

REQUIREMENTS FOR THE INSERTION OF THE SINDBIS ENVELOPE GLYCOPROTEINS INTO THE ENDOPLASMIC RETICULUM MEMBRANE

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ABSTRACT

Previous work has shown that the Sindbis structural proteins, core, the internal protein, and PE₂ and E₁, the integral membrane glycoproteins are synthesized as a polyprotein from a 26S mRNA; core PE₂ and E₁ are derived by proteolytic cleavage of a nascent chain. Newly synthesized core protein remains on the cytoplasmic side of the endoplasmic reticulum while newly synthesized PE₂ and E₁ are inserted into the lipid bilayer, presumably via their amino-termini. PE₂ and E₁ are glycosylated as nascent chains. Here, we examine a temperature-sensitive mutant of Sindbis virus which fails to cleave the structural proteins, resulting in the production of a polyprotein of 130,000 mol wt in which the amino-termini of PE₂ and E₁ are internal to the protein. Although the envelope sequences are present in this protein, it is not inserted into the endoplasmic reticulum bilayer, but remains on the cytoplasmic side as does the core protein in cells infected with wild-type Sindbis virus. We have also examined the fate of PE₂ and E₁ in cells treated with tunicamycin, an inhibitor of glycosylation. Unglycosylated PE₂ and E₁ are inserted normally into the lipid bilayer as are the glycosylated proteins. These results are consistent with the notion that a specific amino-terminal sequence is required for the proper insertion of membrane proteins into the endoplasmic reticulum bilayer, but that glycosylation is not required for this insertion.

KEY WORDS membrane glycoprotein
insertion · Sindbis virus

The biosynthesis of integral membrane glycoproteins has been studied using enveloped viruses such as VSV and Sindbis virus as model systems (10, 15, 24, 21). The advantage of using such systems is that many viruses have few structural proteins, usually only one or two integral membrane proteins and, in some cases, an internal

peripheral protein. In contrast, an average mammalian cell contains several hundred membrane proteins. Further, these viruses do not contain enough genetic information to encode all of the enzymes necessary for their biogenesis and therefore must rely on the host cell for many functions. In general, host cell protein synthesis is inhibited during viral infection, making the study of specific proteins much easier.

Studies with VSV and Sindbis virus have dem-

onstrated that the polysomes synthesizing membrane glycoproteins are bound indirectly to the endoplasmic reticulum membrane via the nascent polypeptide chain (15, 18, 23, 24). Indirect evidence indicates that the amino-terminus of the membrane glycoproteins is involved in the binding of these polysomes to membranes and in the subsequent transfer of the protein into the endoplasmic reticulum lumen (1, 2, 18, 23). The nascent polypeptide chains are glycosylated, presumably in the lumen, by the transfer of a preformed oligosaccharide to the protein chain (9, 21).

Two questions remain unanswered in these studies. (a) Is a specific amino-terminal sequence required for the proper insertion of membrane glycoproteins into the endoplasmic reticulum? (b) Is glycosylation required for this insertion? To answer these questions, we first examine the fate of the viral structural proteins in cells infected with a temperature-sensitive mutant of Sindbis virus. This mutant, ts 2, fails to cleave the structural proteins at the nonpermissive temperature, resulting in the production of a polyprotein of 130,000 mol wt (referred to as the ts 2 protein). The order of the proteins in this polypeptide is presumed to reflect the gene order which is NH₂-core-PE₂-L₁-COOH (5, 6, 19, 22, 14, 17). Therefore, in the ts 2 protein, the amino-terminal sequence of each glycoprotein, E₁ and PE₂ is internal. Secondly, we examined the fate of unglycosylated Sindbis envelope proteins in cells infected with Sindbis virus and treated with the glycosylation inhibitor, tunicamycin (13, 14).

MATERIALS AND METHODS

Cell Preparation and Viral Infection

Secondary cultures of CEF (chicken embryo fibroblasts) were used in all experiments. The cells were grown on plastic tissue culture dishes (100 or 60 mm Diam) or in glass roller bottles (690 cm²). The cultures were transferred 18–24 h before the experiment. Wild-type Sindbis virus was obtained from Dr. B. Sefton and the ts 2 mutant was obtained from Dr. S. Schlesinger. All of the virus used in these experiments was derived from plaque-purified stocks.

For infection, the culture medium was removed and 10–100 plaque-forming units of Sindbis virus or ts 2 virus per cell were added to the monolayer in one-tenth of the normal volume of Minimum Essential Medium (MEM) containing 5% calf serum and 1 μg/ml actinomycin D. After absorption (30 min) at 39°C for the wild-type virus and at 30°C for the ts 2 mutant, the remaining nine-tenths of the medium was added to the monolayers.

In cultures (60-mm dish) which were treated with tunicamycin, the culture medium was removed 2.5 h after infection and replaced with 1.5 ml of the above medium containing 10 μg/ml of tunicamycin (Eli Lilly & Co., Indianapolis, Ind.). Incubation was continued at 39°C.

Radioactive Labeling

Infected cells were labeled as described previously (24). [³⁵S]methionine was purchased from Amersham Corp. (Arlington Heights, Ill.) and had a sp act of 500–1,000 Ci/mmol. [³H]uridine was purchased from Amersham Corp. and had a sp act of 46 Ci/mmol. The details of each experiment are described in the figure legends.

