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SEMLIKI FOREST VIRUS: A PROBE FOR MEMBRANE TRAFFIC IN THE ANIMAL CELL

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I. Introduction

During the past 25 years a considerable body of data has been accumulated, often to atomic resolution, on the structure and function of proteins. In contrast we know far less about the life cycle of these proteins—those processes which put a protein in the part of the cell in which it is to function and the cellular movements (if any) of this protein as it carries out its function. We know even less about those processes which eventually single out the protein for degradation.

The first evidence that the routing of proteins to their correct destination in the cell is encoded in the primary structure of the protein came from work on secretory proteins (Milstein *et al.*, 1972). Mainly through the work of Blobel and associates it was found that the amino-terminal extension of secretory proteins, termed the signal peptide, directs the ribosomal complex to the endoplasmic reticulum (ER), and, as a result, the polypeptide chain is transferred through the ER membrane during synthesis and segregated into the lumen (Blobel and Dobberstein, 1975a,b). Not only secretory proteins but also a number of other proteins are synthesized with amino-terminal signal peptides (see Warren, 1981). Those proteins which have their domiciles in such diverse organelles as the cell surface membrane, lysosomes, and secretory granules are synthesized on membrane-bound ribosomes and are initially found in the same compartment, the ER. From here the proteins are transported

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to the Golgi complex, the organelle which routes the proteins to their final destinations (see Tartakoff, 1980).

The traffic among the cellular compartments is thought to be mediated by membrane vesicles which bud from one compartment and fuse with the next (Palade, 1975). Despite the continuous exchange of membrane components among them, the organelles maintain their characteristic protein and lipid compositions so that the traffic remains selective, thus avoiding intermixing of components. Membrane must also be recycled backward to compensate for loss of membrane in the forward movement. Membrane recycling at the cell surface has been especially studied and it is now thought that the cell membrane of all animal cells is being continuously and rapidly endocytosed (Silverstein *et al.*, 1977). This membrane traffic recycles components from the cell surface to the interior of the cell and back to the cell surface again (Anderson and Kaplan, 1983). Some of the surface membrane components are channeled at least in part to the lysosome to be degraded. In some cells with considerable secretory activity, recycling of surface components back to the Golgi complex has also been detected (Farquhar and Palade, 1981). The membrane traffic between the ER and the cell surface involves a major sorting problem (Rothman, 1981). Little is known of how the animal cell has solved this problem in molecular terms. Such processes are exceedingly difficult to study in the cell in which a multitude of proteins is synthesized simultaneously with a sizable proportion of them initially routed into the ER. *In vitro* systems have been developed to study the first phase of assembly into the ER during protein synthesis (Blobel and Dobberstein, 1975a,b). Attempts to reconstruct other phases in the transport of proteins from the ER to other organelles have begun (Fries and Rothman, 1980) but are still in their infancy. Other simplifications are obviously needed to make possible studies of these processes at the molecular level.

One experimental tool in this direction is provided by some enveloped animal viruses which mature at the cell surface of infected cells (Kääriäinen and Renkonen, 1977; Lenard, 1978). Such viruses include influenza virus, Semliki Forest virus (SFV), Sindbis virus, and vesicular stomatitis virus (VSV). They are extremely simple in makeup and hence are very well characterized. They can be tagged with biochemical probes in many different ways. They infect many animal cells in culture, and after infection turn the cells into factories for the production of virus progeny. The protein-synthesizing machinery of the host cell is programmed by the viral RNA to make viral proteins exclusively and these include the viral surface glycoproteins. These are synthesized with signal peptides and inserted into the ER membrane (Katz *et al.*, 1977; Garoff *et*

al., 1978; Bonatti *et al.*, 1979), from which they are transported to the cell surface via the Golgi complex (Bergmann *et al.*, 1981; Green *et al.*, 1981). The net effect is the same as if the cell were to divert most of its protein-synthesizing capacity to the making of only one or two of its own plasma membrane glycoproteins. This amplification is the key to the use of viral glycoproteins as probes for membrane traffic from the ER. The endocytic route from the cell surface can also be studied with enveloped viruses, because this is the route they use to infect the cell (Helenius *et al.*, 1980).

The purpose of this article is to illustrate the use of enveloped viruses as tools in the study of membrane traffic in the animal cell. We will do this in the context of the life cycle of the virus in the host cell. The article will be concerned mainly with Semliki Forest virus (SFV) which is the virus we have worked with. SFV belongs to the alphaviruses, a genus of the togavirus family. Another well studied and closely related alphavirus is Sindbis virus. For more information on the biology of these viruses see the monograph on togaviruses (Schlesinger, 1980). There are also a number of reviews on the structure and assembly of alphaviruses which overlap but also cover aspects not treated here (Strauss and Strauss, 1977; Kääriäinen and Söderlund, 1978; Garoff *et al.*,1982a).

II. Structure

The alphavirus particle consists of RNA, protein, and lipid. The viral 42 S RNA molecule is single stranded and has a molecular weight of 4.1–4.3 $\times$ 10^6 (see Kääriäinen and Söderlund, 1978; Kennedy, 1980). Together with the capsid protein which has a molecular weight of 29,700 (Garoff *et al.*, 1980a; Rice and Strauss, 1981) it forms the nucleocapsid which is encapsulated by the viral envelope, a lipid bilayer studded with spikes of viral glycoproteins. In SFV each viral spike glycoprotein is formed from three polypeptide chains (molecular weights in parentheses): E1 (50 $\times$ 10^3), E2 (50 $\times$ 10^3), and E3 (10 $\times$ 10^3), each of which contains covalently bound carbohydrates (Garoff *et al.*, 1974; Ziemiecki and Garoff, 1978). In Sindbis virus the viral spike is a two-chain structure containing only E1 and E2 (Schlesinger and Schlesinger, 1972; Rice and Strauss, 1982).

The weight of the SFV particle was, until recently, thought to be about 60 $\times$ 10^6 daltons (Laine *et al.*, 1973; Kääriäinen and Söderlund, 1978). This estimate was based on the chemical composition of the virus and on the molecular weight of the viral RNA. This value has now been shown to be significantly wrong by three independent methods.

1. The sedimentation coefficient ($s_{20,w}$) measured in the analytical ultracentrifuge is 274 ± 2 S. The diffusion coefficient from light scattering measurements ($D_{20,w}$) is 6.35 ± 0.1 × 10^{-8} cm^2 sec^{-1}. These values, together with the partial specific volume of the virus (0.75 ml g^{-1}) give a weight for the viral particle of 42 × 10^6 daltons (Jacrot *et al.*, 1983).
2. From neutron scattering measurements using different concentrations of D_2O, an independent molecular weight estimate of 40.8 × 10^6 has been derived (Jacrot *et al.*, 1983).
3. Mass determination of unstained virus specimens in the scanning transmission electron microscope gives a value of 35 ± 7 × 10^6 daltons (Freeman and Leonard, 1981).

These new estimates of the particle weight change the number of copies of the viral glycoprotein per viral particle. The polypeptides (capsid, E1, E2, and E3) are present in equimolar amounts (Garoff *et al.*, 1974). Since 56.6% of the virus is protein (leaving out the carbohydrate content) the viral particle (using a molecular weight of 41–42 × 10^6) should contain about 180 copies of each protein.

Electron microscopic studies have suggested that the alphavirus particle has icosahedral symmetry (see below). The triangulation number is not certain, however (Murphy, 1980). Previous estimates for the molecular weight were compatible with 240 subunits per virus particle, and electron micrographs appear to show a $T = 4$ surface lattice (von Bonsdorff and Harrison, 1975). More information is now needed to determine the surface organization, since compositional data show fewer than 240 subunits.

A. The Nucleocapsid

The nucleocapsid can be isolated from purified viral particles using mild detergents to solubilize the viral envelope (see Kääriäinen and Söderlund, 1978). It can also be purified from extracts of infected cells. The weight of the SFV nucleocapsid, recently determined by neutron scattering analysis, is 9.46 × 10^6 daltons of which the RNA accounts for 4.1 × 10^6 daltons (Jacrot *et al.*, 1983). This leaves 5.36 × 10^6 daltons for the capsid proteins, which is exactly the value calculated for 180 protein subunits each having a molecular weight of 29.7 × 10^3. These values are in good agreement with the RNA percentage (42%) determined by chemical analysis (Jacrot *et al.*, 1983). The diameter of the nucleocapsid in the virus particle has been determined by low-angle X-ray scattering (Harrison *et al.*, 1971; S. C. Harrison and L. Kääriäinen, unpublished

data). The diameters of the Sindbis virus and SFV nucleocapsid are 400 and 380 Å, respectively. Recent neutron scattering values give a slightly larger value of 410 Å for the diameter of the isolated SFV nucleocapsid (Jacrot *et al.*, 1983). The surface structure of the nucleocapsid appears to be icosahedral. Although the exact organization of the capsid proteins in the nucleocapsid is not yet clear (see Murphy, 1980), the composition is compatible with $T = 3$ icosahedral symmetry. The difficulty in determining the surface lattice of the capsid protein is probably due to the organization of the protein within the nucleocapsid. Neutron diffraction studies suggest that the proteins do not form a shell around the RNA (Jacrot *et al.*, 1983). Instead, the proteins and the RNA appear to be rather uniformly distributed within the particle and this might make the protein subunits rather difficult to visualize in electron micrographs.

The capsid protein is 267 amino acids in length in SFV, and 264 in Sindbis virus (Garoff *et al.*, 1980a; Rice and Strauss, 1981; Boege *et al.*, 1981). Their amino acid sequences have been established both by conventional methods and by DNA sequencing (Fig. 1). The capsid proteins from both viruses contain a striking cluster of lysine, arginine, and proline residues in the amino-terminal third of the polypeptide chain. A number of nucleocapsid proteins from other viruses have similar base sequences and this region is probably involved in the interaction of the protein with the RNA (e.g., Fiers *et al.*, 1978; Shinnik *et al.*, 1981; Kitamura *et al.*, 1981). However, there is less sequence homology in this part of the molecule between the two viruses than in the carboxyl-terminal part of the proteins (residues 166–267) where 76% of the residues are identical and another 6% are conservative substitutions. This carboxyl-terminal region is probably important for other critical functions of the protein such as those protein–protein interactions that stabilize the nucleocapsid structure and those that are formed between the spike proteins and the capsid proteins during viral budding. Serine-219 in the SFV capsid protein is part of a sequence (Gly–Asp–Ser–Gly) characteristic of serine proteases (Boege *et al.*, 1981). The same sequence is found in an analogous position in the Sindbis virus capsid protein. This might be part of the catalytic site of the putative autoprotease activity of the capsid protein (see Section III,B,1). The amino-terminal methionine is acetylated in the Sindbis virus capsid protein (Bell and Strauss, 1981) and in SFV it is also blocked (Kalkkinen *et al.*, 1980). Such blocks are fairly common in the structural proteins of viruses and might protect them against proteolytic degradation by cellular exopeptidases (Jörnvall, 1975).

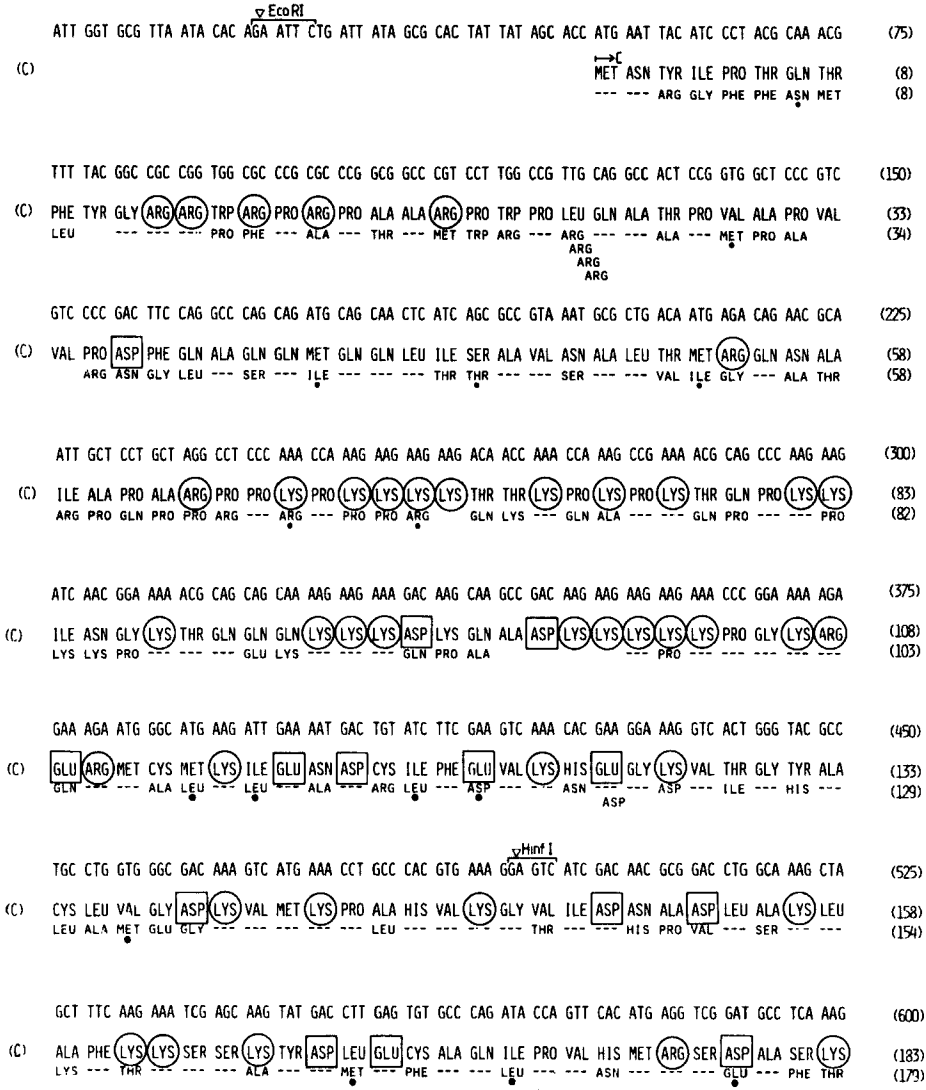

FIG. 1. Nucleotide sequence of the SFV 26 S RNA (top row), the corresponding amino acid sequence (middle row), and the amino acid sequence of the Sindbis virus structural proteins (bottom row). Nucleotides are numbered from the 5′ end of the RNA molecule and all amino acids from the amino terminus of each protein. The amino- and the carboxyl-terminal ends of each protein are indicated by arrows, glycosylation sites by triangles, and membrane-spanning regions of the viral glycoproteins by underlines for Sindbis virus and overlines for SFV. Amino acids in boxes are negatively charged (Asp and Glu), and those circled are positively charged (Lys and Arg). Some restriction endonuclease cleavage sites are shown on the nucleotide sequence. The alignment of the amino acid

