MEMBRANE BIOGENESIS

In Vitro Cleavage, Core Glycosylation, and Integration into Microsomal Membranes of Sindbis Virus Glycoproteins

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ABSTRACT

Sindbis virus 26S RNA has been translated in a cell-free protein-synthesizing system from rabbit reticulocytes. When the system was supplemented with EDTA-stripped dog pancreas microsomal membranes, the following results were obtained: (*a*) Complete translation of 26S RNA, resulting in the production, by endoproteolytic cleavage, of three polypeptides that are apparently identical to those forms of C, PE_2, and E_1 that are synthesized in vivo by infected host cells during a 3-min pulse with [^{35}S]methionine. (*b*) Correct topological deposition of the three viral polypeptides—in vitro-synthesized PE_2 and E_1 forms are inserted into dog pancreas microsomal membranes in an orientation which, by the criterion of their limited (or total) inaccessibility to proteolytic probes, is indistinguishable from that of their counterparts in the rough endoplasmic reticulum of infected host cells; in vitro-synthesized C is not inserted into membranes and therefore is accessible to proteolytic enzymes, like its in vivo-synthesized counterpart. (*c*) Core glycosylation of in vitro-synthesized PE_2 and E_1 forms, as indicated by binding to concanavalin A Sepharose and subsequent elution by α-methylmannoside.

KEY WORDS membrane protein biosynthesis · cell-free translation · in vitro cleavage

Sindbis virus contains three proteins: a capsid protein, C, which is associated with the genomic 42S RNA, and two glycoproteins, E_1 and E_2, both integral proteins of the viral membrane envelope. All three proteins are synthesized by a single polyadenylated species of 26S RNA (20), the most abundant viral RNA present in infected cells. Because 26S RNA contains only one initiation site (6), the three proteins are generated by proteolysis. E_2 is synthesized as a precursor protein (PE_2) which is larger by 10,000 daltons (18). PE_2 is probably converted to E_2 after completion of its intracellular pathway at the plasma membrane (20). By analogy to the closely related Semliki Forest virus, it is widely assumed that the gene order of 26S RNA is C, PE_2, E_1 (7). 26S RNA can be obtained from membrane-bound polysomes (14, 23). Furthermore, in vivo pulse-labeling followed by exposure of cell fractions to chymotrypsin showed that newly synthesized C was degraded, whereas newly synthesized E_1 and PE_2 were largely protected (23). Thus, it appears that the first cleavage product of the nascent chain that comprises its C portion is discharged into the cytosol, whereas the other two cleavage products containing its PE_2 and E_1 portions are integrated into the rough endoplasmic reticulum (RER)[1] membrane.

[1] *Abbreviations used in this paper*: Con A, concanavalin A; MEM, minimum essential medium (Eagle); RER, rough endoplasmic reticulum; SDS, sodium dodecyl sulfate; TPCK, 1-(tosylamido 2-phenyl) ethyl chloromethyl ketone.

To extend the in vivo studies (23) and as a prelude for a detailed analysis of the mechanisms by which a single mRNA is able to direct the synthesis of one soluble protein and two membrane proteins (23), we decided to use a recently developed cell-free protein-synthesizing system supplemented with dog pancreas microsomal membranes (17). This in vitro system has been shown to yield segregation of secretory proteins into microsomal vesicles (13, 17). In this paper, we demonstrate that translation of 26S RNA in the cell-free, membrane-supplemented system results in an apparently faithful reproduction in vitro of all the in vivo events that are presumably coupled to the translation of Sindbis 26S RNA.

MATERIALS AND METHODS

Total Sindbis RNA was prepared from infected chick embryo fibroblasts 7 h postinfection by sodium dodecyl sulfate (SDS)/phenol/chloroform extraction. Single-stranded RNA was precipitated from DNA by an overnight incubation in 2 M LiCl at 4°C. Polyadenylated RNA was prepared by oligo (dT) cellulose affinity chromatography (5). 26S RNA was separated from the 42S viral genome by SDS sucrose gradient centrifugation (14).

