The Oligomerization Reaction of the Semliki Forest Virus Membrane Protein Subunits

Bernd-Uwe Barth, Johanna M. Wahlberg, and Henrik Garoff

Department of Molecular Biology, Center for Biotechnology, Karolinska Institute, Novum, S-141 57 Huddinge, Sweden

Abstract. The Semliki Forest virus (SFV) spike is composed of three copies of a membrane protein heterodimer. The two subunits of this heterodimer (p62 and E1) are synthesized sequentially from a common mRNA together with the capsid (C) in the order C-p62-E1. In this work heterodimerization of the spike proteins has been studied in BHK 21 cells. The results indicate that: (*a*) the polyprotein is cotranslationally cleaved into individual chains; (*b*) the two membrane protein subunits are initially not associated with each other in the endoplasmic reticulum (ER); (*c*) heterodimerization occurs predominantly between subunits that originate from the same translation product (heterodimerization in *cis*); (*d*) the kinetics of subunit association are very fast ($t_{½}$ = 4 min); and (*e*) this heterodimerization is highly efficient. To explain the *cis*-directed heterodimerization reaction we suggest that the p62 protein, which is made before E1 during 26S mRNA translation, is retained at its translocation site until also the E1 chain has been synthesized and translocated at this same site. The mechanism for p62 retention could either be that the p62 anchor sequence cannot diffuse out from an "active" translocation site or that the p62 protein is complexed with a protein folding facilitating machinery that is physically linked to the translocation apparatus.

Most if not all viral spike proteins that are made in animal host cells are oligomeric complexes of several transmembrane polypeptide subunits (Doms et al., 1993). These in general associate with each other in the ER soon after being synthesized and inserted into the ER membrane. However, the oligomerization process itself is still poorly understood. One important question concerns the possible involvement of oligomerization facilitating factors that could operate both on the lumenal and the cytoplasmic side of the ER membrane (Bole et al., 1986; Doms et al., 1993). Another question is whether oligomerization occurs at specific sites of the ER that offer favorable conditions for this process. For instance, it has been suggested that the oligomerization process could be driven by a high local concentration of subunits generated by the translation activity of ribosomes in the same polysome (Boulay et al., 1988). This possibility has recently been tested experimentally using the influenza hemagglutinin (HA)[1] homotrimer as a model (Boulay et al., 1988). This work showed that the HA subunits were recruited for oligomerization randomly from a mixed pool of subunits in the ER. Thus the products made from individual ribosomes of the same polysome are not any "closer" to oligomerize than those made from the ribosomes of separate ones. In the case of several other viruses (e.g., Flavi, Rubella, Alpha, and Bunya virus) the spike complex consists of heterodimeric oligomers of which the subunits are made from a common translation product (Baron and Forsell, 1991; Persson and Pettersson, 1991; Wengler and Wengler, 1989; Ziemiecki and Garoff, 1978). This product is cotranslationally inserted into the ER membrane at a translocation site and separate subunits are generated by proteolytic cleavage events mediated by signal peptidase (Garoff et al., 1978). Because of the mode of synthesis it is reasonable to assume that the spike complex of these viruses would be formed by the association of subunits generated from the same translation product (heterodimerization in *cis*). Such local recruitment of subunits would ensure both fast and efficient oligomerization. Surprisingly this was to the case for the spike heterodimeric complex (G1-G2) of the Uukuniemi virus (a Bunyavirus) when tested experimentally (Persson and Pettersson, 1991). Instead G1 and G2 were found to be separated from each other soon after the synthesis and cleavage of the common translation product. The G1 part matured rapidly into its correct conformation and complexed with earlier made G2 subunits. In contrast the G2 part obtained its correct conformation only slowly and eventually complexed with G1 made at a later time point.

In the present work we have analyzed the oligomerization

J. M. Wahlberg's present address is Department of Biochemistry, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland.

Address all correspondence to H. Garoff, Department of Molecular Biology, Center for Biotechnology, Karolinska Institute, Novum, 14157 Huddinge, Sweden. Ph.: (46) 8 608 9100. Fax: (46) 8 774 5538.

1. *Abbreviations used in this paper*: C, capsid; HA, hemagglutinin; NC, nucleocapsid; NEM, N-ethylmaleimide; SFV, Semliki Forest virus; wt, wild type.

process of the spike heterodimer p62–E1 of the alphavirus Semliki Forest virus (SFV). The two subunits are translated from a 26S mRNA together with the viral capsid (C) protein and a small transmembrane (6K) peptide (Garoff et al., 1980). Translation starts with C, which is able to proteolytically cleave itself from the extending polyprotein, and continues with p62, 6K, and E1. The latter three chains carry topogenic signals that direct their insertion into the ER membrane via the ER translocation machinery for secretory products (Garoff et al., 1990; Lijeström and Garoff, 1991; Melancon and Garoff, 1986). The p62 and the E1 subunits associate into heterodimers which are then routed to the cell surface where they drive virus budding by promoting virus envelope-nucleocapsid (NC) interactions (Green et al., 1981; Suomalainen et al., 1992; Ziemiecki and Garoff, 1978; Ziemiecki et al., 1980). Before being used for virus budding the p62–E1 complexes are proteolytically modified. The p62 subunit is cleaved (after residue 66) into the small peripheral E3 part and the transmembrane E2 part. Furthermore, the heterodimer trimerizes into a homotrimer (E2–E1) × 3, and this constitutes the SFV spike (de Curtis and Simons, 1988; Fuller, 1987; Garoff et al., 1980; Lobigs and Garoff, 1990; Vénien-Bryan and Fuller, 1994; Vogel et al., 1986).

Materials and Methods

Cells, Virus, Plasmids, and Antibodies

BHK 21 cells were grown in Glasgow minimal essential medium (GIBCO BRL, Glasgow, Scotland; MEM [BHK-21], with L-glutamine, without Tryptose phosphate broth) supplemented with 10% tryptose phosphate broth, 5% fetal calf serum, 20 mM Hepes (pH 7.3), and 2 mM glutamine. Penicillin (100 U/ml) and streptomycin (100 μg/ml) were also added to media for passage of cells. Cells were incubated at 37°C and 5% CO_2. For most purposes cells were grown in 35 mm dishes (about 1×10^6 cells).

SFV wild type (wt) virus was the laboratory strain, SFV4, (Liljeström et al., 1991). Plasmids encoding complete or mutant SFV sequences were: (*a*) pSFV4, which contains the wt genome (Liljeström et al., 1991); (*b*) pSFV C-p62*-E1, which contains the complete structural gene region but with a mutated p62* cleavage site (Berglund, 1993); (*c*) pSFV p62*-E1, which contains a C gene deletion in addition to the p62 cleavage site mutation; (*d*) pSFV C-p62, which contains wt C and p62 but no E1 gene; and (*e*) pSFV p62, which contains wt p62 but no C nor E1 genes. In all plasmids the SFV sequences can be transcribed by SP6 polymerase into RNA molecules (the viral genomes) which are competent for replication in cells.

Antibodies used were monoclonal antibody UM 8.139 against E1 subunit and monoclonal antibody UM 5.1 against E2 subunit (Boere et al., 1984; Wahlberg et al., 1989). These were used as mouse ascites preparations.

