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Assembly of the Semliki Forest Virus Membrane Glycoproteins in the Membrane of the Endoplasmic Reticulum *in Vitro*

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A cell-free system has been constructed to study the mechanism by which a single messenger RNA directs the synthesis of proteins destined for two different cellular locations. The Semliki Forest virus (SFV) 26 S mRNA codes for the viral capsid protein (C protein) and the membrane proteins p62 and E1. The three virus proteins are read in this order from the messenger RNA using one initiation site. The C protein is left on the cytoplasmic side and the p62 and the E1 proteins are inserted into the endoplasmic reticulum membrane. Translation of 26 S mRNA in a HeLa cell-free system in the presence of microsomes from dog pancreas reproduced the segregation, and proteolytic processing and glycosylation observed in infected cells. The signal for membrane binding was in the amino-terminal end of p62. The results indicate that the membrane proteins become inserted in the nascent state. The cleavage between p62 and E1 was coupled to membrane insertion. If the membranes were added after a period corresponding to the synthesis of about 100 amino acids of the p62 protein, segregation, glycosylation and cleavage between p62 and E1 failed to occur.

1. Introduction

One essential problem in cell biology is to find out how proteins destined for different locations within the cell reach their destinations. The mechanism by which secretory proteins become segregated into the lumen of the endoplasmic reticulum has been greatly clarified by the construction of cell-free systems capable of specifically transferring secretory proteins into the lumen of rough microsomal vesicles (Blobel & Dobberstein, 1975). Secretory proteins contain a short sequence at their amino terminus termed the signal sequence, which is thought to provide the conditions for vectorial discharge of the nascent polypeptide chain into the cisternal space (see Campbell & Blobel, 1977). An analogous cell-free system has recently been devised for the synthesis and insertion of membrane glycoproteins. The vesicular stomatitis virus glycoprotein was incorporated into the endoplasmic reticulum membrane spanning the bilayer in the correct orientation (Katz *et al.*, 1977). Moreover, a tight coupling between polypeptide synthesis and membrane insertion and glycosylation was shown (Rothman & Lodish, 1977).

Semliki Forest virus (SFV) provides an interesting model system (Simons *et al.*, 1978) to study how a single messenger RNA directs the synthesis of proteins destined for different cellular locations. In cells infected with SFV, all the structural proteins of the virus are read sequentially from a 26 S mRNA using one initiation site (Clegg

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& Kennedy, 1975*b*; Clegg, 1975; Glanville *et al.*, 1976*b*). The proteins are made in the following order: capsid protein (C protein, $M_r = 30 \times 10^3$, Simons & Kääriäinen, 1970), p62 ($M_r = 62 \times 10^3$, Simons *et al.*, 1973) and E1 ($M_r = 49 \times 10^3$, Garoff *et al.*, 1974). The C protein is cleaved from the nascent chain and associates in the cytoplasm with the viral 42 S RNA molecule to form nucleocapsids (Scheele & Pfefferkorn, 1969; Söderlund, 1973). The major part of both membrane proteins is transferred through the rough endoplasmic reticulum membrane to its cisternal side, where they remain attached to the lipid bilayer by their hydrophobic carboxy-terminal ends (Garoff & Söderlund, 1978; Wirth *et al.*, 1977). The p62 protein spans the endoplasmic reticulum membrane and has a small segment of about $M_r = 3 \times 10^3$ at its carboxy-terminal end located on the cytoplasmic side of the membrane. The p62 and E1 proteins are transported from the endoplasmic reticulum to the plasma membrane, probably as a p62–E1 complex. At the plasma membrane they assemble with nucleocapsids into virus particles (Acheson & Tamm, 1967; Richardson & Vance, 1976). Before incorporation into virus particles, the p62 protein is cleaved to form E3 ($M_r = 10 \times 10^3$) and E2 ($M_r = 52 \times 10^3$) (Simons *et al.*, 1973; Garoff *et al.*, 1974). E3 is derived from the amino-terminal end of p62. One chain each of E1, E2 and E3 are associated with one another on the virus surface and form the external glycoprotein projections (Ziemiecki & Garoff, 1978).

To study how the SFV membrane proteins are inserted into the membrane, cleaved and glycosylated, we have constructed a cell-free system in which the viral proteins have been translated from the 26 S mRNA in the presence of microsomes from dog pancreas. The C protein was left outside the microsomal vesicles, while the p62 and the E1 proteins were inserted into the membrane with the correct orientation. Membrane insertion, p62–E1 cleavage and glycosylation failed to occur if the membranes were added later than a critical time occurring shortly after the C protein had been completed and removed.

2. Materials and Methods

(a) *Preparation of 26 S mRNA*

26 S mRNA was isolated from baby hamster kidney cells that had been infected with SFV (Glanville *et al.*, 1976*a*). The isolated RNA was dissolved in water at a concentration of 2 mg/ml and stored at −80°C.

(b) *Preparation of HeLa cell extract*

HeLa cell lysate was prepared in essence as described before (Maxon *et al.*, 1972). Some modifications were made to facilitate the complete translation of the large 26 S mRNA of SFV. Cells of a 3 to 4-l suspension culture (approx. 10^9 cells) were collected by centrifugation and washed 3 times with phosphate-buffered saline (Dulbecco, 1954) and once with a buffer containing 20 mM-HEPES-KOH (pH 7·4), 100 mM-KCl, 2 mM-magnesium acetate and 6 mM-2-mercaptoethanol. The cell pellet was resuspended in 2 volumes of a hypotonic buffer containing 10 mM-HEPES-KOH (pH 7·4), 15 mM-KCl, 1·5 mM-magnesium acetate and 6 mM-2-mercaptoethanol. After swelling for 15 min at 0°C, the cells were broken by 10 strokes in a Dounce homogenizer. One-tenth volume of a buffer containing 20 mM-HEPES-KOH (pH 7·4) and 0·9 M-KCl was added and the homogenate was centrifuged at 40,000 *g* for 20 min. The supernatant was collected and passed through a Sephadex G25 column equilibrated with a buffer containing 20 mM-HEPES-KOH (pH 7·4), 100 mM-KCl, 2 mM-magnesium acetate and 6 mM-2-mercaptoethanol. The fractions emerging in the void volume contained the HeLa lysate used for *in vitro* translations. It

was divided into several portions and stored at −80°C. Before use endogenous mRNA of the lysate was inactivated by nuclease digestion as described below for microsomal preparations.