To label viral proteins, in vivo infected cultures (5 h postinfection) were first starved for 60 min with amino acid-free minimum essential medium (Eagle) (MEM), then incubated for 3 min in amino acid-free MEM containing 100 μCi/ml of [^{35}S]methionine. The cells were quickly chilled, collected, and homogenized in 10 mM Tris HCl, pH 7.4, 150 mM NaCl. A postnuclear supernate was prepared by centrifugation for 10 min at 500 *g* and stored at −20°C.

Conditions for protein synthesis in a cell-free extract of rabbit reticulocytes (15, 17), polyacrylamide slab gel electrophoresis in SDS and subsequent autoradiography (3), preparation of EDTA-stripped microsomal membranes from dog pancreas rough microsomes (4), concanavalin A (Con A) Sepharose affinity chromatography (13), trypsin-1-(tosylamido 2-phenyl)ethyl chloromethyl ketone (TPCK) digestion of proteins in gel slices, and subsequent sample preparation for fingerprint analysis (12) were performed as described previously.

Incubations of in vitro- or in vivo-synthesized viral proteins with trypsin and chymotrypsin are described in the figure legends. [^{35}S]methionine was purchased from Amersham Corporation, Arlington Heights, Ill. Trypsin-TPCK from Worthington Biochemical Corp., Freehold, N. J., and Con A Sepharose from Pharmacia Fine Chemicals, Uppsala, Sweden.

RESULTS

26S RNA isolated from chick embryo fibroblasts infected with Sindbis virus was translated in a cell-free, nuclease-treated reticulocyte lysate system in either the absence or presence of dog pancreas microsomal membranes. To compare the in vitro-synthesized products to their equivalent in vivo counterparts, Sindbis-infected chick embryo cells were briefly pulsed with [^{35}S]methionine 6 h postinfection, and a postnuclear supernate was prepared from cell homogenate. Either directly or after incubation with proteolytic enzymes, the in vitro- and in vivo-synthesized products were analyzed by SDS-polyacrylamide gel electrophoresis and subsequent autoradiography.

On the basis of its apparent mol wt of 30,000 daltons, the capsid protein C can be readily identified as one of the major products which is synthesized in vivo (Fig. 1, Lane 7) and in vitro, either in the absence (Fig. 1, lane 2) or in the presence of microsomal membranes (Fig. 1, lane 4). The other major products synthesized in vivo (Fig. 1, lane 7) were the two viral envelope proteins, PE_2 (60,000 daltons) and E_1 (50,000 daltons). Products with a molecular weight apparently identical to that of in vivo-synthesized PE_2 and E_1 were also synthesized in vitro. However, this occurred only when microsomal membranes were present during translation (Fig. 1, lane 4). In the absence of microsomal membranes, there was synthesis of several distinct bands in the region between 30,000 and 60,000 daltons (Fig. 1, lane 2). Since tryptic peptide mapping of these bands (data not shown) revealed that they all contained peptides characteristic of C, it seems plausible that they represent translation products from which the capsid portion had not been cleaved. As shown in Fig. 1, lane 4, these bands were not apparent when membranes were present during translation, suggesting that there was complete cleavage of all nascent chains at the C-PE_2 site. Furthermore, under these conditions there was complete translation of 26S RNA, as well as cleavage at the PE_2-E_1 site to give rise to PE_2 and E_1. No changes in the pattern shown in Fig. 1, lane 2 were obtained if membranes were added posttranslationally (data not shown). It should be noted that several investigators previously demonstrated the synthesis of C protein in response to 26S RNA of both Sindbis and Semliki Forest virus in numerous cell-free systems (6, 8, 9, 22). After translation of Sindbis 26S RNA in a lysate of rabbit reticulocytes, Simmons and Strauss (19) also reported production of a 100,000-dalton protein, which they interpreted to contain the PE_2 and E_1 sequences. However, no clear evidence for

