Transient Translocation of the Cytoplasmic (Endo) Domain of a Type I Membrane Glycoprotein into Cellular Membranes

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Abstract. The E2 glycoprotein of the alphavirus Sindbis is a typical type I membrane protein with a single membrane spanning domain and a cytoplasmic tail (endo domain) containing 33 amino acids. The carboxyl terminal domain of the tail has been implicated as (a) attachment site for nucleocapsid protein, and (b) signal sequence for integration of the other alphavirus membrane proteins 6K and E1. These two functions require that the carboxyl terminus be exposed in the cell cytoplasm (a) and exposed in the lumen of the endoplasmic reticulum (b). We have investigated the orientation of this glycoprotein domain with respect to cell membranes by substituting a tyrosine for the normally occurring serine, four amino acids upstream of the carboxyl terminus. Using radioiodination of this tyrosine as an indication of the exposure of the glycoprotein tail, we have provided evidence that this domain is initially translocated into a membrane and is returned to the cytoplasm after export from the ER. This is the first demonstration of such a transient translocation of a single domain of an integral membrane protein and this rearrangement explains some important aspects of alphavirus assembly.

Sindbis virus (the prototype of the alphaviruses) is a membrane-bound virus containing 240 copies of each of three structural proteins, E1, E2, and C (capsid) (Anothony and Brown, 1991; Choi et al., 1991; Paredes et al., 1992). E1 and E2 are type I membrane glycoproteins (Schlesinger and Schlesinger, 1986) which are integrated into the virus membrane and organized by protein–protein associations into a T=4 icosahedral lattice (Anthony and Brown, 1991). The capsid protein is organized with the viral RNA into a complementary T=4 icosahedron which is totally enclosed in the viral membrane (Coombs and Brown, 1987; Choi et al., 1991; Paredes et al., 1992). The E2 glycoprotein (423 amino acids) has a significant cytoplasmic (internal) domain of 33 amino acids (Rice and Strauss, 1981a,b; Wirth et al., 1979). Specific associations between this cytoplasmic E2 carboxyl-terminal domain and an unidentified domain in the capsid protein provide the driving force for the envelopment of the nucleocapsid in the glycoprotein-modified plasma membrane of an infected cell (Metsikko and Garoff, 1990; Schefefers et al., 1980).

The three structural proteins of Sindbis virus are synthesized together as part of a large 130-kD polyprotein. The proteins are organized in the order N H3-capid–PE2–6K–El–COOH (Schlesinger and Schlesinger, 1986). The capsid protein proteolytically cleaves itself from the developing polyprotein (Choi et al., 1991; Hahn and Strauss, 1990). This cleavage event exposes a hydrophobic domain in the amino terminus of the PE2 (precursor to E2) portion of the polyprotein which is bound by the signal recognition particle (SRP)1 (Bonatti et al., 1984); this complex translocates the developing polypeptide into the membrane of the ER (Wirth et al., 1979; Liljestrom and Garoff, 1991). The PE2 portion of the polyprotein is a type III membrane glycoprotein and its signal sequence is not cleaved by signal-peptidase but is rather cleaved after processing of the remainder of the polyprotein (Schlesinger and Schlesinger, 1986). Integration of the 6K and E1 portions of the polyprotein create a multimembrane spanning protein which crosses the membrane six times (Liljestrom and Garoff, 1991) (see Fig. 6 a). Liljestrom and Garoff (1991) used an in vitro translation–translocation assay to demonstrate that the signal sequences responsible for membrane translocation of the 6K and E1 proteins reside in the COOH-terminal region of the PE2 (P62) precursor and the 6K protein, respectively. They presented evidence that the integrated polyprotein is processed on the luminal side of ER membranes by signal peptidase at consensus cleavage sites immediately after the signal sequences. These proteins form heterodimers and are exported to the plasma membrane where the final events in virus maturation take place.

