Formation of the Semliki Forest Virus Membrane Glycoprotein Complexes in the Infected Cell

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SUMMARY

In Semliki Forest virus (SFV)-infected cells, all structural proteins are translated from a 26S mRNA using a single initiation site. The capsid protein which is made first is released into the cytoplasm whereas the two membrane proteins, p62 (the precursor for E2 and E3) and E1, are inserted into the rough endoplasmic reticulum membrane. Based on gradient centrifugation and cross-linking studies, it can be seen that the p62 and E1 polypeptides form a complex immediately after synthesis and migrate to the plasma membrane in the form of a p62–E1 complex. The processing of p62 to E2 and E3 is first seen 25 to 30 min after a 10 min pulse of radioactive amino acids. This cleavage can be inhibited by addition of antisera specific for E1 and E3, thus supporting the view that, as in the case of the related Sindbis virus, this cleavage occurs on the external face of the plasma membrane.

Proteolytic digestion of crude vesicle preparations derived from plasma membranes, combined with peptide mapping, indicate that the carboxy-terminal end of E2 spans the cell plasma membrane, there being a portion of mol. wt about 3000 located towards the cytosol.

INTRODUCTION

Semliki Forest virus (SFV) is one of the simplest enveloped viruses known (for reviews, see Kääriäinen & Söderlund, 1978; Simons et al. 1978; Strauss & Strauss, 1977). The virus particles consist of a spherical nucleocapsid containing one RNA molecule, mol. wt. about $4 \times 10^6$ (Strauss & Strauss, 1977) complexed with a lysine-rich protein, mol. wt. about 30000 (Simons & Kääriäinen, 1970). The nucleocapsid is surrounded by a membrane derived from the host cell plasmalemma (Acheson & Tamm, 1967; Richardson & Vance, 1976) with which three virus specified glycopolypeptides E1, E2 (both of mol. wt. about 50000) and E3 (mol. wt. about 10000) are associated (Garoff et al. 1974). These glycopolypeptides form spike-like projections on the external surface of the virus envelope, each spike consisting of one copy of E1, E2 and E3 (Ziemiecki & Garoff, 1978).

Two species of virus mRNA are found in virus-infected cells, 42S RNA and 26S RNA. The 42S RNA is used for synthesis of the virus non-structural proteins, whereas the 26S RNA codes for all the structural proteins (Kääriäinen & Söderlund, 1978). The virus structural proteins are translated sequentially, using one initiation site (Clegg, 1975; Clegg & Kennedy, 1975; Glanville et al. 1976), in the order nucleocapsid polypeptide, envelope glycopolypeptide precursor p62 (E3 and E2) and the envelope glycopolypeptide E1. In vitro studies have shown that translation of the 26S mRNA is initiated on free ribosomes and the...
first product, the nucleocapsid protein, is cleaved from the nascent chain immediately after it is completed (Garoff et al. 1978; Bonatti et al. 1979). The amino terminal end of p62 then directs the mRNA-ribosome complex to the microsomal membrane where further synthesis of the glycoproteins is coupled to membrane insertion and glycosylation. The proteolytic cleavage between p62 and E1 takes place on the nascent chain when p62 has been completed. In the infected cell the newly synthesized virus glycoproteins migrate through the intracellular membrane system to the plasma membrane where they are incorporated into virus particles (Pfefferkorn & Clifford, 1964; Simons et al. 1978).

The generation of the three-chain structure of the SFV spike glycoprotein in the infected cell has been studied. The virus envelope glycopolypeptide precursor p62 and the envelope glycopolypeptide E1 form a complex after they have been synthesized in the rough endoplasmic reticulum and these glycopolypeptides most likely migrate through the cell to the plasma membrane in the form of a p62–E1 complex. Furthermore, these data suggest that cleavage of p62 to E2 and E3 occurs on the external face of the plasma membrane as previously shown for the related Sindbis virus (Bracha & Schlesinger, 1976; Jones et al. 1977) and that the carboxy-terminal end of E2 spans the cell plasma membrane, there being a portion of about 3000 mol. wt. located towards the cytosol which can be removed by chymotrypsin from the cytoplasmic side.

