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# Evidence for a Separate Signal Sequence for the Carboxy-Terminal Envelope Glycoprotein E1 of Semliki Forest Virus

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When Semliki Forest virus temperature-sensitive mutant ts-3 was grown at the restrictive temperature an aberrant nascent cleavage of the 130,000-dalton structural polyprotein took place relatively frequently. This cleavage yielded an abnormal 86,000-dalton fusion protein (p86) consisting of the amino-terminal capsid protein linked to the amino acid sequences of envelope protein p62 (a precursor of E3 and E2). The other cleavage product was the carboxy-terminal envelope protein E1. p86 was not glycosylated and was sensitive to the action of protease in the microsomal fraction, whereas E1 was glycosylated and protected from proteases, indicating that it had been segregated into the cysternal side of the microsomal vesicles. All attempts to show the E1 protein at the cell surface have failed so far, suggesting that it remains associated with intracellular membranes. When ts-3-infected cells labeled at the restrictive temperature were shifted to the permissive temperature the only labeled protein released with the virus particles was E1, indicating that E1, synthesized at the restrictive temperature, was competent to participate in the virus assembly. These results suggest strongly that there are two separate signal sequences for the envelope proteins of Semliki Forest virus. One follows the capsid protein as shown previously, and the other is for the carboxy-terminal E1. Even if the insertion of the amino-terminal envelope protein (p62) fails due to a cleavage defect, the other signal sequence can operate independently to guide the E1 through the endoplasmic reticulum membrane.

The structural proteins of Semliki Forest virus (SFV), an alphavirus, include the following: the capsid protein (30,000 daltons) and envelope glycoproteins E1 (49,000 daltons), E2 (52,000 daltons), and E3 (10,000 daltons) are translated principally as a polyprotein by using a subgenomic 26S RNA as a template (13, 37). The amino-terminal capsid protein is cleaved off the growing polyprotein and becomes rapidly associated with 42S genome RNA to form the viral nucleocapsid in the cytoplasm (3, 19, 35, 40, 44). The primary translational products of envelope proteins, the 62,000-dalton protein p62 (precursor of E3 and E2) (13, 28, 33) and E1, are segregated through the rough endoplasmic reticulum membrane and obtain their mannose-rich oligosaccharide side chains from a dolichol-phosphate lipid intermediate (3, 9, 18, 20, 21, 29, 44). Both these proteins have a carboxy-terminal hydrophobic sequence, which anchors them to the lipid bilayer (10). Only p62 appears to span the lipid bilayer, with a small part of it on the cytoplasmic side of the membrane (3, 9, 44).

Present evidence suggests that p62 and E1 form a complex during their transport to the plasma membrane (5, 12, 28, 34, 46). During their intracellular migration these proteins undergo modification as removal of mannose and addition of galactose, glucosamine, fucose, and sialic acid takes place in the oligosaccharide side chains of these glycoproteins (23, 25, 30, 31, 32). The virus maturation takes place at the plasma membrane when the cytoplasmic nucleocapsid recognizes the envelope proteins at the cell surface and buds through the membrane to the extracellular space (1, 6) bringing with it host cell lipids and about 240 trimers of envelope proteins E1, E2, and E3 (13, 41, 46).

The mode of alphavirus protein synthesis has been elucidated to a large extent by using temperature-sensitive mutants. In the cells infected with Sindbis virus (another alphavirus) mutants of the complementation group C, the cleavage of the whole structural polyprotein is largely inhibited leading to the accumulation of a

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130,000-dalton protein (p130) (27, 36) on the cytoplasmic side of the endoplasmic reticulum membrane (45). We have isolated a similar SFV mutant, ts-3 (15), and have previously analyzed the proteins synthesized in ts-3-infected cells at the restrictive temperature, 39°C, in detail (16, 19). In addition to p130, which has the tryptic peptides of all structural proteins, another prominent protein, p86, containing the amino acid sequences of capsid and p62 proteins, is found in ts-3-infected cells. The third major protein migrates at the position of E1 and has the tryptic peptides of the virion-derived E1 (19).

Here, we show that this E1 in ts-3-infected cells is glycosylated and inserted into the endoplasmic reticulum membrane without simultaneous synthesis of p62.

## MATERIALS AND METHODS

**Virus and cell culture.** The SFV prototype strain and the temperature-sensitive mutant ts-3 isolated from it (15) were used in this study. Propagation of the virus and the characterization of ts-3 have been described previously (15, 16, 19).