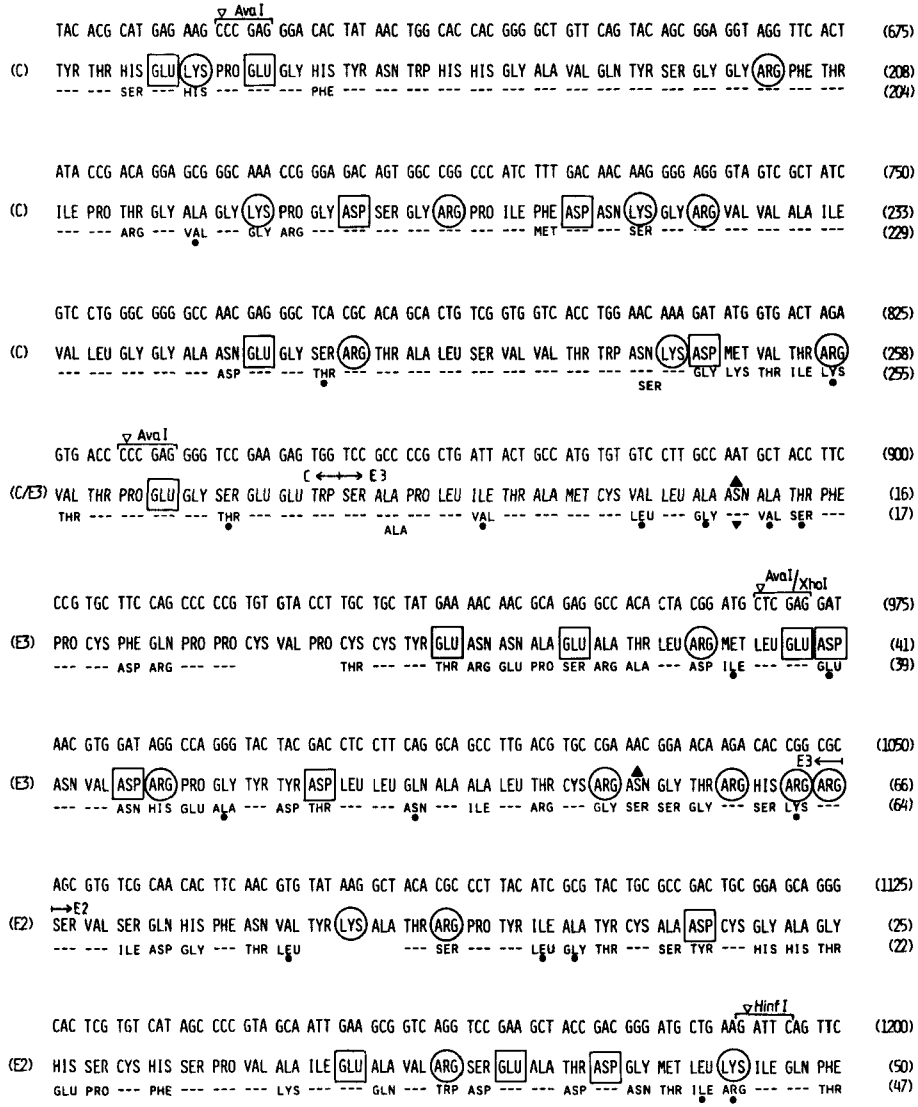

FIG. 1 (*continued*).

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sequences of the two alphaviruses has been made to maximize homology and therefore numerous small deletions (empty spaces) and insertions (amino acids below each other) are present. A dashed line in the position of an amino acid in the Sindbis virus sequence indicates homology with the SFV sequence. A dot under an amino acid in the Sindbis virus sequence indicates a conserved change. From Garoff *et al.* (1982a), with permission.

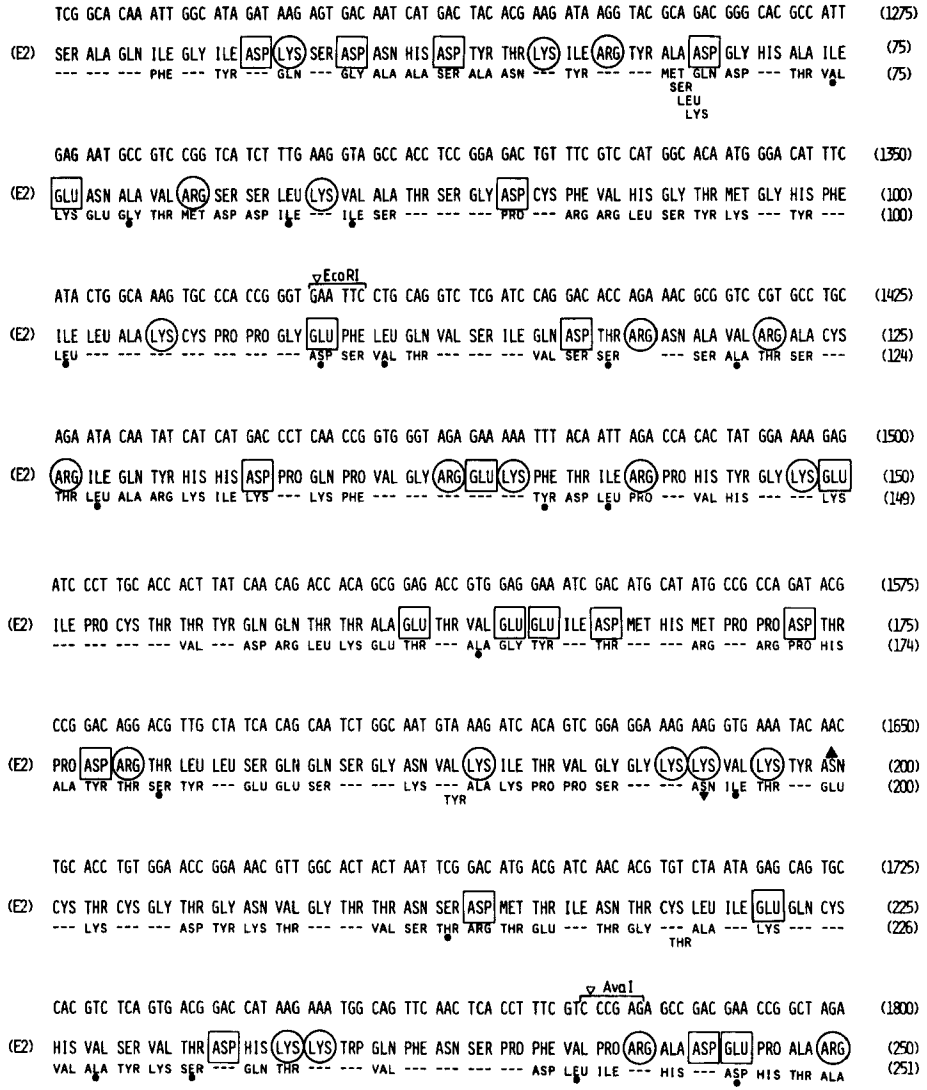

FIG. 1 (*continued*, see legend on pp. 84–85).

B. The Viral Envelope

1. Viral Glycoproteins

The systematic study of how detergents solubilize the viral proteins laid the basis for our understanding of how the viral particle is built. The

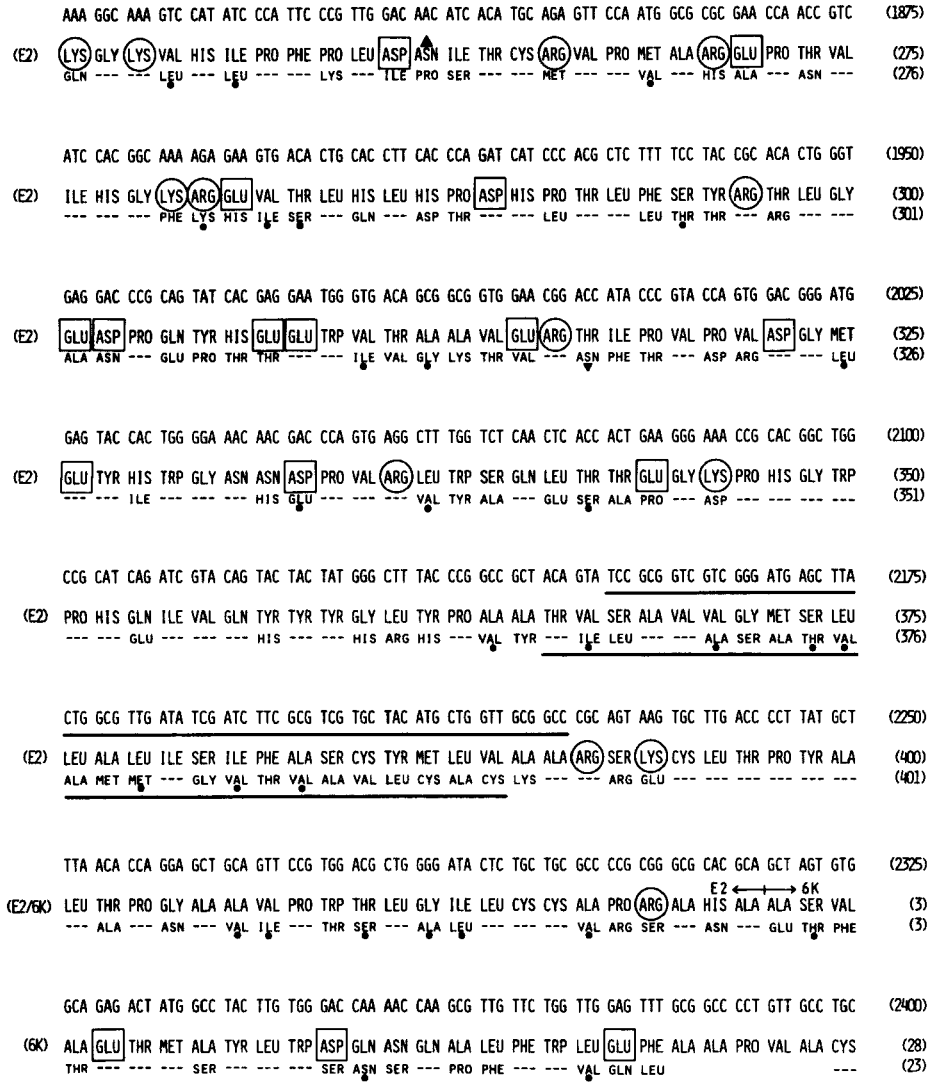

FIG. 1 (*continued*, see legend on pp. 84–85).

results obtained also led to important insights into the mechanisms by which detergents solubilize biological membranes (Helenius and Simons, 1975). These results have been reviewed previously (Simons *et al.*, 1977, 1978) and will not be covered here.

a. Subunit Structure and Topology. The subunit structure of the mem-

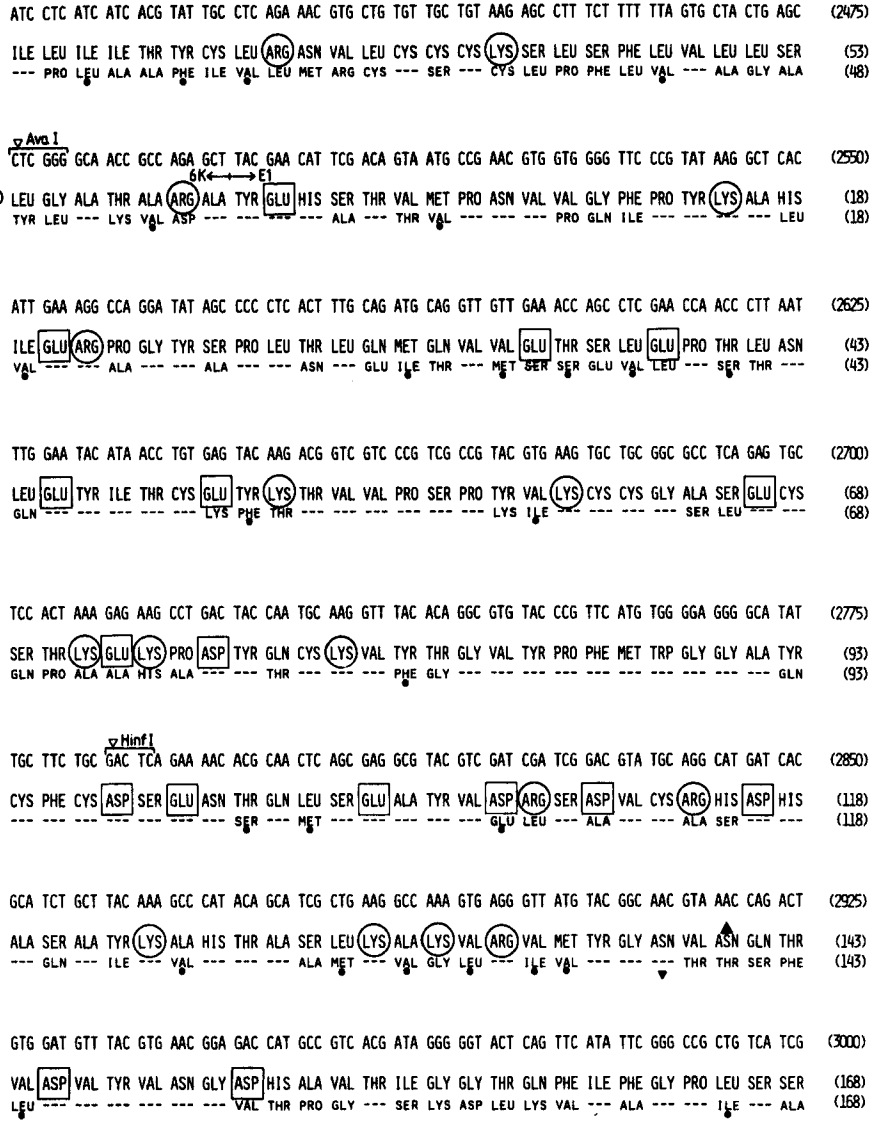

FIG. 1 (*continued*, see legend on pp. 84–85).

brane glycoproteins has been deduced from studies in which the virus is cross-linked with bifunctional, amino-reactive reagents, and the products solubilized with the aid of detergents (Simons *et al.*, 1973a; Ziemiecki and Garoff, 1978; Rice and Strauss, 1982). In SFV the com-

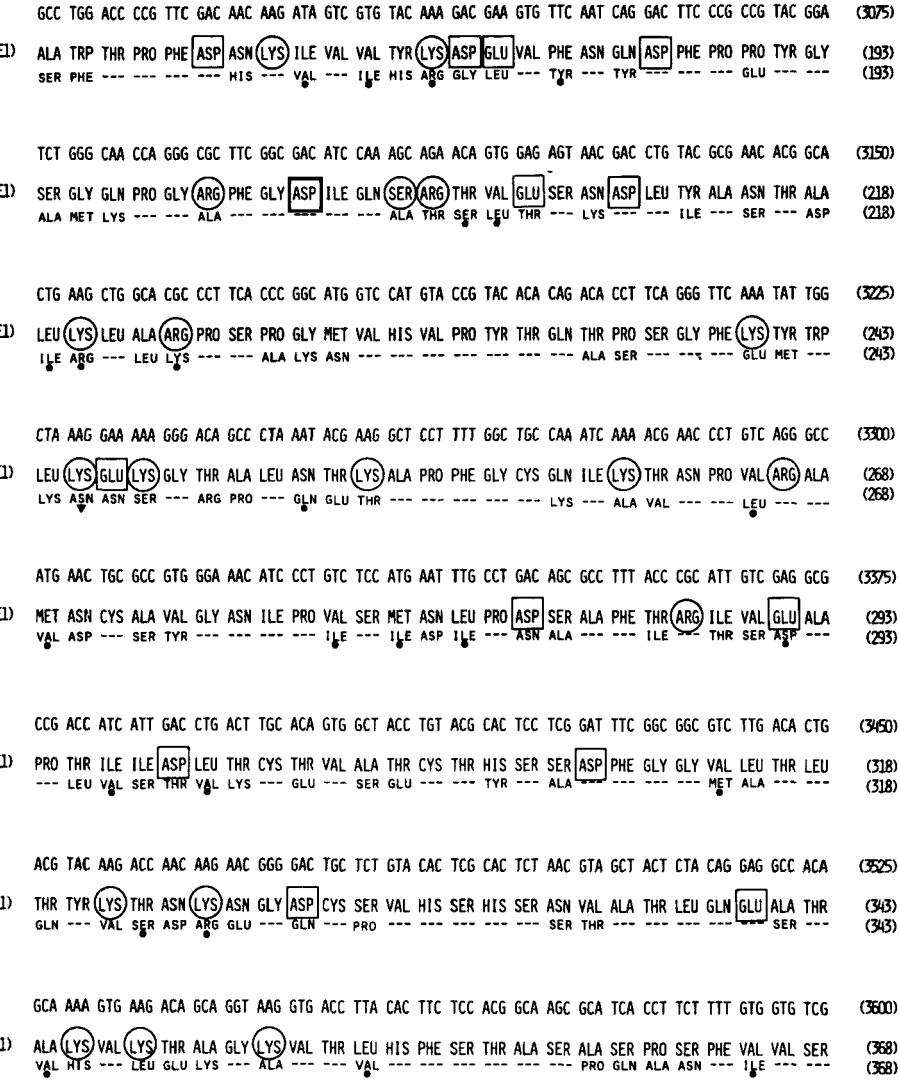

FIG. 1 (*continued*, see legend on pp. 84–85).

plex of E1, E2, and E3 is held together by weak interactions and can be solubilized intact using the mild nonionic detergent Triton X-100. However, if antibodies to either the E1 or the E2 proteins are added, the polypeptide chains dissociate from each other. This separation can also be observed when deoxycholate is used to solubilize the spike glycoproteins (Helenius *et al.*, 1976).