METHODS

Cells and virus. A prototype strain of SFV was grown on monolayer cultures of baby hamster kidney (BHK) cells and purified as described (Kääriäinen et al. 1969). The virus was labelled with 3H-isoleucine, leucine and valine using 20 μCi of each per ml (The Radiochemical Centre, Amersham, U.K.) and purified as described by Kääriäinen et al. (1969). Cells were infected with 50 to 100 p.f.u. of virus per cell in methionine-free Eagle's minimum essential medium (MEM). The infected cells were pulse labelled 4 to 5 h after infection (p.i.) for 10 min with approx. 20 μCi/ml 35S-methionine (500 Ci/mmol, Radiochemical Centre, Amersham). The chase medium contained a 10-fold excess of unlabelled methionine. SFV labelled with 35S-methionine was prepared as described previously (Kääriäinen et al. 1969).

Separation of virus glycoprotein complexes by centrifugation. BHK cells grown in 6 cm Petri dishes and infected with SFV were labelled for 10 min as described above. The cells from one dish were washed once with 5 ml Hanks' PBS and solubilized either directly after the pulse or after a chase of 30 to 60 min in 1 ml disruption buffer (0.01 M-tris, pH 7.5, containing 0.15 M-NaCl, 0.0015 M-MgCl₂ and 0.65% Triton X-100). The lysate was clarified by centrifugation in a bench centrifuge (Wifug, Stockholm, Sweden) and aliquots of the supernatant fluid were applied on to a 5 ml 15 to 30% (w/w) sucrose gradient in TN buffer (0.05 M-tris, pH 7.4, 0.1 M-NaCl) containing 0.1% Triton X-100. The gradients were centrifuged at 50,000 rev/min for 24 h at 4 °C in the 498 rotor of an IEC-B60 ultracentrifuge. Gradients were fractionated from the bottom in 250 μl fractions and aliquots were analysed for radioactivity. 3H-labelled SFV solubilized with Triton X-100 was added to the cell lysate before centrifuging in some experiments to act as internal marker.

Cross-linking and analysis of cell extracts. The samples to be cross-linked, either cell lysates or sucrose gradient fractions, were mixed with an equal vol. of 0.3 M-triethanolamine buffer, pH 8.2 (TEA), containing between 6 and 12 mg/ml dithiobispropionimidate (DTBP). The mixture was incubated for 1 h at room temperature and layered on to a 13 ml 5 to 15% (w/w) sucrose gradient in 0.15 M-TEA buffer containing 0.1% SDS. Gradients were centrifuged at 40,000 rev/min for 20 h at 22 °C in the 488 rotor of an IEC-60 ultracentrifuge. The gradients were fractionated from the bottom into 300 μl fractions and 50 μl amounts were taken for radioactivity determination.
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SDS-polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoretic analysis of proteins was done according to the method of Neville (1971). For optimal E1 and E2 separation no reduction with mercaptoethanol was done. Cross-linked species were cleaved into individual polypeptides by reduction with 1% mercaptoethanol before electrophoresis. The gradient gel system used to analyse NTCB cleavage peptides has been described elsewhere (Garoff & Söderlund, 1978). Fluorography (Bonner & Laskey, 1974) and densitometry of the labelled bands were as described by Ziemiecki & Garoff, 1978.

Membrane vesicle preparation and chymotrypsin digestion. A confluent monolayer of BHK cells in a Falcon flask was labelled with 35S-methionine and chased in the presence of fresh medium for 1 h as described above. The cells were washed once with cold TN buffer and put on ice. The neck of the Falcon flask was sawn off and the cells were suspended by scraping and pipetting with 2 ml of TN buffer. The cell suspension was introduced into a Yeda nitrogen cavitation press (Yeda Research and Development Co. Ltd., Rehovot, Israel) and subjected, while being stirred, to a nitrogen pressure of 50 atmospheres for 30 min at 4°C. The cells were lysed by slowly releasing the pressure by means of a needle valve, thereby extruding the homogenized cell suspension. This suspension was clarified by centrifugation in a bench centrifuge. Aliquots of the final supernatant were brought to 0.5 mg/ml α-chymotrypsin (Worthington, Freehold, N.J., U.S.A.) in TN buffer and incubated for 1 h at 37°C in a shaking water bath. Control aliquots were similarly incubated in the absence of α-chymotrypsin. After the incubation period, the reaction was stopped by addition of an equal volume of 20% TCA at 0°C. The TCA precipitates were collected by gentle centrifugation and resuspended in sample buffer containing 2% SDS. Sodium hydroxide was used to neutralize any remaining TCA.