Secondary, specific-pathogen-free chicken embryo fibroblasts were cultivated on 35- or 50-mm plates (Sterilin; Teddington, Middlesex, England) as described previously (15).

**Radioactive labeling.** Monolayers of chicken embryo fibroblasts on 35- or 50-mm plates were infected with either ts-3 or wild-type virus at a multiplicity of 50 to 100 PFU per cell. After 1 h of adsorption at 39°C, the cultures were washed with prewarmed Hanks balanced salt solution and incubated at 39°C in methionine-free Eagle minimum essential medium (MEM) supplemented with 0.2% bovine serum albumin, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.2), and 1 μg of actinomycin D (a generous gift from Merck, Sharp & Dohme) per ml. At the times indicated in the figure legends, the medium was replaced with 2 or 4 ml of MEM containing [$^{35}$S]methionine (10 to 40 μCi/ml, 900 to 1,280 Ci/mmol; Radiochemical Centre, Amersham, England), and the cultures were incubated for an appropriate time, followed by a chase in MEM containing a 20-fold excess of unlabeled methionine.

**SDS-polyacrylamide gel electrophoresis.** Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out by the method of Neville (24) as described previously (19). The infected cells were washed once with prewarmed phosphate-buffered saline, dissolved into a small volume of SDS-gel sample buffer (1% SDS, 1% 2-mercaptoethanol, 10% glycerol, and 0.04% bromophenol blue in sample buffer, pH 6.1) and passed five times through a fine syringe needle. For analysis of viral proteins in released virus, nonreduced conditions (without 2-mercaptoethanol) were used to get a better separation of envelope proteins E1 and E2 (14).

After electrophoresis the slab gel was fixed and stained by the method of Fairbanks et al. (7) and fluorographed as described by Bonner and Laskey (4).

For quantitation of the radioactivity in the polypeptide bands, the gel was cut in pieces, swollen in water, and solubilized in NCS (Amersham, Searle). The radioactivity was counted in toluene-based scintillation fluid.

**Preparation and protease treatment of microsomes.** CEF ($3 \times 10^7$ cells) were infected as above and incubated at 39°C until 6 h postinfection. Then the cultures were labeled with 40 μCi of [$^{35}$S]methionine per ml for 10 min and chased for 2 or 20 min in the chase medium as above. The cells were put on ice, washed with ice-cold phosphate-buffered saline, and collected by scraping followed by centrifugation at 250 × *g* for 5 min. The cell pellet was suspended in 5 ml of hypotonic buffer (50 mM Tris [pH 7.4], 10 mM NaCl, 1.5 mM $MgCl_2$), swollen for 10 min in an ice bath, and disrupted with 40 strokes of a tight-fitting Dounce homogenizer. Nuclei and cell debris were removed by centrifugation at 250 × *g* for 5 min. The postnuclear supernatant was centrifuged at 15,000 × *g* for 20 min, and the post-mitochondrial supernatant was centrifuged further at 100,000 × *g* for 60 min in a Spinco SW50.1 rotor. The resulting pellet was suspended in 200 μl of an isotonic buffer (50 mM Tris [pH 7.4], 0.14 mM NaCl, and 1.5 mM $MgCl_2$). Samples (50 μl) of the suspension were treated with 200 μl of trypsin-1-tosylamido-2-phenylethyl-chloromethyl-ketone (217 μg/mg; Worthington Diagnostics, Freehold, N.J.) per ml in the presence or absence of 1,000 U of Trasylol (Bayer) per ml or 1% Triton X-100 (BHD Chemical Ltd., Poole, England). After 30 min of incubation at 37°C, Trasylol was added to each sample and incubated for 10 min, and then an appropriate amount of concentrated SDS-gel buffer was added to make the standard concentration of the buffer. The samples were heated at 100°C for 2 min and stored at −70°C until analyzed.

**Isolation of released virus.** After removal of the cell debris by centrifugation (10,000 × *g*, 30 min), the culture medium was layered on top of 0.5 ml of 35% (wt/wt) sucrose in 0.05 M Tris (pH 7.4)–0.1 M NaCl and centrifuged at 40,000 rpm for 2 h at 4°C in a Spinco SW50.1 rotor. The pellet was suspended in 50 μl of SDS-gel sample buffer without 2-mercaptoethanol.

