fig. 1.*

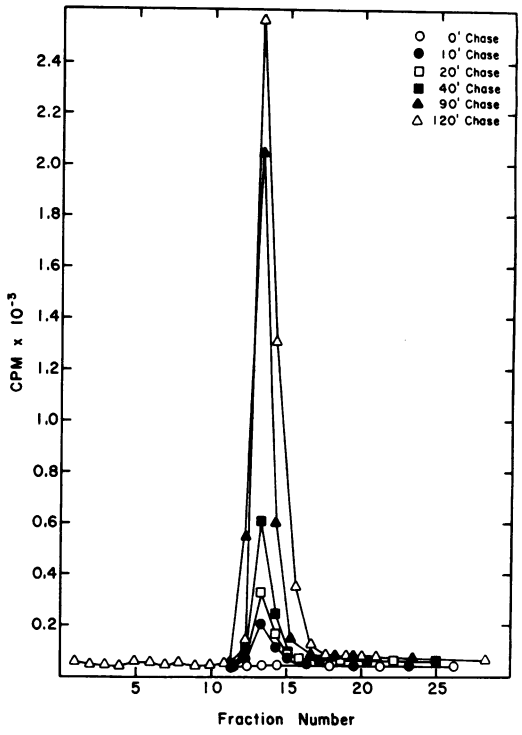

FIG. 3. *Movement of radioactive protein from SVHR-infected BHK-21 cells to mature viruses during the pulse-chase experiment shown in Fig. 2 and described in the text. The chase medium was collected and examined for the presence of labeled virus by density gradient centrifugation, as described in the text.*

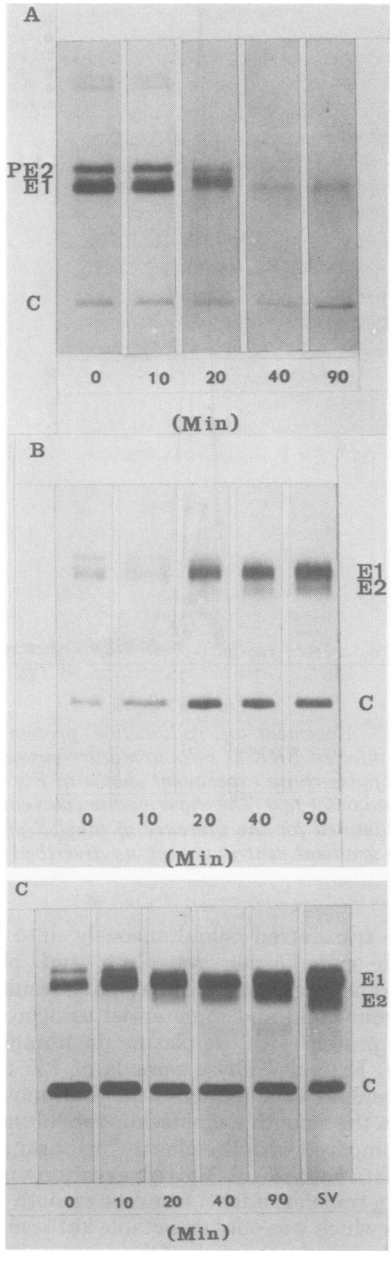

FIG. 4. *Polypeptides associated with cellular membrane fractions from SVHR-infected BHK-21 cells. Pooled fractions from the high-density (A), medium-density (B), and low-density (C) membrane fractions in the experiment described in the legend to Fig. 2 were subjected to electrophoresis in sodium dodecyl sulfate-polyacrylamide slab gels, as described in the text. In these and all other polyacrylamide gels, the positions of polypeptides E_1, E_2, and C were determined by coelectrophoresis of purified [^{35}S]methionine-labeled SVHR virus. The characteristics of this marker virus are shown in (C) (tract SV).*

the viruses produced in short chase periods showed that pulse-labeled capsid protein was incorporated into mature viruses before envelope proteins were incorporated, as described previously (data not shown) (33).