(c) *Preparation of microsomal membranes*

Rough endoplasmic reticulum microsomes were isolated from homogenized cells of dog pancreas (kindly provided by Dr S. Hoyer) by centrifugation in a discontinuous sucrose gradient (Blobel & Dobberstein, 1975). The rough microsomes were collected at the interface of the 1·75 M/2·1 M-sucrose and diluted with ice-cold buffer containing 20 mM-HEPES-KOH (pH 7·7), 50 mM-KCl, 2 mM-magnesium acetate and 2 mM-dithiothreitol, to a concentration of about 100 A_{260} units/ml. Endogenous RNA was digested with micrococcal nuclease (80 units/ml) (Boehringer) in the presence of 1 mM-$CaCl_2$ (Pelham & Jackson, 1976). After incubating for 10 min at 20°C the mixture was cooled to 0°C and nuclease-inactivated by adding EGTA† (pH 7·0) to a final concentration of 4 mM. Ribosomes were dissociated by treating with 10 mM-EDTA (pH 7·0) (Blobel & Dobberstein, 1975) and microsomes were re-isolated by pelleting at 100,000 *g* for 1 h through 5 ml of 0·5 M-sucrose in 20 mM-HEPES-KOH (pH 7·4), 50 mM-KCl, 2 mM-magnesium acetate and 2 mM-dithiothreitol. The pelleted membranes were resuspended by homogenization in 20 mM-HEPES-KOH (pH 7·4), 130 mM-potassium acetate, 2·2 mM-magnesium acetate and 2 mM-dithiothreitol to a final concentration of about 10 mg protein/ml and then divided into several portions which were frozen in liquid nitrogen and stored at −80°C.

Rough microsomal membranes (about 5 to 10 mg protein/ml) of SFV-infected cells were obtained by density-gradient centrifugation as described before (Wirth *et al.*, 1977).

(d) *Cell-free synthesis*

Translation mixtures contained the following final concentrations of substances: 20 mM-HEPES-KOH (pH 7·4), 40 mM-KCl, 100 mM-potassium acetate, 2·2 mM-magnesium acetate, 80 μM-spermine, 2 mM-dithiothreitol, 1 mM-ATP, 50 μM-GTP, 8 mM-creatine phosphate, 40 μg creatine phosphokinase/ml, 25 μM- of all amino acids except methionine. A total of 10 μCi of [^{35}S]methionine (500 Ci/mmol) (Amersham-Searle), 40 μl of nuclease-treated HeLa lysate and 40 μg of mRNA were added per 100 μl of the mixture. Synchronization of translation was accomplished by adding 7-methylguanosine 5′-monophosphate (P-L Biochemicals, Inc.) (1·5 mM) 2 min after starting the incubation (Rothman & Lodish, 1977; Weber *et al.*, 1977). All incubations were for 90 min at 30°C. When membranes had to be present, 10 μl of the nuclease and EDTA-treated pancreatic microsomes were added per 100 μl of translation mixture before, or at various times after starting, the synthesis. The presence of membranes in the translation mixture inhibited protein synthesis by less than 20%.

(e) *Analysis of* in vitro *products*

Proteins made *in vitro* were precipitated in 10% (w/v) trichloroacetic acid at 0°C and analyzed by sodium dodecyl sulphate/polyacrylamide gel electrophoresis as described by Blobel & Dobberstein (1975). The acrylamide concentration of the separating slab gel was 10% (w/v) and the running time was 16 h at 30 mA and at 25°C. Protein bands were visualized by fluorography (Bonner & Laskey, 1974). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate according to Neville (1971) was used to isolate proteins for peptide analyses. The proteins were eluted from the wet slab gel and cleaved at their cysteine residues by treatment with 2-nitro-5-thiocyanobenzoic acid (NTCB, Eastman Kodak) (Jacobson *et al.*, 1973; Degani & Patchornik, 1974). The NTCB peptides were resolved by electrophoresis on 15% to 22·5% gradient gels and visualized by fluorography. Quantitative measurements of [^{35}S]methionine-labelled protein species were made by densitometric tracing of protein bands on the fluorographs using a Joyce–Loebel densitometer. The E1, p62 and C proteins contain 17, 20 and 27 mol methionine residues/thousand moles of amino acids, respectively (Simons & Kääriäinen, 1970; Garoff *et al.*, 1974).

† Abbreviations used: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)*N*, *N*′-tetraacetic acid; NTCB, 2-nitro-5-thiocyanobenzoic acid.

Protease treatment of *in vitro* translation mixtures (10 μl) or rough microsomes of cells infected with SFV (10 μl; approx. 50 μg protein) was carried out by adding 50 μg of chymotrypsin (Worthington) or proteinase K (Merck) in 40 μl of 0·05 M-Tris (pH 7·4), 0·1 M-NaCl and incubating for 60 min (chymotrypsin) or 15 min (proteinase K) at 37°C. After the incubation, 50 μg of either a chymotrypsin inhibitor (tosylphenylalaninechloromethylketone: Sigma) or a proteinase K inhibitor (phenylmethylsulfonylfluoride: Sigma) was added. The proteins that had been protected by the membranes from proteolysis were precipitated by the addition of trichloroacetic acid and analyzed by gel electrophoresis as described above.