To provide the signal for translocation of the 6K protein into the ER and to be cleaved by signal-peptidase, the carboxyl terminus of the PE2 glycoprotein must be exposed in the lumen of ER. To bind capsid protein and initiate envelop-
BHK (BHK-21) cells were used for virus propagation and titration as well as cDNA clone Toto1101 by in vitro transcription and transfection (described by Brown, 1989). Cycloheximide and other chemicals were from Sigma Chemical Co. (St. Louis, MO). 

Materials and Methods

Materials
Na<sup>25</sup>I and [35S]methionine/cysteine were from DuPont New England Nuclear (Boston, MA). A polynucleotide monospecific anti-E2 carboxyl terminus antiserum was produced in a rabbit against a synthetic 33 amino acid peptide corresponding to the 33 amino acid cytoplasmic domain of E2 (see Fig. 1). This antiserum can be used as a marker for the configuration of the tail in the membrane of various intracellular compartments by testing its ability to be radioiodinated by a nonmembrane penetrating reagent. The substitution of tyrosine for serine at this position also creates a new chymotryptic peptide in the E2 tail (Fig. 1). As is shown below, the carboxy terminus of the Sindbis E2 glycoprotein is initially protected from chymotryptic digestion and radioiodination and is subsequently exposed, supporting the hypothesis that the distal carboxyl domain of this polypeptide is transiently translocated into a membrane bilayer.

Cell Culture and Virus

BHK (BHK-21) cells were used for virus propagation and titration as well as RNA transfection and have been described previously (Knüfer and Brown, 1989).

Wild-type Sindbis virus was derived from the full-length Sindbis virus cDNA clone Toto1101 by in vitro transcription and transfection (described below).

Wild-type and mutant virions were purified by isopycnic gradient centrifugation in potassium tartrate as described previously (Coombs and Brown, 1987).

Site-directed Mutagenesis of Toto1101

The procedures of Sarkar and Sommer (1990) were used to generate a mutated fragment of Toto1101 (BstHII [nt 9804]-SpII [nt 10381]) with a codon TAC (Tyr) substituted for TCG (Ser). This fragment was subcloned into the full-length cDNA clone Toto1101. Three primers were used: "upstream" primer a (19mer): 5'GCAGTGATGTTGCGATCAAC; primer b (20mer): 5'TCAAGATTGGCC (wild-type as CTG/wild-type as GACCTAAC (including the desired mutation at 9889 and 9890); "downstream" primer c (19mer): 5'GCAGTGGGCACCAAATC. Primers a and b were used to prime in the first PCR (as described by Sarkar and Sommer, 1990) with AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT). Thermal cycle parameters were as follows: 95°C, 1 min (denaturation); 55°C, 2 min (annealing); 72°C, 1 min (elongation); 27 cycles. Time delay: 95°C, 5 min (initial denaturation); and 72°C for 5 min (extension) at the beginning and end, respectively. The 142-bp product (megaprimer) was purified from the agarose gel by the "freeze-squeeze" method (Tautz and Renz, 1983). The megaprimer was used directly without strand separation in the second PCR with the downstream primer c. The second PCR (Barik and Galinski, 1991) was similar to the first PCR and the product was 681 bp long. A 577-bp fragment obtained after restriction digestion with BstHII and SpII was purified from an agarose gel. Toto1101 (13,638 bp) was digested with BstHII and SpII and the product was purified from a low melting agarose gel. The mutated 577-bp fragment and the purified Toto1101 fragments were ligated by T4 DNA ligase. This reconstructed Toto1101 was transformed into E. coli AG1 strain (Stratagene, La Jolla, CA) by electroporation (Gene Pulser, 25 μF, 200 ohms, 2.5 kV, Bio Rad Laboratories, Hercules, CA) and the cells were plated on ampicillin selection Luria-Bertani (LB) plates. Individual colonies were propagated, and double-stranded DNA was isolated. Clones containing the desired mutations were identified by dideoxynucleotide sequencing (Sequenase kit Version 2.0, US Biochemical Corp., Cleveland, OH) with the upstream oligonucleotide primer a. The mutations were again confirmed by sequence analysis directly from RNA transcripts synthesized in vitro or from purified virion RNA by the dideoxynucleotide chain reaction method (Zimmern et al., 1978) with reverse transcriptase and the upstream primer a.