Analysis of virus proteins associated with fractionated membranes. Samples of the labeled membrane fractions shown in Fig. 2 were subjected to analysis by polyacrylamide gel electrophoresis to determine which virus-specified proteins were associated with the various membrane fractions and to determine what detectable changes in the proteins, if any, occurred with time. Figure 4 shows the results of such an analysis on the pulse-chase data shown in Fig. 2. Each track represents the label obtained from an equal number of cells at the end of the indicated chase period. Although the latitude of the X-ray film did not allow a direct quantitative comparison among proteins in a single gel track, each gel profile indicates the composition, as well as the relative quantity, of the virus proteins present in each fraction. All three membrane fractions contained polypeptides PE_2 and E_1 during the pulse and early chase times. The concentrations of labeled PE_2 and E_1 in the rough endoplasmic reticulum fraction decreased with time, whereas the low concentration of C remained constant throughout. No change in the composition of this membrane fraction was detected. Even when large amounts of the rough endoplasmic reticulum membrane fraction from chase periods of up to 90 min were analyzed (data not shown), only traces of polypeptide E_2 were detected in this membrane fraction. The amount of label detectable by polyacrylamide gel electrophoresis in the smooth endoplasmic reticulum fraction increased as the decrease in the amount of label in the rough endoplasmic reticulum fraction took place. The smooth endoplasmic reticulum membranes initially contained polypeptides PE_2, E_1, and C. The proteins in the smooth endoplasmic reticulum fraction underwent a qualitative change in composition with time. PE_2 was converted quantitatively to E_2 in a processing reaction which was first detected at 20 min postpulse and was nearly complete at 40 min postpulse. The amounts of label in the envelope and capsid protein bands of the smooth endoplasmic reticulum fraction appeared to increase somewhat abruptly just as cleavage of PE_2 to E_2 took place.

The cleavage of PE_2 to E_2 was observed most clearly in the samples derived from the plasma (light) membrane fractions. This conversion was first detected at 20 min postpulse, and (unlike the smooth endoplasmic reticulum fractions) little PE_2 was detected at this time. Conversion of PE_2 to E_2 was complete at 40 min. The amount

of virus glycoprotein in the plasma membranes increased with time, whereas the capsid protein levels associated with this fraction seemed to be very high throughout the experiment, even during the pulse period. This immediate binding of large amounts of capsid protein to the plasma membrane suggested a preferential and rapid association of capsid with plasma membrane which contained nonradioactive proteins synthesized before the pulse period. This rapid and direct association of capsid with plasma membrane accounted for the observation that after short pulse periods the label found in mature viruses was primarily capsid (33; data not shown). The relatively large amount of capsid protein binding to the plasma membrane fraction compared with the amount of capsid protein binding to other membrane fractions implied preferential association of this protein with modified plasma membranes rather than with modified internal membranes.

Intracellular distribution in Sindbis virus proteins in cells infected with temperature-sensitive mutants. BHK cells were fractionated into their component membranes after infection with temperature-sensitive mutants *ts*-20 (complementation group E) and *ts*-23 (complementation group D). Cells were infected and maintained at the nonpermissive temperature (39.5°C) for 5 h, after which they were pulse-labeled with [^{35}S]methionine, as described above. The cells were fractionated immediately after the pulse period and after a 90-min chase period either at 39.5°C or at the permissive temperature, 28°C (shiftdown). The distribution of label in the various membrane fractions at the end of the 7-min pulse was similar to the distribution observed at the end of the pulse in wild-type virus-infected cells (Fig. 2; data not shown).

The distributions of membrane-associated label in *ts*-20-infected cells after 90-min chases at the nonpermissive and permissive temperatures are shown in Fig. 5. After a 90-min chase at either 28 or 39.5°C, cells infected with *ts*-20 showed a redistribution of label from the rough membranes to the smooth and plasma membranes. Although proteins PE_2 and E_1 synthesized in a pulse at the nonpermissive temperature were transported to the plasma membrane fraction in a manner similar to that seen in wild-type virus infections (Fig. 2) and similar amounts of protein were detected on the surfaces of wild-type virus- and *ts*-20-infected cells (Table 1), no PE_2 was processed to E_2 (Fig. 6), and all three membrane fractions contained only PE_2 and E_1 (data not shown). This result was obtained when the chase was carried out at 39 or 28°C (data not shown). Furthermore, no label incorporated