3. Results

(a) *Translation of 26 S mRNA in an HeLa cell-free system in the absence of membranes*

When 26 S mRNA was translated in the HeLa cell-free system, two proteins were synthesized (Fig. 1(a)). One (67% of the protein made) comigrated with the C protein from cells infected with Semliki Forest virus, and the other (33%) comigrated with the 97K protein ($M_r = 97 \times 10^3$). The 97K protein of infected cells is known to be unglycosylated and contain the amino acid sequences of E1 and p62 (Sefton & Burge, 1973; Lachmi *et al.*, 1975). To demonstrate that the "C" protein made *in vitro* was identical with that made in infected cells, the proteins were cleaved at their cysteine residues with NTCB (Jacobson *et al.*, 1973) and the peptides analysed by gel electrophoresis (Fig. 2(b) and (c)). The peptide patterns obtained showed that authentic C protein was produced by the *in vitro* system. NTCB peptides of the 97K protein (Fig. 2(a)) showed almost no resemblance to those of E1 and p62 (Fig. 2(e) and (g)). The fact that E1 and p62 are glycosylated and proteolytically processed most likely changes the mobility of most of their NTCB peptides compared to those of the 97K protein. Taking together the results described below, however, there is little doubt about the nature of the 97K protein. The synthesis of this protein in the time-course study (section (d), below) and the membrane addition experiment (section (e), below) clearly suggest that the 97K protein is read from the same part of the messenger RNA as are the E1 and the p62 proteins.

In comparison to previous *in vitro* systems used for the translation of 26 S mRNA from SFV (or the closely related Sindbis virus) the present HeLa cell-free system gave an appreciable increase in the translation efficiency, as judged by the amount of the 97K protein produced. None or only small amounts of this protein had been detected in previous studies (Glanville *et al.*, 1976*a*,*b*; Simmons & Strauss, 1974; Wengler *et al.*, 1974; Cancedda *et al.*, 1974; Clegg & Kennedy, 1975*a*).

(b) *Translation of 26 S mRNA in the presence of membranes*

When 26 S mRNA is translated in the presence of dog pancreatic microsomes, two new proteins were made in addition to the C protein (65% of the protein made) and the 97K protein (2%). One (8%) comigrated with the E1 protein and the other (25%) comigrated with the p62 protein from infected cells (Fig. 1(b), (d) and (e)). The p62 protein from infected cells and its corresponding *in vitro* product are seen as split bands†. NTCB peptide analyses of the E1 counterpart made *in vitro* showed that this

† We denote the precursor for E3 and E2 as p62 following the nomenclature agreed on at the International alphavirus symposium in Helsinki, 1975. With the high-resolution gel electrophoresis system used here, p62 splits into two bands. The major band has an apparent M_r of 62×10^3 and the minor band has $M_r = 65 \times 10^3$ (see Discussion).

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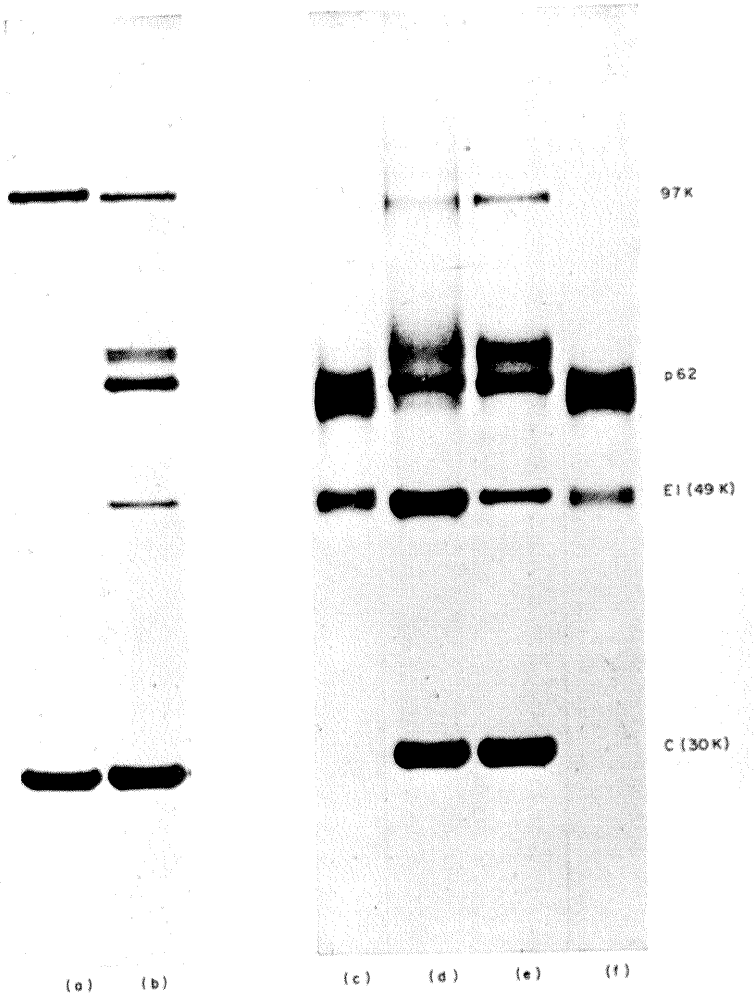


FIG. 1. Translation of 26 S mRNA *in vitro*.