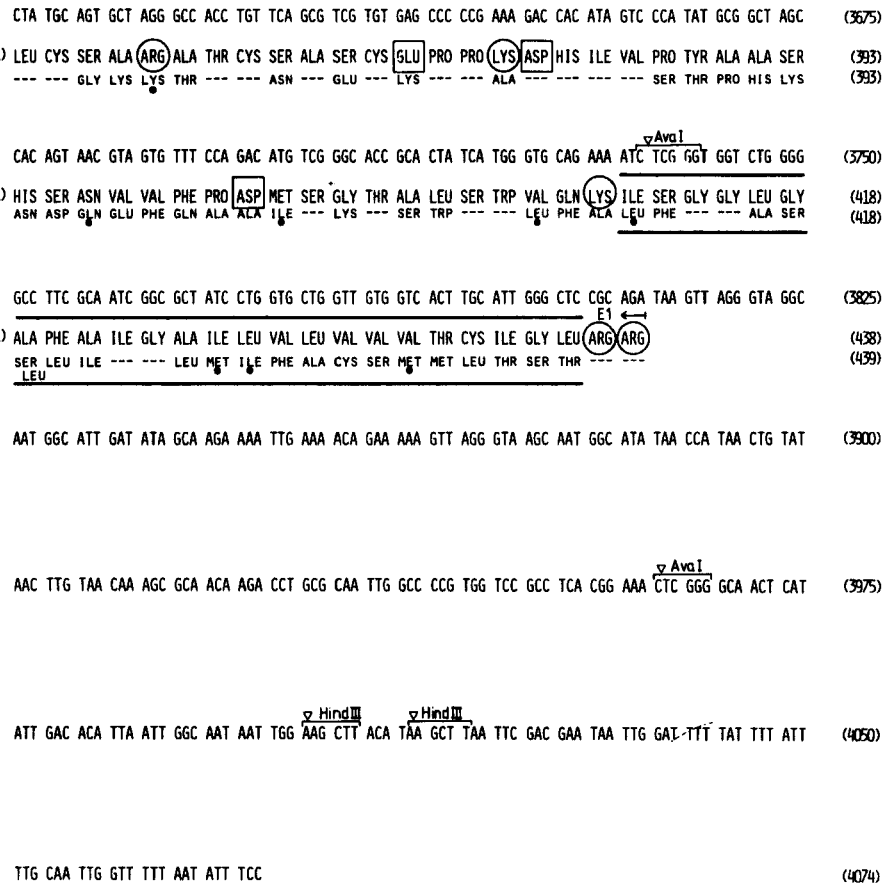

FIG. 1 (*continued*, see legend on pp. 84–85).

If the virus is treated with proteolytic enzymes the fuzzy layer formed by the viral spikes is removed (Osterrieth, 1965; Compans, 1971; Gahmberg *et al.*, 1972; Sefton and Gaffney, 1974; Utermann and Simons, 1974). Remnants of both E1 and E2 are left in the bilayer. These have a hydrophobic amino acid composition, and are soluble in lipid solvents such as chloroform–methanol. The amphiphilic nature of the spike protein is also evident from its capacity to bind Triton X-100 (0.6 g/g protein) which binds to the hydrophobic part to form a water-soluble protein–detergent complex (Simons *et al.*, 1973a). The ability of amphiphilic proteins to bind Triton can be used to separate them from hydrophilic proteins using an extraction procedure recently described

by Bordier (1981). The virus membrane is solubilized with Triton X-114, another detergent of the octylphenolpolyoxyethylene series, and hydrophilic proteins are separated from the amphiphilic ones simply by raising the temperature to 30°C. At this temperature, Triton X-114 separates into a detergent phase containing the viral spike glycoproteins leaving the viral nucleocapsids in the aqueous phase (G. Warren, unpublished observations).

The hydrophobic peptide segments of E1 and E2, which attach the spike protein to the lipid bilayer, can be localized on the polypeptide chains by a mapping procedure first used by Dintzis (1961) to show that the synthesis of polypeptide chains begins at the amino-terminal end. The hydrophobic stubs left in the viral membrane after protease treatment are found at the carboxyl-terminal ends of both the E1 and the E2 polypeptides (Garoff and Söderlund, 1978).

Further studies have shown that not only do the carboxyl-terminal regions of the E1 and the E2 proteins penetrate into the lipid bilayer, but the E2 chain also spans the membrane. When the virus is labeled from the outside and from both sides with formyl[^{35}S]methionyl sulfone methyl phosphate, one additional basic peptide derived from the E2 chain can be labeled (Garoff and Simons, 1974; Simons *et al.*, 1980). This is assumed to be derived from the internal domain of the E2 chain. This internal domain can be demonstrated more directly in vesicles derived from ER membrane after assembly of the viral glycoprotein in the infected cell. These vesicles are "inside out" when compared to the viral particle. Protease digestion of such vesicles removes about 25–30 amino acids from the carboxyl-terminal region of the E2 chain (Garoff and Söderlund, 1978). No comparable evidence has been obtained for the E1 chain. Another approach using the cross-linker dimethyl suberimidate, which cross-links reactive groups that are about 11 Å apart, shows that the spike glycoproteins in the SFV can be cross-linked to the underlying nucleocapsid probably by links between the internal domain of the E2 chain and the capsid protein (Garoff and Simons, 1974). However, it has not been possible to isolate glycoprotein–capsid oligomers, mainly because the basic capsid proteins prefer to cross-link with each other forming large polymers that do not penetrate into polyacrylamide gels (Garoff and Simons, 1974; Richardson and Vance, 1978a). But nucleocapsids cross-linked to glycoproteins can be isolated by density gradient centrifugation after detergent treatment. These contain up to 65% of the spike proteins in the original viral particle. With Sindbis virus, bifunctional amino-reactive reagents have not led to cross-linking of the spike proteins with the nucleocapsid (Rice and Strauss, 1982). However, treatment of the virus with formaldehyde results in such cross-links,

suggesting that similar interactions also exist in Sindbis virus (Brown *et al.*, 1974).

More evidence that the spike proteins are attached to the nucleocapsid can be obtained using mild detergents that solubilize the lipids from the viral particles but leave most of the spike proteins still attached to the nucleocapsid (Helenius and Kartenbeck, 1980). When SFV is treated with 22 m*M* octyl β-D-glucoside at neutral pH and at low ionic strength (10 m*M* NaCl), 80% or more of the spike proteins remain bound to the nucleocapsid. The bound spikes can still be seen after negative staining in the electron microscope. When either the pH is increased or the ionic strength is raised above 50 m*M*, the spike proteins dissociate from the nucleocapsid. This sensitivity to pH and to salt concentration suggests that the interaction of the spike proteins with the nucleocapsid depends on charged groups.

If the pH is lowered to about 6.0 the SFV particle undergoes a dramatic decrease in diameter of about 70 Å which is due to the contraction of the nucleocapsid (Söderlund *et al.*, 1972; von Bonsdorff, 1973). The viral membrane apparently adheres to the nucleocapsid during the contraction, and excess membrane is extruded in the form of blebs. Interestingly, few spike proteins are seen on these blebs suggesting that they contain only lipid and that the spike proteins remain bound to the nucleocapsid during shrinkage.

These observations are all in keeping with the postulated interaction of the spike protein with the capsid protein (Garoff and Simons, 1974), and though they do not show exactly how these proteins interact, it is probable that each capsid protein binds one spike protein via the internal domain of the E2 chain.

b. Primary Structure. The complete amino acid sequence (Fig. 1) of each of the viral glycoproteins has now been established (Garoff *et al.*, 1980b; Rice and Strauss, 1981). They have been deduced from the sequence of the DNA complementary to the 26 S RNA messenger which codes for the structural proteins of the virus (see Section III,B). The coding region for the different proteins was localized from the amino-terminal and carboxyl-terminal amino acid sequences determined by conventional methods (Bell *et al.*, 1978; Bonatti and Blobel, 1979; Kalkkinen, 1980; Boege *et al.*, 1981; Kalkkinen *et al.*, 1980; Welch *et al.*, 1981; Garoff *et al.*, 1982b). The results confirm and extend previous results showing that the genes for the structural proteins are arranged on the RNA in the order 5′–capsid–E3–E2–E1–3′ (Clegg, 1975; Lachmi *et al.*, 1975; Garoff and Söderlund, 1978).

The E3 chain is composed of 66 amino acid residues in SFV, and 64 in Sindbis virus. The E2 protein is 422 amino acids in length in SFV and

423 in Sindbis. The E1 protein is slightly longer being 438 amino acids in SFV and 439 in Sindbis. The overall homology between the structural proteins of the two alphaviruses is striking; 47% of the residues are identical while another 12% represent conservative substitutions.

The most gratifying aspect of these amino acid sequences is that they are fully consistent with the biochemical evidence on the organization of the E1, E2, and E3 glycoproteins with respect to the lipid bilayer. Using the criteria proposed by Segrest and Feldmann (1974) to search for hydrophobic segments that could be embedded in lipid bilayers, three such segments can be found in the SFV protein sequences. Two of these are in the same hydrophobicity range and of the same length as the transmembrane segment of glycophorin (more than 25 residues in length and uninterrupted by charged amino acids) (Tomita and Marchesi, 1975). One is located in the carboxyl-terminal region of the E2 protein between glutamine-352 and alanine-391 in SFV and between proline-364 and cysteine-390 in Sindbis virus. The other is in the carboxyl-terminal region of the E1 protein between isoleucine-413 and leucine-436 in SFV and between threonine-405 and threonine-437 in Sindbis. In all of these segments there is a cluster of basic amino acids marking the carboxyl-terminal end of the hydrophobic peptide, and this is followed in SFV E2 by 31 more residues before the carboxyl terminus is reached, and in Sindbis E2 by 33 residues. This internal domain contains a lysine residue in position 440 in SFV (corresponding to an arginine in Sindbis) which is presumably the lysine which was labeled by formyl[^{35}S]methionyl sulfone methyl phosphate or cross-linked to the capsid protein by dimethyl suberimidate (Garoff and Simons, 1974; Simons *et al.*, 1980). This domain also contains a tyrosine at position 443, a likely cleavage site for chymotrypsin in experiments in which 25–30 amino acids were cleaved from the carboxyl-terminal region of E2 in microsomal vesicles (Wirth *et al.*, 1977; Garoff and Söderlund, 1978). This internal domain in the E2 protein shows strong homology between SFV and Sindbis virus, and is probably involved in the interaction with the capsid protein in the viral particle. In the E1 protein there are only two arginine residues on the carboxyl-terminal side of the hydrophobic segment. This explains why the approaches used to detect the internal domain of the E1 protein failed. Although formal evidence is lacking it seems most likely that the hydrophobic segment of the E1 chain also spans the membrane and the two arginine residues are on the internal side of the bilayer.

Further confirmation for the location of the membrane-spanning domains of the E1 and of the E2 polypeptide chains has come from studies of Sindbis virus. After chymotrypsin digestion of the viral particle, the

hydrophobic stubs left in the membrane have been isolated and sequenced. As expected, the amino-terminal amino acid sequence showed that the E1 and the E2 proteins had been cleaved on the amino-terminal external side of the hydrophobic segments at phenylalanine-398 in E1 and at tyrosine-359 in E2 (Rice *et al.*, 1982).

A number of putative transmembrane segments have been sequenced in several viral and cellular glycoproteins and a comparison of these sequences reveals certain common features (see Warren, 1981; Garoff *et al.*, 1982a; Rice *et al.*, 1982). Each segment has at least 20 residues and contains predominantly hydrophobic amino acids. Charged amino acids (Asp, Glu, Lys, Arg) are excluded as is Pro. These rules are probably most useful in showing which parts of a polypeptide could not span a lipid bilayer. However, they give no indication as to which residues of a putative spanning segment are actually within the lipid bilayer. This can be illustrated for the putative spanning sequence of E1 for both SFV and Sindbis. By the above criteria the spanning segment would be eight residues longer in Sindbis than in SFV (Rice *et al.*, 1982). Whether more of the Sindbis E1 chain is actually located within the lipid bilayer than of the SFV E1 will demand a more direct method of analysis.