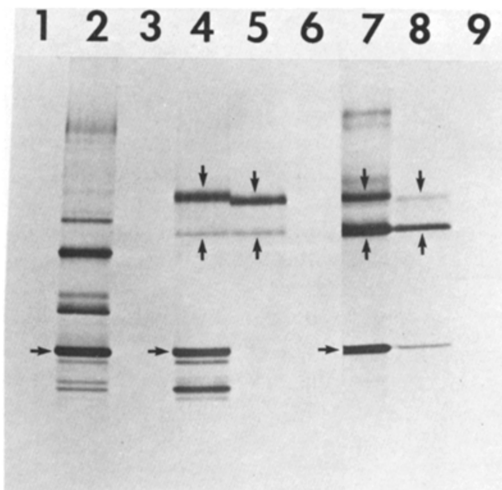

FIGURE 1 Comparison of in vivo-labeled viral proteins with in vitro-synthesized translation products. 26S RNA was translated in the staphylococcal nuclease-treated rabbit reticulocyte lysate (15), in the absence or presence of nuclease-treated dog pancreas microsomal membranes (17). 5-μl aliquots of the translation products (lanes 1–6) and 20-μl aliquots (0.1 A_{260} U) of the postnuclear supernate (lanes 7–9) obtained from infected cells pulse-labeled for 3 min with [^{35}S]methionine were prepared for polyacrylamide slab gel electrophoresis (17), either directly or after proteolytic digestion. Lanes 7–9 derive from a separate slab gel and were aligned by identical protein standards present in each slab gel. The incubation mixture for translation (25 μl) contained 12 μCi of [^{35}S]methionine, 0.02 A_{260} U of 26S RNA (lanes 2–6), and either no microsomal membranes (lanes 1–3) or 0.125 A_{260} U (measured in the absence of detergents) of microsomal membranes (lanes 4–6). Incubation was carried out for 60 min at 29°C. Lanes 1, 2, 4, and 7: aliquots directly processed for electrophoresis. Lanes 3, 5, and 8: aliquots were incubated at 0°C for 20 min with a final concentration of 300 μg/ml each of trypsin and chymotrypsin; digestion was terminated by the addition of Trasylol (Delbay Pharmaceuticals, Inc., Kenilworth, N. J.) to a final concentration of 1,000 U/ml and SDS to 2%, and by incubation at 100°C for 5 min. Lanes 6 and 9: aliquots were incubated with proteolytic enzymes as above but in the presence of 1% Triton X-100. Downward- and upward-pointing arrows indicate PE_2-PE_2' (see text) and E_1 forms, respectively, labeled in vivo or synthesized in vitro. Rightward-pointing arrows indicate C protein. It is not clear why some of the in vivo-synthesized C is resistant to proteolysis (lane 8) in contrast to in vitro-synthesized C which is totally degraded (lane 5).

the synthesis of a major 100,000-dalton protein was obtained in our studies.

To provide more definitive evidence (i.e., other than electrophoretic comigration) for the identity of the in vitro-synthesized forms of PE_2 and E_1 with their in vivo-synthesized counterparts, we performed one-dimensional analysis of their tryptic peptides by high-voltage electrophoresis. The characteristic spots for the in vivo-synthesized PE_2 (Fig. 2, lane 4) and E_1 (Fig. 2, lane 2) which also occur in the in vitro-synthesized counterparts (shown in adjacent lanes 3 and 4) indicate chemical similarity between the in vitro- and in vivo-synthesized forms of these two proteins.

By analogy to previous data on in vitro integration and core-glycosylation of the G glycoprotein of vesicular stomatitis virus (11), we reasoned that the in vitro-synthesized forms of PE_2 and E_1 (Fig. 1, lane 4) might be core-glycosylated and membrane-integrated in a form similar to their equivalent forms which are synthesized in vivo in a 3-min pulse (Fig. 1, lane 7).

Proteolytic enzymes were used to compare the membrane orientation of in vivo- and in vitro-synthesized PE_2 and E_1. Incubation of the postnuclear supernate from Sindbis-infected pulse-labeled chick embryo cells with trypsin and chymotrypsin showed (Fig. 1, lane 8) that a significant percentage of PE_2, E_1, and C was resistant to proteolysis. However, in the case of PE_2, proteolysis generated a distinctly faster-moving derivative – PE_2' – which by its mobility can be estimated to be ~3,000 daltons smaller than PE_2. There was no protection when proteolysis was carried out in the presence of Triton X-100 (Fig. 1, lane 9). Similar observations were reported recently by Wirth et al. (23). Posttranslational incubation of the in vitro-synthesized products with trypsin and chymotrypsin gave analogous and more clear-cut results. As expected, all products that were synthesized in the absence of microsomal membranes were degraded (Fig. 1, lane 3). Among the products which were synthesized in the presence of microsomal membranes (Fig. 1, lane 5), all of the C molecules were degraded, all of the E_1 molecules were apparently protected, and all of the PE_2 molecules were reduced by ~3,000 daltons each, exactly like their in vivo-synthesized counterparts. Protection was completely abolished when proteolysis was performed in the presence of Triton X-100 (Fig. 1, lane 6). It is clear from these data that newly synthesized C does not enter the microsomal vesicles, whereas newly synthesized PE_2 and E_1 ere integrated into the microsomal vesicles in an orientation which, when probed by proteolytic enzymes, is indistinguisha-