**Immunoprecipitation.** For detection of envelope proteins at the cell surface, infected, labeled cells were treated with envelope antiserum at 0°C for 1 h. After four washes with phosphate-buffered saline the cells were solubilized in 1% Triton X-100 in Dulbecco phosphate-buffered saline containing 0.5% bovine serum albumin and 100 U of Trasylol per ml and centrifuged at 1,000 × *g* to remove the nuclei. Precipitation with formaldehyde-fixed *Staphylococcus aureus* (17) was carried out for 250-μl samples of the cytoplasmic extracts as described previously (40).

For examination of the reactivity of intracellular proteins with antiserum prepared against 29S envelope protein octamers (26) and antisera prepared against isolated E1, E2, and E3 proteins (Väänänen et al., manuscript in preparation), the labeled cells were dissolved in 2% SDS, and 10- to 20-μl samples were diluted in 1 ml of NET buffer (0.4 M NaCl, 0.005 M EDTA, 0.05 M Tris [pH 8.0], 1% Triton X-100) and treated first with 20 μl of normal rabbit serum at 20°C for 30 min followed by incubation for 1 h at 20°C with

200 μl of a 10% suspension of staphylococci. After pelleting of the bacteria, the supernatant was treated with 20 μl of the appropriate antiserum followed by precipitation with the staphylococci as above. The bacteria were washed three times with NET buffer containing 1, 0.4, and 0.15 M NaCl, respectively, and finally with gel electrophoresis sample buffer, and the proteins were eluted into 50 μl of 2% SDS. The bacteria were sedimented, and the supernatant was analyzed in polyacrylamide gels as described above.

## RESULTS

**Identification of the E1 protein in ts-3-infected cells.** Figure 1 shows a polyacrylamide gel analysis of the proteins synthesized at 39°C in ts-3-infected cells as compared with the SFV wild-type-infected cells. The identity of the protein in ts-3-infected cells migrating at the position of E1 was confirmed by immunoprecipitation. Extracts of ts-3 and wild-type-infected cells were treated with immune sera prepared against isolated envelope protein octamers (26) and purified E1, E2, and E3 proteins followed by formaldehyde-inactivated staphylococci. In SFV wild-type-infected cells E1 protein was specifically precipitated by antiserum against purified E1 protein (Fig. 2, lane b). Both p62 and E2 proteins were precipitated by antiserum against E2 (Fig. 2, lane c), whereas antiserum made against E3 precipitated the p62 protein (Fig. 2, lane d), which is the precursor of E2 and E3 (13, 28, 33). In ts-3-infected cells antiserum against E1 precipitated a protein which migrated at the position of protein E1 (Fig. 2, lane f). The same protein was precipitated also by immune serum prepared against envelope protein octamers, which include E1, E2, and E3 (26) (Fig. 2, lane e).

Neither p62 nor E2 was precipitated from the cytoplasm of ts-3-infected cells by any of the immune sera (Fig. 2, lanes e to h). Precipitation with normal rabbit serum followed by treatment with staphylococci resulted in specific loss of the

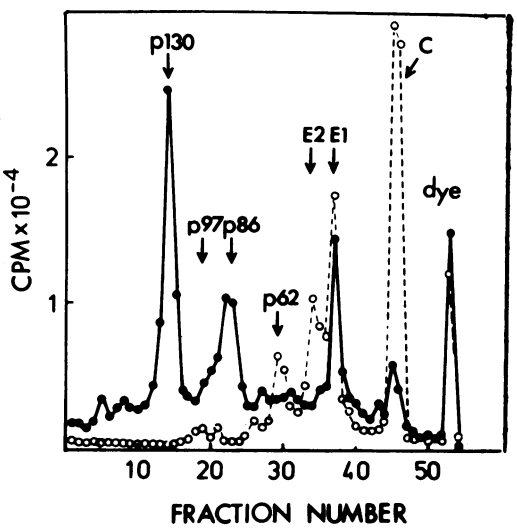


FIG. 1. *Gel fractionation pattern of proteins synthesized at 39°C in cells infected with ts-3 (●) and wild-type (○) SFV. Cultures of CEF cells on 50-mm plates were infected with ts-3 or wild-type SFV with 50 PFU/cell. At 5 h postinfection the cultures were labeled with 10 μCi of [$^{35}$S]methionine per ml for 30 min and chased thereafter for 90 min in MEM containing a 20-fold excess of unlabeled methionine. At harvest the cultures were washed with PBS, and the cells were solubilized in 0.5 ml of SDS-electrophoresis buffer and heated at 100°C for 2 min. Equal volumes of both samples were applied to a slab gel. After electrophoresis the gel was fixed, stained, and fractionated to give 2-mm slices with stacked razor blades. The slices were solubilized in NCS and counted in toluene-based scintillation fluid for radioactivity. The major proteins observed in ts-3-infected cells were the large proteins p130 (contains all structural protein sequences) and p86 (contains the sequences of capsid protein and p62) together with a protein migrating at the position of wild type E1. p97 denotes a protein containing all envelope protein sequences, and C denotes capsid protein.*