(a) and (b) The proteins synthesized *in vitro* in the absence (a) and in the presence (b) of membranes.

(c) and (d) The proteins found in the rough microsomal fraction of SFV-infected cells before (d) and after (c) chymotrypsin treatment. Infected cells were pulse-labelled for 10 min with [^{35}S]methionine 4 h after infection.

(e) and (f) The proteins found after *in vitro* synthesis in the presence of membranes when analyzed before (e) and after (f) chymotrypsin treatment. Samples (10 μl) containing about 5×10^4 to 10×10^4 cts/min of ^{35}S radioactivity were analyzed by sodium dodecyl sulphate/polyacrylamide gel electrophoresis. In the case of the chymotrypsin-treated samples, double the sample volume was analyzed.

C is the capsid protein ($M_r = 30\times10^3$) and E1 ($M_r = 49\times10^3$) and p62 are the membrane proteins of SFV. 97K is a protein containing the amino acid sequences of E1 and p62. If 26 S mRNA was omitted from the *in vitro* translation mixture none of these proteins was made.

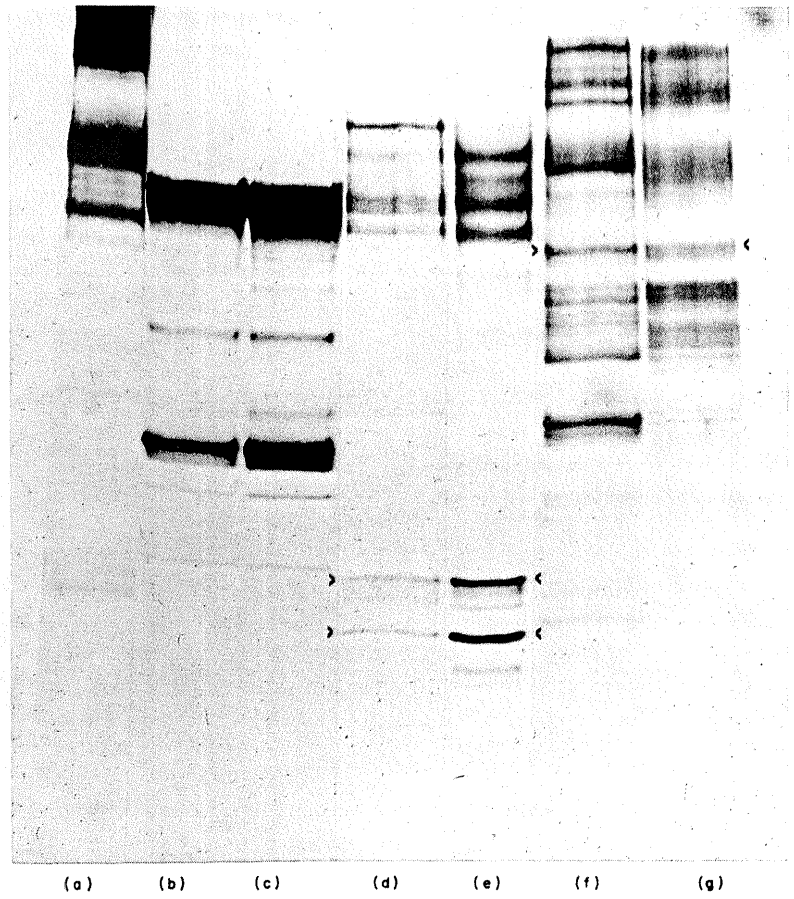
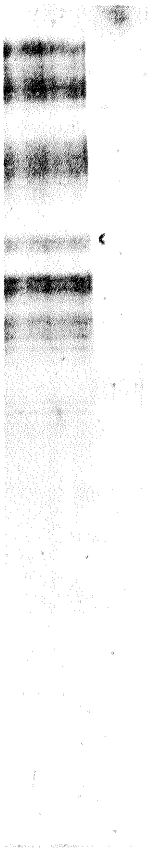


FIG. 2. Identification of *in vitro* products.

Proteins of *in vitro* translates and of microsomes from infected cells were isolated by sodium dodecyl sulphate/polyacrylamide gel electrophoresis, cleaved with NTCB and analyzed by electrophoresis on a 15% to 22·5% gradient gel. The lower arrowhead in lanes (d) and (e) indicates the amino-terminal peptide ($M_r = 5{\cdot}5 \times 10^3$) of E1 and the upper arrowhead indicates a peptide ($M_r = 7 \times 10^3$) characteristic for the carboxy-terminal end of the E1 protein. Arrowheads in lanes (f) and (g) indicate a peptide ($M_r = 25 \times 10^3$) characteristic for the carboxy-terminal end of p62 (Garoff & Söderlund, 1978).

The lanes show NTCB peptides of: (a) 97K protein made *in vitro*; (b) C protein from microsomes of infected cells; (c) C proteins made *in vitro*; (d) E1 protein made *in vitro*; (e) E1 protein from microsomes of infected cells; (f) p62 made *in vitro*; (g) p62 from microsomes of infected cells.



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protein represents the E1 polypeptide (Fig. 2 (d) and (e)). Both the amino-terminal 5·5K NTCB peptide of E1 and the 7K NTCB peptide characterizing its carboxy terminus (Garoff & Söderlund, 1978) are present in this product. Analysis of the NTCB peptides of p62 and its corresponding *in vitro* product showed similar patterns. Some migration differences were seen however. These might be explained by variations in the degree of glycosylation of these proteins. The p62 protein of infected cells may undergo some carbohydrate processing during its labelling and isolation which does not take place *in vitro* (Tabas *et al.*, 1978). It should be noted that probably all the large NTCB peptides of p62 seen in Figure 2 represent only partially cleaved products, each of which contains at least one of the three or four oligosaccharide side-chains of the p62 protein (Mattila *et al.*, 1976; Garoff, unpublished results). The authenticity of the *in vitro* made p62 protein, however, is strongly suggested by its double banded appearance on polyacrylamide gels (Fig. 1(e)) and its correct location in the microsomal membrane (see below).