The third hydrophobic region found by the Segrest and Feldmann criteria is in the E1 protein (Garoff *et al.*, 1980b). This segment is located between valine-80 and cysteine-96 both in SFV and Sindbis virus. The segment is more highly conserved than the spanning segment of the E1 and the E2 proteins. It does not conform to the criteria for spanning sequences because it is interrupted by a proline residue in both viruses. The function of this segment is not known but it may involve the fusion activity which appears to be a function of the E1 protein (see Section III,A,4).

c. Carbohydrate Side Chains. The oligosaccharides bound to the E1, E2, and E3 proteins are of the *N*-glycosidic type with *N*-acetylglucosamine attached to the amide nitrogen of asparagine. Both high-mannose type and complex oligosaccharides are found in the SFV and Sindbis virus proteins (Sefton and Keegstra, 1974; Keegstra and Burke, 1977; Burke and Keegstra, 1979; Mattila *et al.*, 1976; Mattila and Renkonen, 1978; Pesonen and Renkonen, 1976; Pesonen *et al.*, 1979). They have a common Man-(1 → 6)-Man-(1 → 3)-Man-(1 → 4)-GlcNAc-(1 → 4)-GlcNAc pentasaccharide core. The viral polypeptides are glycosylated by host cell enzymes, and carbohydrate side chains with similar structures are found in both cellular and viral glycoproteins (see Staneloni and Leloir, 1982). The ratio of high-mannose type to complex glycans in the SFV and Sindbis proteins varies with the host cell (Keegstra *et al.*, 1975; Kääriäinen and Pesonen, 1982). The glycosylation sites are determined by the structure of the protein. Not all of the asparagines can

be glycosylated in part because potential sites must conform to the sequences Asn–X–Ser or Asn–X–Thr (Neuberger *et al.*, 1972).

The E1 protein has a single glycosylation site in SFV, which is glycosylated (Garoff *et al.*, 1974; Mattila *et al.*, 1976; Garoff *et al.*, 1980b). When synthesized in BHK-21 cells it appears to contain a two-branched complex oligosaccharide chain, whereas the glycans of the E1 protein made in chick embryo fibroblasts are heterogeneous, consisting of multibranched and two-branched complex chains as well as of high-mannose chains (Mattila *et al.*, 1976; Rasilo and Renkonen, 1979; Kääriäinen and Pesonen, 1982). The cause of this heterogeneity is not known. In Sindbis virus the E1 protein has two potential sites, asparagine-139 and -245, both of which are glycosylated (Burke and Keegstra, 1976; Rice and Strauss, 1981). Both of the oligosaccharides are of the complex type when the virus is grown in BHK-21 cells, but in chick embryo fibroblasts only one is complex whereas the other is of the high-mannose type (Sefton and Keegstra, 1974).

The Sindbis virus E2 protein has two potential sites, asparagine-196 and -318 (Rice and Strauss, 1981). The former has a complex chain, whereas asparagine-318 carries a high-mannose-type oligosaccharide (Sefton and Keegstra, 1974; Burke and Keegstra, 1976). The complex side chain is a two-branched structure of the type that is found also in other proteins (see Staneloni and Leloir, 1982). The E2 protein of SFV also has two potential sites, asparagine-200 and -264, both of which are glycosylated (Garoff *et al.*, 1980b; Mattila *et al.*, 1976; Rasilo and Renkonen, 1979). In BHK-21 cells, the E2 protein has one complex and one high-mannose side chain whereas in chick embryo fibroblasts both seem to be of the high-mannose type (Kääriäinen and Pesonen, 1982).

The E3 protein in Sindbis virus has one glycosylation site (asparagine-14) which is glycosylated (Welch and Sefton, 1979; Rice and Strauss, 1981). This protein, which in contrast to SFV is shed into the extracellular medium of infected cells, contains a single complex oligosaccharide. The E3 protein in SFV has two potential glycosylation sites, asparagine-13 and -60, and it carries only complex glycans. At least asparagine-13 appears to be glycosylated (Garoff *et al.*, 1980b; Kalkkinen *et al.*, 1980).

2. *Lipids*

The lipids in the viral envelope are taken from the host cell. Pfefferkorn and Hunter (1963) had already shown that the viral phospholipids are largely derived from cellular phospholipids synthesized before infection. Subsequent studies of the phospholipid, glycolipid, and cholesterol content of the alphaviruses have shown that the lipid composi-

tions are very similar if not identical to that of the host cell plasma membrane (Renkonen *et al.*, 1971; Laine *et al.*, 1972; Quigley *et al.*, 1971; Hirschberg and Robbins, 1974). By growing the viruses in different host cells, large differences can be obtained in the viral lipid composition (Luukkonen *et al.*, 1976). The small differences observed between the lipid compositions of the viral envelope and of the host plasma membrane can in general be attributed to the contamination of the plasma membrane preparations which cannot be purified to the same extent as those of the virus. Whether alphaviruses assert any selectivity on the set of lipids they take with them from the host cell plasma membrane is therefore difficult to ascertain. On the other hand, the lack of demonstrable specificity is consistent with what is generally known of protein–lipid interactions in biological membranes (Chapman *et al.*, 1979; Seelig and Seelig, 1980). With present methods, specificity cannot usually be demonstrated. It is therefore reasonable to conclude that viral lipids are more or less passively incorporated into the viral particle during budding from the plasma membrane.

The viral lipids are organized into a bilayer about 50 Å in width (Harrison *et al.*, 1971). The distribution of the different phospholipids between the two monolayers has been studied with SFV grown in BHK-21 cells (van Meer *et al.*, 1981). The phospholipids are localized by using phospholipid exchange proteins, by digestion with phospholipases, and by labeling with trinitrobenzenesulfonate. Phosphatidylcholine appears to be about equally distributed whereas phosphatidylethanolamine and sphingomyelin are enriched in the inner monolayer. Phosphatidylserine has not yet been localized. Altogether, 30% of the viral phospholipids can be assigned to the outer monolayer and 50% to the inner monolayer; 20% (phosphatidylserine and some minor phospholipids) have not yet been assigned. Apart from the phospholipids (48 mol% of the total lipid), SFV grown in BHK-21 cells contains 48 mol% cholesterol and 4 mol% glycolipids (Renkonen *et al.*, 1971). Comparable experiments using VSV grown in BHK-21 cells have given similar distributions of lipids in the two halves of the bilayer (see Patzer *et al.*, 1979).

Table I gives a compilation of the molecular composition of SFV grown in BHK-21 cells, based on the revised weight for the viral particle of $41–42 \times 10^6$ daltons (Jacrot *et al.*, 1983). If one assumes that each phospholipid–cholesterol pair takes up a surface area of about 90–100 $Å^2$ (Israelachvili and Mitchell, 1975) and each glycolipid about 55 $Å^2$ (Pascher and Sundell, 1977), then about 80% of the surface area in the bilayer is occupied by the lipids, leaving about 20% for the spanning proteins. This is somewhat more than would be expected if 180 spike proteins span the bilayer, each having two transmembrane α helical segments.

TABLE I
Molecular Composition of Semliki Forest Virus Based on a T = 3 Symmetry Model

Component	MW of component	Molecules per virion	Total MW $\times 10^{-6}$
Nucleocapsid	—	—	9.4
RNA	4.1×10^6	1	4.1
Protein	29.7×10^3	180	5.3
Envelope proteins			
E1	49×10^3	180	8.8
E2	52×10^3	180	9.4
E3	10×10^3	180	1.8
Lipids			
Phospholipids	775	10,000	7.8
Cholesterol	385	10,000	3.9
Glycolipids	1,200	650	0.8
Virion	—	—	41–42

The underlying assumptions in Table I are that the nucleocapsid is built according to $T = 3$ icosahedral symmetry, and that the symmetric arrangement of the spike proteins would be dictated by the direct interaction of one spike glycoprotein with one capsid protein. This interaction is assumed to be the basic structural design of the viral particle. There is no evidence that the nucleocapsid penetrates into the lipid bilayer and interacts with the lipids directly. The isolated nucleocapsid does not bind Triton X-100 and its primary structure shows no obvious hydrophobic regions (Helenius and Söderlund, 1973; Garoff *et al.*, 1980a).

To prove the basic design of the viral particle, the structure of the virus would have to be determined to high resolution. This has not yet been possible. Electron micrographs of thin sections from pellets produced by ultracentrifugation of SFV have shown that the regular arrays of particles seen represent three-dimensional crystals, the largest being up to 5 μm on one side (Wiley and von Bonsdorff, 1978). The probable space group was found to be $F23$. The diffraction pattern of the electron micrographs of the embedded and sectioned crystals of SFV revealed crystalline order to only 100 Å resolution. R. Leberman (EMBL) was able to crystallize SFV by conventional methods. However, X-ray diffraction analyses of these crystals showed that they were difficult to handle and they did not diffract to high resolution. The length of the unit cell edge was 890 Å indicating a nearest neighbor distance between viral

particles of 630 Å (F. Winkler, unpublished observations). The particles seemed to be packed into a face-centered cubic lattice with specific neighbor contacts, but there was only short-range order. Thus particles separated by more than a few unit cells did not scatter coherently to better than about 40 Å resolution.

III. The Life Cycle of Semliki Forest Virus

The structure of an alphavirus particle is simpler than that of all known cellular organelles, but it is built according to the same principles. This is because the viral genome is small and the virus must use for its construction those cellular components normally engaged in the biogenesis of host cell membranes. This means that studies of viral replication can be exploited to study cellular functions at the molecular level. Naturally viral infections also perturb cellular physiology, but there is usually enough time early in infection for studies to be carried out before cellular malfunction becomes a source of error.

The life cycle of SFV is initiated by the delivery of the viral RNA into the cytoplasm of the host cell. The viral RNA is then transcribed into new 42 S RNA molecules and into 26 S messenger RNA molecules which are translated into viral structural proteins. Nucleocapsids are formed in the cytoplasm from the 42 S RNA molecules and capsid proteins. The viral glycoproteins are assembled in the ER membrane, then modified and transported via the Golgi complex to the cell surface. The newly made nucleocapsids bind to the cytoplasmic face of the plasma membrane via the viral glycoproteins, and function as a template for binding more spike glycoproteins. The plasma membrane becomes modified as it wraps around the nucleocapsid and is finally released into the extracellular medium. Only a few functions needed for virus replication are specified by the viral RNA; for the most part the virus exploits the normal function of the host cell. What is special about alphaviruses (and some other enveloped viruses) is that they have specifically adapted the mechanisms by which they enter and leave the host cell to existing routes of membrane traffic connecting the internal cellular compartments with the cell surface.

A. Infection

1. Binding to the Cell Surface

The first phase in the entry of the virus into the cell is its binding to the cell surface. SFV can infect a wide variety of cultured cells of mammalian, avian, or invertebrate origin, suggesting that the virus must recog-

nize surface structures common to many different cell types (Mussgay *et al.*, 1975). For BHK-21 cells, SFV binds with an apparent binding constant of $3 \times 10^{10} M^{-1}$ at pH 6.8; the apparent number of sites is 50×10^3 (Fries and Helenius, 1979). Using the SFV spike proteins isolated in a water-soluble form as (E1, E2, E3) octamers, binding to the H2 and HLA cell surface glycoproteins can be demonstrated (Helenius *et al.*, 1978). However, more recent studies show that these common cell surface antigens cannot be the sole receptors for SFV. There are murine cell lines which do not express major H2-histocompatibility antigens but the cells can nevertheless be infected with SFV (Oldstone *et al.*, 1980). The major problem confronting researchers studying virus–receptor interactions is the multivalency of the binding which leads to tight attachment of the virus to the cell surface even if the interaction between one individual spike protein and one cell surface receptor is of low affinity. The low affinity makes biochemical studies of cell surface receptors for SFV difficult, and which surface molecules can function as receptors for the virus remains unknown. The interaction does not seem to involve sialic acid, since this sugar is not found in mosquito cells (Stollar *et al.*, 1976).

Morphological studies show that SFV particles bound to BHK-21 cells are preferentially associated with the microvillar projections of the cell surface membranes (Helenius *et al.*, 1980). Many of the virions which are not bound to microvilli (5% of all the cell surface viruses) are located in coated pits. The coated pits are invaginations of the plasma membrane, with a characteristic electron-dense coat composed of clathrin and other proteins on the cytoplasmic face (Pearse and Bretscher, 1981). Many of the coated pits are localized close to the base of microvilli.

2. *Endocytosis*

Binding to the cell surface proceeds at 0°C, but the cells are not infected (Helenius *et al.*, 1980). When the cells are warmed to 37°C the virus is rapidly removed from the cell surface and infection ensues. In general there are two ways to envisage the entry of enveloped viruses into cells—either by penetration directly through the plasma membrane, or by endocytosis (engulfment by a plasma membrane-derived vesicle) (see Lonberg-Holm and Philipson, 1974). In both cases delivery of the nucleocapsid with the RNA would have to involve a fusion reaction between the viral envelope and either the cell surface membrane or the vesicle membrane. Paramyxoviruses are known to fuse their envelopes with the plasma membrane (see Hosaka and Shimizu, 1977). However, whether this process leads to productive infection has not yet been settled.

Careful studies by electron microscopy have shown that SFV enters

the cell by endocytosis (Helenius *et al.*, 1980). No evidence for fusion at the cell surface has ever been obtained. The cell-bound viruses are rapidly (within seconds) internalized at 37°C into coated vesicles which form by invagination of the coated pits and usually carry only one viral particle. After 1 minute or longer, viruses can be observed accumulating into larger, irregularly shaped vacuoles. These prelysosomal vacuoles have been named endosomes (see Marsh *et al.*, 1983) or receptosomes (see Pastan and Willingham, 1981). The endosomes are devoid of lysosomal enzymes, and are probably important in membrane recycling. From these vacuoles some viruses find their way into lysosomes, as demonstrated by lysosome-specific staining procedures (Helenius *et al.*, 1980).

The internalization process can be followed quantitatively by using radiolabeled virus and the susceptibility of bound virus to protease treatment; proteinase K at 0°C removes surface-bound viruses but not internalized viruses (Helenius *et al.*, 1980). The uptake process is extremely efficient. The half-life of a virus particle on the cell surface is less than 20 minutes (Marsh and Helenius, 1980). The uptake is saturable at high virus concentrations. However, the saturation observed is due to saturation of binding and not of endocytosis. The highest average rate of uptake measured is around 2000 viral particles per minute per cell. Since, on average, 1.3 viral particles are internalized per coated vesicle, the measured uptake of 2000 viral particles per minute means that, on average, 1600 coated vesicles internalize viruses from the cell surface per minute. This uptake is not induced by the virus. The maximal rate of virus uptake corresponds to the ongoing endocytic rate of BHK-21 cells measured by [^{3}H]sucrose uptake. Thus, SFV uptake must occur by a continuous cellular process. This process is clearly distinguished from phagocytosis which is blocked by cytochalasin B, does not involve small coated vesicles, shows different kinetics, and is induced by the particle to be phagocytosed (Silverstein *et al.*, 1977). Instead, the SFV uptake into BHK-21 cells has exactly the same characteristics as receptor-mediated endocytosis (Goldstein *et al.*, 1979). This interpretation is supported by a large number of similarities between SFV uptake and the endocytosis of physiological ligands such as low-density lipoproteins, asialoglycoproteins, epidermal growth factor, α_2-macroglobulin, and lysosomal enzymes (Kaplan, 1981; Anderson and Kaplan, 1983). These similarities include temperature dependence; kinetics; the involvement of coated pits, coated vesicles, endosomes, and lysosomes; and the effect of inhibitors. The only efficient way to inhibit receptor-mediated endocytosis is to lower the temperature below 10°C. Inhibitors of oxidative phosphorylation also have a marked inhibitory effect on the rate of endocytosis, at least in BHK-21 cells. As shown for a number of physiological ligands,

the number of receptors on the cell surface does not decrease by adding saturating amounts of SFV and letting the viruses endocytose. The cell surface receptors are probably taken into the cell with their ligands which then dissociate, and the receptors are then recycled back to the surface within a matter of minutes (Pearse and Bretscher, 1981).