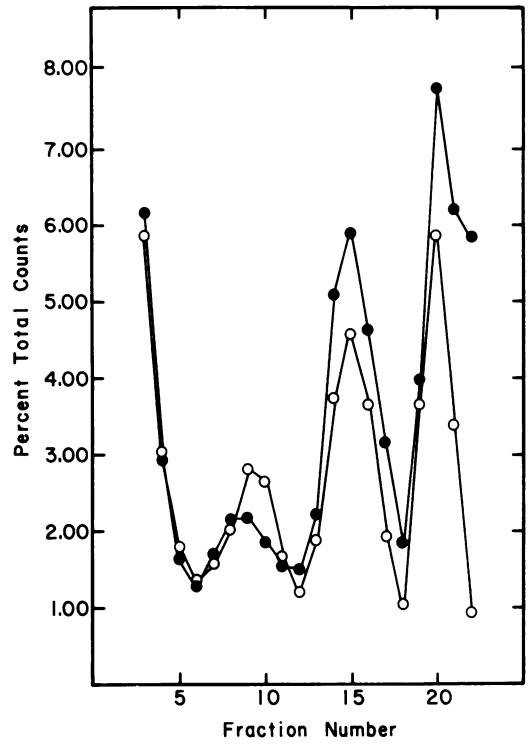

FIG. 5. *Association with cellular membranes of proteins synthesized at the nonpermissive temperature by Sindbis virus temperature-sensitive mutant ts-20. Symbols: ●, pulse-labeled at 39.5°C for 7 min and chased at 39.5°C for 90 min; ○, pulse-labeled at 39.5°C for 7 min and chased at 28°C for 90 min. Cells were infected, labeled, and fractionated as described in the text. Peak fractions correspond to those shown in Fig. 1.*

into proteins at 39.5°C could be recovered in viruses after *ts*-20-infected cells were shifted to 28°C for extended periods of time (Fig. 7) (11, 38).

Cells infected with Sindbis virus mutant *ts*-23 pulse-labeled for 7 min and fractionated immediately produced a profile of membrane-associated label similar to that found in pulsed *ts*-20- or SVHR-infected cells (data not shown). Cells infected with *ts*-23 produced a profile different from that of *ts*-20-infected cells after a chase period of 90 min at 39.5 or 28°C (Fig. 8). Unlike *ts*-20 proteins, *ts*-23 proteins produced under nonpermissive conditions were incorporated primarily into smooth membranes on chasing. In *ts*-20-infected cells, the distribution of counts among the three membrane peaks was 15, 40, and 45% for the rough endoplasmic reticulum, smooth endoplasmic reticulum, and plasma membrane fractions, respectively; for the comparable fractions derived from *ts*-23-infected cells, these values were 28, 48, and 24%, respec-

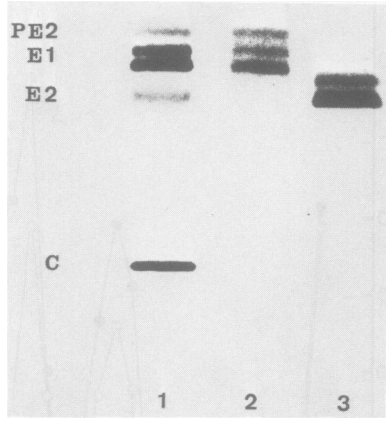

FIG. 6. *Membrane-associated virus proteins synthesized in BHK-21 cells infected with SVHR (lane 1), ts-20 (lane 2), or SVHR in the presence of tunicamycin (lane 3). The crude membrane fraction prepared as described in the text was analyzed by polyacrylamide gel electrophoresis. The positions of virus proteins PE_2, E_1, E_2, and C were determined by labeled marker virus, as described in the legend to Fig.*

tively (Fig. 5 and 8). No cleavage of PE_2 to E_2 took place at either chase temperature (Fig. 9), nor was any label incorporated at 39.5°C incorporated into mature viruses after an extended chase at 28°C (Fig. 7).