Harvesting and Subcellular Fractionation

Monolayers were washed twice in ice-cold, phosphate-buffered saline and once in ice-cold SS buffer. The cells were scraped from the roller bottle with a rubber policeman tip (Arthur H. Thomas Co., Philadelphia, Pa.). The suspension was pelleted at 1,000 g for 5 min, resuspended in SS buffer, and swelled for 10 min in an ice bath. The cells were broken with 40 strokes of a tight-fitting Dounce homogenizer (Kontes Co., Vineland, N. J.). Nuclei were removed by centrifugation at 1,000 g for 4 min. The postnuclear supernate was layered onto a linear 15–40% wt/wt sucrose gradient (30 ml) with a 5-ml cushion of 55% wt/wt sucrose. The gradients were centrifuged for 4.5 h at 4°C and at 26,500 rpm in an SW27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). SS Buffer (0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid, pH 7.5, 0.01 M NaCl, 0.0015 M MgCl₂).

In some experiments, the gradients were collected into 1.5-ml fractions, and aliquots were taken for the determination of acid-insoluble radioactivity and for subsequent analysis. In other experiments, the membrane band found at the interface of the 40/55% sucrose was collected with a syringe and needle, and diluted with SS buffer. Membranes were recovered by centrifugation for 1 h at 4°C and 26,500 rpm in an SW27 rotor. The membrane pellet was resuspended in SS buffer and homogenized 10 times with a tight-fitting Dounce homogenizer before subsequent treatment.

SDS Sucrose Gradient

Aliquots of the initial sucrose gradient sedimentation were diluted with SS buffer mixed with SDS (to a final concentration of 1%), and incubated at 37°C for 10 min. The material was layered onto a 15–50% sucrose gradient in SDS buffer. The gradients were centrifuged for 18 h at 24,000 rpm at 22°C. Fractions were collected and acid-insoluble radioactivity was determined.

Liquid Scintillation Counting

For the determination of acid-insoluble radioactivity, the TCA-precipitable material was collected onto a GF/

A filter (Whatman), rinsed with 5% TCA, and dried under an infrared lamp. Each filter was immersed in 5 ml of scintillation fluid and counted in a Beckman LS-250 scintillation counter. For the determination of total radioactivity, fractions were collected directly into scintillation vials, mixed with 2 ml of water and 16 ml of Aquasol (New England Nuclear, Boston, Mass.), and counted in a Beckman LS-250 scintillation counter. In double-label experiments, standards were prepared and counted under identical conditions.

Polyacrylamide Gel

Electrophoresis (PAGE)

Proteins were resolved by polyacrylamide electrophoresis (see reference 24) with the modification that the sample buffer contained 0.1 M dithiothreitol and 10% β -mercaptoethanol. The separating gel was 10% acrylamide, and the stacking gel was 3.6% acrylamide. The stacking gel contained 0.1 mg of riboflavin instead of ammonium persulfate; it was polymerized for 40 min, 15 cm from a fluorescent bulb. Electrophoresis was carried out for 4.5 h at 20 mA.

The gels were fixed with 10% acetic acid and 25% isopropanol, stained with Coomassie Brilliant Blue, and dried under vacuum onto a sheet of 1-MM paper (Whatman). The radioactive profile was determined by autoradiography with Kodak SB-54 X-ray film. The autoradiographs were quantitated with a gel scanner (Biomedical Research & Development Labs, Inc., Rockville, Md.).

RESULTS

Subcellular Location of *ts2* 26S mRNA

In wild-type Sindbis-infected cells, we have shown that the 26S mRNA is localized in membrane-bound polysomes and is directing the synthesis of core and the two envelope proteins. To determine the subcellular location of the 26S mRNA which was synthesizing the *ts2* protein, cells were infected with *ts2* mutant of Sindbis virus and incubated at both the permissive and nonpermissive temperature; the viral-specific RNA was labeled with [³H]uridine. The cells were lysed and fractionated on a linear sucrose gradient, with a dense sucrose cushion. In this system, the membranes migrate to the 55/40% sucrose interface, and the soluble material, including the polysomes, is distributed in the linear sucrose gradient. As shown in Fig. 1, the majority of the viral-specific RNA from cells infected with the *ts2* mutant of Sindbis virus at the nonpermissive temperature was membrane bound. A similar observation was made when the infected cells were grown at the permissive temperature.