3. The Endocytic Route Leads to Infection

The first indication that the endocytic route leads to infection came from inhibitor studies. Five well-characterized lysosomal inhibitors (chloroquine, NH_4Cl, amantadine, tributylamine, and methylamine) block infection by SFV (Helenius *et al.*, 1980, 1982). These agents have no direct viricidal effect, and no effect on cell viability during the span of the experiments. Moreover, they do not block binding or endocytosis of the virus. They prevent release of the RNA from an intracellular vacuole into the cytoplasm. This release can be assayed by lysing cells and using ribonuclease as a probe for RNA location; when the nucleocapsid has penetrated into the cytoplasm the viral RNA becomes susceptible to ribonuclease attack. The common target of these lipophilic bases is the lysosome. Being weak bases they rapidly accumulate in this acidic organelle, and raise the lysosomal pH (De Duve *et al.*, 1974; Poole and Ohkuma, 1981). Inhibition of SFV infection can be achieved using concentrations of the inhibitors which have been shown to increase the pH of the lysosomes from 4.8 to around 6.

Recent results indicate that not only do the lysosomes have an acidic pH, but that the endosomes are acidic as well (Tycho and Maxfield, 1982). Thus, lysosomal inhibitors could also assert their inhibitory effect on SFV infection by raising the pH of the endosomes. In fact, it seems likely that this is the case (Marsh *et al.*, 1983). Since lysosomal inhibitors like chloroquine and NH_4Cl raise the lysosomal pH within seconds, they can be used to pinpoint the time at which infection can no longer be inhibited. Such studies show that addition of the inhibitor within 4 minutes after viral entry from the cell surface gives virtually complete inhibition of viral infection whereas after 6 minutes the agent is largely ineffective in preventing the penetration of the viral nucleocapsid into the cytoplasm. These times are more consistent with the effect being localized to endosomes since morphological studies show that viruses do not reach the lysosomes within 6 minutes after leaving the cell surface (Helenius *et al.*, 1980; Marsh *et al.*, 1983). More data have been obtained using other ligands. Dunn *et al.* (1980) have shown that in perfused livers the passage of asialoglycoprotein from endosomes to lysosomes can be prevented by simply lowering the temperature to 20°C. Endocytic uptake into the endosomes is slowed but not inhibited. At this temperature

Marsh *et al.* (1983) have shown that SFV can still infect BHK-21 cells. Moreover, the release of nucleocapsid into the cytoplasm (as measured by the ribonuclease assay) proceeds, albeit 40% more slowly than at 37°C. Thin-section electron microscopy confirms that SFV particles are not transferred to lysosomes at this temperature.

Although strongly suggestive, these experiments do not prove that the normal pathway of infection involves only the endocytic route. Infection could also occur by fusion of the virus with the plasma membrane. This was shown not to be the case by allowing uptake of SFV into BHK-21 cells for 10 minutes at 37°C in the presence of inhibitory concentrations of NH_4Cl (Helenius *et al.*, 1982). All of the viruses left on the cell surface were then removed by proteinase K digestion at 0°C, and after removal of the inhibitor, the incubation continued at 37°C. The intracellular viruses were shown to infect the cells almost as efficiently as in control cells.

4. Penetration by Fusion

One would assume that the mechanism for delivery of the nucleocapsid through the membrane of the intracellular vacuole has to be provided by the virus. There are no known precedents in normal cell physiology for the passage of macromolecular assemblies like the viral nucleocapsids into the cytoplasm. The most likely mechanism would be fusion of the viral envelope with the vacuolar membrane and subsequent release of the nucleocapsid into the cytoplasm. But if penetration occurs by fusion why would this occur intracellularly and not at the cell surface? The clue comes from the low pH dependence of the infection.

Low pH has been shown to induce an extremely efficient membrane-fusion activity (Helenius *et al.*, 1980). The fusion activity of SFV is expressed first at pH values of 6 or lower. The most important tool to study this fusion process has been a quantitative assay based on the ribonuclease (RNase) sensitivity of the nucleocapsid RNA. Liposomes filled with RNase and mixed with SFV below pH 6 degrade the viral RNA introduced into the liposome interior after fusion of the viral envelope with the liposomal membrane. The viral glycoproteins are integrated into the lipid bilayer with the same orientation as in the viral particle; the spikes project from the external surface of the liposome. This assay has shown that besides low pH, the fusion reaction requires the viral spike glycoproteins and cholesterol (optimally one molecule per two phospholipid molecules) in the target membrane (White and Helenius, 1980). The fusion reaction takes place within seconds, does not require divalent cations, is not leaky (if the virus is not damaged, for instance, by freezing and thawing), and is more than 90% efficient.

Although fusion between SFV and the plasma membrane normally does not occur, cell surface-bound viruses can be induced to fuse simply by decreasing the extracellular pH below 6 for a few seconds (White *et al.*, 1980; Väänänen *et al.*, 1981). As a result of its fusion activity SFV can hemolyze red blood cells at pH 5.8 (Väänänen and Kääriäinen, 1979, 1980). However, the lysis occurs only with virus damaged by freezing and thawing. Cells can also be made to fuse with each other using SFV at low pH (White *et al.*, 1981).

The membrane fusion activity is probably a function of the E1 protein, since the hemolytic activity of Sindbis virus can be inhibited by monoclonal antibodies specific for E1 (Chanas *et al.*, 1982). Moreover, studies using cDNA molecules coding for the spike proteins have shown that if the spike protein is expressed at the cell surface, fusion between cells is induced at low pH. However, when the p62 protein is expressed alone, no fusion occurs (Kondor-Koch *et al.*, 1983).

Although the evidence is still incomplete, Sindbis virus seems to enter its host cells by the same mechanism as SFV (Talbot and Vance, 1980, 1982). Moreover, this mechanism is more general since influenza virus and VSV also enter canine kidney cells (MDCK) by endocytosis and by low-pH-mediated fusion (Matlin *et al.*, 1981, 1982). Influenza virus is especially interesting in this context. The major spike glycoprotein, the hemagglutinin, is responsible for the fusion process (Maeda and Ohnishi, 1980; Huang *et al.*, 1981; White *et al.*, 1981). The protein consists of two polypeptide chains HA1 and HA2 (see Simons and Garoff, 1980). The amino-terminal end of the HA2 polypeptide chain is hydrophobic (Skehel and Waterfield, 1975; Porter *et al.*, 1979), and it is this part of the molecule which seems to be involved in the low-pH-induced fusion activity (Richardson *et al.*, 1980). Bromelain treatment releases a water-soluble spike of the hemagglutinin molecule from the viral particle, leaving a short hydrophobic stub in the viral membrane (Brand and Skehel, 1972). The bromelain-released spike protein has been crystallized, and its three-dimensional structure has been determined to 2.8 Å resolution (Wilson *et al.*, 1981). From this structure it is known that the amino-terminal end of the HA2 polypeptide is not available for direct interaction with a target lipid membrane. If, however, the water-soluble spike is subjected to a pH below 6, a drastic conformational change is induced which can be followed by circular dichroism (Skehel *et al.*, 1982). As a result of the low-pH treatment, the spike protein becomes hydrophobic; it binds Triton X-100, it aggregates to form protein micelles, and it attaches to liposomes. Model building based on the spike protein structure suggests that the amino-terminal end of the HA2 protein can be exposed by a conformational change so

as to participate in hydrophobic interactions. Through this interaction the viral membrane and the target membrane might be brought into the close proximity needed to initiate the fusion reaction between the two lipid bilayers.

The viral fusion systems provide a unique opportunity to study membrane fusion at the molecular level. It would be surprising if the mechanism of spike protein-mediated fusion did not have features in common with the mechanisms involved in the fusion reactions occurring during membrane vesicle traffic in the cell. It may even be that the viral fusion proteins have evolved from cellular fusion proteins. The trigger for cellular fusion would of course be different and other proteins would be needed to specify which membranes are to fuse together.

Analysis of SFV entry has thus shown that the virus binds to receptors on the cell surface and moves by lateral diffusion into coated pits to be internalized by coated vesicles. The endocytosed virus is delivered into endosomes. Here presumably, the viral envelope is activated by the low pH prevailing in this compartment to fuse with the vacuolar membrane. This results in the release of the viral nucleocapsid into the cytoplasm. During normal infection, the virus might not enter into lysosomes although SFV particles have been identified in this compartment using the large loads of virus needed to visualize the entry process by electron microscopy. Even if this were to happen normally, the viral nucleocapsid would escape destruction because of the rapidity of the fusion mechanism.

B. Synthesis

After the nucleocapsid has been expelled into the cytoplasm, the RNA must be released from the capsid proteins to allow the viral 42 S RNA to function as a messenger. The uncoating of the nucleocapsid is probably induced by some mechanisms related to the penetration event. The incoming nucleocapsid must be changed in some way to make it different from newly made nucleocapsids, which, after assembly in the cytoplasm, form new virions. It might be that the low pH is the crucial factor. Low pH has a drastic effect on the SFV nucleocapsid causing it to shrink from a diameter of about 400 to 320 Å (Söderlund *et al.*, 1972). This conformational change may induce uncoating of the RNA, although clearly some other factor is also required since uncoating does not occur by low pH alone. The Sindbis virus nucleocapsid does not undergo a similar shrinkage, although the lowest pH tested was 6 and the pH in the endosomes and in the lysosomes is lower than this (Söderlund *et al.*, 1979).

The incoming 42 S RNA serves as a messenger RNA for several non-

structural proteins which are translated from one initiation site at the 5′ end of the molecule (Cancedda *et al.*, 1975; Glanville *et al.*, 1976a; Kääriäinen and Söderlund, 1978). The 3′ end of the 42 S RNA contains the genes for the structural proteins but these are not translated. The nonstructural proteins are subunits of one or more RNA-dependent RNA polymerases which are needed for the replication of the viral RNA (Clegg *et al.*, 1976; Lachmi and Kääriäinen, 1976; Ranki and Kääriäinen, 1979; Sawicki and Sawicki, 1980; Lehtovaara *et al.*, 1980). Two major species of RNA molecules, new 42 S and 26 S RNA, are formed during viral infection (see Strauss and Strauss, 1977). The 26 S RNA molecule is a subgenomic copy of the 3′ end of the 42 S RNA and serves as a messenger RNA for the structural proteins (Kennedy, 1976; Wengler and Wengler, 1976; Schlesinger and Kääriäinen, 1980).

The viral RNA polymerase first transcribes the incoming positive-stranded 42 S RNA into negative-stranded 42 S RNA, which in turn serves as a template for the synthesis of both new positive-stranded 42 S RNA molecules and 26 S RNA molecules. The transcription of the 26 S RNA is initiated internally on the negative-stranded 42 S RNA. RNA replication seems to take place on membranes in characteristic cytoplasmic vacuoles, called cytopathic vacuoles I (CPV I), which appear soon after infection and are not seen in uninfected cells (Grimley *et al.*, 1968, 1972; Friedman *et al.*, 1972). The detailed mechanism of the RNA replication will not be dealt with here (for reviews see Strauss and Strauss, 1977; Kääriäinen and Söderlund, 1978; Kennedy, 1980).

The nucleotide sequence of the 26 S RNA has been determined for both SFV and Sindbis virus (Garoff *et al.*, 1980a,b; Rice and Strauss, 1981; Riedel *et al.*, 1982). In addition, the junction where the 5′ end of the 26 S RNA resides on the 42 S RNA has been sequenced and shows that all three reading frames are efficiently blocked before the first gene of the structural proteins is reached (Riedel *et al.*, 1982). There is thus no overlap between viral genes for the nonstructural and the structural proteins. The 26 S RNA has one initiation site for protein synthesis located 50 nucleotides from the 5′ end (Clegg, 1975; Cancedda *et al.*, 1975; Glanville *et al.*, 1976a; Garoff *et al.*, 1982a; Riedel *et al.*, 1982). The four different structural proteins of the virus are generated by proteolytic cleavage occurring both during and after translation (Fig. 2).

The sequences flanking the initiator AUG in the SFV 26 S RNA are accommodated within the structure $CAXXAUG^{G}_{A}$ that has been considered a possible consensus sequence for a eukaryotic initiation site for translation (Kozak, 1981). Downstream from the initiation codon by 7 bases is a sequence of 11 nucleotides (AUCCCUACGCA), 9 of which (those underlined) are complementary to the purine-rich tract close to

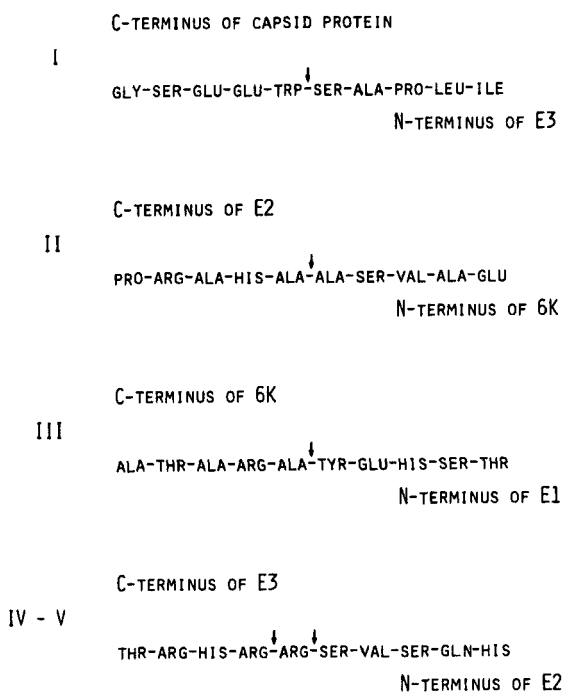

FIG. 2. Proteolytic cleavages involved in the formation of the SFV structural proteins. Cleavages I–III take place during translation of the 26 S RNA, and cleavages IV–V during intracellular transport.

the 3′ end of the ribosomal 18 S RNA (UAGGAAGGCGU) (Riedel *et al.*, 1982). From the initiation codon there is one open reading frame 3760 nucleotides long. The 3′ untranslated region in the SFV 26 S RNA is 264 nucleotides long, not including the poly(A) tail which is 60–70 nucleotides in length (Clegg and Kennedy, 1974; Kääriäinen and Söderlund, 1978).

The gene order on the 26 S RNA has been established by a number of methods, and is now conclusively known from the nucleotide sequence (Clegg, 1975; Lachmi *et al.*, 1975; Garoff *et al.*, 1982a). The gene nearest the 5′ end is the capsid gene followed by the genes for E3, E2, and E1. All the other genes are contiguous except for E2 and E1. These have a segment of 180 nucleotides between them coding for a polypeptide 60 amino acids long, which has been named the 6 K peptide in SFV (Welch and Sefton, 1980; Garoff *et al.*, 1980b). In Sindbis virus it is 55 amino acids long (Welch and Sefton, 1979; Rice and Strauss, 1981). This 6K

peptide has been detected in infected cells, and presumably functions as a signal peptide for the E1 protein (see Section III,B,2).