Immunoprecipitation of virus glycoproteins from membrane vesicle preparations. The membrane vesicle preparations after TCA precipitation and solubilization in 2% SDS (see above) were diluted with PBS to give a final concentration of 0.1% SDS. To 100 μl of this lysate containing 250 x 10^3 to 500 x 10^3 cpm/min of 35S-radioactivity, 10 μl of antiserum was added. We used either antiserum reacting with glycopolypeptide E1 or with both glycopolypeptides E2 and E3. The mixture was allowed to react for 1 h at room temperature and then 50 μl of an approx. 10% suspension of protein A-containing Staphylococcus aureus (Cowan strain) in PBS containing 0.1% SDS were added. This mixture was allowed to stand for 30 min with periodic agitation. The bacteria were pelleted, washed with 1 ml of PBS containing 0.1% SDS and the washed bacterial pellet was resuspended in 50 μl of sample buffer containing 2% SDS. The bacteria were re-pelleted and the supernatant used for electrophoretic analysis.

2-nitro-5-thiocyanobenzoic acid (NTCB) cleavage of proteins. The 35S-methionine-labelled polypeptides present in control and chymotrypsin-treated membrane vesicle preparations were resolved by slab-gel electrophoresis, the slabs were dried directly and the protein bands were detected by autoradiography. The desired bands were cut out of the dried slab and the proteins eluted as described previously (Garoff & Söderlund, 1978). The purity of the eluted proteins was checked by electrophoresis. Proteins were cleaved at their cysteine residues by treatment with 2-nitro-5-thiocyanobenzoic acid (NCTB, Eastman Kodak; Jacobson et al. 1973; Degani & Patchornik, 1974). The NTCB-peptides were resolved by electrophoresis on 15 to 22.5% acrylamide gradient gels (Garoff & Söderlund, 1978) and the bands were detected by fluorography.

Antisera production. Antisera were raised in rabbits as described previously (Ziemiecki & Garoff, 1978). The E1, E2 and E3 polypeptides were purified by SDS-hydroxyapatite chromatography (Garoff et al. 1974).
RESULTS
The cleavage of p62 takes place at the plasma membrane

The cleavage of p62 to E2 and E3 is a late event in the morphogenesis of SFV. It occurs shortly before the spike glycoproteins are incorporated into the virus at the plasma membrane of infected cells. For Sindbis virus this cleavage has been shown to take place at the external surface of the plasma membrane of the cell (Bracha & Schlesinger, 1976; Jones et al. 1977). To find out whether this is also the case in SFV we used the same strategy as in the previous studies, in which cleavage was blocked by antibodies to the spike protein added extracellularly to infected cells.

Infected cells were pulsed for 10 min with $^{35}$S-methionine and then chased in the absence or presence of antiserum against the spike polypeptides in medium containing excess unlabelled methionine. After the chase the labelled polypeptides were analysed by SDS–gel electrophoresis. Fig. 1(a) shows the samples which were chased for different times in the absence of antiserum. Immediately after the pulse, p62, E1 and the nucleocapsid (C) polypeptides were present. The cleavage of p62 to E2 and E3 was first detected after a 20 to 25 min chase. If the chase was carried out in the presence of antiserum against the spike polypeptides there was an almost total inhibition of processing of p62 (Fig. 1b). Antiseras against E1, E2 plus E3, or all the spike polypeptides were effective in blocking the cleavage. Barring the possibility that the antibodies could have entered the intracellular compartments through which the spike glycopolypeptides are transported to the surface, these results suggest that most of the p62 is cleaved to E2 and E3 at the external plasma membrane. This confirms the results obtained with Sindbis virus (Bracha & Schlesinger, 1976).