The glycosylation of the *in vitro* made membrane proteins was assessed from their migration in polyacrylamide gels. In cells infected with SFV, the p62 and the E1 proteins become glycosylated through the transfer of sugar core units of about $M_r = 2 \times 10^3$ from lipid intermediates to the polypeptide chains (Sefton, 1977; Krag & Robbins, 1977). Three to four such units are thought to be added to the p62 protein and one to the E1 protein (Mattila *et al.*, 1976). If the formation of the lipid–sugar intermediate is inhibited in the cell by adding tunicamycin, then completely non-glycosylated forms of the p62 and the E1 proteins are made (Garoff & Schwarz, 1978). These forms of the membrane proteins are easily distinguishable from their glycosylated counterparts on polyacrylamide gels. The unglycosylated p62 protein runs as a split band with an apparent M_r of 54×10^3 to 57×10^3 and that of E1 as a band with $M_r = 47 \times 10^3$. As only bands corresponding to the glycosylated forms of the viral membrane proteins were seen during *in vitro* translation in the presence of microsomes, we suggest that glycosylation of p62 and E1 does occur in this system.

(c) *Segregation of* in vitro *products*

Protease treatment of the translation mixture was used to study the segregation of the *in vitro* products. Previous studies have shown that protease treatment of microsomes of cells infected with SFV results in complete degradation of the C and the 97K proteins and in the removal of about $M_r = 3 \times 10^3$ from the carboxy-terminal end of the p62 protein (Wirth *et al.*, 1977). The rest of the p62 protein and all of the E1 protein remain intact. Figure 1(f) shows the effect of protease treatment on the viral proteins that have been synthesized *in vitro* in the presence of membranes. The C and the 97K proteins were completely degraded, whereas the E1 protein and the p62 protein were protected from proteolysis. The mobility of the p62 protein showed the same small increase in mobility after protease treatment as seen for p62 in protease-treated microsomes isolated from infected cells (Fig. 1(c)). The chymotrypsin-treated p62 is seen as a broad band in Figure 1(c) and (f). When proteinase K was used instead of chymotrypsin the broad band was split into two bands. These results show that the p62 and E1 proteins made *in vitro* segregate into the microsomes, and that the p62 protein (and probably also E1) becomes correctly oriented in the membrane. Some losses of p62 and E1 (about 25%) were observed

after protease treatment. This is most likely due to the presence of some unsealed vesicles (Sabatini & Blobel, 1970). If the membranes were solubilized with 0·5% Triton X-100 before protease treatment, the p62 and E1 proteins were completely degraded.

(d) *Coupling of protein synthesis with membrane insertion, glycosylation and p62–E1 cleavage*

Having established an *in vitro* system in which the viral membrane proteins become correctly inserted into the lipid bilayer and are cleaved and glycosylated, we went on to study how these events were temporally correlated with the synthesis of these proteins. In this experiment 26 S mRNA was translated in the presence of membranes and two samples were taken every second minute after initiation of synthesis. One sample was analyzed directly by gel electrophoresis and the other after protease treatment. Figure 3 shows the sequential translation of the viral proteins. The C protein appeared eight minutes after initiation of protein synthesis (not shown). The p62 protein appeared as a split band after 24 minutes and the E1 and the 97K protein appeared together after 32 minutes. Thus the whole 26 S mRNA was translated into protein of about $M_r = 130 \times 10^3$ in 32 minutes. Assuming a constant rate of translation, we can calculate that the capsid protein ($M_r = 30 \times 10^3$) should be made in 7·4 minutes and the p62 (M_r of the protein part 54×10^3 to 57×10^3) in 13·3 to 14 minutes; that is, about 21 minutes after initiation. This means that the cleavage of both the capsid and the p62 proteins occurs very soon after completion.

The glycosylation of p62 and E1 must also occur before or soon after chain completion, since no band corresponding to unglycosylated forms of p62 or E1 was detected in this experiment. A more exact timing of the addition of the oligosaccharide chains to the p62 and E1 proteins was attempted. Addition of Triton X-100 to a translation mixture containing membranes inhibits the glycosylation but not polypeptide chain elongation (Rothman & Lodish, 1977). If glycosylated or partially glycosylated forms of a glycoprotein are found when Triton X-100 is added to a translation mixture before chain completion, then glycosylation must have occurred on the nascent chain. This approach was only partially successful, since in the case of SFV, Triton X-100 treatment inhibited the p62–E1 cleavage if added before p62 completion. The 97K protein which was found under these conditions showed more slowly migrating forms in polyacrylamide gels, which might reflect nascent glycosylation of the "p62" part (not shown).

Analyses of the samples after chymotrypsin treatment (Fig. 3) show that the earliest detectable p62 protein (after 24 minutes of synthesis) and E1 protein (after 32 minutes) are already protected. The membrane proteins must therefore be inserted into the microsomal membranes either very soon after completion or, what seems more likely, they are continuously transferred across the membrane during synthesis. If the polyacrylamide gel containing the 10 to 22-minute samples after protease treatment were autoradiographed for a longer time than shown in Figure 3, protected material was seen as diffuse bands in the region between the C and the p62 proteins (Fig. 4). The bands increase in size from the 10-minute sample to the 22-minute sample. In the 24-minute sample much less of these bands was seen and in later samples almost none. It is possible that this material represents nascent chains of the p62 protein that have been extruded across the membrane. However, this has to be confirmed by peptide analyses.