1. Formation of the Nucleocapsid

About 1.5–2 hours after infection the first viral structural proteins can be detected in cells efficiently infected by SFV (see Kääriäinen and Söderlund, 1978). By 3–4 hours the synthesis of host cell proteins is shut off. The mechanism of host protein shutoff is not yet known, but studies by van Steeg (1982) suggest that it is the viral capsid protein itself which is responsible for the selective inhibition of host protein synthesis. The capsid protein seems to reduce the activity of the initiation factors eIF–4B and CAP binding protein below levels necessary for the formation of the 80 S initiation complex from host mRNA. Translation of the viral 26 S RNA is, however, unaffected. More studies are clearly needed to substantiate this interesting possibility.

After the shutoff of host protein synthesis, the cell has essentially been converted into an assembly line for the production of new viral particles. To assemble new nucleocapsids only two components are needed: capsid proteins and 42 S RNA. The capsid protein is the first to be translated from the 26 S RNA and is cleaved from the nascent polypeptide chain soon after it has been completed (Clegg, 1975; Garoff and Söderlund, 1978; Garoff *et al.*, 1978). The proteolytic cleavage may be catalyzed by the capsid protein itself (Aliperti and Schlesinger, 1978). The newly synthesized capsid protein first associates with the large ribosomal subunit before it binds to the 42 S to form nucleocapsids (Glanville and Ulmanen, 1976; Ulmanen *et al.*, 1976; Söderlund and Ulmanen, 1977). This process is fast and efficient (Söderlund, 1973). Only completed nucleocapsids can be detected in the infected cell (see Kääriäinen and Söderlund, 1978). Neither empty capsids nor partially completed aggregates of RNA and capsid proteins have been identified. Apparently, not only protein–protein interactions, but also RNA–protein interactions play a decisive role not only in initiating the encapsidation process but also in stabilizing the nucleocapsid. Ribonuclease treatment leads to contraction of the nucleocapsid, and, in combination with EDTA treatment, the structure of the SFV nucleocapsid is destroyed (Kääriäinen and Söderlund, 1971; Söderlund *et al.*, 1975).

Wengler *et al.* (1982) were able to assemble nucleocapsids of the correct density and size from 42 S RNA and from isolated capsid proteins. Surprisingly, the interaction between the nucleic acid and the capsid protein was found to be fairly unspecific since it was possible to substitute the viral RNA with RNA and DNA molecules ranging in size from

100 to 6000 nucleotides (e.g., tRNA and fdDNA). In the infected cell there must be some mechanism to prevent the 26 S mRNA from being encapsidated. This *in vitro* assay may make it possible to study the specificity and the mechanism of the assembly process in detail.

2. Assembly of the Viral Glycoproteins in the Endoplasmic Reticulum

The ribosome that translates the capsid protein continues synthesis of the E3, E2, and E1 polypeptide chains. Studies of infected cells show that these proteins are assembled in the membrane of the ER (Wirth *et al.*, 1977; Garoff and Söderlund, 1978). They are also glycosylated during (or soon after) synthesis (Sefton, 1977). The glycosylation is performed by host enzymes. The biosynthesis of N-glycosidic oligosaccharides involves the en bloc transfer of a preformed glycan from an oligosaccharide diphosphate dolichol intermediate to the nascent polypeptide chain (see Kääriäinen and Pesonen, 1982; Staneloni and Leloir, 1982). The oligosaccharide chain consists of $(Glc)_3(Man)_9(GlcNAc)_2$.

The assembly of the virus proteins into the ER membrane can be studied in more detail *in vitro*. Early translation studies *in vitro* usually showed only capsid protein and small amounts of the membrane proteins with aberrant molecular weights (Cancedda and Schlesinger, 1974; Simmons and Strauss, 1974; Glanville *et al.*, 1976b; Clegg and Kennedy, 1975a,b). Only after the introduction of the *in vitro* translation system, supplemented with microsomal vesicles (Blobel and Dobberstein, 1975a,b), did it become possible to translate the 26 S RNA into authentic products representing the structural proteins of the virus (Garoff *et al.*, 1978; Bonatti and Blobel, 1979; Bonatti *et al.*, 1979).

By using an HeLa cell-free system together with microsomes from dog pancreas, four proteins can be made from the 26 S RNA: the capsid protein, the E1 protein, a protein with an M_r of 62,000 (the p62 protein), and small amounts of a large protein, the 97K protein (M_r = 97,000) (Garoff *et al.*, 1978). Exactly the same proteins are seen in infected cells labeled with a short pulse of radioactive amino acids. The p62 protein is a precursor for the E3 and the E2 proteins (Schlesinger and Schlesinger, 1972, 1973; Simons *et al.*, 1973b; Garoff *et al.*, 1974), whereas the 97K protein contains the sequences for all the viral membrane proteins (Lachmi *et al.*, 1975). If the microsomes are left out of the translation system, only two proteins are made: the capsid protein and the 97K protein. In this laboratory the 97K protein has been found to be nonglycosylated both *in vitro* and *in vivo* (Garoff and Schwarz, 1978; Garoff *et al.*, 1978). This apparently represents an aberrant product which is not processed to form the authentic membrane proteins. In cells infected with Sindbis virus there have been claims that the equivalent of the 97K

protein, the B protein (Schlesinger and Schlesinger, 1972; Simmons and Strauss, 1974) is glycosylated (Hakimi and Atkinson, 1982). However, no evidence has been presented showing that the product analyzed was indeed the 97K protein and not a dimer of the E1 protein or another protein (see Kaluza and Pauli, 1980).

With the *in vitro* system the time course of assembly can be followed. The proteins are translated sequentially; capsid protein is followed by the p62 protein and then by the E1 protein (Garoff *et al.*, 1978). The assembly of the polypeptide chains can be monitored by protease treatment (Wirth *et al.*, 1977). Microsomal vesicles are impermeable to proteases, and only structures on the outside (the cytoplasmic side) are accessible to proteolytic degradation. Most of the p62 and all of the E1 polypeptide chains are inaccessible to protease as soon as they can be detected, whereas the capsid protein and the 97K protein are completely degraded. It is the carboxyl-terminal end of the p62 protein that is degraded as expected since the E2 part spans the membrane (Garoff and Söderlund, 1978). Transfer of the p62 and E1 chains through the ER membrane takes place concomitantly with translation. If microsomes are added to the *in vitro* system after translation of the polypeptide chains is completed, no transfer takes place. By synchronized translation experiments modeled according to Rothman and Lodish (1977), it can be shown that the microsomal membranes must be added before about 100 amino acids of the p62 chain have been translated if subsequent assembly of the protein into the membrane is to occur (Garoff *et al.*, 1978). Thus the signal peptide responsible for the initiation of transfer to the ER must be located in the amino-terminal end of the p62 protein in the E3 part.

From these experiments it is not possible to find out whether the E1 protein has its separate signal peptide, mainly because of the low yields of the E1 protein in the synchronized translation experiments. However, evidence for such a peptide has come from studies with a temperature-sensitive mutant of SFV in which the cleavage between the capsid and the p62 protein is blocked (Hashimoto *et al.*, 1981). In cells infected with this mutant at the restrictive temperature, the E1 protein is assembled in the correct orientation into the membrane of the ER. The uncleaved protein containing the capsid and the p62 sequences (M_r = 87,000) is left in the cytoplasm. These findings suggest that the E1 protein has its own signal peptide which might be located in the 6K protein.

Studies with VSV and SFV were the first to show that membrane glycoproteins make use of the same mechanisms used by secretory proteins to become segregated into the ER lumen (Katz *et al.*, 1977; Rothman and Lodish, 1977; Wirth *et al.*, 1977; Lingappa *et al.*, 1978;

Toneguzzo and Ghosh, 1978; Garoff *et al.*, 1978; Bonatti *et al.*, 1979). A signal peptide at the amino-terminal end of the nascent polypeptide to be segregated is recognized by what we now know is a cytoplasmic signal recognition protein which binds the ribosome. This halts translation until the complex binds to a docking protein in the ER membrane so that transfer of the polypeptide chain across the membrane can then take place (Walter and Blobel, 1982a,b; Meyer *et al.*, 1982). However, unlike most secretory and membrane proteins the signal peptide is not removed by a signal peptidase from the E3 protein (Bonatti and Blobel, 1979; Garoff *et al.*, 1982a). Ovalbumin is another exception (Braell and Lodish, 1982; Meek *et al.*, 1982). The putative signal peptide for E1, the 6K peptide, is unusually large for a signal peptide (60 amino acids) (see Kreil, 1981), and exactly when and how it is excised during translation is not yet clear.

In contrast to secretory proteins which are delivered into the lumen of the ER, membrane glycoproteins are assembled into the ER membrane. Membrane anchorage is a function of the hydrophobic peptide segments at the carboxyl-terminal end of the proteins (see Sabatini *et al.*, 1982). The polypeptide chain is apparently transferred into the lumen of the ER until the hydrophobic spanning segment prevents further transfer. There is also another class of plasma membrane glycoproteins and viral glycoproteins which are attached to the membrane by their amino-terminal ends, which are presumed to be their signal peptides (Blok *et al.*, 1982; Desnuelle, 1979; Fields *et al.*, 1981; Hauri *et al.*, 1982).

Those features of the carboxyl-terminal hydrophobic segment needed to attach the SFV proteins to the membrane have been studied by Garoff *et al.* (1983). They constructed a series of deletion mutants from the cDNA (copied from the 26 S SFV RNA), in which the DNA sequences coding for the carboxyl-terminal end of the E2 protein have been shortened, and the 6K and the E1 regions deleted. These DNA molecules containing the genes for the capsid and the shortened p62 protein have been inserted, under control of the early SV40 promoter, in vectors designed for expression of cDNA molecules in animal cells. The DNA molecules have been introduced into the nucleus of BHK-21 cells by microinjection, and the expression of the p62 protein is studied by immunofluorescence using antibodies specific to the E2 protein. Their results show that a shortened gene coding for only three of the amino acids of the internal (cytoplasmic) domain of the E2 protein (together with seven extra amino acids provided by a stop-linker nucleotide) is expressed normally, assembled into the ER, and transported to the cell surface. However, when the deletion extends into the region coding for

the hydrophobic domain, the E2 protein precursor appears not to become membrane bound but is instead secreted into the ER lumen.

The spike protein complex of the alphaviruses appears to be assembled in the ER. Cross-linking studies of Triton X-100-solubilized viral proteins have shown that the p62 and E1 proteins are linked together to form a complex in the ER (Ziemiecki *et al.*, 1980). The topology of these complexes is the reverse of that in the viral particle; the spikes are within the lumen of the ER and the internal domain of E2 is on the cytoplasmic side of the ER membrane.

Glycosylation of the p62 and E1 proteins is not needed for correct assembly in the ER, since in tunicamycin-treated cells, the assembly of these nonglycosylated proteins proceeds normally (Garoff and Schwarz, 1978). The drug tunicamycin blocks the assembly of the dolichol-linked oligosaccharide intermediate so that no transfer of oligosaccharides to nascent protein is possible (Tkasz and Lampen, 1975).

C. Intracellular Transport of the Viral Glycoproteins

1. Posttranslational Modifications

It takes about 30–60 minutes for the spike protein complex to reach the cell surface (Scheele and Pfefferkorn, 1969; Green *et al.*, 1981) and during intracellular transport the viral glycoproteins become modified—the carbohydrate units are trimmed and extended (Kääriäinen and Pesonen, 1982), fatty acid acylation occurs (Schmidt and Schlesinger, 1980), and the p62 protein is cleaved to form the E3 and the E2 proteins (Fig. 3) (Schlesinger and Schlesinger, 1972; Simons *et al.*, 1973b). The topology of the spike proteins appears to remain the same as in the ER; only the internal domain of the E2 chain is exposed on the cytoplasmic side whereas the spike proteins project into the lumen of those intracellular compartments through which the proteins pass during transport to the cell surface (Ziemiecki *et al.*, 1980).

a. Carbohydrate Processing. Most of the data for the biosynthesis and processing of the carbohydrate side chains for membrane glycoproteins have been derived from studies of viral glycoproteins (Hubbard and Ivatt, 1981; Staneloni and Leloir, 1982); VSV has been used for most of these studies (Kornfeld *et al.*, 1978). For the alphaviruses the evidence is not as complete, but it seems to correspond to the pattern established for VSV. After transfer of a primary high-mannose-type oligosaccharide $[(Glc)_3(Man)_9(GlcNAc)_2]$ to the polypeptide chain during translation, the glucose residues are removed. This is followed by the removal of the four α-(1 → 2)-linked mannose residues within 20–30 minutes after trans-

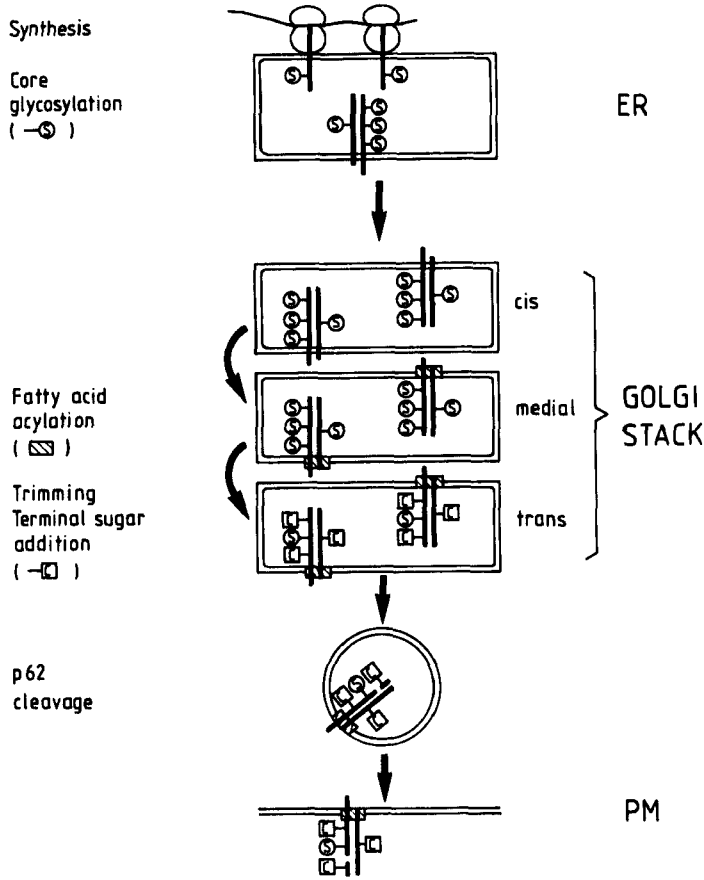

FIG. 3. Intracellular transport route of the SFV spike glycoproteins from the endoplasmic reticulum (ER), over the Golgi apparatus, to the plasma membrane (PM). The cis cisternae do not react positively for acid phosphatase or thiamin pyrophosphatase, and do not label with ricin in thin frozen sections. The medial cisternae do not react positively for thiamin pyrophosphatase or acid phosphatase, but label with ricin. The trans cisternae are positive for all of these markers.

fer (Robbins *et al.*, 1977; Hubbard and Robbins, 1979). Two or more mannose residues are then removed, but only after an *N*-acetylglucosamine residue has been linked to the internal pentasaccharide (Kornfeld *et al.*, 1978; Harpaz and Schachter, 1980a,b). Terminal carbohydrate residues are then added to construct the complex oligosaccharide side chains. The conversion of high-mannose type to complex oligosaccharides can be monitored conveniently using the enzyme endo-β-*N*-acetylglucosaminidase H which cleaves the bond between the two *N*-

acetylglucosamine residues in the high-mannose-type oligosaccharide (Tarentino and Maley, 1974; Tarentino *et al.*, 1978). Complex oligosaccharides are resistant to this enzyme. The alphavirus glycoproteins become resistant to endo-β-*N*-acetylglucosaminidase H about 20–30 minutes after synthesis (Robbins *et al.*, 1977; Green *et al.*, 1981).

b. Fatty Acid Acylation. Both Sindbis virus and SFV E1 and E2 proteins have been shown to contain covalently attached fatty acids (Schmidt *et al.*, 1979; Schmidt, 1982). These are detected by labeling infected cells with [^{3}H]palmitate. Either one or two molecules of palmitate are attached to the E1 polypeptide and five or six molecules to E2 in Sindbis virus. These are located in the carboxyl-terminal hydrophobic stubs obtained after treating Sindbis virus with chymotrypsin to remove the external spikes (Rice *et al.*, 1982). The fatty acids are believed to be linked to serine and threonine residues through an ester bond. These bonds are labile to transesterification and to hydroxylaminolysis, but in no instance has the amino acid to which the fatty acid is attached been directly identified. A number of other glycoproteins both of cellular and viral origin are now known to be acylated (Magee and Schlesinger, 1982). The addition of the fatty acids to the alphavirus polypeptide chain occurs some 20 minutes after synthesis, but before the oligosaccharide chains are processed into the complex form (Schmidt and Schlesinger, 1980; Quinn *et al.*, 1983).

c. Cleavage of the p62 Protein. Four proteolytic cleavages are needed to produce the viral structural proteins of the alphaviruses from the polypeptide chain translated from the 26 S RNA (Fig. 2). The cleavages releasing the capsid protein, the p62 protein, and the E1 protein occur during and not after synthesis (Garoff *et al.*, 1978). The enzymes that excise the 6K peptide are unknown. It could be that the cleavage between the 6K peptide and the amino terminus of the E1 protein is effected by the signal peptidase (see Kreil, 1981). The required specificity, if the bond to be cleaved is X—Y, is that X must be an amino acid with a short side chain. The carboxyl terminus of the 6K peptide is alanine.