Analysis of virus proteins associated with the membranes of Sindbis virus temperature-sensitive mutant-infected cells. An analysis of the virus polypeptides associated with the fractionated membranes of cells infected with Sindbis virus mutants *ts*-20 and *ts*-23 was carried out by polyacrylamide gel electrophoresis, as described above. This analysis produced the predicted result, based on previous studies; namely, no polypeptide E_2 could be generated from PE_2 produced at nonpermissive temperatures, regardless of length of the chase period or whether the chase was at the permissive or nonpermissive temperature (11, 38).

Although this analysis produced the predicted result and is not presented in complete form, some new information was obtained (Fig. 6 and 9). When wild-type and *ts*-20 virus proteins were synthesized at the nonpermissive temperature (39.5°C), the membrane proteins demonstrated an unusual pattern of migration (Fig. 6). This unusual pattern appeared as a doublet of the E_1 polypeptide. This banding pattern was eliminated when the proteins were generated in the presence of the drug tunicamycin (an inhibitor of glycosylation [40] (Fig. 6) and thus, most likely, resulted from atypical glycosylation that occurred at high temperatures in our particular line of BHK-21 cells. Mutant *ts*-23 membrane proteins did not demonstrate the atypical gel profile when synthesized at 39.5°C (Fig. 9). Thus, mutant *ts*-23 differed from mutant *ts*-20 and from the parental strain SVHR in the susceptibility of the E_1 protein to atypical glycosylation at high temperatures.

Although most of the *ts*-23 membrane protein synthesized at the nonpermissive temperature was incorporated into the smooth membrane fraction with extended chasing at the permissive and nonpermissive temperatures, some label was recovered in the plasma membrane fraction (Fig. 8 and 9 and Table 1). An analysis of this fraction revealed a difference in the electrophoretic mobilities of the limited amounts of PE_2 and E_1 in the plasma membranes of *ts*-23-infected cells compared with the rough and smooth membrane fractions of the same cells or cells infected with either *ts*-20 or wild-type virus. Plasma membrane proteins of *ts*-23 had higher-than-usual molecular weights. Thus, although *ts*-23 pre-

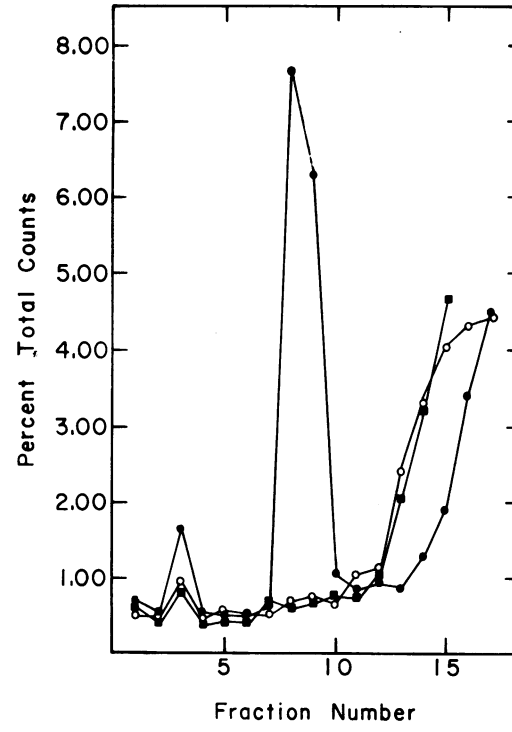

FIG. 7. *Incorporation of wild-type virus and temperature-sensitive mutant virus proteins synthesized at the nonpermissive temperature into virus particles. BHK-21 cells infected with SVHR (●), ts-20 (○), or ts-23 (■) were pulse-labeled for 7 min at 39.5°C and chased for 3 h at 28°C. The medium was collected, and virus particles were purified as described in the text. The data are presented as percentages of the total nondialyzable counts per minute in a linear potassium tartrate gradient.*

vented the migration of most of the virus membrane protein from the smooth membranes to the plasma membrane, some transfer did occur. The defective PE_2-E_1 complexes which did reach the cell surface were of higher-than-usual molecular weight. This may have resulted from an increased level of glycosylation of these proteins.