As shown in Fig. 2A and B, the membrane fraction from the *ts2*-infected cells grown at the nonpermissive temperature contained both 42S and 26S RNA. Less than 5% of the total viral-specific RNA in membranes was 42S. In a similar experiment, with cells infected with wild-type virus, 15% of the total viral-specific RNA in membranes was 42S. One possible source of 42S RNA in the membrane fraction is nucleocapsid structures which are bound to the membrane. The

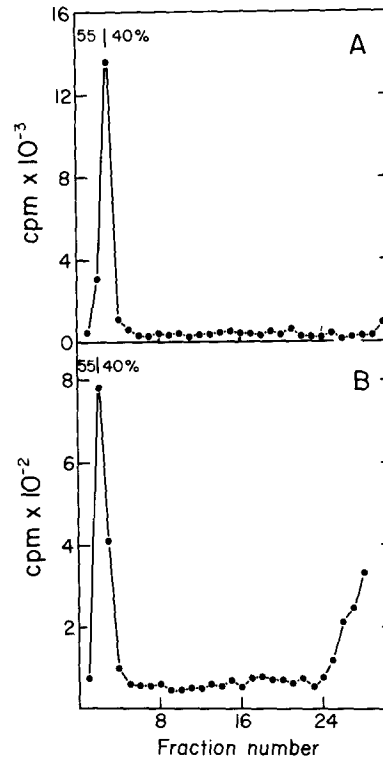


FIGURE 1 Subcellular fractionation of Sindbis-specific RNA. Two roller bottles of CEF (8×10^7 cells/bottle) were infected with Sindbis *ts2* virus at an m.o.i. of 10–50 in medium containing 5% calf serum and 1.5 μ g/ml of actinomycin D. After absorption (30 min) and an incubation at 30°C, one of the infected cultures (panel A) was shifted to 41°C; the other remained at 30°C (panel B). [³H]uridine (5 μ Ci/ml) was added to each culture. The cells were harvested 4.5 h postinfection, lysed by Dounce homogenization, and the postnuclear supernate was layered onto a 15–40% linear sucrose gradient with a 55% sucrose cushion in SS buffer. (15–40%/55%). After a 4.5-h centrifugation at 26,500 rpm in an SW 27 rotor, the gradients were fractionated. 1.5-ml fractions were collected and the TCA-insoluble radioactivity was determined for 100- μ l aliquots of each fraction. Sedimentation is from right to left.

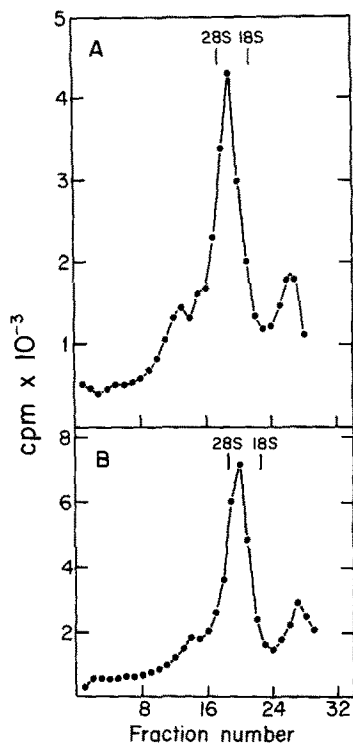


FIGURE 2 Sindbis-specific RNA from the membrane fraction. CEF were infected with ts 2 virus or wild-type virus, labeled with [³H]uridine, and fractionated as described in Fig. 1. The material sedimenting at the 40/55% interface (membrane fraction) was isolated; the membranes were dissolved with SDS, and the RNA was fractionated on a linear 15–30% sucrose gradient containing SDS. After centrifugation at 26,550 rpm for 15 h, the gradient was fractionated and the TCA-insoluble radioactivity was determined for each fraction. The direction of sedimentation is from right to left. (A) Wild-type Sindbis-infected cells grown at 41°C. (B) ts 2-infected cells grown at 41°C.

decreased level of 42S RNA found in the membranes isolated from ts 2-infected cells may be explained by the absence of nucleocapsids in these cells (3, 4, 19, 24). Thus, on the basis of the subcellular location of the mRNA, the infected cells grown at the nonpermissive temperature appear much like wild type.

Subcellular Location of the ts-2 Protein

Cells infected with the ts 2 mutant at the nonpermissive temperature were pulse-labeled with [³⁵S]methionine and fractionated as described above. The majority of the radioactivity is associ-

ated with the membrane fraction. The labeled proteins from the membrane and soluble fractions were resolved by PAGE as shown in Fig. 3. By comparing the amount of the ts 2 protein in the total lysate with the amount found in the membrane fraction, it was determined that 85% of the radioactivity in the ts 2 protein is associated with the membrane fraction. The ts 2 protein has been