Infection and Transfection of Cells

Wt virus was used to infect BHK 21 cells at a multiplicity of infection (m.o.i.) of 50. Infection was done by incubating cells at 37°C in a 35 mm Petri dish with virus suspension in 500 μl MEM medium (MEM with Earle's Salts, without L-glutamine; GIBCO BRL) for 1 h. After this, 2 ml of BHK medium was added and incubation continued.

Transcription of RNA from plasmid DNA was done in a 50 μl mixture as described in Liljeström et al. (1991). One third of the mixture (~17 μl) was used to transfect 5×10^6 BHK cells by electroporation in a 0.4 cm cuvette (850 V/25 μF) (Liljeström et al., 1991). Efficient transfection was achieved by thoroughly suspending cells beforehand by trypsinization and by gentle mixing. After transfection the cells were diluted 20-fold with BHK medium, distributed into six 35 mm plates and incubated at 37°C in a 5% CO_2 atmosphere.

Metabolic Labeling of Viral Proteins

For the labeling of one dish of cells ~50 μCi of [^{35}S]methionine (Amersham Intl., sp. act. >37TBq/mM, >1,000 Ci/mM) was used. Labeling was carried out for different amounts of time in 500 μl of MEM lacking methionine (GIBCO BRL; MEM with Earle's Salts, without L-glutamine, without L-methionine). The incubation of cells in this media was started 30 min before labeling, to prestarve cells of methionine. Labeling was terminated by changing the medium to MEM containing 10 times concentrated unlabeled methionine. Chase in this medium was continued for different amounts of time. Cells infected with wt virus were labeled 5 h after starting the infection. Transfected cells were labeled 7 h after electroporation.

Preparation of Cell Lysates and Media

After pulse-chasing of cells these were put onto ice, the medium was removed, cells were washed with cold PBS and solubilized with 300 μl of NP-40 (1%) lysate buffer (Wahlberg et al., 1989), containing PMSF (10 μg/ml) and NEM (N-ethylmaleimide) (20 mM). The lysate was centrifuged at 5,000 *g* for 5 min at 4°C. In some cases cells were also solubilized in SDS lysis buffer (1% SDS, 50 mM Tris/HCl, pH 7.4, 2 mM EDTA, 10 μg/ml PMSF). SDS lysis was done at room temperature, the SDS lysate was passed five times through a gauge (20G1½ 9 × 40) needle, heated to 70°C for 5 min and finally centrifuged for 5 min at 5,000 *g*. The supernatant was transferred to a fresh tube. The medium of the cell culture was separated from any released cells by centrifugation for 5 min at 5,000 *g*.

Immunoprecipitation

One third (100 μl) of an NP-40 lysate sample was used for protein A-mediated immunoprecipitation of viral membrane proteins as described previously (Wahlberg et al., 1989). When virus particles were precipitated from medium, NP-40 was omitted in all steps of the immunoprecipitation protocol (Ekström et al., 1994). Final precipitates were solubilized into 80 μl SDS gel sample buffer of which 20 μl was applied onto a 10% gel (Cutler and Garoff, 1986) (mighty small gel system; Hoefer, San Francisco, CA). When gels were run under reducing conditions the loading buffer was adjusted to 50 mM DTT and the sample was heated 5 min at 95°C before it was loaded onto the gel.

Centrifugation in Density Gradient

Cell lysate (250 μl) was layered onto a 5–20% sucrose gradient (wt/wt) in TNE (30 mM Tris, 100 mM NaCl, 1.25 mM EDTA, pH 7.4) containing 0.1% NP-40 and 10 μg/ml PMSF and centrifuged in an SW41 rotor at 39,000 rpm for 24 h at +4°C (Wahlberg and Garoff, 1992). The gradients were fractionated from below into 43 fractions (300 μl). Proteins were recovered by TCA precipitation. This was done by adding 40% TCA to a final concentration of 10%, incubating on ice for 30 min, and finally pelleting the precipitate at full speed for 30 min in an Eppendorf benchtop centrifuge at 4°C. Each precipitate was taken up into 30 μl SDS gel sample buffer containing 50 mM DTT by incubating 5 min at 95°C and sonicating the sample.

Other Techniques

Fluorography was carried out as described in Wahlberg et al. (1989). Quantitation of radioactive viral protein subunits was done from corresponding bands in PAG using a Fuji phosphoimager (type FUJIX BAS 2000 TR). The radioactivity found in each band was normalized to the methionine content of corresponding polypeptide before being used for calculations. Calculations are described in footnotes to Table I. $t_{½}$ value for heterodimerization expresses the time required for oligomerization of about 50% of p62 and E1 subunits. The value given includes 1/2 of pulse time plus chase time.

Results

Cotranslational Processing of the SFV Structural Polyprotein

The generation of the p62 and E1 chains during 26S mRNA translation was studied in SFV infected BHK-21 cells using a 2-min pulse time with [^{35}S]methionine and chase times ranging from 0–10 min. Fig. 1 *a* shows the labeled proteins in such cell samples. These have been solubilized in SDS lysis buffer and reduced before analysis by SDS-PAGE. The three structural proteins, C (33 kD), p62 (62 kD), and E1 (50 kD) can clearly be identified. In addition, there are significant amounts of labeled material which correspond in

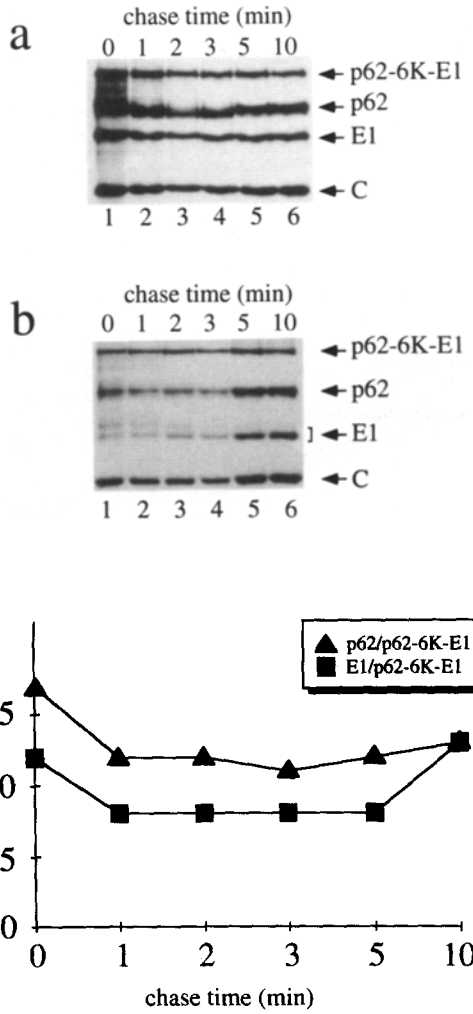

Figure 1. Time course of membrane polyprotein cleavage. BHK cells in dishes were infected with wt SFV. 5 h after infection, proteins were metabolically labeled with [^{35}S]methionine for 2 min. After chase times for 0, 1, 2, 3, 5, and 10 min cells were lysed either with a SDS lysis buffer (*a*) or with a NP-40–based buffer (*b*). The samples in *a* were reduced before analysis by SDS PAGE (10%) whereas those in *b* were not. In *c* radioactivity of protein bands in *a* have been quantitated and the p62/p62-6K-E1 and E1/p62-6K-E1 ratios calculated for each time point of the chase.