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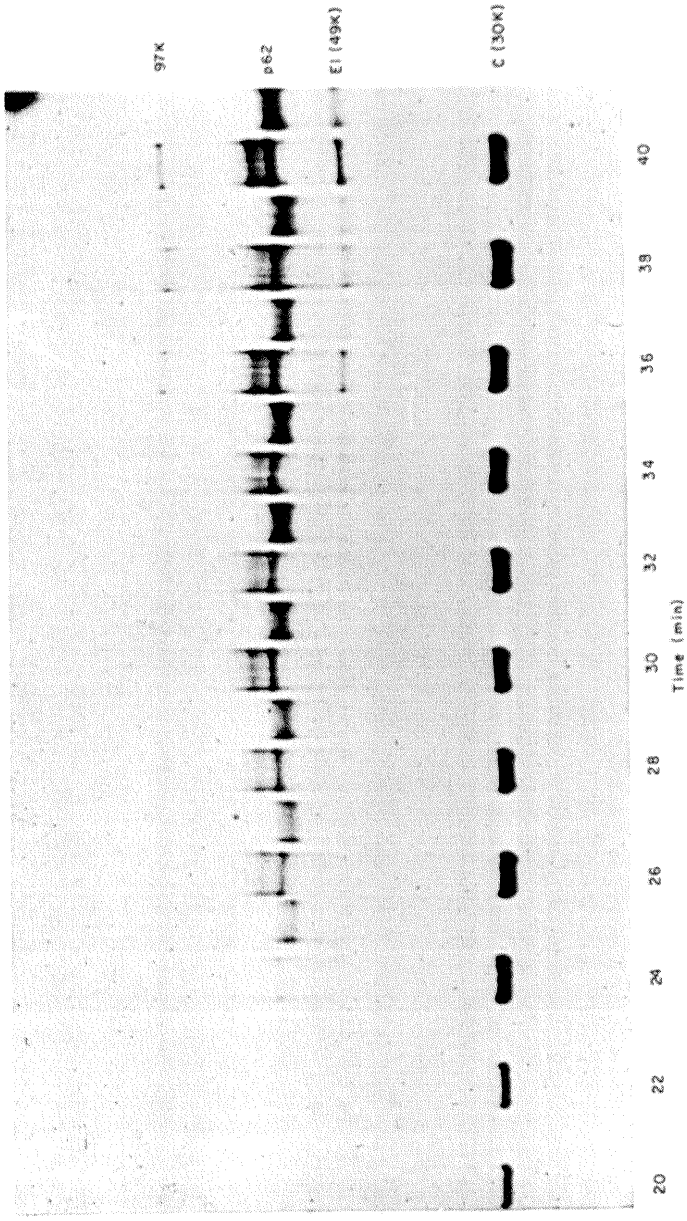

FIG. 3. Coupling of membrane protein synthesis with insertion, cleavage and glycosylation.

The 26 S mRNA was translated in the presence of membranes. Every second minute (see base of Figure) 2 samples were taken for electrophoretic analysis, one (10 μl) directly (lane above the time) and the other (20 μl) after protease treatment (lane to the right). The positions of p62, E1 ($M_r = 49\times10^3$), C protein ($M_r = 30\times10^3$) and the 97K protein are indicated.

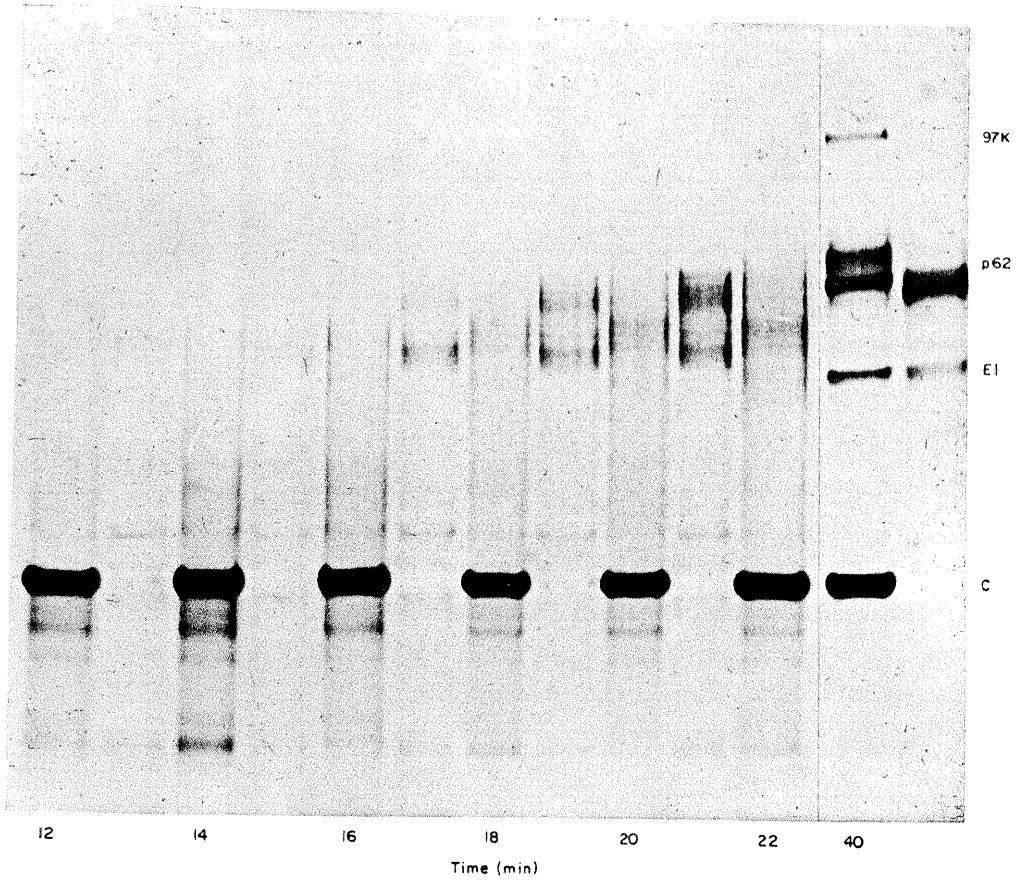

FIG. 4. Coupling of membrane protein synthesis with insertion.
The 12 to 22 minute samples of Fig. 3 are here autoradiographed for 1 week more to show what appears to be protected nascent chains of the p62 protein.