In Vitro Transcription and RNA Transfection

Plasmid DNA containing the full-length cDNA copy of Sindbis virus RNA was linearized and used for runoff transcription reaction with SP6 RNA polymerase as described previously (Rice et al., 1987). The RNA transcripts were introduced into BHK-21 cells by cationic Liposome-mediated transfection (Lipofectin<sup>TM</sup> as described previously (Polo et al., 1991). Transfected cells were incubated at 37°C until cytopathic effect was observed (~24 h); the media was then harvested and after low speed centrifugation it was aliquoted and frozen for stock virus.

Cell-free Membrane Vesicles

BHK cells (two 75 cm<sup>2</sup> flasks, ~4 x 10<sup>7</sup> cells) infected with mutant or wild-type virus were removed from the flask in 10 ml lifting buffer (15 mM KCl, 10 mM Hepes, 2 mM EDTA) and pelleted by centrifugation at 500 rpm for 5 min. The supernatant was discarded and the cells were resuspended in 2 ml of homogenization buffer (20 mM Hepes-KOH pH 7.2, 1 mM EDTA, 1 mM EGTA, 0.25 M sucrose). The cells were then homogenized by passing the suspension 5–10 times through a 27-gauge needle. Cell disruption was monitored by light microscopy after Trypan blue staining. More than 95% of the cells were disrupted by this procedure. Nuclei and intact cells were pelleted by centrifugation at 2,000 rpm for 10 min. The supernatant contained membrane vesicles of intracellular organelles and plasma membrane.

Radioiodination

Virions or membrane vesicles were radioiodinated with Na<sup>25</sup>I by IODO-BEAD<sup>TM</sup> iodination reagent (Pierce Chemical Co., Rockford, IL), which is N-chloro-benzenesulfonylamine (sodium salt) immobilized on nonporous polystyrene beads. Before iodination, IODO-BEADS were washed twice in PBS-D buffer and air dried. IODO-BEADS resuspended in a 1.5 ml microcentrifuge tube with 600 μl PBS-D buffer and 400 μCi (4 μl) Na<sup>25</sup>I were incubated at room temperature for 5 min, then 400 μl of virions or
membrane vesicles were added to the reaction mixture. After 10 min incubation, the beads were removed to terminate iodination.

**Immunoprecipitation and PAGE**

Immunoprecipitations of radiolabeled viral proteins were carried out with antisera conjugated to cyanogen bromide-activated Sepharose 4B beads in lysis buffer: 0.5% NP-40, 0.02 M Tris, 0.05 M NaCl, 0.2 mM PMSE, 0.2 mM TPCK and 0.02 mM TLCK as described previously (Presley and Brown, 1989). The immunoprecipitated bead-antibody-protein complexes were washed three times with lysis buffer and then solubilized in SDS-PAGE sample buffer consisting of 12% glycerol, 4% SDS, 50 mM Tris, 5% mercaptoethanol, 0.02% bromophenol blue. The samples were heated for 3 min at 95°C, and electrophoresis was carried out on a 16.5% T 3.5% Tricine-SDS-PAGE to detect the 2.5 K peptide (described in text) using the buffer system of Schagger and Jagow (1987) and Amersham rainbow molecular weight markers (Amersham Corp., Arlington Heights, IL) (46,000 to 2,350). Gels were run for 16 h at 5 W. 10.8% SDS-PAGE as described previously (Presley and Brown, 1989) was used to observe the PE2 shift after the proteinase K treatment. Gels were dried in a Hoefer SE1200 Gel Drier. Fluorography was performed as described (Bonner and Laskey, 1974) and gels were exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY).