Virus glycoprotein complexes in the infected cell

The virus glycoproteins are synthesized in the rough endoplasmic reticulum in the form of p62 (precursor to E2 and E3) and E1. Several studies with the closely related Sindbis virus have suggested the presence of a p62–E1 complex within infected cells (Bracha & Schlesinger, 1976; Jones et al. 1977; Smith & Brown, 1977). Such a complex could be formed either directly after synthesis of p62 and E1 in the rough endoplasmic reticulum during transport of the glycoproteins to the plasma membrane or at the plasma membrane. In the mature virus particle the glycoproteins are present as an E1–E2–E3 complex (Ziemiecki & Garoff, 1978).

Several experiments were carried out firstly to ascertain whether a p62–E1 complex can be detected and secondly to determine when the complex is first formed. In these experiments it was assumed that solubilization of cells with Triton X-100 will not result in the dissociation of any p62–E1 complexes present. Triton X-100 is a mild non-ionic detergent and as such should preserve protein–protein interactions (Helenius & Simons, 1975). The E1–E2–E3 glycoprotein complex found in virus particles can be solubilized intact with Triton X-100 and can be isolated by centrifugation in Triton X-100 containing sucrose density gradients (Simons et al. 1973). Furthermore, the cleavage of p62 to E2 and E3 has been used as a marker for the arrival of p62 at the plasma membrane (Fig. 1).

BHK cells infected with SFV were pulse-labelled with $^{35}$S-methionine for 10 min and chased for different periods (0, 30 and 60 min). After the chase, the cells were washed and solubilized with buffer containing Triton X-100. Aliquots of the lysates were mixed with Triton X-100-solubilized $^3$H-labelled SFV and centrifuged in sucrose gradients containing Triton X-100. Immediately after the 10 min pulse and after 30 and 60 min chase there was a peak of $^{35}$S-methionine activity in approximately the same place in the gradients. This peak co-migrated with the $^3$H-peak obtained with Triton X-100-disrupted $^3$H-labelled virus
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Fig. 1. SDS-gel electrophoresis of samples from pulse-chase experiments (a) in the absence and (b) in the presence of specific antisera. Infected cells (6 cm Petri dishes) were labelled for 10 min with ³⁵S-methionine and either chased for various times (0' to 30') in the absence of specific antibody or chased for 1 h in the presence of antisera specific for E1 (AE1), E2 and E3 (AE2) or E1, E2 and E3 (AEnv); 200 µl of antiserum with 1.3 ml chase medium. The bottom portion of the gel was exposed longer to visualize the poorly labelled E3.

(Fig. 2). The ³⁵S-methionine-labelled polypeptides present in the peak fractions (10 to 15) of each gradient are shown in Fig. 3. Immediately after the pulse (0') the peak fractions consisted of p62 and E1 in approximately equimolar amounts as determined by densitometry. The 97 K protein containing E1, E2 and E3 (Garoff et al. 1978) was also seen in varying amounts. After a 30 min chase (30') some of the p62 had been cleaved into E2 and E3. The E3 protein is not seen in Fig. 3 because much longer exposure times were needed to visualize this small protein. After a 60 min chase (60'), almost all the p62 had been cleaved into E2 and E3.

Taken together these results indicate that the virus glycoproteins form a Triton X-100-resistant complex immediately after synthesis in the rough endoplasmic reticulum, and that they migrate as a complex to the cell plasma membrane where the p62 cleavage occurs, generating the E1–E2–E3 virus spike complex.