DISCUSSION

It has been shown previously that the membrane proteins of Sindbis virus and other enveloped viruses are synthesized at polyribosome complexes which are associated with the rough endoplasmic reticulum of infected cells (8, 17, 18, 21, 22, 27, 42). Integration of the proteins into rough endoplasmic reticulum membranes accompanies synthesis and is not prevented by agents which inhibit glycosylation of these proteins (17, 42). Since maturation of Sindbis virus polypeptides into mature virions occurs primarily at the plasma membranes of infected cells

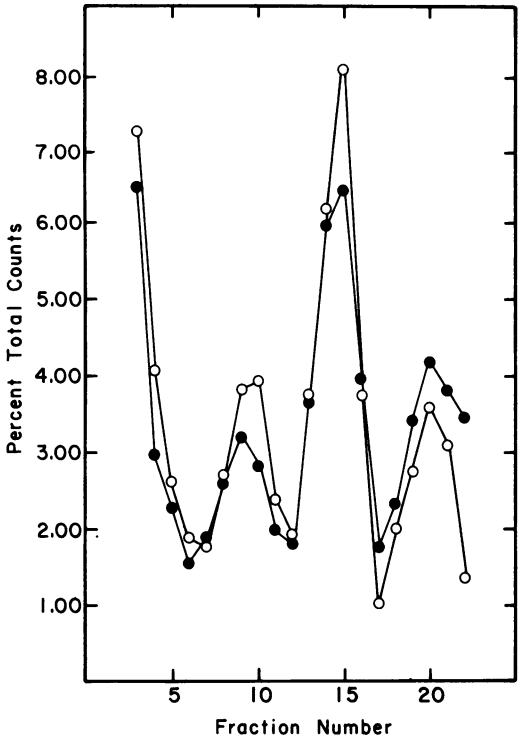

FIG. 8. *Association with cellular membranes of proteins synthesized at the nonpermissive temperature by Sindbis temperature-sensitive mutant ts-23. Symbols, ○, pulse-labeled at 39.5°C for 7 min and chased at 39.5°C for 90 min; ●, pulse-labeled at 39.5°C for 7 min and chased at 28°C for 90 min. Cells were infected, labeled, and fractionated as described in the text. Peak fractions correspond to those shown in Fig. 1.*

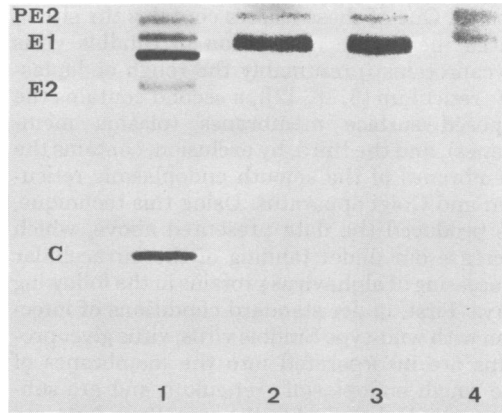

FIG. 9. *Polypeptides associated with cellular membrane fractions from BHK-21 cells infected with ts-23, labeled, and chased at the nonpermissive temperature. Lane 1, Proteins associated with the combined membranes (crude fraction) of BHK-21 cells infected with SVHR (See also Fig. 6, lane 1); lane 2, proteins of ts-23-infected cells associated with the high-density membrane fraction (Fig. 8); lane 3, proteins of ts-23-infected cells associated with the medium-density membrane fraction (Fig. 8); lane 4, proteins of ts-23-infected cells associated with the low-density membrane fraction (Fig. 8). Proteins were identified as described in the legends to Fig. 4 and 6.*

(11, 13, 23, 32, 36, 38), virus membrane proteins must be transported to cell surfaces from their site of integration through internally situated cellular membranes. It has been demonstrated previously that certain temperature-sensitive defects can prevent the appearance of virus membrane proteins on the surfaces of cells and, consequently, preclude the production of virions (3, 14, 38).