**Results**

**Construction of the Mutant TYR<sup>420</sup>**

Sindbis virus genomic RNA (49S) contains 11,703 nucleotides plus a poly A tail at the 3' terminus (Strauss et al., 1984). The complete sequence of cDNA derived from the genomic RNA has been determined (Strauss et al., 1984), and a full-length cDNA clone (Totoll01) has been constructed by Rice and co-workers (Rice et al., 1987). The clone contains an SP6 promoter which can be easily transcribed in vitro, producing infectious RNAs. Using the PCR, two nucleotides were changed in the eDNA clone, resulting in a codon change at nucleotides 9,889 and 9,890 from TCG into BHK-21 cells by cationic liposome-mediated (Lipofectin<sup>TM</sup>) transfection (see Materials and Methods). Cytotoxic effect (CPE) was detected at 24 h of incubation at 37°C and high yields of virus were produced. RNA sequence analysis from in vitro transcripts of purified virion RNA confirmed the substitution in the progeny virus. Subsequent passage indicated that the substitution of tyrosine for serine at position 420 has little effect on virus replication.

**Characterization of the Sindbis Virus TYR<sup>420</sup> Mutant**

The substitution of tyrosine for serine at position 420 in the cytoplasmic domain of the E2 glycoprotein creates a new chymotryptic cleavage site as well as a new site for radiiodination (Fig. 1). This substitution predicts that radioiodination of the mutant TYR<sup>420</sup> should result in the production of a new labeled chymotryptic peptide with a mol wt of ~2.5 kD. This prediction was tested by iodination of the proteins of gradient purified mutant and wild-type virus after solubilization of the virus in 0.5% Nonidet P-40 (NP-40). The proteins were digested with chymotrypsin and the digest was immunoprecipitated with a polyclonal monospecific antibody produced against a synthetic peptide corresponding to the 33 amino acid wild-type E2 cytoplasmic domain (Fig. 1) (see Materials and Methods). Equal counts/min of the precipitated peptides were resolved by PAGE. As seen in Fig. 2, the mutant TYR<sup>420</sup> contains an iodinated chymotryptic peptide of the molecular weight predicted for the 20 amino acid domain shown in Fig. 1. This peptide is not found in wild-type virus.

**Orientation of the PE2/E2 Carboxyl Terminus in the Membranes of Infected Cells**

The following strategy was employed to determine the organization of the PE2/E2 cytoplasmic (endo) domain relative to cell membranes. Cells infected with Sindbis virus wild-type, or mutant TYR<sup>420</sup>, were placed in the agent carbonyl cyanide m-chlorophenylhydrazone (CCCP) which blocks export of proteins from the rough endoplasmic reticulum (Balch et al., 1986; Kaarinen et al., 1980; Tartakoff, 1986). Alternatively, infected cells were treated with cycloheximide, an inhibitor of protein synthesis, and allowed to export viral glycoproteins from the rough endoplasmic reticulum to later compartments in the cell (Golgi/plasma membrane) (Erwin and Brown, 1980). In each case, cells were disrupted by homogenization (see Materials and Methods) and the nuclei were removed by centrifugation, creating a post-nuclear supernatant (PNS).

To demonstrate that viral glycoproteins are integrated into the membrane vesicles in the PNS with their cytoplasmic (endo) domains exposed, protease sensitivity assays were used. Infected cells were labeled for 30 min with [35S]-cysteine/methionine in the presence of CCCP. These conditions block export of proteins from the ER and label only the PE2 (precursor to E2) polyprotein and E1, which are in endo-H sensitive forms typical of the proteins when they are located in the ER (Knipfer and Brown, 1989). The labeled infected cells were then disrupted and the PNS was produced and treated with proteinase K. After proteinase treatment, the

![Figure 2. Radioiodination of Sindbis virus wild-type and mutant TYR<sup>420</sup> glycoprotein E2. Virus produced from cells transfected with RNA from cloned mutant and wild-type cDNA were lysed with NP-40 and iodinated, as described in the text. Proteins were digested with chymotrypsin and immunoprecipitated peptides were separated by PAGE, as described in the text. TYR<sup>420</sup> shows an iodinated peptide of mol wt 2.5 kD which is not found in wild-type virus. (S) SVHR; (T) mutant TYR<sup>420</sup>. Equal counts/min were loaded in each lane.](image-url)
Proteinase K