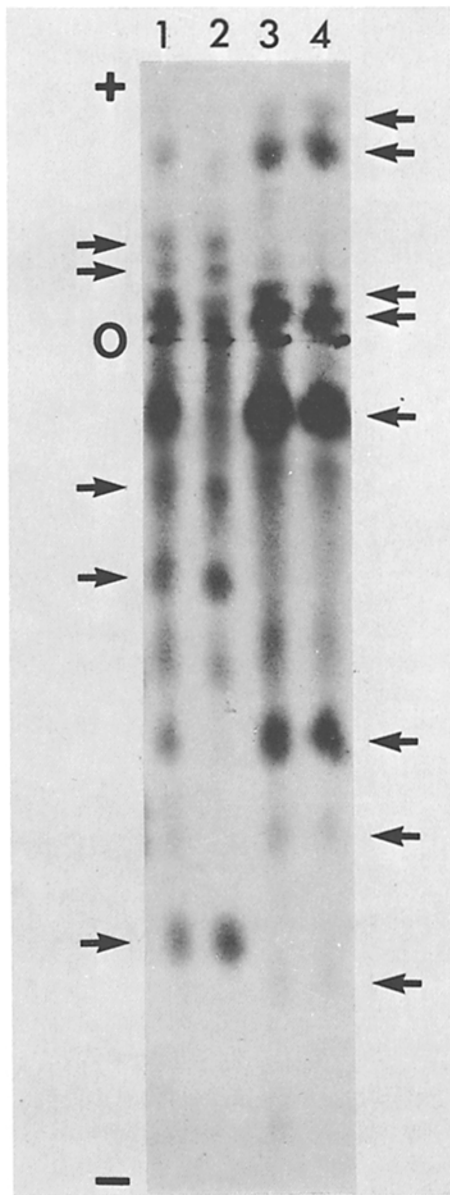

FIGURE 2 Analysis by high-voltage paper electrophoresis of [^{35}S]methionine-labeled tryptic peptide fragments obtained from in vivo-labeled and in vitro-synthesized PE_2 and E_1 forms. A 250-μl aliquot of translation products (lane 4, Fig. 1) and a 200-μl aliquot of postnuclear supernate (lane 7, Fig. 1) were processed for slab gel electrophoresis (17). The relevant bands were excised and processed for trypsin-TPCK digestion as described (12). Finally, peptides were resuspended in electrophoresis buffer (5% acetic acid, 0.5% pyridine in water, pH 3.5) containing 2 mM EDTA, and aliquots containing ~30,000 cpm were spotted onto Whatman 3 MM paper. Electrophoresis was carried out for 1.5 h at 4 kV, and the labeled peptides were revealed by autoradiography. Lanes 1 and 3: tryptic peptides from E_1 and PE_2 forms, respectively, synthesized in vitro. Lanes 2 and 4: tryptic peptides from E_1 and PE_2 forms, respectively, labeled in vitro. O = origin; arrows indicate characteristic spots of the in vivo-labeled glycoproteins present in their in vitro-synthesized counterparts. In vitro-synthesized E_1 was slightly contaminated by PE_2.

ble from that of their in vivo synthesized counterparts.