size (~100 kD) to unglycosylated and unprocessed membrane polyprotein p62-6K-E1 (Garoff et al., 1978). Host proteins are not labeled due to the efficient shut-off of host mRNA translation by SFV infection (Garry, 1994). At the 0 min time point there are also several bands with intermediate mobility. These most likely correspond to incompletely elongated C, E1, p62, and p62-6K-E1 chains. Such chains are to a less extent also present after 1 and 2 min chase times. Fig. 1 *b* shows the corresponding analysis of cell samples which have been solubilized in an NP-40 lysis buffer. In this case samples for SDS gel analysis were not reduced. The overall picture is very similar to that of Fig. 1 *a*. However, at early times after synthesis a slower migrating E1 form is seen in addition to the mature one. This could represent a folding intermediate of E1 with aberrant disulfide bond(s) (Marquardt and Helenius, 1992). A quantitation of the p62-6K-E1, p62, and E1 material in the analysis shown in Fig. 1 *a* shows that there are very similar ratios of p62/

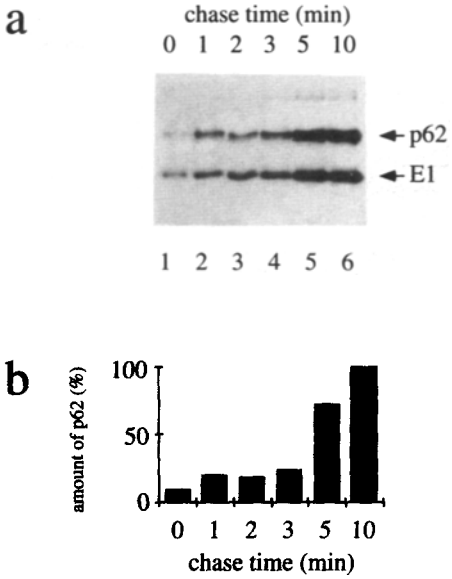

Figure 2. Time course of heterodimerization: coimmunoprecipitation analysis. Cells in dishes were infected, pulse-labeled and chased as described in legend to Fig. 1. After lysis with NP-40 containing buffer the p62–E1 heterodimers were captured with anti-E1 monoclonal antibody. The precipitates were analyzed by SDS-PAGE (10%). The corresponding autoradiograph is shown in *a*. Note that in this case samples have not been reduced before analysis. Quantitation of heterodimers is shown in *b*; the values represent co-precipitating p62 subunits in relation to p62 found in the sample chased for 10 min.

p62-6K-E1 and E1/p62-6K-E1 at all time points during the chase (Fig. 1 *c*). This suggests that the p62 and E1 chains are produced cotranslationally rather than posttranslationally. If the latter was true it would be expected that the p62-6K-E1 product would predominate immediately after the pulse and then become cleaved into individual subunits during the chase. Thus the present results support our earlier in vitro studies of the SFV 26S mRNA translation. In there we have clearly shown that most p62 and E1 chains are cleaved during ongoing elongation, translocation across the ER membrane, and glycosylation (Garoff et al., 1978). A minor fraction of the in vitro translation product was also found to remain untranslocated, uncleaved, and nonglycosylated.

Kinetics of Heterodimerization

Coimmunoprecipitation Analysis. To study the kinetics of the p62 and E1 heterodimerization reaction we first followed the immunoprecipitation of heterodimers with a monoclonal anti-E1 antibody (UM 8.139; Wahlberg et al., 1989) from cells which have been infected, pulse labeled, chased, and lysed with NP-40 as described above. The result of the heterodimer extraction is shown in Fig. 2 *a*. One can see that p62–E1 heterodimers can be captured from all cell samples, including the one solubilized immediately after the pulse. However, quantitation (Fig. 2 *b*) shows that very little p62 and E1 is present in such complexes immediately after the pulse. About 5-min incubation time is required before a maximum of p62–E1 heterodimers are captured in this assay.

Sedimentation Analysis. To see whether the newly synthesized p62 and E1 chains, which did not precipitate with