Figure 3. Orientation of Sindbis glycoprotein PE2/E2 in membrane vesicles produced from infected cells. BHK-21 cells were infected with Sindbis virus, radiolabeled and held in the ER by treatment with CCCP (lanes a and b) or exported from the ER in the presence of cycloheximide (lanes c and d), the labeled cells were disrupted by homogenization (described in text) and the nuclei-free homogenate was exposed to proteinase K (lanes b and d). Immunoprecipitation with a polyclonal antiserum specific to PE2/E2, E1 was followed by PAGE. Proteinase treatment of intact vesicles removes a peptide of ~3 kD from the PE2 (lane b) or E2 (lane d) protein. Lane m shows proteins from purified virions.

Proteinases were immunoprecipitated with a polyclonal antiserum specific for glycoproteins E1 and E2. Fig. 3, lane a, shows that only PE2 and E1 are precipitated by this antiserum, confirming that E2 has not been produced. Proteinase K treatment of the vesicles in the PNS increases the electrophoretic mobility of the PE2 protein by an amount expected by the removal of the 33 amino acid cytoplasmic tail (lane b) (Wirth et al., 1979). The E1 glycoprotein shows no change in mobility, reflecting the fact that its cytoplasmic tail contains only two amino acids. If membranes are first treated with NP-40 and subsequently treated with proteinase K, the PE2 and E1 proteins are totally degraded (not shown). If the labeled viral proteins are chased in the presence of cycloheximide and in the absence of CCCP for 40 min, most of the PE2 label is converted to E2, indicating export from the rough ER (Fig. 3, lane d) (Knipfer and Brown, 1989). The residual PE2 and the E2 in the vesicular membranes (produced as described in Methods) shows a similar sensitivity to protease, indicating that the ecto domains of both proteins are protected by their position in the interior of the vesicle (Fig. 3, lane d). Densitometer tracing of the bands representing PE2, E1, and E2 show no significant reduction in density after proteolysis of the vesicle containing supernatant (data not shown). These data show that the glycoproteins PE2, E1 and E2 are incorporated into vesicles which completely protect their ecto domain, that vesicles are not wrong side out, and that there is no nonmembrane-associated glycoprotein.

The protection of the glycoproteins from digestion with proteinase K (Fig. 3) suggests that these proteins are integrated into the ER membranes. This integrated state was confirmed by attempting to remove these proteins by washing the membranes (prepared as in Fig. 3) with 100 mM sodium carbonate (Na2CO3) at pH 11.5, as described by Fujiki et al. (1982). The proteins sedimented quantitatively with the membranes confirming their integrated state (data not shown).

We next tested whether or not the PE2 protein which is integrated in the endoplasmic reticulum has its carboxyl terminus exposed to the cell cytoplasm. BHK-21 cells infected with wild-type, or mutant TYR420 for 90 min were placed in the drug CCCP (0.3 mM) for 30 min at 37°C. Cells were then disrupted, as described above, and the PNS was radioiodinated (see Materials and Methods) with or without 0.5% NP-40 using the nonmembrane penetrating IODO-BEAD procedure. Iodination was followed by treatment with chymotrypsin. The reaction mixture was then immunoprecipitated with an antiserum prepared against the E2 tail (see Methods) and equal counts/min of each sample were subjected to SDS-PAGE. This protocol was chosen as it results in precipitation of a peptide from the E2 tail (see Fig. 1) which contains the amino acid 420. That this antibody does not precipitate iodinated nonvirial peptides of similar molecular weight is demonstrated by controls in which the antibody is used to precipitate peptides from cells infected with wild-type virus (see below). The results of this experiment (Fig. 4) show that Tyr at position 420 can only be iodinated if the vesicles are first solubilized with 0.5% NP-40 (lane d). Wild-type virus, as expected, does not show the presence of the labeled peptide under any of the conditions employed. We have previously shown that in the absence of CCCP the viral glycoproteins are almost quantitatively converted to endo-H resistant forms in 17 min postsynthesis and that PE2 is converted to E2 in 30 min. In the presence of CCCP, PE2 is not converted to E2 (see above) and the glycoproteins are not converted to endo H-resistant forms (data not shown). These data suggest that upon integration into the membranes of the ER, the carboxyl terminal domain of the PE2 protein is organized such that the protein domain containing Tyr420
is not accessible from the cytoplasmic side of the membrane vesicle.