(e) *Critical timing for membrane addition*

Membranes have to be present during translation of the 26 S mRNA for membrane protein insertion, cleavage, and glycosylation to occur. If membranes are added after completion of translation and the mixture further incubated, the 97K protein made is neither inserted into the membrane nor cleaved to p62 and E1. To determine the latest time at which membranes can be added after protein synthesis has been started for membrane insertion to occur, translation of the 26 S mRNA was synchronized with 7-methylguanosine-5′-phosphate. At two-minute intervals after synchronous translation of 26 S mRNA had been started in the absence of microsomes, two samples were removed from the translation mixture. One sample was electrophoresed directly to monitor the rate of protein synthesis. Membranes were added to the other and the translation completed. The samples which were completed in the presence of membranes were analyzed by electrophoresis either as such or after protease treatment. This approach has been used to study the membrane requirements for synthesis of the G protein of vesicular stomatitis virus *in vitro* (Rothmann & Lodish, 1977). Figure 5 shows that the C protein of SFV was completed in less than eight minutes.

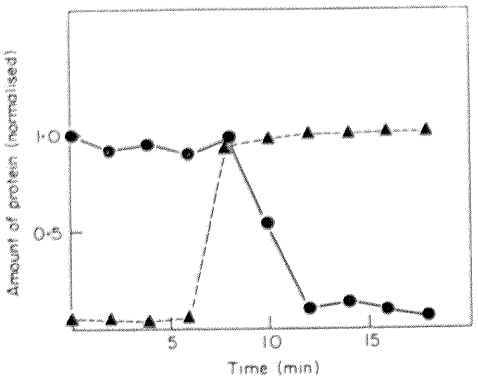

FIG. 5. The critical time for membrane addition.

The 26 S mRNA was synchronously translated in the absence of membranes (see Materials and Methods). At the start and at intervals of 2 min thereafter, two 10-μl samples were taken: one for direct electrophoretic analysis and another for further incubation in the presence of membranes. The membrane-containing samples after completion of translation were analysed on sodium dodecyl sulphate/polyacrylamide gels directly and after protease treatment. The quantities of C protein made at different times after starting synthesis (– ▲ – ▲ –), and the amount of protected p62 protein that was present after completion of synthesis when membranes were added at various times after initiation (— ● — ●—) are shown. The amounts were measured by densitometric tracing of the corresponding fluorographs. The amount of C protein is given as a fraction of the final amount made and that of p62 is given as a fraction of the amount protected p62 made when membranes were added at the start of synthesis.

After this time-point little increase in the amount of C protein occurred, demonstrating that translation was synchronized. If the membranes were added to the translation mixture within the first eight minutes of translation, the amount of p62 (and E1, not shown) made was the same as if the membranes had been present from the beginning of synthesis. Membrane addition at ten minutes resulted in a dramatic reduction in the amount of p62 (and E1) produced and, if the microsomes were added still later, only C and 97K proteins were made. All 97K protein made was susceptible to protease. Membrane insertion is thus possible only during the short period between the time after the capsid protein has been completed and removed, and until the amino terminus of p62 has been extended to about 100 residues (calculated from the rate of protein synthesis observed under these conditions).

4. Discussion

An interesting feature of the Semliki Forest virus 26 S mRNA is that it directs the translation of proteins destined for both the cytoplasm and the endoplasmic reticulum membrane. To study how this segregation is achieved, we have constructed a cell-free system in which the protein-synthesizing machinery originates from HeLa cells and the microsomes from dog pancreas. This heterologous system is capable of reproducing the protein segregation and the proteolytic processing and glycosylation occurring during Semliki Forest virus protein synthesis in the infected cell.

The sequence of events suggested by our results is summarized in Figure 6. The C protein is translated first by ribosomes in the free state. As soon as the C protein is completed it is released from the growing chain. No membranes are required for its synthesis. In the infected cell the C protein has been shown to attach to the 60 S