More evidence for complex formation between p62 and E1 was obtained by cross-linking studies with the cleavable cross-linking reagent DTBP. Cells infected with SFV, labelled with ³⁵S-methionine and chased for different periods of time were washed and solubilized with Triton X-100 before cross-linking with DTBP. After cross-linking, the products were analysed on 5 to 15% sucrose gradients containing 0.1% SDS. Fig. 4 shows the radioactivity profiles of such gradients immediately after the pulse and after a chase of 30 min and 60 min, respectively. The curve at the bottom of Fig. 4 shows the profile of ³⁵S-methio-
nine-labelled SFV solubilized with Triton X-100 and cross-linked with DTBP. In all gradients there were two major peaks, a faster sedimenting peak (arrowed), sedimenting in approximately the same position as the 100000 mol. wt. E1–E2 complex observed in the cross-linked virus (Ziemiecki & Garoff, 1978), and a slower sedimenting peak in the position of virus E1 and E2 monomers. E3 when present is not cross-linked and stays on top of the gradient (Ziemiecki & Garoff, 1978). De-cross-linking of the fractions from the arrowed peak in Fig. 4 and analysis of the constituent polypeptides on SDS-gels (not shown) revealed patterns similar to those seen in Fig. 3. Immediately after the pulse, the major cross-linked component consisted of p62 and E1. After a 30 min chase, the major cross-linked species consisted of p62, E2 and E1 and after a 60 min chase the major cross-linked component consisted mostly of E2 and E1 with a small amount of p62. Identical results were seen when the peak fractions (10 to 15) in Fig. 2 were cross-linked with DTBP and analysed as described above.
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Fig. 4. Analysis of DTBP-cross-linked Triton X-100 lysates in SDS-containing sucrose gradients. Triton X-100 lysates of cells labelled for 10 min (0' chase), labelled for 10 min and chased for 30 min (30' chase) and labelled for 10 min and chased for 60 min (60' chase) were cross-linked using DTBP, solubilized in SDS and analysed on SDS-containing sucrose gradients. SFV was similarly treated. The arrow indicates the position of the major cross-linked species. The top of the gradient is indicated.

The orientation of the virus glycoprotein in the plasma membrane of infected cells

The topology of newly made virus glycoproteins in the rough endoplasmic reticulum is such that the major part of the proteins, including their amino-terminal ends, are on the lumenal side of the rough endoplasmic reticulum (Garoff & Söderlund, 1978). The carboxy-terminal ends of E1 and p62 attach the proteins to the membrane. The p62 spans the membrane. About 3000 mol. wt. can be cleaved from the carboxy-terminal end of the 62 protein when microsomes from cells infected with SFV are treated with protease (Garoff & Söderlund, 1978). In the following experiments we have investigated the topology of the virus glycoproteins in the plasma membrane by treating membrane vesicles from infected cells with chymotrypsin. As in the preceding section, the cleavage of p62 to E2 and E3 is assumed to occur first at the plasma membrane.

Infected cells were labelled with $^{35}$S-methionine for 10 min and chased for 60 min in the presence of excess unlabelled methionine. After the 60 min chase most of the labelled p62
Fig. 5. SDS-gel electrophoresis after chymotrypsin treatment of crude membrane vesicles. The SFV-infected cells were labelled with °S-methionine and chased for 1 h before homogenization. The sample to the left was the untreated control, the sample in the middle treated with chymotrypsin and the sample to the right was solubilized with Triton X-100 before chymotrypsin digestion. The filled circles indicate the polypeptide generated by chymotrypsin treatment.

Fig. 6. SDS-gel electrophoresis after immunoprecipitation of crude vesicle preparations similar to those shown in Fig. 5. Vesicles either treated or not treated with chymotrypsin were solubilized in SDS and reacted with antisera specific for E1 (AE1) or for E2 and E3 (AE2/3). The immunoprecipitations were done using protein A-bearing Staph. aureus, solubilized and electrophoresed. Labelled material is seen at the top of the gels. This is due to the fact that the samples were not reduced. Reduction with mercaptoethanol leads to impaired separation of E1 and E2 (A. Ziemiecki, unpublished observations).

has been cleaved to E2 and E3 (Fig. 1 and 3). The cells were washed and membrane vesicles were made by nitrogen cavitation. Such crude vesicle preparations were treated with chymotrypsin and the protein profiles of these were compared with untreated vesicles. Two populations of plasma membrane vesicles are possible when cells are homogenized by nitrogen cavitation: 'right-side-out' vesicles with the cytoplasmic face to the inside and 'inside-out' vesicles with the cytoplasmic face turned to the outside. If, on the plasma membrane of infected cells, E1 and E2 are oriented so that the major portions of each protein are
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Fig. 7. SDS-gel analyses of NTCB peptides from E1 and E2 glycopolypeptides isolated from untreated and chymotrypsin-treated crude vesicle preparations. C and N indicate peptides that are derived from the carboxy (C) or the amino (N) terminal region of the polypeptide chain (Garoff & Söderlund, 1978).