The proteolytic cleavage of one of the two polypeptides (cleavage of PE_2 to E_2) appears to be essential to the process of envelopment. Genetic (temperature-sensitive mutants) or other circumstances which block the cleavage of PE_2 to E_2 invariably prevent envelopment (11, 12, 24, 28, 38). Although the pathway of processing of Sindbis virus polypeptides in cells infected with wild-type virus and temperature-sensitive mutants is well understood, little is known regarding the process of intracellular migration of these polypeptides. Although a large body of circumstantial evidence (38) suggests that the precursor polypeptide PE_2 appears in the plasma membranes of infected cells before cleavage to E_2 and incorporation into mature virus, the presence of PE_2 in plasma membranes has never been demonstrated directly by any technique.

We have developed a technique for fractionating the membranes of Sindbis virus-infected BHK-21 cells grown in monolayers into three

classes. One of these classes contains the site of initial membrane association of Sindbis virus glycoproteins (presumably the rough endoplasmic reticulum [8, 18, 42]), a second contains the exposed surface membranes (plasma membranes), and the third, by exclusion, contains the membranes of the smooth endoplasmic reticulum and Golgi apparatus. Using this technique, we produced the data presented above, which increase our understanding of the intracellular processing of alphavirus proteins in the following ways. First, under standard conditions of infection with wild-type Sindbis virus, virus glycoproteins are incorporated into the membranes of the rough endoplasmic reticulum and are subsequently transferred to the smooth and plasma membrane fractions. The cleavage of PE_2 to E_2 is initiated at 20 min after synthesis, is complete at 40 min after synthesis, and may occur in the smooth (medium-density) or plasma (low-density) membrane fractions, but not in the rough membranes. The rapid and preferential association of nucleocapsid protein with plasma membranes (primarily with membrane proteins synthesized before pulse-labeling) dramatically demonstrates the independence of maturation of capsid protein relative to membrane protein. This agrees with the observations of Schlesinger and Schlesinger (33) and with our observation (data not shown) that in a short pulse period, capsid protein is the only labeled protein found in mature viruses.

Second, the Sindbis virus temperature-sensitive mutant *ts*-20 produces proteins at the nonpermissive temperature which are processed through the various cell membrane components in a fashion similar to the processing of wild-type virus proteins. In agreement with previous studies (3, 12, 14, 38), virus structural proteins synthesized at the nonpermissive temperature appeared on the surfaces of infected cells, but they were not transferred into viruses, nor was there any detectable cleavage of PE_2 to E_2. This situation was not changed by shifting cells pulse-labeled at the nonpermissive temperature to permissive temperatures for extended periods of time. Polypeptide E_1 which was synthesized by mutant *ts*-20 or strain SVHR at 39.5°C (but not 37°C) migrated on polyacrylamide gels as two bands, which were eliminated by tunicamycin treatment. This suggests that a high temperature may produce a conformational change in polypeptide E_1, allowing atypical glycosylation mediated by cellular enzymes. The unusual banding patterns demonstrated by SVHR and *ts*-20 at the nonpermissive temperature were not observed consistently, and we believe that the presumed atypical glycosylation shown in Fig. 6 was dependent upon unknown physiological factors associated with our strain of BHK cells. This unusual glycosylation did not affect the pattern of intracellular migration of proteins in either wild-type virus- or mutant-infected cells or the production of virus in wild-type virus-infected cells (data not shown).