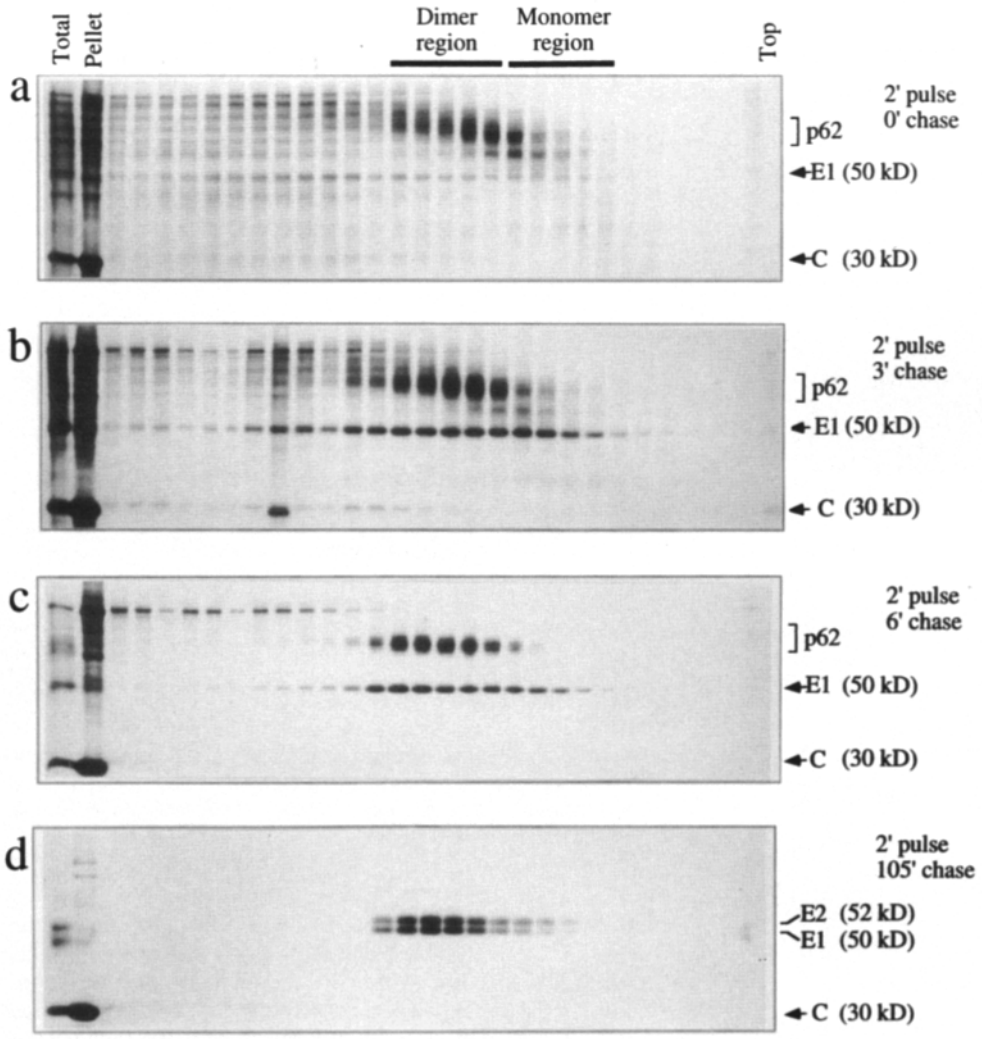

Figure 3. Time course of heterodimerization: sedimentation analysis. BHK cells in dishes were infected with wt SFV, pulse-labeled, and then lysed as described in legend to Fig. 2. Chase times were 0, 3, 6, and 105 min. Lysates were run on a 5–20% sucrose gradient (wt/wt) in an SW41 TI rotor at 4°C and at 39,000 rpm for 24 h. The gradients were fractionated, proteins precipitated and samples analyzed by SDS-PAGE (10%) under reducing conditions. Totals represent 4% of sample loaded on gradient. Sedimentation standards for p62–E1 heterodimers and the corresponding monomers were obtained from NP-40 solubilized viral membranes from untreated or acid-treated SFV particles (Wahlberg et al., 1989). These were analyzed in parallel and the fractions containing dimers and monomers are indicated on top of the panels. Viral structural proteins p62 (*62 kD*), E1 (*50 kD*), E2 (*52 kD*) and C (*33 kD*) are indicated. Note that autoradiographs shown in *a* and *b* have been exposed three times longer than those shown in *c* and *d*.

the monoclonal anti-E1 antibody were separated or together we performed sedimentation analysis of NP-40 lysates in sucrose gradients at conditions previously described to separate heterodimers and monomers (Wahlberg and Garoff, 1992). For this purpose BHK-21 cells were infected and pulse-labeled (2 min) as before and then chased for 0, 3, 6, and 105 min. As controls heterodimeric and monomeric forms of the spike proteins, which were derived from SFV particles solubilized with NP-40 in neutral and acid buffer, respectively (Wahlberg et al., 1989), were analyzed in parallel gradients. Fig. 3 *a* shows the labeled proteins from cells which have not been chased, as analyzed by SDS-PAGE after sedimentation and fractionation of the gradients. At the earliest time point most of the p62 and the E1 chains which are found appear in different regions of the gradient. The E1 chains fractionate predominantly into the pellet and into the bottom half of the gradient. In contrast, the p62 chains are found mostly in a region between control E1 and E2 dimers and monomers. Several other bands with intermediate migration properties as compared to p62, E1, and p62–6K–E1 proteins are also seen in this analysis. These could correspond to the afore mentioned incomplete translation products of the viral proteins. Most of these latter products appear in the lower half of the gradient. At a time point 3 min after the pulse (Fig. 3 *b*) the majority of the p62 and E1 chains are seen to cofractionate as apparent heterodimers. However, significant amounts of E1 is still present in large complexes. Some E1 appears also to be present in the monomer fraction. After a 6-min chase the heterodimerization reaction seems to be complete (Fig. 3 *c*). After additional chase time the p62 subunit is proteolytically processed into the E2 form (Fig. 3 *d*). The C protein is almost totally pelleted in all samples. This is consistent with rapid formation of nucleocapsid structures (140S) in infected cells (Söderlund and Ulmanen, 1977). Thus, these results show that the p62 and E1 chains are made as separate units which have the capacity to heterodimerize fast and efficiently. The half time for this can be estimated to about 4 min.

Heterodimerization in cis Versus trans

Experiments were designed to determine whether the SFV spike heterodimer is formed predominantly by p62 and E1 subunits originating from the same polyprotein translation product (heterodimerization in *cis*), or from a pool of subunits originating from different translation products (heterodimerization in *trans*). Two different genetic variants of SFV, C-p62*-E1 and C-p62 were used in this study. The

former directs the synthesis of p62 and E1 from a wt-like common coding unit, but the p62 encoded by this genome does not undergo processing into the E2 form due to a mutation at the cleavage site (Berglund, 1993; Salminen et al., 1992). The SFV C-p62 variant directs the synthesis of wt-like p62, but no E1 (Barth, B.-U., and H. Garoff, unpublished data). Fig. 4, *a* and *c*, show pulse-chase analyses of viral membrane protein heterodimers in cells which have been transfected separately with C-p62 and C-p62*-E1 RNA, respectively. Subunits p62* and E1 encoded by the C-p62*-E1 genome efficiently formed heterodimers in the ER, as judged by coimmunoprecipitation of p62* by the monoclonal anti-E1 antibody (Fig. 4 *c*, lane *1*). As expected, the p62* was not converted to E2 during the chase. The p62*-E1 heterodimers were however, transported to the cell surface since they became efficiently incorporated into virus particles (Fig. 4 *c*, lane *5*). C-p62 RNA directed the synthesis of p62 subunits, which were normally processed to E2 upon transport to the PM (Fig. 4 *a*). However, while E2 in the wt E2-E1 heterodimer retains one of its two asparagine-linked sugar units in an untrimmed and endo H-sensitive form, the E2 which is expressed without E1 is trimmed at both units (Mattila, 1979; Barth, B.-U., and H. Garoff, unpublished results). This completely endo H-resistant form of E2 (E2′) migrates in SDS-PAGE in an intermediate position between p62 and normal E2 (Fig. 4, *a* and *e*, lane *2*). Note also that there is significant degradation of the p62/E2′ products during the 3-h chase.

To determine whether the SFV spike complex is formed by subunits originating from the same polyprotein translation product or from a mixed pool of subunits, the C-p62*-E1 and C-p62 RNAs were cotransfected into cells. If heterodimerization occurs in *cis*, E1 subunits in the co-transfected cells complex with p62* only, whereas if heterodimers are formed from a mixed pool of subunits, both p62*-E1 and p62-E1 heterodimers are formed (in proportions dependent on the p62* versus p62 concentrations). The p62*-E1 and p62-E1 heterodimers can be distinguished by the latter being converted into E2-E1 forms during the chase. Fig. 5 *a* shows pulse-chase analyses of viral proteins in C-p62*-E1 RNA and C-p62 RNA co-transfected cells. SFV spike proteins were immunoprecipitated from cell lysates by monoclonal anti-E1 or -E2 antibodies. The analysis shows extensive coimmunoprecipitation reactions involving the E1, p62, and E2 subunits. This suggests the formation of both noncleavable p62*-E1 and cleavable p62-E1 heterodimers in the cells. In addition we can detect much p62-derived E2′ in the chased samples (seen as a broadening of p62 band in lanes *2–4*, *right*). The E2′ doesn't seem to complex with E1 as it is not coimmunoprecipitated with the anti-E1 antibody (see lack of E2′ in the αE1 antibody assay).