We next determined if the cytoplasmic domain of the PE2/E2 glycoprotein undergoes a topological rearrangement which exposed the carboxyl terminal region of the protein to the cytoplasm during export to the plasma membrane. BHK cells infected with wild-type or mutant TYR<sub>420</sub> for 3.5 h at 37°C were placed in cycloheximide (75 µg/ml) to block further protein synthesis and incubated for an additional 2 h. We have previously shown that these conditions result in the complete conversion of viral glycoproteins from endo-H sensitive to endo-H resistant forms (indicating that they have passed through the early Golgi compartments) and that all PE2 is processed to E2 (Erwin and Brown, 1980; Knipfer and Brown, 1989). Cells were disrupted and post-nuclear supernatants were radioiodinated, treated with chymotrypsin and immunoprecipitated as described in Fig. 4. Fig. 5 shows that in contrast to the proteins contained in the ER membrane, exported (chased) PE2/E2 has its carboxyl terminal region exposed and accessible from the cytoplasmic side of the membrane (lane b) as indicated by its ability to be conjugated to iodine in the absence of NP-40.

The possibilities that the peptide containing TYR<sub>420</sub> is labeled because the protein was freed from vesicles during the process of cell disruption or because the vesicles are wrong side out are ruled out by two observations: (a) the ectodomain of the protein (in the form of E2) is protected from protease degradation as is the nonexported form (see above) indicating that the proteins are primarily incorporated into right side out vesicles; (b) the amount of label incorporated into the gel fraction containing the iodinated TYR<sub>420</sub> peptide is not increased when the lysate is treated with detergent before iodination (data not shown). It would be expected that the amount of label would have increased if the peptide was protected in the interior of a vesicle, as is the case in Fig. 4.

**Discussion**

The data presented above suggest that a dramatic rearrangement of the carboxyl-terminal domain of the Sindbis virus glycoprotein PE2/E2 takes place during intracellular transport. Fig. 6 shows a model representing the orientation of maturing Sindbis virus glycoproteins in cellular membranes. The model suggests that the PE2-6K-E1 polyprotein is translocated into the membranes of the rough ER with the carboxyl terminus of PE2 located in the lumen of the rough ER (Fig. 6 a) (see Liljestrom and Garoff, 1991). Recent evidence suggests that initially, the membrane-spanning regions of the proteins are located in aqueous channels (Simon and Blobel, 1991) formed by proteins in the translocating apparatus. Completion of translation and translocation results in the release of these proteins from the protein pore to become integrated into the membrane by layer (Chuck and Lingappa, 1992). The viral proteins examined in this study (Fig. 4) have completed translation and translocation and are protected from protease degradation and are not removed by high salt, high pH wash. In the extended chase in the presence of CCCP it is expected that these proteins would be release from the protein channel and be integrated into the membrane bilayer of the ER, as shown in Fig. 6 c. Cleavage by signal-peptidase produces PE2, which is initially a type III

**Figure 5.** Release of the carboxyl terminus of the PE2/E2 glycoprotein from membranes during intracellular transport. Membrane vesicles were prepared from BHK cells infected with Sindbis virus wild-type (S) or mutant TYR<sub>420</sub> (T) which were placed in cycloheximide for 2 h to stop protein synthesis and to allow transport to plasma membrane, as described in Fig. 3. Vesicle-associated proteins were iodinated and immunoprecipitated with or without chymotrypsin treatment, as in Fig. 4. Tyr<sub>420</sub> is now available for iodination in the absence of NP-40 treatment (lane b). Equal counts/min were loaded in each lane.