Third, Sindbis virus mutant *ts*-23 produces polypeptides PE_2, E_1, and C at the nonpermissive temperature, as does *ts*-20. Virus proteins are not detected at the cell surface in large quantities (Fig. 1), nor are they incorporated into virus. The distribution of *ts*-23 membrane proteins in fractionated cell membranes differs strikingly from the distribution of *ts*-20-infected cell membrane proteins. Like SVHR and *ts*-20, *ts*-23 proteins first appear in the rough endoplasmic reticulum membranes of the cell. *ts*-23 proteins synthesized at the nonpermissive temperature move in a pulse-chase experiment from the rough membranes to the smooth membranes, but not to the plasma membrane fraction. Therefore, *ts*-23 is a transport-defective mutant altered such that it is incapable of undergoing processing from the smooth membranes to the plasma membranes of an infected cell. A limited amount of virus protein was transported to plasma membranes in cells infected with *ts*-23 at the nonpermissive temperature, and this label consisted primarily of what appeared to be forms of PE_2 and E_1 having higher-than-normal molecular weights. These altered molecular weights may reflect limited excessive glycosylations of nonfunctional *ts*-23 proteins. These glycosylations may specifically favor the transport of these polypeptides to cell surfaces. The transfer of cells containing proteins which were synthesized at a high temperature to growth conditions at the permissive temperature did not alter this pattern, nor did it alter the observation that no PE_2 could be converted to E_2 during an extended chase period.

ts-23 proteins also differ from the proteins produced at 39.5°C by wild-type and *ts*-20 viruses in that *ts*-23 proteins never demonstrate the unusual pattern of glycosylation of E_1. This supports the suggestion that the temperature-sensitive alterations in *ts*-23 proteins reside in polypeptide E_1 (12, 38, 42, 44).

A major question in understanding the process of maturation of alphaviruses from host membranes remains unanswered in this study. Namely, does PE_2 appear in the cell plasma membrane before envelopment, or is it cleaved at some internal site and transported to the plasma membrane as E_2? Although the fractionated membrane preparations described here showed PE_2 in the fraction designated as containing the cell surface, this material could be present as contaminating rough or smooth mem-

branes. All labeled PE_2 is converted to E_2 within 40 min after the labeling period, and E_2 remains associated with the plasma membranes after very long chase periods. We have shown that this cell-associated E_2 label cannot be accounted for as mature virus trapped in the plasma membranes during fractionation. Thus, PE_2 can be cleaved to E_2 without being converted into free mature virus. However, it remains possible that this label is associated with partially enveloped virions of the type morphologically demonstrated by Brown et al. (13). Some of these structures may, for reasons not yet understood, complete envelopment only very slowly or never.

Studies with mutant *ts*-20 clearly demonstrate the presence of PE_2 in the plasma membrane fraction. No PE_2 label is converted to E_2 during a 90-min chase period at either the permissive or the nonpermissive temperature, and the amount of PE_2 labeled in the plasma membrane fraction is too high to be accounted for by contamination from other membrane fractions at this extended chase period. Thus, it is clear that PE_2 can arrive at the plasma membrane without conversion to E_2 as suggested previously (38). However, it may still be argued that the irreversible defect in this protein results from its erroneous incorporation into the plasma membrane in the underprocessed state. The question regarding the incorporation of uncleaved PE_2 into plasma membranes during infection with wild-type virus must be resolved by development of procedures for purifying plasma membranes free of contamination by other cellular components.

ts-23 proteins produced at nonpermissive temperature are similar to those produced under identical conditions by *ts*-20 in that under no circumstances can PE_2 produced in a high-temperature "pulse" be converted to E_2. The majority of *ts*-23 proteins are transported only as far as the smooth membranes and are not transported to the cell surface. Is the cleavage of PE_2 to E_2 a necessary precursor to incorporation into the plasma membrane? The answer is not provided in this study; the data presented here, as well as observations by others, suggest that the *ts*-23 defect is in polypeptide E_1 and not polypeptide PE_2. Since this mutant complements *ts*-20, it may by argued that the PE_2 polypeptide in *ts*-23 is normal, and that the failure to transport the PE_2-E_1 complex (11, 23, 45) beyond the smooth membranes lies in the alteration of some configuration in the E_1 polypeptide essential for transport out of the smooth membrane fraction. The temperature-sensitive defect in *ts*-23, which apparently blocks the normal intracellular migration of the PE_2-E_1 complex from the medium-density fraction to the plasma membrane fraction, may result from a single amino acid change in the E_1 polypeptide. Experiments leading to the determination of the location of this substitution and its effects on the conformation of the E_1 polypeptide at the nonpermissive temperature are in progress and may elucidate the mechanism by which cells are able to direct newly synthesized proteins to particular cellular locations.

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