The relative efficiencies of the *cis* versus *trans* heterodimerization reactions were studied by quantitative analysis. The relative concentrations of the pulse-labeled p62* and p62 were first measured in the 5-min chase sample as follows. The amount of p62* was assumed to be equivalent to that of E1, which was made from the same coding unit and was possible to quantitate by the precipitation with anti E1 monoclonal antibody. The p62 amount was obtained by first quantitating the p62 and p62* together as recovered by precipitation with the anti-E2 monoclonal antibody and then subtracting from this sum the amount of p62*. The results

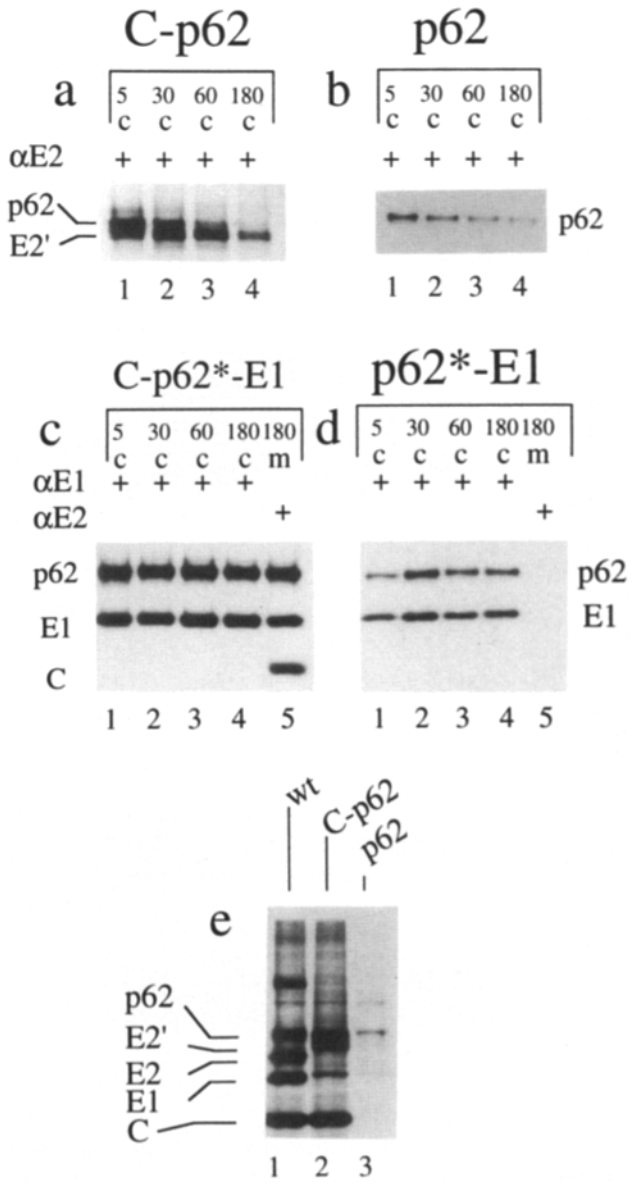

Figure 4. Phenotypes of SFV spike subunits produced by SFV C-p62, SFV p62, SFV C-p62*-E1, and SFV p62*-E1 variants. BHK cells were transfected with the indicated RNAs (*a–d*) and plated on several dishes. At 7 h after transfection the cells were labeled with [^{35}S]methionine for 15 min and chased for 5, 30, 60, and 180 min, respectively. At the end of the chase period media was collected and the cells lysed with NP-40. Aliquots of the cell lysates and 180 min chase media were extracted with monoclonal antibodies against E1 (*αE1*) or E2 (*αE2*) as indicated, and the immunocomplexes were analyzed by SDS-PAGE followed by fluorography. Note that *b* and *d* have been exposed six to seven times longer than *a* and *c*. In *e* the cells were transfected individually with SFV-4 RNA (containing the wt genome), SFV C-p62, and SFV p62 RNA, and then labeled, chased for 30 min, and lysed as above. A 10-μl aliquot of the cell lysates was mixed directly with 2 × SDS sample buffer and analyzed by SDS-PAGE followed by fluorography.

show that the membrane protein products of the two coding units (p62* and p62) are expressed at approximately 1.5:1 ratio (Table I). If subunits would be recruited for heterodimerization randomly from a common pool of monomeric p62*,

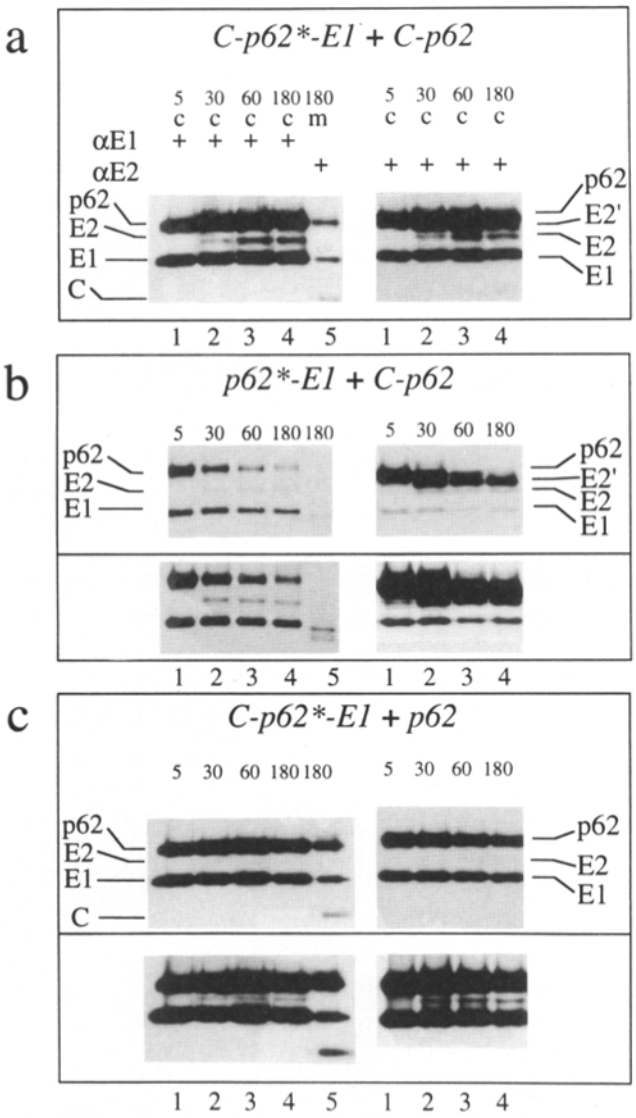

Figure 5. Cis and *trans* heterodimerization of SFV membrane protein subunits. BHK cells were co-transfected with SFV C-p62*-E1 and SFV C-p62 RNAs (*a*), SFV p62*-E1, and SFV C-p62 RNAs (*b*), or with SFV C-p62*-E1 and p62 RNAs (*c*), and grown on several dishes. The cells were pulse-labeled and chased as described in the legend to Fig. 4. Aliquots of the cell lysates and the 180 min media samples were extracted with either anti-E1 or -E2 monoclonal antibodies as indicated. Note that bands indicating p62 in figures refer to both wt p62 and mutant p62*. Note also that the left panel in (*b*) has been exposed 10 times longer than the right panel. Over-exposures of *b* and *c* are shown in the lower part of corresponding panel.

p62, and E1 subunits it is expected that noncleavable and cleavable heterodimers would form in a 6:4 ratio, i.e., there should be 60% p62*-E1 and 40% p62-E1 complexes present. This assumes that all of the E1 subunits are used for complex formation. When the co-immunoprecipitated p62*, p62, and E2 subunits were quantitated and compared with the precipitated E1 after the anti-E1 monoclonal antibody reaction (Fig. 5 a, *left*) it was evident that indeed, virtually all E1 subunits have been involved in heterodimerization reactions (Table I). However, when the noncleavable and cleavable heterodimer fractions were measured after a chase for 60 min, which should ensure almost complete conversion of all p62-E1 forms into E2-E1 forms (Zhao and Garoff, 1992), only 10% of E2-E1 heterodimers were found, the rest being noncleavable p62*-E1 complexes. Considering the given subunit concentrations the results suggest that most heterodimerization reactions must have occurred in *cis*, that is between the p62* and E1 subunits that have been generated from the same polyprotein translation. However, a small but significant fraction of the E1 subunits generated from this translation is also able to complex in *trans* with p62 made from C-p62 RNA at these conditions of subunit concentrations.