The p62 protein is cleaved to generate the E3 and the E2 proteins about 30–35 minutes after translation, shortly after the carbohydrate side chains have been processed to their complex form (Jones *et al.*, 1974; Kaluza, 1976; Ziemiecki *et al.*, 1980; Green *et al.*, 1981). The cleavage site is Arg–Arg–Ser in SFV (Garoff *et al.*, 1980b) and Lys–Arg–Ser in Sindbis virus (Rice and Strauss, 1981). In SFV the last arginine is excised leaving the penultimate arginine residue as the carboxyl terminus of E3 (Kalkkinen, 1980). Such a combined action of a trypsin-like endopeptidase with a carboxypeptidase B-like exopeptidase has been found to be involved in the intracellular processing of a large

number of prohormones and proproteins into their mature forms at a late stage of secretion (see Docherty and Steiner, 1982). The proteases responsible for the dibasic cleavages have not been characterized but different enzymes seem to be involved because the same hormone precursor can be processed differently by different cells.

d. Antigenic Changes. Antibodies have also been used to detect changes in the SFV glycoproteins during intracellular transport. Kaluza *et al.* (1980) have shown that it is possible to obtain antibodies which recognize immature forms of the p62 and E1 proteins. This was done by absorbing polyclonal antisera against the SFV glycoproteins. The immature forms of the proteins recognized by these antibodies disappear about 10–15 minutes after translation. Burke *et al.* (1983) have obtained monoclonal antibodies against the E2 protein that recognize the reverse event—the appearance of a new antigenic determinant in the E2 part of the p62 protein resulting from modifications taking place during intracellular transport. Bonatti and Cancedda (1982) have found that the apparent molecular weight of the E1 protein of Sindbis virus increases shortly after synthesis. This effect was seen in SDS–polyacrylamide gels, but not in those run in the presence of urea, suggesting a change in protein conformation. The precise nature of all of these modifications is still unknown but it would be useful to identify their trigger since changes in protein conformation might well be important in intracellular transport.

e. Other Modifications. The glycoproteins of Sindbis virus are also known to become sulfated (Pinter and Compans, 1975) and phosphorylated (Tan and Sokol, 1974; Waite *et al.*, 1974) during intracellular transport. Where and why these modifications occur are not known.

2. *The Intracellular Transport Route*

Secretory glycoproteins are known to move from the ER to the Golgi complex where their carbohydrate side chains are trimmed and further modified (see Palade, 1975; Tartakoff, 1980; Farquhar and Palade, 1981). In most secretory cells the proteins are then concentrated into condensing vacuoles which store the secretory proteins until they are discharged by exocytosis through a fusion reaction between the vacuolar membrane and the plasma membrane. In other secretory cells, like plasma cells, proteins are continuously secreted and they appear to leave the Golgi complex within vesicles without being concentrated before exocytosis.

The central feature of the Golgi complex is a stack of flattened cisternae which has, in many secretory tissues, a clearly recognizable polarity with the cis side facing the nucleus (see Farquhar and Palade, 1981).

Secretory proteins seem to enter at the cis side and leave the Golgi stack from the trans side, but the route taken through the stack has not been delineated. For membrane proteins there was not until recently any direct evidence that they took the same route from the ER as secretory protein. However, since they carried carbohydrate side chains similar to those present on secretory proteins, it was generally assumed that they would have to pass through the Golgi complex where the enzymes responsible for carbohydrate processing were known to be located. Autoradiographic evidence at the ultrastructural level (Hall *et al.*, 1969; Fambrough and Devreotes, 1978) and immunofluorescence studies with the light microscope (Kääriäinen *et al.*, 1980; Saraste *et al.*, 1980b) suggested that this was indeed the case, but the resolution was not high enough to show that the membrane glycoproteins passed through the stack.

Direct evidence for the involvement of the stacks of Golgi cisternae in the intracellular pathway to the cell surface for membrane glycoproteins came once again from studies with viral glycoproteins (Bergmann *et al.*, 1981; Green *et al.*, 1981). The most extensive studies have been carried out with SFV. BHK-21 cells infected with SFV have been treated with cycloheximide to stop further synthesis of the viral proteins (Green *et al.*, 1981). The viral glycoproteins can then be localized at different times in thin frozen sections of the cells using antibodies against the spike protein labeled indirectly with ferritin or gold particles. This immunocytochemical method allows precise and quantitative localization of the antigens at the ultrastructural level (Tokuyasu, 1980; Griffiths *et al.*, 1983a). Before the addition of cycloheximide the spike proteins are found throughout the membranes of the ER, in all cisternae of the Golgi stacks, and at the cell surface. After the addition of cycloheximide the spike proteins move from the ER through the Golgi stack to the cell surface. Membrane carrier vesicles between the ER and the Golgi, and between the Golgi and the cell surface, could not be identified with certainty. The spike proteins spent about 15 minutes in the ER after the cycloheximide block, and another 15 minutes in the Golgi stack before being routed to the cell surface. Parallel biochemical studies show that many of the oligosaccharides in the viral spike proteins are modified to the complex forms at the same time that the proteins pass through the Golgi stacks. Cell fractionation studies reveal the same pattern; the proteins pass from the ER to the plasma membrane via a vesicle fraction. This fraction must be derived at least in part from the Golgi complex, because it was isolated according to its content of two Golgi markers, galactosyltransferase, an enzyme involved in the formation of complex oligosaccharide chains, and an antigen (M_r = 135,000) specifically localized in the Golgi complex (Green *et al.*, 1981; Louvard *et al.*, 1982). Further studies have shown

that the Golgi stack in BHK-21 cells can be divided into at least three distinct parts, the cis, medial, and trans compartments, each comprising one and at most two Golgi cisternae (Griffiths *et al.*, 1982, 1983b; Quinn *et al.*, 1983). These three compartments have been defined by cytochemical and immunocytochemical criteria (Fig. 3). The pattern of cytochemical labeling is not changed during SFV infection. About 5 hours after infection, rod-shaped structures covered with viral nucleocapsids appear in the infected cells. These have been observed earlier and have been called cytopathic vacuoles II (CPV II) (Acheson and Tamm, 1967; Grimley *et al.*, 1968). In cross section the rods appear as membrane vesicles with nucleocapsids around their outer surface. These membranes are always labeled with ricin and with antibodies to the spike protein. Their function is unclear but they could be aberrant products caused by the massive transport of spike proteins through the cell. These structures are usually found on the trans side of the Golgi stack, and have been used to define the polarity of the stack, which is otherwise difficult to do in BHK-21 cells (Griffiths *et al.*, 1982). When cycloheximide is added to infected cells to stop viral protein synthesis, the labeling with ricin of the trans cisternae decreases by about 50% after the spike proteins have been shown to leave the Golgi stacks. Since ricin labels carbohydrate side chains only after they have been trimmed and galactose has been added, these data suggest that the spike proteins acquire their galactose residues in the trans part of the Golgi in which galactosyltransferase has recently been found to be localized in other cells (Roth and Berger, 1982). The viral proteins in the cis part of the Golgi are not labeled with ricin; their carbohydrate side chains have thus not yet been processed to the complex type. These results are in keeping with the movement of the spike proteins from the cis to the trans side of the Golgi stack.

3. Inhibition of Intracellular Transport

A successful tool in the early studies of metabolic pathways was blocking the pathway at some specific point. This could be done by the use of either mutants or inhibitors. Schekman *et al.* have isolated a number of yeast mutants with blocks in their secretion pathway (Schekman, 1982). It is not yet known which proteins these mutations affect, but this is clearly a most promising approach for identifying those components involved in transport. In animal cells there are no cellular mutants with blocks in the intracellular transport of protein from the ER to the cell surface. There are, however, genetic diseases which affect the routing of lysosomal enzymes to the lysosomes (Neufeld *et al.*, 1975; Sly and Fischer, 1982). For viruses it has been possible to isolate temperature-sensitive mutants in which a mutation in the viral glycoprotein arrests

the protein in different cellular locations at the nonpermissive temperature (see Pfefferkorn, 1977; Saraste *et al.*, 1980a; Zilberstein *et al.*, 1980). A number of inhibitors have also been found that inhibit intracellular transport at different intracellular sites (see Tartakoff, 1980).

a. Temperature-Sensitive Mutants. Burge and Pfefferkorn (1966a,b) were the first to characterize a number of temperature-sensitive mutants of Sindbis virus. A mutant was found in which the viral glycoprotein appears to accumulate in the ER at the nonpermissive temperature, and, after shifting down to the permissive temperature, is transported to the cell surface (Bell and Waite, 1977; Smith and Brown, 1977; Saraste *et al.*, 1980a). Kääriäinen and co-workers have characterized a number of SFV mutants (Keränen and Kääriäinen, 1974; Saraste *et al.*, 1980b; Pesonen *et al.*, 1981). In one mutant, *ts-1*, the glycoproteins are arrested in the ER at the restrictive temperature. The mutation is reversible; transport of the spike glycoproteins to the cell surface occurs when the temperature is lowered to 28°C. Spike glycoproteins from *ts-1* mutants have oligosaccharide side chains exclusively of the high-mannose type at 39°C, but after a shift to 28°C, about 35% of the oligosaccharides are converted to complex glycans in keeping with a transport defect which arrests the proteins in the ER.

Saraste *et al.* (1980b) have postulated the existence of "transport signals" carried by the viral glycoproteins (see also Blobel, 1980). These signals should be recognized by the cellular mechanisms responsible for sorting proteins from the ER to the Golgi complex, and from the Golgi complex to the cell surface. Since the cytoplasmic domain of the SFV spike glycoproteins can be essentially deleted (Garoff *et al.*, 1983) without affecting the transport of the protein to the cell surface, one would assume that the signals for sorting are localized either in the transmembrane domain or in the hydrophilic portion of the spike. However, the basic cluster at the cytoplasmic end (see Section II,B,1) still remained in these deletion mutants, and it could, of course, be involved in transport.

b. Effect of Inhibitors. i. Tunicamycin. In the presence of tunicamycin, the N-glycosylation of nascent proteins is efficiently inhibited. This drug blocks the glycosylation of the p62 and the E1 proteins (Leavitt *et al.*, 1977; Schwarz *et al.*, 1976; Garoff and Schwarz, 1978). The nonglycosylated viral proteins are not transported to the cell surface. Instead, they appear to aggregate in the ER and cannot be extracted with Triton X-100, in contrast to their glycosylated counterparts. Tunicamycin does not always block intracellular transport in this way. A number of other nonglycosylated membrane and secretory proteins are transported to the cell surface normally (see Gibson *et al.*, 1981). Thus, the lack of glycosylation in the case of the alphavirus glycoproteins changed their

solubility in such a way that they aggregated. The carbohydrate side chains seem not to be a necessary requirement for intracellular transport to the cell surface, and cannot therefore be an essential target for the sorting mechanisms responsible for transport of the protein from the ER and from the Golgi apparatus.

ii. Uncoupling agents. Transport of secretory proteins both from the ER to the Golgi complex and from there to the cell surface is blocked by inhibitors or uncouplers of oxidative phosphorylation (see Palade, 1975). Kääriäinen *et al.* (1980) have used the uncoupler carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) to show that the intracellular transport of the SFV glycoproteins is also energy dependent.

iii. Drugs affecting the cytoskeleton. Microfilaments and microtubules do not play a decisive role in cellular secretion (see Tartakoff, 1980). Inhibitors affecting these cytoskeletal components do not block secretion, which continues though often at a slower rate. The same results have been obtained using SFV (Richardson and Vance, 1978a,b; Kääriäinen *et al.*, 1980). Cytochalasin B, which disrupts actin-containing microfilaments, does not affect spike glycoprotein transport. Colchicine and vinblastine, which cause the disappearance of microtubules, decrease the rate of transport of the spike glycoproteins to the cell surface by about 50%.

iv. Ionophores. Monensin, the Na^+ and K^+ ionophore, inhibits the intracellular transport of secretory as well as membrane glycoproteins (see Tartakoff, 1980). This is also true for the alphavirus glycoproteins (Johnson and Schlesinger, 1980; Kääriäinen *et al.*, 1980; Pesonen and Kääriäinen, 1982). Monensin appears to block transport at some point in the Golgi complex but the precise site has been difficult to localize because monensin destroys the characteristic morphology of the Golgi stacks; the flattened cisternae become swollen and separated from each other. Interestingly, in cells infected with SFV or Sindbis virus, some of the swollen Golgi cisternae are found to be covered with nucleocapsids bound to the cytoplasmic face. Apparently, the accumulation of viral spike proteins in these swollen cisternae leads to the binding of numerous nucleocapsids. This observation has made it possible to determine the site at which monensin blocks the transport of SFV glycoproteins in BHK-21 cells. Observations from a variety of cytochemical and immunocytochemical experiments suggest that this site is located between the medial and the trans cisternae in the Golgi stack (Griffiths *et al.*, 1983b; Quinn *et al.*, 1983). In infected BHK-21 cells treated with monensin there was no significant trimming of the high-mannose residues or conversion of the carbohydrate side chains to their complex forms. These functions therefore presumably reside in the trans cisternae, although in

another cell type it has been claimed that mannose trimming occurs proximal to the trans cisternae (Dunphy *et al.*, 1981). Cleavage of the p62 protein in E3 and E2 is also blocked by the drug (cf. Oda and Ikehara, 1982). However, fatty acid acylation of the E1 and the p62 proteins continues during monensin treatment, suggesting that this modification might be a cis or a medial Golgi function. Earlier studies have suggested that acylation does not occur in the ER (Schmidt and Schlesinger, 1980). Due to the increased density of the medial cisternae caused by the binding of the dense nucleocapsids, it is possible to separate them from the cis and the trans Golgi cisternae membranes by density gradient centrifugation (Quinn *et al.*, 1983). If the nucleocapsids are then detached using conditions known to disrupt the interactions between spike proteins and nucleocapsid (pH 8, 0.1 *M* NaCl; see Section II,B,1,a), the membranes lose the bound nucleocapsids and regain the density of Golgi membranes from cells not treated with monensin.