on the outside, then proteolytic digestion of sealed right-side-out vesicles would lead to loss of the major portions of both E1 and E2 (and E3). On the other hand, proteolytic digestion of sealed inside-out vesicles would result in digestion of only those parts of the proteins originally located in the cell cytoplasm and protection of those major portions originally located outside the cell. Thus, this approach circumvents the necessity of separating and isolating the two kinds of vesicles.

The result of chymotrypsin digestion of crude vesicle preparations is shown in Fig. 5.
Digestion resulted in almost total disappearance of E2 whereas E1 and the nucleocapsid protein were still seen. A new protein band was detected migrating faster than E2 (Fig. 5 indicated by filled circles). Triton X-100 disruption of vesicles before chymotrypsin treatment resulted in digestion of all the virus proteins (Fig. 5).

The nature of the new protein was investigated with antisera specific for the virus glycoproteins. Control and chymotrypsin-treated vesicle preparations were TCA-precipitated and the pellets resuspended in PBS containing 2% SDS. The resuspended pellets were diluted with buffer to give a final SDS concentration of 0.1% and aliquots were reacted with the specific antiserum. Antigen-antibody complexes were isolated using protein A-bearing *Staphylococcus aureus*, eluted with 2% SDS and analysed on SDS-gels (Fig. 6). Antiserum specific for glycoprotein E1 precipitated E1 from both control and chymotrypsin-treated vesicles. Antiserum specific for glycoprotein E2 and E3 precipitated E2 and E3 from control vesicles and the new protein and some E3 from chymotrypsin-treated vesicles. Some undigested E2 was also observed in such precipitates (Fig. 6). From these results we conclude that the new protein band observed in chymotrypsin-treated vesicle preparations is derived from E2. To exclude the possibility that the new protein (designated E2 chy) was derived from mature viruses attached to the surface membrane, a control experiment was performed. SFV labelled with 35S-methionine was treated in the same way by nitrogen cavitation and digested with chymotrypsin as in Fig. 5. Chymotrypsin removed E1 and E2, whereas the C protein was not digested. No band in the position of E2 chy was seen in SDS-gel electrophoresis (not shown). Thus, if virus particles were present in the vesicle preparations the E2 chy band would not have been derived from these.

In order to establish from which end of the E2 polypeptide chymotrypsin had cleaved a fragment, its NTCB peptides were compared with those of intact E2. Virus glycoproteins from control and chymotrypsin-treated vesicle preparations were isolated, cleaved with NTCB and analysed in SDS gels. (Fig. 7). The NTCB-peptide pattern of E1 was the same before and after chymotrypsin digestion. The peptide patterns of E2 and E2 chy were not identical. By comparing the gels with those of E2 from an earlier study where the and amino-terminal NTCB-peptides of E2 were identified, it was seen that the carboxy-terminal peptides of E2 are not present in E2 chy. These NTCB-peptide shifts are also seen with p62 when microsomes derived from infected cells are treated with chymotrypsin (Garoff & Söderlund, 1978). As indicated in Fig. 7, chymotrypsin digestion had no effect on the mobility of the amino-terminal NTCB peptides of E2. These results indicate that chymotrypsin digestion of the crude vesicle preparations results in digestion of the carboxy-terminal end of E2 only.