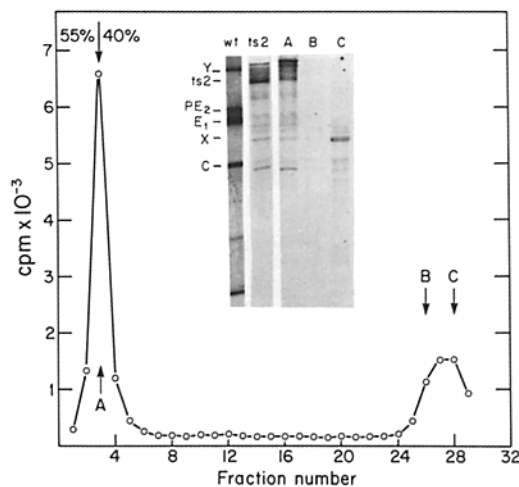


FIGURE 3 The ts 2 protein is membrane-associated. CEF (5×10^6 cells/100-mm plate) were infected with ts 2 virus at an m.o.i. of 10, in MEM containing 5% calf serum and 1 μ g/ml actinomycin D. After absorption (30 min) and a 1-h incubation at 30°C, the infected monolayers were transferred to 41°C. At 4.5 h after infection, the culture medium was removed and the monolayer washed twice with MEM containing 2% dialyzed calf serum but without methionine. The cultures were incubated for 10 min at 41°C in the above medium containing 20 μ Ci/ml of [³⁵S]methionine. The cells were harvested, lysed by Dounce homogenization, and the post-nuclear supernate was layered onto a 15–40%/55% sucrose gradient in SS buffer. After centrifugation for 4.5 h at 26,500 rpm in an SW27 rotor at 4°C, the gradient was fractionated. 1.5-ml fractions were collected and the TCA-insoluble radioactivity from 100- μ l aliquots was determined. The direction of sedimentation is from right to left. *Inset*: The proteins in the fractions indicated were concentrated by acetone precipitation and resolved by 10% PAGE. Shown is an autoradiograph of the dried gel. Exposure time: 7 days. X and Y indicate the positions of host cell proteins. wt, Proteins made in wild-type-infected cells after a 10-min pulse of [³⁵S]methionine. ts 2, Cytoplasmic extract of cells infected with ts 2 and labeled with [³⁵S]methionine for 10 min at 41°C. A–C, Proteins contained in the membrane and soluble fractions of the cytoplasmic extract of cells infected with ts 2 as described above.

identified by peptide mapping (not shown).

What is the topological relationship between the ts 2 protein and the isolated membrane vesicles? The ts 2 protein contains the amino acid sequences of the soluble core protein and the two envelope glycoproteins. It could be localized on the cytoplasmic side of the endoplasmic reticulum bilayer as is the core protein, it could be entirely inserted into the lipid bilayer as are the envelope proteins, or it could be arranged as are the normal wild-type proteins—the core being on the cytoplasmic side of the endoplasmic reticulum and the envelope proteins inserted into it. To test these possibilities, membrane vesicles were isolated as described above from cells infected with ts 2 at 41°C and pulse-labeled with [³⁵S]methionine for 10 min before cell lysis. We have previously shown that in vesicles isolated from the endoplasmic reticulum, the polysomes are found on the outside of the vesicles while the lumen is inside (24). These isolated vesicles were digested with low levels of chymotrypsin. If the ts 2 protein is entirely on the cytoplasmic side of the endoplasmic reticulum, it should be removed by proteolytic digestion. If the entire protein is inserted through the bilayer into the lumen, then it should be resistant to proteolysis, unless the membrane vesicles are destroyed by pretreatment with detergent. If the protein is partially inserted, then it should be cleaved to a smaller size by proteolysis.

Fig. 4 shows that the ts 2 protein is absent after proteolytic digestion of the membrane vesicles from pulse-labeled infected cells. No smaller fragments are found after proteolysis, which could account for the radioactivity associated with the ts 2 protein before proteolysis. As can be seen in Fig. 4, there are proteins in ts 2-infected cells which comigrate with the structural proteins from cells infected with wild-type virus. These proteins may be the result of a low level of cleavage activity in cells infected with the ts 2 mutant at the nonpermissive temperature or they may be unrelated to the Sindbis structural proteins.

Since the actual identity of these proteins was not clear, they could not be used as internal controls for the proteolysis experiment. Cells were coinfecting with wild-type Sindbis and the ts 2 mutant at the nonpermissive temperature. As can be seen in Fig. 4, in coinfecting cells, the ts 2 protein, PE₂, E₁, and core protein are synthesized. If membrane vesicles from [³⁵S]methionine-pulse-labeled cells are digested with chymotrypsin, some 90% of the radioactivity associated with the ts 2