In this interpretation it is assumed that membrane protein subunits are expressed in the calculated 1.5 (p62*):1.0 (p62) ratio in all cells of the sample. This assumption is likely to be true because separate transfections with the same amount of RNA as used in co-transfection experiments resulted in almost complete host cell shut-off in total cell sample (see Fig. 4 *e*, lane *2*) and (Ekström et al., 1994). This is only possible if the RNA replication occurs in all of the cells.

Subunit Concentration Effect on Heterodimerization Reaction in cis Versus trans

To confirm our results that heterodimerization occurs predominantly in *cis* we performed two further heterodimerization analyses. In these we analysed the effect of subunit concentration on the heterodimerization reactions. The expectation is that reactions in *trans* should be sensitive to subunit concentration changes whereas reactions in *cis* should be insensitive. For this purpose we introduced two new SFV variants, SFV p62 and SFV p62*-E1 (Barth, B.-U., and H. Garoff, unpublished data). Both of these constructs lack the C gene of SFV. This results in about 10-fold lower subunit expression than SFV replicons containing C (Sjöberg et al., 1994). This is due to a region within the first 100 bases of the C gene, which enhances translation initiation and because of this effect it was possible to change the subunit concentrations in the cells without changing the conditions of efficient RNA transfection. In Fig. 4 *e* we have compared the expression efficiencies of wt SFV RNA, the C-p62 RNA and p62 RNA. The p62 and E2′ expression level of C-p62 transfected cells is shown to correspond to that of p62 and E2 of wt RNA-transfected cells whereas the concentration of p62 in p62 RNA transfected cells is considerably lower. Note that although much less p62 is produced in the latter case there is no concomitant increase in labeling of host proteins. This shut off of host protein synthesis is a good indication of efficient RNA transfection and virus RNA production in all cells of the sample. Fig. 4 *b* shows a pulse-chase analysis of p62 which is made in cells transfected with p62 RNA. In this chase the p62 material appears to be rapidly degraded without giving rise to any detectable E2′ forms. In Fig. 4 *d* a similar analysis is shown for the p62* and E1 products made in p62*-E1 RNA transfected cells. The two membrane protein subunits are shown to heterodimerize efficiently into noncleavable p62*-E1 complexes although these are now made at 10-fold lower concentration than when produced from C-p62*-E1 transfected cells (compare Fig. 2 *c*). This points to an efficient heterodimerization reaction in *cis*.

Table I. Cis and Trans Heterodimerization Efficiencies of E1 in Transfected Cells

Coding units*	Relative protein level‡ p62*/p62	Heterodimerization efficiencies of E1 (%)§ 5′ *cis*	5′ *trans*	30′ *cis*	30′ *trans*	60′ *cis*	60′ *trans*
C-p62*-E1	NA	NA	NA	NA	NA	NA	NA
C-p62*-E1 + C-p62	1.5:1	75	(1.2)	79	(7.3)	90	9.9
p62*-E1 + C-p62	1:7	84	(1.2)	59	(12)	50	15
C-p62*-E1 + p62	10:1	70	(1.8)	95	(4.1)	97	3.3

* RNA molecules used for transfection of cells.

‡ Bands with viral proteins in gels shown in Fig. 5 (*a–c*) were measured for radioactivity using a Fuji phosphoimager. The numbers were normalized for methionine content of respective subunit. The amount of p62* and p62 subunits were measured in the 5 min chase sample. As p62* and E1 are made in equimolar amounts both from the C-p62*-E1 and p62*-E1 coding unit, the amount of E1 subunits was used as a measure for p62*. The amount of E1 subunits, and hence also p62* subunits was determined by precipitation with the anti-E1 monoclonal antibody. In order to obtain the p62 concentration we first measured the total p62 and p62* concentration, by precipitating with the anti-E2 monoclonal antibody and then we deducted the p62* amount from this sum.

§ The E1 fraction that complexed with a p62 subunit from another coding unit (heterodimerization in *trans*) was obtained by measuring the amount of E2 subunits which was co-precipitated with E1 from the 60 min chase samples when extracted with the anti-E1 monoclonal antibody. At 60 min after synthesis most p62–E1 heterodimers are cleaved into the E2–E1 form (Zhao and Garoff, 1992) and hence the yield of E2–E1 heterodimers reflect the amount of originally made p62–E1 heterodimers. The table also includes the fraction of E1 in complex with E2 after shorter chase times (in brackets). These values are not representative for the true *trans* heterodimerization efficiency but merely indicate that fraction of heterodimers formed in *trans*, which have undergone maturation cleavage at the indicated chase times. The E1 fraction that heterodimerized with a p62* subunit originating from their common translation product (heterodimerization in *cis*) was obtained by first measuring the total amount of p62, p62*, E2, and E2′ which coimmunoprecipitated with E1 in the 5 to 60 min chased samples, and then deducting the amount of *trans*-heterodimerizing E1 (above) from this sum.

The co-transfection of p62*-E1 and C-p62 RNAs into BHK cells (Fig. 5 *b* and Table I) resulted in the production of p62* and p62 subunits in a ratio of 1:7. If a common pool of monomeric subunits is assumed to be present in the endoplasmic reticulum and all E1 is heterodimerizing, this should yield about 14% noncleavable and 86% cleavable heterodimers. However, our results indicate a different scenario. In the cell sample chased for 60 min as much as 50% of non-cleavable heterodimers (p62*-E1) are found together with only 15% cleavable (E2-E1) ones and about 35% of the E1 subunits are unassociated in this sample. The likely reason for the free E1 is that a fraction of the heterodimers have undergone degradation in p62/E2 subunit during the chase, because most of E1 is shown to be heterodimerizing in the cell sample chased for the shortest time (Table I) (see also Zhao and Garoff, 1992). Thus, these results suggest that most E1 heterodimerization reactions occur in *cis* that is with the p62* subunit originating from the same polyprotein translate.

The final cotransfection experiment involved the C-p62*-E1 and the p62 RNA. The heterodimer analyses are shown in Fig. 5 *c*. Quantitations show that the p62* to p62 ratio is 10:1 in this experiment (Table I). Heterodimerization using subunit recruitment from a random pool should yield 89% noncleavable and 11% cleavable heterodimers. Our quantitation gives 97 and 3%, respectively and again the results are in agreement with a very efficient heterodimerization reaction in *cis*.