**DISCUSSION**

Previous work has shown that the spike proteins in the mature virus particle are three-chain structures in which most of the protein is external to the lipid bilayer (Gahmberg *et al.* 1972; Utermann & Simons, 1974; Garoff & Söderlund, 1978; Ziemiecki & Garoff, 1978). In this study the formation of the three-chain spike protein structure in the infected cell has been followed. Earlier studies have shown that the three chains are generated by proteolytic cleavages. The first cleavages already occur during translation of the chains, starting with the cleavage between the capsid protein and the amino-terminus of p62 (the precursor for E2 and E3) followed by the cleavage between p62 and E1 (Garoff *et al.* 1978). The pulse-chase experiments in this paper suggest that p62 forms a complex with E1 as soon as these have been synthesized and inserted into the membrane of the rough endoplasmic reticulum (Fig. 2, 3 and 4). After their formation the p62–E1 complexes are transported from the rough endoplasmic reticulum to the surface of the cell. The first complexes appear to
reach the surface within about 30 min after synthesis, as judged by the appearance of E2 and E3 in these and earlier pulse-chase experiments (Kaluza, 1976) and from earlier studies on the kinetics of spike protein incorporation into mature virus particles (Scheele & Pfefferkorn, 1969). Antisera reacting specifically with either E1 or both E2 and E3 inhibit the cleavage of p62 (Fig. 1). Similar results localizing this cleavage to the external surface of the cell have been obtained with Sindbis virus (Bracha & Schlesinger, 1976; Jones et al. 1977). The fact that antiserum against E1 also blocks the cleavage agrees with our finding that p62 and E1 are associated as a complex. It was not possible to detect significant amounts of p62 on the surface of the infected cell by surface labelling methods. Not only were lactoperoxidase and chloramine T-catalysed protein iodination used but also diazotized sulphamic acid to label surface proteins and galactose oxidase-borohydride to label carbohydrate moieties of glycoproteins (unpublished observations). Only E1, E2 and E3 could be labelled, not p62. We therefore favour the view that the p62 cleavage is a rapid process occurring as soon as the p62–E1 complexes have been inserted into the plasma membrane. This cleavage generates the final three-chain structure of the SFV spike glycoprotein. It seems unlikely that p62, if present in substantial amounts on the surface, would be inherently inaccessible to labelling by all these reagents. The alternative view that the hydrophilic domains of the p62 (including the E3 part, most of E2 and the carbohydrate side chains) would be buried in the bilayer is difficult to reconcile with present notions on membrane protein structure.

The transmembrane topology of the virus spike glycoprotein complexes on the plasma membrane was also studied. Our results, although not conclusive, suggest that E2 spans the membrane with a segment from the carboxy-terminal end protruding towards the cytosol. This is the same orientation that has previously been found for E2 in virus particles (Simons et al. 1980) and for the p62 proteins in the endoplasmic reticulum (Garoff & Söderlund, 1978; Wirth et al. 1977). Thus the transmembrane organization of the SFV spike glycoproteins into three separate protein domains (external, transmembrane and internal) is already established during insertion into the endoplasmic reticulum and is not changed during transport to the cell surface.

The cytoplasmic part of the spike proteins is likely to play a major role in the budding of the nucleocapsid through the plasma membrane. We have postulated earlier that the budding process is mediated by transmembrane binding of the spike glycoproteins to the nucleocapsid (Garoff & Simons, 1974). The nucleocapsid is bound to the cytoplasmic (internal) domains of the spike proteins and budding proceeds by more spike proteins diffusing into the budding site and being trapped by the binding. When all the binding sites have been occupied (spike protein–capsid protein stoichiometry in the mature virus is 1:1) the virus particle is released. The budding may be facilitated by lateral interactions between the spike glycoproteins. Bonsdorff & Harrison (1978) have recently shown by freeze-etch electron microscopy of Sindbis virus and of glycoprotein arrays derived from Sindbis virus membranes by non-ionic detergent treatment that the local geometry of glycoprotein–glycoprotein interaction does not depend on the presence of nucleocapsid. The protein contacts formed presumably reflect reasonably strong glycoprotein bonding which could be part of the driving force favouring budding. The reason why budding occurs mainly at the cell surface and not in intracellular membranes could be a consequence of the p62 cleavage. This cleavage may change the conformation of the spike protein such that budding is facilitated. Also, the carbohydrate side-chains of the glycoproteins could mediate the formation of protein–protein contacts (McCarthy & Harrison, 1977). Thus, intracellular incomplete carbohydrate side-chains may hinder the formation of the precise lateral interactions favouring budding.
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