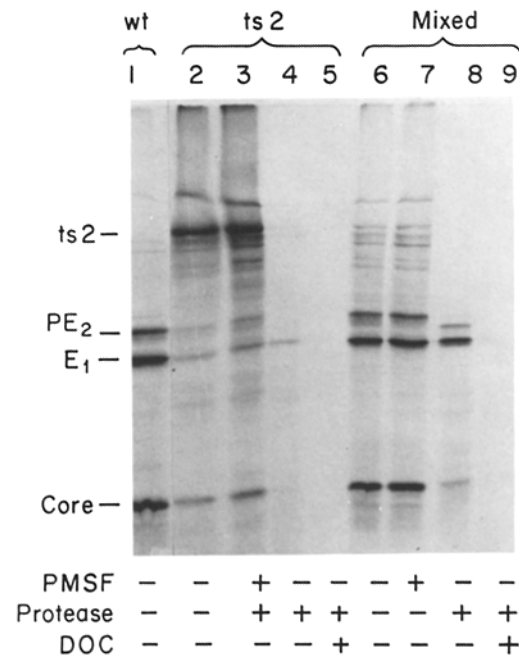


FIGURE 4 Topology of ts 2 protein in infected cells. One culture of CEF (5×10^6 cells/100-mm dish) was infected with ts 2, as described in Fig. 3. A second culture was infected with both ts 2 and wild type at an m.o.i. of 10 for each virus. After absorption (30 min) and a 1-h incubation at 30°C, the culture was shifted to 41°C. At 4.5-h postinfection, the culture was pulse-labeled with [³⁵S]methionine (10 min, 50 μ Ci/ml) and fractionated, as described in the legend to Fig. 3. The membrane fraction sedimenting at the 40/55% sucrose interface was isolated, diluted with PBS buffer, and pelleted for 30 min at 4°C (26,500 rpm in an SW27 rotor). The membrane fractions were resuspended in PBS, and aliquots were incubated as described below. All incubations were carried out for 30 min at 30°C. Chymotrypsin (Worthington Biochemical Corp., Freehold, N. J., final concentration 10 μ g/ml), phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, Mo., final concentration 20 μ g/ml), and deoxycholate (Sigma Chemical Co., final concentration 1% wt/vol) were added to the reactions as indicated by "+". At the end of the incubation period, the reactions were heated for 5 min at 100°C. The proteins were concentrated by acetone precipitation and resolved by 10% PAGE. Shown is an autoradiograph of the dried gel.

and core proteins is destroyed. Over 80% of the radioactivity associated with PE₂ and E₁ is protected from proteolysis. PE₂ has an increased electrophoretic mobility, as described previously (24). If the vesicles are lysed with detergent before proteolysis, all of the proteins are digested.

On the basis of these results, we conclude that the ts 2 protein is located on the cytoplasmic side of the endoplasmic reticulum membrane. Although the envelope sequences are present in this protein, they are not inserted into the bilayer and thus not protected from proteolysis.

Subcellular Location of Unglycosylated Envelope Proteins

Leavitt and co-workers (13, 14) showed that unglycosylated forms of the Sindbis envelope proteins were produced in infected cells treated with tunicamycin. These unglycosylated proteins could be identified by an increased electrophoretic mobility on polyacrylamide gels as shown in Fig. 5. In the presence of tunicamycin, the cleavage of PE₂ to E₂ is inhibited. Upon further examination of the properties of these unglycosylated proteins,

Leavitt and co-workers (14) showed that the envelope proteins sedimented at 100,000 g. This property does not necessarily imply membrane association, however, since the proteins also existed in a relatively insoluble form in the cell and may have sedimented in the form of protein aggregates.

As shown in Fig. 5, two protein bands of higher electrophoretic mobility are found in the envelope protein region when infected cells which have been treated with tunicamycin are labeled with [³⁵S]methionine. These two proteins contain the chymotryptic peptides (from cells labeled with [³⁵S]methionine) of PE₂ and E₁ from untreated cells (data not shown). Two lines of evidence indicate that these proteins represent the unglycosylated forms of PE₂ and E₁. First, the two proteins labeled PE₂' and E₁' (see Fig. 5) comigrate in PAGE with the deglycosylated forms of PE₂ and E₁ produced by treatment of cell extracts with endo β-N-acetylglucosaminidase H (D. F. Wirth, R. A. Van Etten, H. F. Lodish, and P. W. Robbins, manuscript in preparation). Secondly, if tunicamycin-treated infected cells are labeled with [³H]mannose, incorporation into protein is <5% of the control, untreated infected cells. Polyacrylamide gel electrophoretic analysis of infected cells labeled with [³H]mannose shows that PE₂ and E₁ are the only labeled proteins present in these cells (not shown). Thus, incorporation of [³H]mannose into Sindbis-specific proteins is reduced to <5% of the normal level.

To determine whether the unglycosylated envelope proteins (designated PE₂', E₁') were membrane bound, cells infected with Sindbis virus and treated with tunicamycin were pulse-labeled with [³⁵S]methionine, lysed by Dounce homogenization, and the postnuclear supernate was mixed with 65% sucrose and overlaid with a sucrose equilibrium gradient. The membranes migrated to the interface of 50 and 30% sucrose, while soluble material remained in the 65% sucrose. The proteins associated with the membrane and soluble fractions were isolated and resolved by gel electrophoresis. The unglycosylated envelope proteins were membrane associated. In a control experiment, glycosylated envelope proteins were also membrane associated (data not shown).

Are the unglycosylated envelope proteins inserted into the endoplasmic reticulum and thus protected from proteolysis as are glycosylated Sindbis envelope proteins? To answer this question, membrane vesicles were isolated from cells

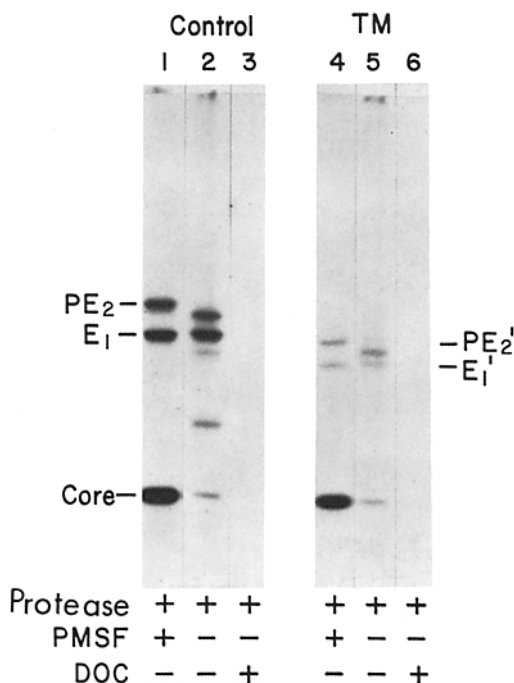


FIGURE 5 Topology of unglycosylated E₁ and PE₂ in infected cells. Two cultures of CEF were infected with Sindbis, m.o.i. of 10 in MEM containing 5% calf serum and 1 μg/ml actinomycin D. After absorption (30 min) and a 2-h incubation at 39°C, tunicamycin (Eli Lilly & Co.) was added to one culture (final concentration 10 μg/ml). At 4.5 h postinfection, the cultures were pulse-labeled with [³⁵S]methionine and fractionated as described in the legend to Fig. 4. The resuspended membranes were incubated as described in the legend to Fig. 4. The proteins were resolved by 10% PAGE.

