Deletions in the Transmembrane Domain of a Sindbis Virus Glycoprotein Alter Virus Infectivity, Stability, and Host Range

Raquel Hernandez, Christine Sinodis, Michelle Horton, Davis Ferreira, Chunning Yang, and Dennis T. Brown*

Department of Molecular and Structural Biochemistry, North Carolina State University, Raleigh North Carolina 27695

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The alphaviruses are composed of two icosahedral protein shells, one nested within the other. A membrane bilayer derived from the host cell is sandwiched between the protein shells. The protein shells are attached to one another by protein domains which extend one of the proteins of the outer shell through the membrane bilayer to attach to the inner shell. We have examined the interaction of the membrane-spanning domain of one of the membrane glycoproteins with the membrane bilayer and with other virus proteins in an attempt to understand the role this domain plays in virus assembly and function. Through incremental deletions, we have reduced the length of a virus membrane protein transmembrane domain from its normal 26 amino acids to 8 amino acids. We examined the effect of these deletions on the assembly and function of virus particles. We found that progressive truncations in the transmembrane domain profoundly affected production of infectious virus in a cyclic fashion. We also found that membrane composition effects protein-protein and protein-membrane interactions during virus assembly.

Sindbis virus is an alphavirus and a member of the arboviruses, a group of viruses which are propagated in nature via a complicated life cycle involving insect vectors and mammalian hosts. Sindbis virus is simple in its composition but complex in its structure (42). The virion contains three structural proteins, E1, E2, and Capsid (C). These proteins are organized into two geometrically identical T=4 icosahedral shells (32). The outer protein shell is composed of the glycoproteins E1 and E2, organized into trimers of heterodimers (2, 9, 34), and surrounds the inner shell, composed of protein C, which is assembled around the viral RNA. A host-derived membrane bilayer is situated between the concentric shells and is penetrated by the transmembrane (TM) domain anchors of each glycoprotein (19, 36, 41). The E2 glycoprotein contains a 33-amino-acid endodomain which specifically interacts with a hydrophobic cleft in the capsid protein (17, 18, 30, 31). The interaction between E2 and C gives stability to the structure of the virus and plays a critical role in the formation of the outer virus protein shell around the preformed inner protein shell as the process of envelopment takes place (13). The membrane glycoprotein E1 is incorporated into the icosahedral outer protein shell in a highly constrained, energy-rich conformation (9, 28). This stored energy likely drives the process of cell penetration during infection. Physical and chemical treatments of the virus result in the conversion of the protein to a low-energy, non-native configuration, which results in the loss of virus infectivity (3, 12, 28).

The three virus structural proteins are synthesized from a subgenomic polycistronic message in the sequence NH-C-PE2 (E3,E2) 6K-E1-COOH (42). The C protein is removed by autoproteolysis after translation on cytoplasmic ribosomes, revealing the signal sequence for the glycoproteins. The remainder of the polyprotein is synthesized after association of the RNA-ribosome complex with the endoplasmic reticulum (ER). Synthesis on the ER results in the incorporation of the protein as a multipass membrane protein with six membrane-spanning domains (19) (see Fig. 1). The protein is processed into PE2, 6K, and E1 by signal peptidase (19), and E1 and E2 are folded and assembled into heterotrimers prior to their export to the cell surface (9). En route to the cell surface, PE2 is processed to E2 and E3 by the furin protease which resides in the transgolgi network (26). During transport of the trimeric complexes to the plasma membrane, the membrane-spanning portion of the E2 endodomain (see Fig. 1, domain 3) is retracted from the membrane and becomes exposed to the cytoplasm (20). At the plasma membrane the E1-E2 heterotrimers are recruited into the outer protein shell by association of the E2 endodomain with the icosahedral nucleocapsid (30).