Discussion

The co-expression experiments carried out in this work demonstrate that the two subunits of the p62–E1 heterodimer are preferentially derived from the same 26S mRNA translation product and not from a mixed pool of subunits in the ER. If a common pool was used as source of heterodimer subunits then it would be expected that heterodimerization of p62 and p62* with E1 would occur according to their relative concentrations in the endoplasmic reticulum after synthesis. This is clearly not the case, for instance at a high p62/p62* ratio much more p62*–E1 heterodimers are formed than p62–E1 heterodimers. Thus, most E1 heterodimerization reactions are in *cis*. However, our present experiments also demonstrate that *trans* heterodimerization is possible. In the case where the p62 and the p62* subunits are both present at a high concentration, close to that during normal infection, the *trans* heterodimerization efficiency was about 10%. This corresponds to a probability of about 1 out of 10 E1 molecules made from the p62*–E1 coding unit to meet p62 from the other coding unit. However, this value is probably not representative for the *trans* heterodimerization efficiency during a normal infection. In that case all subunits are expressed from p62–E1 coding units and there would be no coding units giving rise to a high concentration of separate p62 subunits as detailed in our present experiment. During infection the likely probability of *trans* complementation is $0.1 \times 0.1 = 0.01$ (~1%). Such a low *trans* heterodimerization efficiency is supported by the fact that ts mutants of alphavirus, with amino acid changes in the membrane protein subunits of p62 and E1, show very inefficient complementation in mixed infections (Strauss et al., 1976). Therefore, our conclusion is that during wt virus infection the two membrane protein subunits p62 and E1 are almost exclusively heterodimerizing in *cis*, i.e., using subunits from the same translation. This feature of alphavirus spike subunit oligomerization is therefore clearly different from that of the spike proteins of the Bunya virus and influenza virus (Boulay et al., 1988; Persson and Pettersson, 1991). Thus, the alphavirus spike heterodimer represents so far the sole example of a protein complex, which is made of subunits synthesized at the same site in the ER, rather than originating from a common ER pool.

This unique feature of the heterodimerization reaction of the SFV spike subunits could also explain its rapid kinetics and high efficiency. For example 3 min after the 2 min pulse a major portion of the p62 and E1 subunits have been orga-

nized into heterodimers and within 3 additional minutes most subunits exist as such complexes. This is considerably faster than the oligomerization rate observed for the Vesicular Stomatitis virus G protein subunit ($t_{½}$ = 6–8 min) and that of the influenza HA ($t_{½}$ = 7–10 min) (Copeland et al., 1986; Doms et al., 1987; Gething et al., 1986).

The most efficient way of generating a p62–E1 heterodimer in *cis* would be one where the membrane polyprotein chain would be cleaved posttranslationally at a time point when initial p62–E1 interactions have already been formed. However, this is not supported by our present results. Firstly, the pulse–chase analysis of p62 and E1 biosynthesis support a cotranslational mode of precursor protein cleavage. Secondly, our sedimentation analysis of viral proteins in NP-40 solubilized cells indicate that newly synthesized E1 and p62 chains are separated into different structures immediately after synthesis. The E1 was found mainly in very large complexes whereas corresponding p62 was monomeric or in very small sized complexes. Thirdly we have demonstrated that p62 and E1 subunits have at certain conditions the capacity to undergo a significant heterodimerization reaction in *trans* in addition to the *cis* reaction. This *trans* heterodimerization most likely involves newly synthesized and still uncomplexed p62 and E1 subunits rather than subunit exchange between heterodimers that have originally been formed in *cis* and separate p62/E2′ subunits, because we cannot observe any E2′-E1 heterodimers in our experiments (see Fig. 5). This suggests that there is no subunit exchange on the plasma membrane between E2′ subunits and p62–E1 complexes nor E2–E1 complexes. Furthermore, the p62–E1 complexes, once formed in the ER, are known to be very stable structures (Wahlberg et al., 1989). Thus, we interpret our results to indicate that the p62 and E1 subunits are translocated into the ER lumen as individual chains and not as a precursor molecule.

But how can one then explain the efficient *cis*-directed heterodimerization reaction? At present we have no answer to that question. However, we can suggest two mechanisms for this. Both are based on the assumption that the same translocation site (translocon) is used for the translocation of both the p62 and the E1 chains of a 26S mRNA translation product. If this is the case then it is possible that the p62 subunit which is translated first from the 26S mRNA is kept in the translocation site via its transmembrane (stop transfer) peptide until all of the E1 chain has been made. Thus this would keep the two subunits together and thereby explain the preferential *cis* heterodimerization. Another explanation is that the folding facilitating machinery in the ER is not only functionally linked to an active translocon, as suggested in several recent studies (Klappa et al., 1991; Mayinger and Meyer, 1993; Nicchitta and Blobel, 1993; Sanders et al., 1992; Simon et al., 1992; Vogel et al., 1990) but also physically. In the case of SFV this would mean that p62 and E1 subunits which are made from the same 26S mRNA translation product could be received by the same folding facilitating complex. Both models assume that the two subunits mature very rapidly into oligomerization competent forms because the completion of the polyprotein synthesis will probably initiate reactions which will dissociate the translocon and its postulated association with a folding facilitating machinery. This kind of model could also explain why the Uukuniemi G1 and G2 products do not heterodimerize in *cis*. In this case one of the products has a very slow maturation into oligomerization competence hence it cannot use the *cis*-directed mechanism.

We thank Ingrid Sigurdson for typing, Jimena Parga Rios for help with the cell culture, and Helena Andersson and Roger Hewson for critical reading of the manuscript.

This work was supported by the Swedish Natural Science Research Council (B-BU 09353-306) and the Swedish Cancer Society (3277-B93-02XBB).

Received for publication 17 May 1994 and in revised form 18 October 1994.

References

Baron, M. D., and K. Forsell. 1991. Oligomerisation of the Structural Proteins of Rubella Virus. *Virology.* 185:811–819.

Berglund, B., M. Sjöberg, H. Garoff, G. J. Atkins, J. Brian, B. J. Sheahan, and P. Liljeström. 1993. Semliki Forest virus expression system: production of conditionally infectious recombinant particles. *Bio/Technology.* 11:916–920.

Boere, W. A., T. Harmsen, J. Vinjé, T. B. Benaissa, C. A. Kraaijeveld, and H. Snippe. 1984. Identification of distinct antigenic determinants on Semliki Forest virus by using monoclonal antibodies with different antiviral activities. *J. Virol.* 52:575–582.

Bole, D. G., L. M. Hendershot, and J. F. Kearney. 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol.* 102:1558–1566.

Boulay, F., R. W. Doms, R. G. Webster, and A. Helenius. 1988. Posttranslational oligomerization and cooperative acid activation of mixed influenza hemagglutinin trimers. *J. Cell Biol.* 106:629–639.

Copeland, C. S., R. W. Doms, E. M. Bolzau, R. G. Webster, and A. Helenius. 1986. Assembly of influenza hemagglutinin trimers and its role in intracellular transport. *J. Cell Biol.* 103:1179–1191.

Cutler, D. F., and H. Garoff. 1986. Mutants of the membrane-binding region of Semliki Forest virus E2 protein. I. Cell surface transport and fusogenic activity. *J. Cell Biol.* 102:889–901.

de Curtis, I., and K. Simons. 1988. Dissection of Semliki Forest virus glycoprotein delivery from the trans-Golgi network to the cell surface in permeabilized BHK cells. *Proc. Natl. Acad. Sci. USA.* 85:8052–8056.