infected with Sindbis virus which had been pulse-labeled with [³⁵S]methionine in the presence of tunicamycin. The vesicles were digested with chymotrypsin, and the proteins were resolved in PAGE. As can be seen in Fig. 5, under conditions where 90% of the core protein is removed from the vesicles, the unglycosylated forms of the PE₂ and E₁ are protected from proteolytic digestion. PE₂' shows an increased electrophoretic mobility. The electrophoretic mobility of E₁' remains unchanged. If the membranes are pretreated with detergent, all three of the structural proteins are equally digested.

In a control experiment (see Fig. 4), some 80% of the radioactivity associated with the glycosylated envelope proteins remains after proteolytic digestion under these conditions. In the case of tunicamycin-treated cells, only 50–60% of the radioactivity associated with PE₂' and E₁' remains. The reasons for this difference are not clear. It may be caused by side effects of tunicamycin pretreatment such as leakiness of the membrane vesicles. Also, some of the radioactivity which was associated with PE₂' before proteolysis retains its original electrophoretic mobility after proteolysis. This may represent PE₂' which is completely inserted into membrane vesicles, or residual glycosylated E₁ which comigrates with PE₂'.

From these experiments, we conclude that glycosylation is not required for proper insertion of the envelope proteins into the endoplasmic reticulum membrane.

Stability of Aberrant Proteins

To determine whether post-translation cleavage of the ts 2 polyprotein could occur followed by insertion and glycosylation of the envelope proteins, cells infected with ts 2 were pulse-labeled at the nonpermissive temperature and chased with unlabeled medium at the permissive and nonpermissive temperature. The ts 2 protein remained stable at both temperatures during a 4-h chase period. No increase was found in the amount of labeled envelope or core proteins during the chase period. The ratio of the ts 2 protein to the core protein was determined by densitometer tracings of the exposed autoradiograph, and the results are shown in Table I.

DISCUSSION

As we have previously shown, the Sindbis 26S RNA directs the synthesis of the viral structural

TABLE I
Stability of ts 2 Protein

Chase	Temperature	Core	ts 2 Protein
	°C		
0 min	41	9	85
1.5 h	41	6	73
4.0 h	41	7	78
1.5 h	30	5	68
4.0 h	30	6	70

Each plate (60 mm) was pulse-labeled with [³⁵S]methionine as described in the legend to Fig. 3. The labeled plates which were to be chased were washed twice with MEM containing 5% calf serum and 1 μg/ml actinomycin D which had been prewarmed to the appropriate temperature. After the chase period, the cells were lysed in sample buffer, and equal aliquots of protein were resolved on a 10% polyacrylamide gel. An autoradiograph of the dried gel was scanned with a soft laser gel scanner (Biomedical Research & Development Labs, Inc.). The figures given represent the area (mm)² under the respective peaks.

proteins, core, PE₂ and E₁. Evidence from cell-free translation (5, 6, 22) and mutant studies have shown that the 26S mRNA has only one initiation and one termination site for protein synthesis (19, 22); core, PE₂ and E₁ are derived by proteolytic cleavage of the nascent polypeptide. On the basis of these and other results, we proposed a model for the interaction of the Sindbis 26S polysomal mRNA with the endoplasmic reticulum membrane during the synthesis of viral structural proteins. A ribosome begins translating the core protein at the 5' end of a free 26S mRNA. As soon as the core protein is finished, it is removed by a protease, thus exposing the amino-terminus of the nascent envelope protein, presumably PE₂. The amino-terminus of the PE₂ protein initiates an interaction with the membrane, and is subsequently transferred into and across the membrane presumably through a protein channel. This interaction leads to the binding of the polysome to the endoplasmic reticulum. As the ribosome continues to transverse the mRNA, completed PE₂ is cleaved and remains imbedded in or sequestered by the membrane. E₁ is then translated and inserted into the membrane, again attaching the polysome to the membrane (24).

The class C temperature-sensitive mutants of Sindbis virus, typified by ts 2 mutation, are defective in the cleavage of the nascent structural protein polypeptide (19). Cells infected with ts 2 virus at the nonpermissive temperature produce a protein of 130,000 mol wt which contains the

peptides of the three structural proteins, presumably in the gene order which is NH₂-core-PE₂-E₁-COOH (6, 19). The amino-terminal sequence of the ts 2 protein is presumably the same as that of the core protein.