**Figure 6.** Proposed conformational changes in the PE2/E2 glycoprotein of Sindbis virus during membrane integration and intracellular transport. (a) PE2-6K-E1 polyprotein showing all six membrane-spanning domains. Black dots represent glycosylation sites, * indicates amino acid position 420 (see Fig. 1); (b) cleavage of PE2-6K and 6K-E1 junctions by signalase; (c) release of the amino terminus of PE2 from ER membrane; (d) proteolytic cleavage of PE2 to produce E2 and E3; position amino acid 420 is still in the lumen of the ER or early transport vesicles; (e) return of amino acid 420 and carboxyl terminus of E2 to cytoplasm.
membrane glycoprotein with its signal sequence embedded in the membrane bilayer and two additional transmembranal domains located near its carboxyl terminal end (Fig. 6 b). Release of the signal-peptide produces a type III membrane protein with the amino and carboxyl termini in the lumen of the ER (Fig. 6 e). Export of this polypeptide from the rough ER is accompanied by the conversion of PE2 to E2 and withdrawal of the carboxyl terminus from the membrane bilayer to be exposed in the cytoplasm (Fig. 6, d and e). At this time, the protein has a single transmembranal domain and is organized as a typical type I membrane protein.

This model is supported by the data presented above and is important for the understanding of a number of events occurring during the processing of an alphavirus proteins and virus maturation. We have previously shown that the cytoplasmic tail of the PE2/E2 protein is responsible for interacting with capsid protein in assembled nucleocapsids to bind the capsids to the cytoplasmic side of the membrane (Scheefers et al., 1980; Erwin and Brown, 1980). This repeated interaction drives the process of engulfment (budding). Experiments employing synthetic peptides corresponding to the 33 amino acid cytoplasmic domain of alphavirus proteins have suggested that the most distal portion of this peptide is essential for nucleocapsid binding (Mettskko and Garoff, 1990). Sequence analysis of the polypeptide containing PE2-6K-E1 has shown that the carboxyl terminal domain of PE2 is hydrophobic (Rice and Strauss, 1981b), allowing it to be integrated into the membrane and that the junction between PE2 and 6K is capable of being processed by signal-peptidase (a protease resident in the lumen of the ER) (Gierasch, 1989). Rice and Strauss (1981b) suggested that the PE2/6K junction is first exposed to the lumen of the endoplasmic reticulum and that subsequent to cleavage, the tail is withdrawn from the membrane to be available for nucleocapsid binding on the cytoplasmic side of the membrane. More recently, Lilljestroem and Garoff (1991) have demonstrated that the E2 tail contains a hydrophobic sequence which is responsible for integration of the tail into the membrane of the endoplasmic reticulum and have provided direct evidence for the cleavage of the PE2/6K junction by signal-peptidase.

The data presented in this paper also provide an explanation for the fact that in our previous studies of Sindbis virus assembly, nucleocapsids were never found to bind to membranes of the ER containing PE2 and E1 (Erwin and Brown, 1980). Nucleocapsids were only recovered bound to plasma membrane fractions containing either PE2 or E2 (Erwin and Brown, 1980; Scheefers et al., 1980). The data presented above suggest that the capsid binding domains in the tail of the PE2/E2 protein are buried and unavailable at the endoplasmic reticulum membrane and are exposed and available for capsid attachment only at the plasma membrane.

The transient incorporation of the PE2/E2 tail into the membrane is directly demonstrated for the first time in the experiments described above. To our knowledge, this is the first demonstration of a transient translocation into cellular membranes of the cytoplasmic domain of any transmembranal protein. The transient burial of the cytoplasmic (endo) domain of membrane proteins may be of general importance as a mechanism for masking a functional region of a protein until it is completely processed, reaches an appropriate cell destination, or is stimulated by interaction with an appropriate ligand at some other domain. The mechanism by which the carboxyl-terminal domain of the PE2/E2 protein is released from the membrane bilayer is currently under investigation.

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