Although monensin appears to block the transport of the SFV glycoproteins at a fairly specific site in BHK-21 cells, the findings cannot be generalized. Different results have been obtained in other cells. Monensin seems to inhibit transport at a number of points along a number of pathways and its effects differ depending on the cell type (Johnson and Schlesinger, 1980; Smilowitz, 1980; Basu *et al.*, 1981; Tartakoff *et al.*, 1981). As a specific example, we can consider chick embryo fibroblasts infected with SFV (Pesonen and Kääriäinen, 1982). In these cells, monensin does not inhibit p62 cleavage, and some conversion of simple to complex oligosaccharides is found to take place (and some intermediate forms are found that are not present normally), but the appearance of the spike proteins on the cell surface is efficiently blocked. In these cells the monensin block would appear to be distal to the medial cisternae, perhaps even after the Golgi stack.

Previously the p62 cleavage has been thought to occur at the cell surface (Bracha and Schlesinger, 1976; Jones *et al.*, 1977; Ziemiecki *et al.*, 1980). The results using monensin show that at least in chicken embryo fibroblasts the cleavage can take place intracellularly. The evidence that the p62 cleavage is a cell surface event is based on the finding that antibodies to the spike glycoproteins applied externally block the cleavage. However, since these antibodies may enter the cell by endocytosis and exert their effect intracellularly, these experiments do not rule out an intracellular cleavage. The p62 cleavage takes place about 5 minutes after the viral spike glycoproteins become resistant to endoacetylglucosaminidase H (Green *et al.*, 1981). This timing would be compatible with the cleavage occurring either in the trans Golgi or on the post-Golgi pathway to the cell surface.

D. Budding

1. Assembly of the Viral Particle at the Cell Surface

The first spike proteins can be detected at the cell surface about 2 hours after infection (Birdwell and Strauss, 1974; Kääriäinen *et al.*, 1980). It takes about 1 hour more before mature viral particles are released extracellularly. The virus is released from the cell by a budding outward of the cell membrane. In this process the nucleocapsid binds to the plasma membrane which wraps around the nucleocapsid and the bud is expelled from the cell (Acheson and Tamm, 1967).

The lipids of the viral envelope are derived from the plasma membrane (see Section II,B,2), but practically all of the host proteins are excluded from the bud. At least 99.5% of the protein in the alphavirus particle is viral (capsid, E1, E2, and E3) (Strauss, 1978). The central problem in budding at the molecular level is to understand how the nucleocapsid and the viral spike proteins recognize each other at the cell surface (Simons and Garoff, 1980). The realization that the SFV spike glycoproteins span the membrane led to the proposal that the binding of the spike proteins to the nucleocapsid was the major driving force in the budding process (Garoff and Simons, 1974). Another interaction facilitating the assembly may be the formation of lateral contacts between the spike proteins (McCarthy and Harrison, 1977; von Bonsdorff and Harrison, 1978). The alphavirus spike glycoproteins move by lateral diffusion after insertion into the cell surface (Birdwell and Strauss, 1974). Their diffusion coefficients have been measured by fluorescence photobleaching recovery experiments (Johnson *et al.*, 1981), and are approximately 5×10^{-10} cm^2 sec^{-1}, which is in the range reported for other cell surface glycoproteins (Peters, 1981). However, the longer infection proceeds, the larger the fraction of the spike proteins that become immobile on the time scale of the measurements; 7 hours after infection 14% are mobile and after 10 hours only about 1%. The immobile fraction may be due to the formation of spike protein aggregates in the membrane plane (see however Johnson *et al.*, 1981, for an alternative explanation). There is evidence that budding may occur in patches especially at the cell periphery (Brown *et al.*, 1972; Birdwell *et al.*, 1973), whereas other areas of the cell surface are devoid of budding figures when examined in the electron microscope. However, it should be pointed out that in other cells there seems to be no clustering of budding figures.

Budding is probably initiated by the viral nucleocapsid binding to a cluster of spike proteins at the cell surface. The binding must be mediated by the cytoplasmic domain of the E2 protein attaching to a capsid

protein in the nucleocapsid. Wrapping of the plasma membrane around the nucleocapsid proceeds when more spike proteins move in and become attached to the underlying nucleocapsid. When the nucleocapsid is completely enclosed by the modified plasma membrane, it may pinch off simply from the strain of the curvature imposed by the nucleocapsid–spike protein interactions. The resealing of the disrupted lipid bilayer would occur spontaneously. In keeping with this self-assembly model for budding, the process seems to be independent of metabolic energy (Waite and Pfefferkorn, 1970; Waite *et al.*, 1972). If cells infected with Sindbis virus are treated with low salt (ionic strength 0.105) further budding is arrested; the nucleocapsids bind to the plasma membrane but do not bud. Treatment of arrested cells with metabolic inhibitors does not affect the subsequent release of viral particles after restoring the cells to normal ionic conditions. The budding process can also be inhibited by antibodies to the spike proteins (Bracha and Schlesinger, 1976). In thin section electron micrographs of such cells, cross-linked clusters of spike proteins can be seen on the external cell surface apposed to clusters of nucleocapsids on the opposite side of the membrane (Smith and Brown, 1977).

The reason that host proteins are excluded from the plasma membrane segment enclosing the nucleocapsid is due probably to their lack of affinity for the capsid protein (Garoff and Simons, 1974). The apposition of the spike proteins on the external side of the bilayer and the close proximity of the nucleocapsid to the cytoplasmic face of the bilayer may effectively prevent host proteins from becoming included in the viral particle.

If self-assembly is the mechanism for budding of alphaviruses, why does budding occur mainly at the cell surface, and not intracellularly? The cytoplasmic domain of the spike protein should be available for interaction with the nucleocapsid during intracellular transport. This is due probably to the low concentration of spike protein intracellularly which may not allow the formation of spike protein clusters. Saraste *et al.* (1980b) studied *ts*-7, the reversible mutant of SFV. In cells infected with *ts*-7, the spike proteins accumulate in the Golgi complex at the restrictive temperature. After lowering the temperature to 28°C, the spike proteins presumably resumed their native conformation. Within 10 minutes, binding of nucleocapsids to intracellular membrane was observed, followed by budding into an intracellular membrane compartment, probably the Golgi. By 60 minutes later, budding was seen mostly at the cell surface, and no longer inside the cell. Intracellular budding can be observed also in monensin-treated cells (Johnson and Schlesinger, 1980; Pesonen and Kääriäinen, 1982; Griffiths *et al.*, 1983b). In these cells, the

spike proteins accumulate in the Golgi stack, and budding can frequently be observed into the swollen Golgi cisternae.

These results suggest that the affinity of the nucleocapsid for the spike proteins is fairly low, and a critical concentration of spike protein is required before assembly can proceed. BHK-21 cells infected with SFV are synthesizing about 100,000 spike proteins per minute between 4 and 6 hours after infection. Quantitation in thin, frozen sections shows that the spike proteins spend on average about 15 minutes in both the ER and in the Golgi compartments (Green *et al.*, 1981). Hence at any time there should be about 1,500,000 spike proteins in each of these membrane systems. The surface areas of the ER and the Golgi membranes in infected cells have been determined morphometrically so that the density of spike proteins can be calculated. These amount to about 85 and 800 spike proteins/μm^2 for ER and Golgi membranes, respectively. Since a typical concentration of spanning proteins in many biological membranes is about 20,000/μm^2, it is clear that at no time during intracellular transport do the spike proteins constitute more than 1 in 250 endogenous ER or 1 in 25 endogenous Golgi proteins. At the cell surface, the spike proteins accumulate and completed viral particles contain about 30,000 spike proteins/μm^2. The low concentrations of spike proteins in transit probably ensure that budding does not take place intracellularly. Other factors may also be operative. The cytoplasmic domain of the E2 chain may not be available for interactions with the nucleocapsid at all stages of intracellular transport. Also the cleavage of the p62 protein which takes place shortly before budding may facilitate the molecular interactions involved in the budding process.

2. *Analogies between Viral Budding and Protein Sorting during Intracellular Transport*

The problem we have not yet touched upon is how components can specifically move from one cellular component to another. Both the entry and the exit of SFV spike proteins are dependent on a number of such cellular processes. The newly synthesized spike proteins move from the ER to the Golgi complex and then to the cell surface. The cell surface membrane is continuously retrieved by endocytosis into endosomes. From here the endocytosed membrane components probably recycle back to the cell surface, but some components may also be channeled into lysosomes for degradation. Especially in cells with secretory activity, the recycling pathway from the cell surface also includes the Golgi complex (see Farquhar and Palade, 1981).

With the exception of coated vesicles, which endocytose surface membrane into endosomes, little is known about the membrane vesicles medi-

ating traffic between the different cellular components (see Farquhar and Palade, 1981). There also are no data on how proteins move through the Golgi stack. Coated vesicles have also been implicated as membrane carriers of viral glycoproteins from the ER to the Golgi stack and from the Golgi to the plasma membrane (Rothman and Fine, 1980). However, because at any time a very small fraction of the protein undergoing intracellular transport is present in the carriers shuttling components from one organelle to another, identification of the carriers has proven to be exceedingly difficult.

It is clear that the membrane carriers cannot remove membrane components randomly from one compartment and move them to another. There has to be some sorting device involved which leaves the proteins belonging to the organelle behind. When a membrane vesicle is formed from one organelle and transported to fuse with another, there must also be replenishment of membrane lipids lost. This cannot in most cases be due to the synthesis of new lipids, but is probably due to a compensating backward traffic of membrane. The membrane vesicles mediating the traffic in both directions must recognize their target membranes, and carry a fusion mechanism for delivery of membrane from one organelle to another.

The difficulty in studying sorting at the molecular level is the present lack of assays. *In vitro* systems need to be worked out, and there are already some promising beginnings in this direction (Fries and Rothman, 1980; Rothman and Fries, 1981). Since molecular studies of cellular sorting processes still seem some way off, it might be useful to consider whether there are any other analogous processes more amenable to experimental study. In principle, SFV budding is such a sorting process. The nucleocapsid is the sorter; the affinity between the capsid protein and the spike protein enables the capsid to sort the viral glycoprotein into a membrane vesicle, the viral particle. The topology of the viral vesicle is reversed compared to intracellular membrane carrier vesicles; the viral vesicle has the cytoplasmic side of the membrane toward the inside, whereas the carrier vesicle has it on the outside. In the viral particle the nucleocapsid functions as a scaffold from the inside, whereas in coated vesicles the clathrin coat forms a polyhedral basket on the outside (Pearse and Bretscher, 1981). In viral budding the specificity of the sorting process is tight. Essentially no host proteins are included and only if a cell is infected with two different alphaviruses, e.g., Sindbis virus and eastern equine encephalitis virus, does the viral particle contain spike glycoproteins from both viruses (Burge and Pfefferkorn, 1966c).

The budding process of more complicated enveloped viruses such as

VSV bears even more resemblance to intracellular sorting. In this virus there is a layer of protein composed of the M protein. This layer lies between the nucleocapsid and the lipid bilayer (which contains one species of a spanning glycoprotein, the G protein) (see Simons and Garoff, 1980). The budding of VSV occurs at the cell surface and is most likely driven by an interaction between the G protein and the internal M protein, although an interaction between the G protein and the nucleocapsid proteins cannot be excluded at present. However, unlike alphaviruses, there is no precise stoichiometry between the G and the M proteins (Lodish and Porter, 1980a). The ratio between them can vary by a factor of about six whereas the ratio between the M protein and nucleocapsid proteins stays constant. In addition, the specificity is not as strict as with the alphaviruses. Mixed phenotypes, that is viruses produced from doubly infected cells and containing the spike proteins of two viruses but only one nucleocapsid, can easily be produced (Zavada, 1982). These contain the VSV nucleocapsid, the VSV M protein, and VSV G proteins mixed with the spike glycoproteins of the other virus used for double infection. Viral particles containing the nucleocapsid and M proteins from the other virus, with a mixture of spike proteins, may also be formed. VSV can form mixed phenotypes with alphaviruses (but not the reverse), RNA tumor viruses, influenza viruses, and parainfluenza viruses. Cellular glycoproteins are excluded from the viral envelope (see, however, Lodish and Porter, 1980b). The inclusion of the foreign viral spike glycoproteins could be due to an interaction either directly with the VSV M protein or with a critical amount of VSV G protein through which association with the M protein could occur (see Witte and Baltimore, 1977). The VSV M protein plays an essential role in the budding process and it can be considered analogous to the capsid protein in SFV, but very little is known of how it functions. A closer study of the VSV M protein in viral spike glycoprotein interactions might be of considerable interest now that methods based on cDNA technology are available which could be used for this purpose.

IV. Perspectives

Studies of the alphavirus life cycle have revealed how heavily the virus relies on cellular processes for replication. The paucity of functions that seem unique to the virus is striking. The binding of the virus to the cell surface and the fusion of its membrane intracellularly depend on the viral spike glycoproteins. RNA-dependent RNA polymerases specific for the virus catalyze the replication of the viral RNA. Exit from the cell requires the interaction of the viral spike proteins with the viral capsid

protein. Close scrutiny of some of these processes may disclose that they are not unique after all. They may, in fact, have evolved from cellular counterparts.

The heavy reliance on the normal function of the host cell is the key to the use of viruses as tools to study the molecular mechanisms of the animal cell. In cell biology the use of viruses as tools has only started. A number of new approaches are emerging. Polarized epithelial cells infected with enveloped viruses distribute the spike glycoproteins to either the apical or the basolateral membrane domain of the cell surface (Rodriguez *et al.*, 1980). There are other enveloped viruses that bud intracellularly; bunyaviruses probably assemble in the Golgi apparatus (Bishop and Shope, 1979; Madoff and Lenard, 1982; Pesonen *et al.*, 1982), whereas coronaviruses mainly bud into the ER (McIntosh, 1974). Their glycoproteins can probably be used as models for the assembly of these organelles. There are viruses like rabies and herpes which enter neurons at peripheral nerve endings and move into the central nervous system probably by retrograde axonal transport (Wolinsky and Johnson, 1980). These examples are by no means exhaustive. There is a plethora of different viruses each of which has adapted to a combination of cellular functions for its own selfish purposes.

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