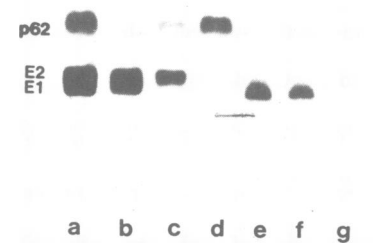


FIG. 2. *Immunoprecipitation of labeled proteins from cells infected with wild-type SFV and ts-3 mutant with antisera prepared against 29S envelope protein octamers and isolated E1, E2, and E3 proteins. The infected cells were labeled at 6 h postinfection for 5 min with 100 μCi of [$^{35}$S]methionine per ml and chased thereafter for 5 min in a 20-fold excess of methionine. At harvest the cells were dissolved in 2% SDS. Immunoprecipitation was performed as described in the text. Lanes a to d: proteins precipitated from wild-type-infected cells with anti-29S (a), anti-E1 (b), anti-E2 (c), and anti-E3 (d). Lanes e to h: proteins precipitated from ts-3-infected cells with anti-29S (e), anti-E1 (f), anti-E2 (g), and anti-E3 (h).*

larger capsid protein containing proteins p130 and p86 as indicated by polyacrylamide gel analysis (data not shown).

**E1 protein in ts-3-infected cells is glycosylated.** The fact that in ts-3-infected cells E1 protein has the same mobility as the E1 in wild-type-infected cells suggests that ts-3-induced E1 is glycosylated. To prove this we used tunicamycin, which is a specific inhibitor of the synthesis of oligosaccharide-lipid intermediate (38, 39). In tunicamycin-treated cells the glycosylation of Sindbis (18, 20, 21) and SFV (8, 29) envelope proteins is completely prevented. As shown previously the mobilities of p62 and E1 of SFV are changed (8, 29), as evidenced also here by a 30-min labeling with [$^{35}$S]methionine (Fig. 3, lanes a and b). The only protein in ts-3-infected cells which showed an altered mobility was E1, implying that only E1 is glycosylated (Fig. 3, lanes c and d).

**E1 protein in ts-3-infected cells is in the lumen of microsomal vesicles.** The glycosylation of alphavirus envelope proteins is connected with their penetration through the membrane into the cysternal side of the endoplasmic reticulum (2). We isolated the microsome fraction from ts-3-infected cells after labeling the proteins with [$^{35}$S]methionine. The resuspended microsomes were exposed to trypsin to digest proteins which were on the cytoplasmic side (2, 3, 9, 44, 45). Microsomes from wild-type-infected cells served as a control (Fig. 4). Parts of the digestions were carried out in the presence of Triton X-100 (Fig. 4, lanes d and e) and Trasylol (Fig. 4, lanes c and f) to control the specificity of the reaction.

In the wild-type-infected cells E1, E2, and p62 were protected from the protease (Fig. 4, lane g). There was a slight mobility change of p62, which

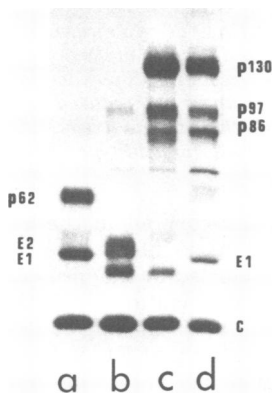


FIG. 3. *Analysis of proteins synthesized in cells infected with ts-3 and wild-type SFV in the presence of tunicamycin. CEF monolayers were infected as described for Fig. 1. Starting after the 1-h adsorption period and throughout the following incubations one set of the cultures was incubated in medium containing 0.5 μg of tunicamycin per ml. At 4 h 20 min after infection the medium was replaced with MEM containing 335 M NaCl. After 40 min of incubation the cultures were labeled with 10 μCi of [$^{35}$S]methionine per ml for 30 min in methionine-free medium. At harvest the cultures were washed with prewarmed PBS, and the cells were scraped into 250 μl of SDS-gel sample buffer. Samples (50 μl) were analyzed by SDS-polyacrylamide (7.5%) gel electrophoresis. After electrophoresis the gel was stained, dried, and fluorographed as described in the text. Lanes a and b: wild-type-infected cells with (b) and without (a) tunicamycin. Lanes c and d: ts-3-infected cells with (c) and without (d) tunicamycin.*