In this study, we have shown that the newly synthesized ts 2 protein is not inserted into the endoplasmic reticulum membrane, but remains on the cytoplasmic side. In this respect, the ts 2 protein resembles the core protein and not PE₂ or E₁ from cells infected with wild-type virus. Although the envelope protein sequences are present, they are not inserted into the membrane. This result is consistent with the notion that the proteolytic cleavage between core and PE₂ exposes a sequence, presumably at the amino-terminus of PE₂, which is essential for proper insertion of PE₂ into the endoplasmic reticulum membrane. At least one other proteolytic cleavage does not occur in ts 2-infected cells: that between PE₂ and E₁. Likewise, it may be the prevention of this second cleavage that prevents the proper insertion of E₁ into the rough endoplasmic reticulum.

How can a single mutation affect the two proteolytic cleavages? One possibility is that the primary lesion in ts 2 mutation is the cleavage between core and PE₂: at high temperatures, this proteolytic clip may be prevented by the conformation at the cleavage site or by a temperature-sensitive viral protease (19). As a result of this lack of cleavage between PE₂ and core, PE₂ is not in the proper conformation to permit cleavage between PE₂ and E₁. It is also possible that the cleavage between PE₂ and E₁ occurs on the luminal side of the endoplasmic reticulum. Since PE₂ is not inserted into the endoplasmic reticulum in ts 2-infected cells, the PE₂-E₁ junction is not accessible to the cleaving enzyme.

The majority of the 26S mRNA is bound to membranes in cells infected with ts 2 at the nonpermissive temperature. This mRNA binding could be the result of at least two interactions: (a) the binding of the mRNA directly to the membranes, (b) the indirect binding of the mRNA via the nascent ts 2 protein which, itself, binds to membranes (due to its content of hydrophobic sequences). These experiments cannot differentiate between these two possibilities. The binding of mRNA to membranes may, in fact, be only indirectly related to function. In the case of the ts 2 26S mRNA, there is no detectable insertion of the protein into membranes, yet the mRNA exhibits characteristics similar to those of the wild-

type 26S RNA where insertion of the envelope proteins does occur. Thus, the presence of mRNA in membrane polysomes may accompany the insertion of a nascent polypeptide into the endoplasmic reticulum, but binding alone is not sufficient to direct this insertion (11, 12).

It is interesting to note that in cells coinfecting with the ts 2 mutant and wild-type Sindbis virus, the amount of radioactivity associated with the ts 2 protein was decreased relative to the amount found in cells infected with ts 2 alone. There could be several explanations for this observation. One possibility is that the defect in the ts 2 mutant is in the cleavage enzyme and that this activity is supplied by wild-type virus. The ts 2 protein would not accumulate because it is cleaved as a nascent polypeptide to form the structural proteins. Other explanations include the following: (a) the wild-type 26S mRNA is produced at a higher rate than the ts 2 26S RNA, (b) the 26S RNA from the wild-type genome is more efficient in translation than the ts 2 26S RNA (this assumes that the cellular components for translation are limiting).

We have also demonstrated that unglycosylated Sindbis envelope proteins made in the presence of tunicamycin are inserted into the endoplasmic reticulum bilayer as are glycosylated PE₂ and E₁. This result argues that glycosylation is not a necessary step for membrane protein insertion. The initial proteolytic cleavages which result in the production of core, PE₂, and E₁ occur normally in tunicamycin-treated cells (13, 14). After the core protein is removed from the growing polypeptide by proteolytic cleavage, the amino-terminus of the first envelope protein, PE₂, is exposed and thus available for insertion into the endoplasmic reticulum membrane.

Although glycosylation often accompanies the insertion of a membrane glycoprotein or secretory protein into the endoplasmic reticulum lumen, it is not a requirement for this process. In this regard, there are several secretory proteins which are not glycosylated, for example immunoglobulin light chain (1). Further, in the case of bovine ribonuclease, the protein is secreted in both the glycosylated and unglycosylated form (17). Several studies using inhibitors, tunicamycin, 2-deoxyglucose and glucosamine, have demonstrated that, in general, glycosylation is not required for secretion (for review, see reference 16). On the other hand, glycosylation appears to be required for the maturation of some enveloped viruses but not others (7, 20).

The role of glycosylation may be in the transport of some proteins through the intracellular matrix of the endoplasmic reticulum and Golgi complex to the cell surface. Leavitt et al. (13, 14) reported that in tunicamycin-treated cells infected with VSV or Sindbis, the unglycosylated envelope proteins did not reach the cell surface. Recent work by the same investigators (8) has shown that VSV virions containing unglycosylated G protein are produced from tunicamycin-treated, infected cells. This level of virus production is low when compared to that of untreated cells; however, that virus which is produced is infectious. One interpretation of these data is that, although intracellular migration of the VSV G protein is impaired in tunicamycin-treated cells, some small portion of the unglycosylated protein does reach the surface and bud into virions. In addition, glycosylation may contribute to the stability of certain proteins, perhaps by covering proteolytic sites. Olden and co-workers (22) found that accumulation of the chicken surface protein (CSP) in chicken fibroblasts was decreased in tunicamycin-treated cells. This decreased level of CSP was explained by increased degradation of the unglycosylated protein. The rates of translation and secretion of CSP in tunicamycin-treated cells was comparable to the rates found in control cells.

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REFERENCES

1. BLOBEL, G., and B. DOBBERSTEIN. 1975. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. *J. Cell Biol.* **67**:835-851.