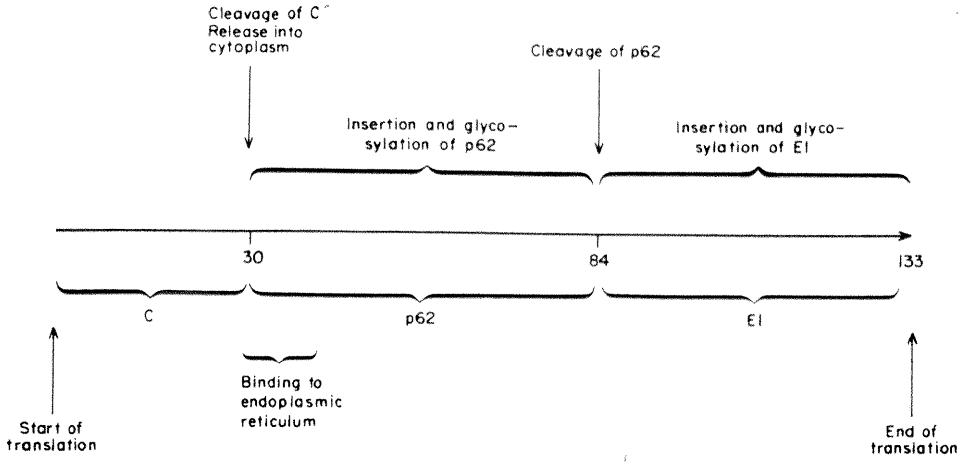

FIG. 6. A schematic representation of the sequence of events during the translation of 26 S mRNA in the presence of membranes. 30 is the molecular weight $\times 10^{-3}$ of the C protein, 84 is the combined molecular weight $\times 10^{-3}$ of the C and the p62 proteins, and 133 is the combined molecular weight $\times 10^{-3}$ of the C, p62 and E1 proteins. The membrane insertion and glycosylation of the membrane proteins are here assumed to take place when these are still in the nascent state.

ribosomal subunit before association with the viral 42 S RNA into nucleocapsids (Söderlund & Ulmanen, 1977). The fate of the Semliki Forest virus membrane proteins is decided shortly after C protein cleavage. If microsomes are added to the growing polypeptide chain, before about 100 amino acid residues of p62 have been made, segregation occurs and the membrane proteins become inserted in the microsomal membrane. Presumably a signal analogous to those found on secretory proteins (see Campbell & Blobel, 1977) resides in the amino-terminal end of p62 which binds the ribosomes to the microsomal membrane as previously suggested by Wirth *et al.* (1977).

Both our results and the study reported by Rothman & Lodish (1977) on vesicular stomatitis virus glycoprotein synthesis show that membrane insertion is tightly coupled to protein translation. In the case of the glycoprotein of vesicular stomatitis virus, the timing of membrane addition was such that two minutes after beginning of translation, membrane insertion was no longer possible. In the SFV system the critical period of membrane addition was later, between 10 and 12 minutes after initiation of protein synthesis at the time when the amino terminus of p62 was being made. In both cases the requirements for establishing a functional ribosome–membrane junction are so stringent that after about 80 to 100 amino acid residues (calculated from the rate of protein synthesis) have been made of the region containing the signal for membrane binding, the capacity for membrane insertion of the nascent chain is lost. It is possible that the nascent chain undergoes a conformational change after elongation has proceeded beyond a critical chain length which inhibits the interaction of the signal peptide with the membrane. Alternatively, too long a nascent chain may obstruct functional binding by preventing direct ribosome–membrane interactions (Unwin, 1977).

If the ribosome translating the 26 S mRNA fails to bind to the microsomes, then membrane proteins seem to be translated as one large 97K polypeptide, which

remains outside the microsomal vesicles (see Figs 1 and 3). This protein is also formed in infected cells and has been shown to be unglycosylated (Sefton & Burge, 1973). Whether the 97K protein is made or not must depend on the efficiency of ribosome binding, and this may vary with different host cells (Strauss *et al.*, 1969). One source of variation could be the number of appropriate ribosome attachment sites available in the endoplasmic reticulum of the cell. The fact that more C protein is usually made during infection than p62 and E1 proteins is at least partially explained by 97K protein formation.

The cleavage between p62 and E1 is coupled to membrane insertion, and occurs as soon as the p62 chain has been completed. We do not know on which side of the microsomal membrane this cleavage takes place. Wirth *et al.* (1977) have proposed that in infected cells the cleavage takes place on the cytoplasmic side of the endoplasmic reticulum membrane. This cleavage would expose the amino terminus of E1 which might function as a signal for new membrane attachment. Another possibility is that the carboxy terminus of p62 not only spans the bilayer but that the chain loops back again into the membrane bringing its carboxy-terminal end to the cisternal side of the endoplasmic reticulum, where the cleavage would occur as soon as the loop has been inserted. No new signal would be required in this case, as the amino terminus of E1 would already be inserted into the membrane and its extrusion could continue as chain elongation proceeds. This mechanism would imply that proteins which traverse the membrane more than once (Henderson & Unwin, 1975) could be synthesized by membrane-bound ribosomes and inserted into the membrane in the nascent state.

The heterogeneity of the p62 protein seen by electrophoresis (Figs 1 and 3) seems not to be due to differences in glycosylation. Also in tunicamycin-treated cells infected with Semliki Forest virus, two bands of the precursor are seen with reduced molecular weights (Garoff & Schwarz, 1978). The heterogeneity is most probably in the amino terminus of the p62 protein, since proteinase K was found to cleave the carboxy-terminal fragment from the p62 protein inserted into the microsomal membrane without affecting the heterogeneity. An incomplete proteolytic removal of a presumptive signal peptide of p62 could explain the two bands of p62.

Whether any cellular mRNAs will use the strategy employed by the 26 S mRNA to code for both a cytoplasmic polypeptide and a membrane polypeptide with a signal for segregation inserted between them remains to be seen. This strategy could be used to synchronize the synthesis of a transmembrane protein with the synthesis of a peripheral protein with which the transmembrane protein interacts on the cytoplasmic membrane face. This interaction could take place in a different cellular compartment, as is the case in cells infected with Semliki Forest virus where the nucleocapsid binds to the spike protein at the plasma membrane before budding (Garoff & Simons, 1974).

The sites in the microsomal membranes to which the ribosomes with the nascent polypeptide chains bind and through which polypeptides are transferred are still uncharacterized. It will be important to find out whether secretory proteins, surface membrane glycoproteins and other organelle proteins use the same or different sites for ribosome attachment. The many functions involved in protein transfer imply that a number of rough microsomal proteins may play a role in these processes.

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