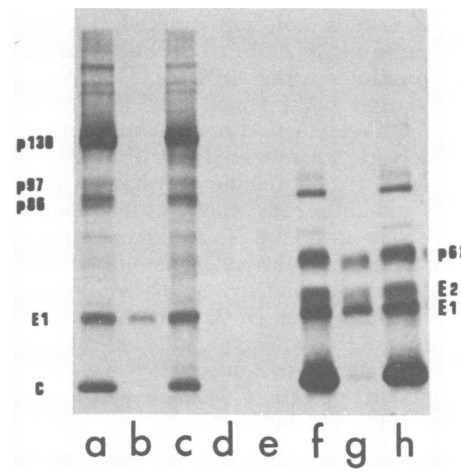


FIG. 4. *Protease treatment of microsome vesicles isolated from cells infected with ts-3 and wild-type SFV. Cultures of CEF on 50-mm plates were infected with 100 PFU/cell and incubated at 39°C. At 6 h postinfection the cultures were labeled with 40 μCi of [$^{35}$S]methionine per ml for 10 min and chased for 20 min in MEM containing a 20-fold excess of unlabeled methionine. After the chase the cultures were placed on ice and washed with ice-cold PBS, and the cells were collected by centrifugation. The microsome vesicles were prepared from the cell pellets as described in the text. Samples of microsome suspensions were incubated for 30 min at 37°C in the presence of trypsin-1-tosylamido-2-phenylethyl-chloromethyl-ketone with or without Trasylol (1,000 U/ml) or Triton X-100 (1%, vol/vol). After treatment the samples were dissolved in SDS-gel sample buffer, heated at 100°C for 2 min, and analyzed by SDS-polyacrylamide (7.5%) gel electrophoresis. The picture shows fluorographs of dried gels. Lanes a to d, ts-3; lanes e to h, wild type; lanes a and h, control microsomes; lanes b to g: microsomes treated with trypsin alone (b, g), in the presence of Trasylol (c, f), or in the presence of Triton X-100 (d, e).*

is probably due to the digestion of the cytoplasmic carboxyterminal part of this protein (3, 9, 44).

All other proteins except E1 (e.g., p130, p97, p86, and capsid proteins) in microsomes from ts-3-infected cells were digested with trypsin (Fig. 4, lane b) indicating that they are located on the cytoplasmic side of the vesicles. There was no detectable mobility change of E1 before and after trypsin treatment (ts-3, Fig. 4, lanes a and b; wild type, Fig. 4, lanes g and h).

The envelope proteins p62 and E1 of wild-type-infected cells as well as the E1 of ts-3-infected cells were protected also in microsomes derived from tunicamycin-treated cells (data not shown). This indicates that these proteins are segregated into the cysternal side of the vesicles also in the absence of glycosylation, as has been reported previously (9, 45).

**E1 of ts-3 is not transported to plasma membrane at 39°C, but can be released in virus particles after shift to 28°C.** We have reported that ts-3-infected cells do not show surface fluorescence with envelope antiserum and fluorescein-conjugated anti-IgG (26). The same result was obtained even when incubation periods and antiserum concentrations were varied. We also used another technique for detection of E1 specific antibodies possibly bound to the cell surface, namely, iodinated protein A from *S. aureus* (12a). Of the $^{125}$I-labeled protein A bound to the wild-type infected-cells, only 0.7% was bound to ts-3-infected cells.

Immunoprecipitation with envelope antiserum administered before the disruption of the cells with Triton X-100 was carried out by using formaldehyde-fixed staphylococci. No evidence of specific precipitation of E1 protein was obtained. In contrast, both E1 and E2 were precipitated in wild-type-infected cells (data not shown).

On the basis of these experiments we conclude that E1 made in ts-3-infected cells was not transported to the plasma membrane during incubation at the restrictive temperature.