2. BLOBEL, G., and B. DOBBERSTEIN. 1975. Transfer of proteins across membranes. II. Reconstitution of functional rough microsomes from heterologous components. *J. Cell Biol.* **67**:852-862.
3. BURGE, B. W., and E. R. PFEFFERKORN. 1966. Isolation and characterization of conditional lethal mutants of Sindbis virus. *Virology*. **30**: 204-213.
4. BURGE, B. W., and E. R. PFEFFERKORN. 1968. Functional defects of temperature-sensitive mutants of Sindbis virus. *J. Mol. Biol.* **35**:193-205.
5. CANCEDDA, R., L. VILLA-KOMAROFF, H. F. LODISH, and M. J. SCHLESINGER. 1975. Initiation sites for translation of Sindbis virus 42S and 26S messenger RNAs. *Cell*. **6**:215-222.
6. CLEGG, J. C. S., and S. I. T. KENNEDY. 1975. Initiation of synthesis of structural proteins of SFV. *J. Mol. Biol.* **97**:401-411.
7. DUDA, E., and M. J. SCHLESINGER. 1975. Alterations in Sindbis viral envelope proteins by treating BHK cells with glucosamine. *J. Virol.* **13**: 1055.
8. GIBSON, R., R. LEAVITT, S. KORNFELD, and S. SCHLESINGER. 1978. Synthesis and infectivity of Vesicular Stomatitis virus containing nonglycosylated G-protein. *Cell*. **13**:671-680.
9. KIELY, M. L., G. S. MCKNIGHT, and R. T. SCHIMKE. 1976. Studies on the attachment of carbohydrate to ovalbumin nascent chains in hen oviduct. *J. Biol. Chem.* **251**:5490-5495.
10. KNIPE, D. M., D. BALTIMORE, and H. F. LODISH. 1977. Separate pathways of maturation of the major structural proteins of VSV. *J. Virol.* **21**:1128-1139.
11. KREIBICH, G., B. L. ULRICH, and D. D. SABATINI. 1978. Proteins of rough microsomal membranes related to ribosome binding. I. Identification of ribophorins I and II, membrane proteins characteristic of rough microsomes. *J. Cell Biol.* **77**:464-487.
12. KREIBICH, G., C. M. FREIENSTEIN, B. N. DEREYRA, B. L. ULRICH, and D. D. SABATINI. 1978. Proteins of rough microsomal membranes related to ribosome binding. II. Cross-linking of bound ribosomes to specific membrane proteins exposed at the binding sites. *J. Cell Biol.* **77**:488-506.
13. LEAVITT, R., S. SCHLESINGER, and S. KORNFELD. 1977. Tunicamycin inhibits glycosylation and multiplication of Sindbis and vesicular stomatitis virus. *J. Virol.* **21**:375-385.
14. LEAVITT, R., S. SCHLESINGER, and S. KORNFELD. 1977. Impaired intracellular migration and altered solubility of nonglycosylated glycoproteins of VSV and Sindbis virus. *J. Biol. Chem.* **252**:9018-9023.
15. LODISH, H. F., and S. FROSHAUER. 1977. Binding of viral glycoprotein mRNA to endoplasmic reticulum membranes is disrupted by puromycin. *J. Cell Biol.* **74**:358-364.
16. OLDEN, K., R. M. PRATT, and K. M. YAMADA. 1978. Role of carbohydrates in protein secretion and turnover: Effects of tunicamycin on the major cell surface glycoprotein of chick embryo fibroblasts. *Cell*. **13**:461-474.
17. PLUMMER, T. H. 1968. Glycoproteins of bovine pancreatic juice. *J. Biol. Chem.* **253**:5961-5966.
18. ROTHMAN, J. E., and H. F. LODISH. 1977. Synchronized transmembrane insertion and glycosylation of a nascent membrane protein. *Nature (Lond.)*. **269**:775-780.
19. SCHLESINGER, M. S., and S. SCHLESINGER. 1973. Large molecular weight precursors of Sindbis virus proteins. *J. Virol.* **11**:1013-1016.
20. SCHWARTZ, R. T., J. M. ROHRSCHEIDER, and M. F. G. SCHMIDT. 1976. Suppression of glycoprotein formation of SFV, influenza and ASV by tunicamycin. *J. Virol.* **19**:782-791.
21. SEPTON, B. M. 1977. Immediate glycosylation of Sindbis virus membrane proteins. *Cell*. **10**:659-668.
22. SIMMONS, D. T., and J. H. STRAUSS. 1974. Translation of Sindbis virus 26S RNA and 49S RNA in lysates of rabbit reticulocytes. *J. Mol. Biol.* **86**:397-409.
23. TONEGUZZO, F., and H. P. GHOSH. 1978. *In vitro* synthesis and insertion into membranes of vesicular stomatitis virus membrane glycoprotein. *Proc. Natl. Acad. Sci. U. S. A.* **75**:715-719.
24. WIRTH, D. F., F. KATZ, B. SMALL, and H. F. LODISH. 1977. How a single Sindbis virus mRNA directs the synthesis of one soluble protein and two integral membrane glycoproteins. *Cell*. **10**:253-263.