Next, we examined whether the in vivo- and in vitro-synthesized forms of PE_2 and E_1 were glycosylated. Because the in vivo-synthesized forms of PE_2 and E_1 were those which were labeled during a 3-min pulse of [^{35}S]methionine, it is likely that they represent intermediate forms that were still located in the RER. Unlike their mature forms, these intermediates may contain an incompletely processed core from which glucose and some of its mannose residues have not yet been removed (21) and to which peripheral sugars have not been added. As shown in Fig. 3, lanes 3 and 7, both forms of PE_2 and E_1 bound to Con A Sepharose and were specifically eluted by α-methyl mannoside. Although these data do not yield any information as to the nature of the core, they do show that core sugars are present in both the in vitro- and the in vivo-synthesized forms of PE_2 and E_1. As expected, neither in vitro- nor in vivo-synthesized C bound to Con-A Sepharose (Fig. 3, lanes 2 and 6). Finally, the PE_2 and E_1 forms obtained by proteolytic treatment of in vitro- or in vivo-synthesized products bound to the Con A Sepharose (Fig. 3, lane 4 and 8). This behavior is consistent with the finding that the core-glycosylating enzymes are located on the cisternal side of the microsomal vesicles (16).

DISCUSSION

Our results indicate that most, if not all, of the processes that are likely to be coupled to translation of Sindbis 26S RNA in vivo can be faithfully reproduced in vitro in a reticulocyte lysate system which is supplemented with dog pancreas microsomal membranes. The results which were obtained with this in vitro system are: (*a*) complete translation of the entire 26S RNA; (*b*) endoproteolytic cleavage of the translation products on at least two specific sites to yield three polypeptides that are closely related or identical to those forms of C, PE_2, and E_1 which are initially synthesized

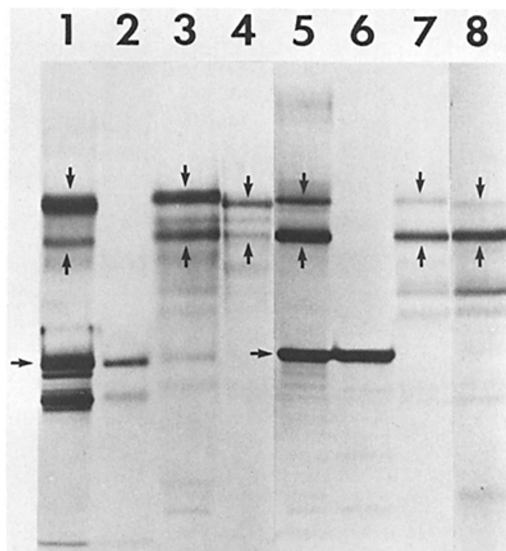

FIGURE 3 Core-glycosylation of in vivo- and in vitro-synthesized viral proteins. 45-μl aliquots of translation products (lane 4, Fig. 1) and 90-μl aliquots of postnuclear supernate (lane 7, Fig. 1) were subjected to Con A Sepharose affinity chromatography and subsequent slab gel electrophoresis as detailed elsewhere (13). All lanes come from a single slab gel. Lanes 1–4 refer to in vitro translation products. Lanes 5–8 refer to postnuclear supernate. Lanes 1 and 5: total products as applied to the Con A column. Lanes 2 and 6: aliquots of unbound material. Lanes 3 and 7: aliquots of bound and α-methylmannoside-eluted material. Lanes 4 and 8: aliquots of bound and α-methylmannoside-eluted material of samples previously digested with trypsin and chymotrypsin (see lanes 5 and 8, Fig. 1). Arrows as in Fig. 1.

in vivo during a 3-min incubation with [^{35}S]methionine; (*c*) proper topological deposition of newly synthesized C, PE_2, and E_1. The newly synthesized C is located in the incubation medium (equivalent of cytosol), while the newly synthesized forms of PE_2 and E_1 are integrated into the dog pancreas microsomal membranes in an orientation which, by the criterion of their accessibility to proteolytic probes, is indistinguishable from that of their equivalent forms in the RER of virus-infected host cells; (*d*) core-glycosylation of newly synthesized PE_2 and E_1.

It is likely that each of these processes is tightly synchronized with distinct phases in the translation of 26S RNA and, moreover, that these processes are coupled to each other in a sequential fashion, whereby the product of one reaction provides the substrate for another. The first cotranslational reaction would be the cleavage of the nascent chain at the C/PE_2 site, presumably a single endoproteolytic cleavage which would result in the removal of C from the nascent chain. Our data here confirm previous observations (20) that this cleavage can take place in cell-free systems. However, both the cleavage site on the nascent chain and the cleavage activity itself remain to be defined. Evidence has been provided recently (1) that newly synthesized C may act in an autoproteolytic activity to effect its own cleavage from the nascent chain.