We also examined whether E1 made in ts-3-infected cells at the restrictive temperature (39°C) can be incorporated into mature virus released after shift to the permissive temperature. Ts-3-infected cells were first incubated at 28°C for 8 h, shifted to 39°C for 30 min, and labeled for 30 min, followed by a 30-min chase. The cultures were shifted back to 28°C and incubated for 14 h to accumulate enough released virus. The virus was purified, and the labeled viral proteins were analyzed by polyacrylamide gel electrophoresis (Fig. 5). Almost equal amounts of the three major structural proteins were recovered in the virus released from the wild-type-infected cells. Essentially only E1 was labeled in the virus released from the ts-3-infected cultures, which had been shifted to 28°C after labeling at 39°C.

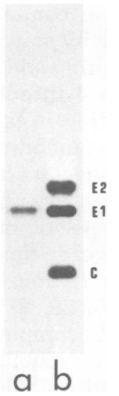


FIG. 5. *Analysis of viral proteins in released virus after shift-down. The infected cultures were first incubated at 28°C in MEM supplemented with 0.2% bovine serum albumin for 5 h. Then the medium was replaced with methionine-free MEM containing 1 μg of actinomycin D per ml, and the cultures were incubated for 3 h at 28°C. At 8 h postinfection the cultures were shifted to 39°C and incubated 30 min and labeled with [$^{35}$S]methionine (20 μCi/ml) for 30 min followed by a 30-min chase with excess unlabeled methionine. Then the cultures were transferred back to 28°C and incubated in the chase medium for 14 h before harvest. The released virus in the culture fluid was collected as described in the text and the proteins in it were analyzed on SDS-polyacrylamide (10%) gel electrophoresis under nonreduced conditions. After electrophoresis the gel was fixed, stained, and fluorographed. (a) ts-3; (b) wild-type SFV.*

This must mean that E1 synthesized at 39°C is competent to become incorporated into the virions together with unlabeled structural proteins synthesized during incubation at the permissive temperature.

## DISCUSSION

The alphavirus structural proteins are translated as a polyprotein in the order $NH_2$-capsid-E3-E2-E1-COOH. If the 26S mRNA is translated in an in vitro system without added microsomal membranes, the translational products are the capsid protein and a nonglycosylated protein, p97, which contains the amino acid sequences of all three envelope proteins (19, 27). When membranes are added to the in vitro system, capsid protein, p62, and E1 are produced. Both p62 and E1 are glycosylated and segregated into the lumen of the rough endoplasmic reticulum (3, 9, 44). If the addition of

membranes is delayed so that a certain number of amino acids of p62 following the capsid protein sequence have been translated, no insertion or cleavage of the envelope proteins occurs. This means that there is a critical period in the translation when the nascent p62 protein can be directed to the lumenal side of the membrane. These experiments have led to the hypothesis suggesting that after the cleavage of the capsid protein a specific signal sequence (2, 11, 22) is revealed which enables the ribosomes to bind to the membrane and segregate the nascent envelope protein to the lumen of the rough endoplasmic reticulum.

Since all of the envelope proteins are translated as parts of the same polyprotein one would assume that a single signal sequence is sufficient for the segregation of both p62 and E1. Our results with ts-3, however, suggest that there is a separate signal sequence for E1. In ts-3-infected cells E1 clearly is synthesized in excess compared with p62 even after shorter pulse (16), excluding the possibility that the latter would be able to direct the E1 protein through the endoplasmic reticulum membrane. The existence of an abnormal fusion protein, p86, consisting of capsid protein and p62 (19) explains the absence of p62 in the ts-3-infected cells.

The p86 protein is most probably not glycosylated, since a protein of similar size was found in tunicamycin-treated cells (Fig. 3). It is probably located on the cytoplasmic side of the microsomal membranes as evidenced by its sensitivity to added trypsin. This would mean that in ts-3-infected cells the cleavage between p86 and E1 takes place at the cytoplasmic side of the endoplasmic reticulum. To explain the segregation and glycosylation of E1 we must assume that there is a separate signal sequence for E1.

In light of these findings with ts-3 we suggest that in SFV-infected cells, after the translation of p62, a nascent cleavage takes place revealing the signal sequence for E1. This signal sequence probably precedes the $NH_2$ terminus of the mature E1 since the sequence of the first 20 amino acids of purified E1 does not show features typical of known signal sequences (14).

A 6K virus-specific protein has recently been isolated from microsomes of SFV-infected cells (43). It has been proposed that this protein is derived as a result of the processing of the structural polyprotein, e.g., by cleavage of a signal sequence. It is tempting to assume that the 6K polypeptide is indeed the signal sequence which precedes E1 protein. Since a similar polypeptide (4.2K) has been isolated from Sindbis virus-infected cells (42) a second signal sequence could be expected with this virus as well.

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