Doms, R. W., D. S. Keller, A. Helenius, and W. E. Balch. 1987. Role for adenosine triphosphate in regulating the assembly and transport of vesicular stomatitis virus G protein trimers. *J. Cell Biol.* 105:1957–1969.

Doms, R. W., R. A. Lamb, J. K. Rose, and A. Helenius. 1993. Folding and assembly of viral membrane proteins. *Virology.* 193:545–562.

Ekström, M., P. Liljeström, and H. Garoff. 1994. Membrane protein lateral interactions control Semliki Forest virus budding. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:1058–1064.

Fuller, S. D. 1987. The T=4 envelope of Sindbis virus is organized by interactions with a complementary T=3 capsid. *Cell.* 48:923–934.

Garoff, H., K. Simons, and B. Dobberstein. 1978. Assembly of the Semliki Forest virus membrane glycoproteins in the membrane of the endoplasmic reticulum in vitro. *J. Mol. Biol.* 124:587–600.

Garoff, H., A. M. Frischauf, K. Simons, H. Lehrach, and H. Delius. 1980. Nucleotide sequence of cDNA coding for Semliki Forest virus membrane glycoproteins. *Nature (Lond.).* 288:236–241.

Garoff, H., D. Huylebroeck, A. Robinson, U. Tillman, and P. Liljeström. 1990. The signal sequence of the p62 protein of Semliki Forest virus is involved in initiation but not in completing chain translocation. *J. Cell Biol.* 111:867–876.

Garry, R. F. 1994. Sindbis virus-induced inhibition of protein synthesis is partially reversed by medium containing an elevated potassium concentration. *J. Gen. Virol.* 75:411–415.

Gething, M. J., K. McCammon, and J. Sambrook. 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. *Cell.* 46:939–950.

Green, J., G. Griffiths, D. Louvard, P. Quinn, and G. Warren. 1981. On the passage of viral membrane proteins through the Golgi complex. *J. Mol. Biol.* 152:6638-698.

Klappa, P., P. Mayinger, R. Pipkorn, M. Zimmermann, and R. Zimmermann. 1991. A microsomal protein is involved in ATP-dependent transport of presecretory proteins into mammalian microsomes. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:2795–2803.

Liljeström, P., and H. Garoff. 1991. Internally located cleavable signal sequences direct the formation of Semliki Forest virus membrane proteins from a polyprotein precursor. *J. Virol.* 65:147–154.

Liljeström, P., S. Lusa, D. Huylebroeck, and H. Garoff. 1991. In vitro mutagenesis of a full-length cDNA clone of Semliki Forest virus-the small 6,000-molecular-weight membrane protein modulates virus release. *J. Virol.*

65:4107–4113.
Lobigs, M., and H. Garoff. 1990. Fusion function of the Semliki Forest virus spike is activated by proteolytic cleavage of the envelope glycoprotein p62. *J. Virol.* 64:1233–1240.
Mattila, K. 1979. Separation of the integral membrane glycoproteins E1 and E2 of Semliki Forest virus by affinity chromatography on concanavalin A-Sepharose. *Biochem. Biophys. Acta.* 579:62–72.
Marquardt, T., and A. Helenius. 1992. Misfolding and aggregation of newly synthesized proteins in the endoplasmic reticulum. *J. Cell Biol.* 117:505–513.
Mayinger, P., and D. I. Meyer. 1993. An ATP transporter is required for protein translocation into the yeast endoplasmic reticulum. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:659–666.
Melancon, P., and H. Garoff. 1986. Reinitiation of translocation in the Semliki Forest virus structural polyprotein: identification of the signal for the E1 glycoprotein. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1551–1560.
Nicchitta, C. V., and G. Blobel. 1993. Lumenal proteins of the mammalian endoplasmic reticulum are required to complete protein translocation. *Cell.* 73:989–999.
Persson, R., and R. F. Pettersson. 1991. Formation and intracellular transport of a heterodimeric viral spike protein complex. *J. Cell Biol.* 112:257–266.
Salminen, A., J. M. Wahlberg, M. Lobigs, P. Liljeström, and H. Garoff. 1992. Membrane fusion process of Semliki Forest Virus II: cleavage-dependent reorganization of the spike protein complex controls virus entry. *J. Cell Biol.* 116:349–357.
Sanders, S. L., K. M. Whitfield, J. P. Vogel, M. D. Rose, and R. W. Schekman. 1992.Sec61p and BiP directly facilitate polypeptide translocation into the ER. *Cell.* 69:353–365.
Sjöberg, E. M., M. Suomalainen, and H. Garoff. 1994. A significantly improved Semliki Forest Virus expression system based on translation enhancer segments from the viral capsid gene. *Bio/Technology.* 12:1127–1131.
Söderlund, H., and I. Ulmanen. 1977. Transient association of Semliki Forest virus capsid protein with ribosomes. *J. Virol.* 24:907–909.
Simon, S. M., C. S. Peskin, and G. F. Oster. 1992. What drives the translocation of proteins? *Proc. Natl. Acad. Sci. USA.* 89:3770–3774.
Strauss, E. G., E. M. Lenches, and J. H. Strauss. 1976. Mutants of sindbis virus. I. Isolation and partial characterization of 89 new temperature-sensitive mutants. *Virology.* 74:154–168.
Suomalainen, M., P. Liljeström, and H. Garoff. 1992. Spike protein-nucleocapsid interactions drive the budding of alphaviruses. *J. Virol.* 66: 4737–4747.
Vénien-Bryan, C., and S. D. Fuller. 1994. The organization of the spike complex of Semliki Forest virus. *J. Mol. Biol.* 236:572–583.
Vogel, J. P., L. M. Misra, and M. D. Rose. 1990. Loss of BiP/GRP78 function blocks translocation of secretory proteins in yeast. *J. Cell Biol.* 110:1885–1895.
Vogel, R. H., S. W. Provencher, C.-H. von Bonsdorff, M. Adrian, and J. Dubochet. 1986. Envelope structure of Semliki Forest virus reconstructed from cryo-electron micrographs. *Nature (Lond.).* 320:533–535.
Wahlberg, J. M., and H. Garoff. 1992. Membrane fusion process of Semliki Forest Virus I: low pH-induced rearrangement in spike protein quaternary structure precedes virus penetration into cells. *J. Cell Biol.* 116:339–348.
Wahlberg, J. M., W. A. Boere, and H. Garoff. 1989. The heterodimeric association between the membrane proteins of Semliki Forest virus changes its sensitivity to low pH during virus maturation. *J. Virol.* 63:4991–4997.
Wengler, G., and G. Wengler. 1989. Cell-associated West Nile Flavivirus is covered with E+Pre-M protein heterodimers which are destroyed and reorganized by proteolytic cleavage during virus release. *J. Virol.* 63:2521–2526.
Zhao, H. X., and H. Garoff. 1992. Role of cell surface spikes in alphavirus budding. *J. Virol.* 66:7089–7095.
Ziemiecki, A., and H. Garoff. 1978. Subunit composition of the membrane glycoprotein complex of Semliki Forest virus. *J. Mol. Biol.* 122:259–269.
Ziemiecki, A., H. Garoff, and K. Simons. 1980. Formation of the Semliki Forest virus membrane glycoprotein complexes in the infected cells. *J. Gen Virol.* 50:111–123.