The removal of C from the nascent chain would expose a new amino-terminal sequence that has been proposed (14, 23) to serve as a signal sequence. This sequence would establish a ribosome-membrane junction and thereby initiate cotranslational transfer of nascent PE_2, amino terminus first, into the cisternae of the RER (4). Unlike secretory proteins, however, the transfer of nascent PE_2 across the membrane would be interrupted at a specific point during translation by a "stop transfer" sequence (2) leaving the carboxy-terminal portion (or a portion of it, see below) of PE_2 exposed on the outside of the vesicle. The synthesis of PE_2 would then be completed by cleavage of the nascent chain at the PE_2/E_1 site. Our in vitro data provide several lines of evidence for the deposition of PE_2 in dog microsomal membrane in an asymmetric orientation which is indistinguishable from that of its in vivo-synthesized counterpart: (*a*) the bulk of both in vitro- and in vivo-synthesized forms of the PE_2 molecule is resistant to proteolysis except for a small portion amounting to ~3,000 daltons; (*b*) the resistance of the bulk fragment of the molecule (PE_2') to proteolysis depends on the integrity of the membrane; resistance is abolished when proteolysis is carried out in the presence of detergents. It should be noted that a similarly sized portion of the newly synthesized envelope glycoprotein (G) of vesicular stomatitis virus can be removed by proteolysis when G is synthesized in a cell-free system supplemented with dog pancreas microsomal membranes (11). A comparison of the amino-terminal sequence of PE_2 and PE_2' is now underway; sequence identity between these two molecules would constitute proof of asymmetric orientation with a cisternal amino terminus and a cytosol-exposed portion near the carboxy terminus.

A cytosol-exposed carboxy-terminal portion of PE_2 would pose the intriguing problem of how membrane transfer of the remainder of the nas-

cent chain, i.e., of its E_1 portion, is reinitiated. As suggested by Wirth et al. (23), it is possible that reinitiation of transfer is coupled to cleavage at the PE_2/E_1 site, analogous to the triggering of transfer of PE_2 by a cleavage at the C/PE_2 site. In this case, cleavage would presumably occur in the cytosol by a protease that is either soluble or associated with the cytosol aspect of the RER. Alternatively, it is possible that cleavage takes place on the cisternal site of the RER and only after reinitiation of transfer. For example, it is conceivable that the PE_2/E_1 cleavage site corresponds to a signal peptidase site (10).

If this were the case, an "internal" signal sequence (as opposed to an amino-terminal one) would reinitiate transfer of the E_1 portion of the nascent chain; after cleavage by signal peptidase, this internal signal sequence would then end up as the carboxy terminal portion of PE_2. Unlike an amino-terminal signal sequence, the reinitiation of chain transfer by an internal signal sequence would require its insertion into the membrane as a hairpin loop. Because of the fact that signal peptidase is a latent RER enzyme (i.e., its active site is not exposed on the cytosol aspect of the membrane [10]), it is likely that insertion of the loop would result in a transmembrane localization of the internal signal sequence, spanning the membrane, with its carboxy-terminal residues (containing the signal peptidase site) on the cisternal side. It is, of course, entirely possible that not only internal but also amino-terminal signal sequences insert into the membrane in this hairpin looplike fashion, a mechanism which would constitute an alternative to a previous proposal for the initiation of chain transfer by the signal sequence (3). In any case, from the above considerations it should be evident that newly synthesized PE_2 may loop back and forth across the RER membrane. By extrapolation then, it is conceivable that a series of stop-transfer sequences and of uncleaved signal sequences (13), alternating with each other, may provide a possible mechanism by which integral membrane proteins that loop back and forth across the membrane many times could be inserted into the membrane cotranslationally.

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Note Added in Proof: Similar findings have been reported for the closely related Semliki Forest Virus, by H. Garoff, K. Simons, and B. Dobberstein. 1978. *J. Mol. Biol.* **124:**587–600.

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