Sindbis virus, like all arboviruses, is vectored in nature by blood-sucking insects and thus must be capable of replicating and assembling in two phylogenetically unrelated genetic and biochemical environments (5). It is generally accepted that these viruses are insect viruses which have expanded their host range to include vertebrates and have thus adapted to engage the biochemical apparatus of each diverse host (40). The virus particles are hybrid structures, with proteins and genomic RNA that are the product of virus genetic information while carbohydrate side chains on their glycoproteins and the membrane are produced by host cell synthetic machinery. There are vast differences in the biochemistry and cell biology of insect and mammalian cells. Particularly dramatic are the differences in the structure and composition of mammalian and insect...
membranes. In mammalian cells, membranes of the secretory pathway are rich in cholesterol. Cholesterol is distributed differentially in these membranes in a concentration gradient from low levels in the ER to high concentrations in the plasma membranes (4). Membranes of the exocytic pathway employ these varying concentrations of cholesterol to alter the physical and chemical properties of these membranes. Membrane viscosity, ion permeability, and bilayer thickness vary relative to the amounts of cholesterol present in the membrane. Insects have no pathway for the synthesis of cholesterol (4, 10, 11), and it is anticipated that insect membranes will differ from mammalian membranes in the properties imparted by the presence of this sterol. In addition, insect membranes have been demonstrated to have a lipid composition different from that used in mammalian membranes, most notably containing more phosphatidyl-ethanolamine and lipids of shorter chain length (22).

The membrane proteins of Sindbis virus and, indeed, all arboviruses must interact with cellular membranes for assembly of the virus glycoproteins to take place. The primary association is the integration and proper orientation of the polyprotein membrane-spanning domains into membranes of the endoplasmic reticulum (19) (see Fig. 1). We have hypothesized that the differences in the composition and physical properties of insect and mammalian membranes may place upon the virus glycoproteins specific constraints for proper assembly into host membrane bilayers. This hypothesis developed from the observation that a Sindbis virus mutant (ΔK391), in which the E2 endodomain is truncated by the removal of a nonconserved lysine at the cytoplasmic interface of the membrane bilayer, displayed a host range phenotype (14). This mutant grew to high titer in insect cells but grew very poorly in mammalian cells. We proposed that this host restriction was a result of the ability of the viral proteins assembled in the insect membranes to compensate for the truncated endodomain. The insect membrane might allow for compensating conformations of the viral proteins which in turn allow proper virus assembly.

In the experiments described below, we have systematically truncated the TM domain of the Sindbis virus E2 glycoprotein to explore the interactions of this domain with host cell membranes. We found that installing deletions in this domain specifically (i) produces alterations in protein association with the membrane bilayer, (ii) disrupts virus assembly, and (iii) alters protein interactions which result in virus instability and a loss of infectivity. In some instances these effects were influenced by the host in which the mutant was grown.

MATERIALS AND METHODS

Cell culture, plaque assay, and virus. Baby hamster kidney cells (BHK-21) were described previously (35). These cells were grown and maintained in minimal essential medium containing Earl’s salts (Invitrogen, Carlsbad Calif.) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 5% tryptose phosphate broth, and 2 mM glutamine as described previously (35). The Aedes albopictus clones were subclones derived from Singh’s original larval isolates (24). The U4.4 cells were cloned from cells originally provided by Sonya Buckley (Yale Arbovirus Research Unit, New Haven, Conn.). This line has been adapted to growth in MEM as described above or in Mitsuhashi and Maramorosch medium with 20% FBS (25). The C7-10 line has been described in reference 24 and is maintained in minimal essential medium containing Earl’s salts as described previously or in MEM containing Hanks salts.

The virus strain chosen for mutagenesis was produced from the cDNA Toto 1101 clone (37) containing a substitution in E2 at position 420 changing a serine...
to a tyrosine, Y420, and has been described previously (20). Y420 serves as the wild-type and all virus mutants were constructed using this cDNA.

Titrations of virus produced were done using BHK-21 or C7-10 cells as indicator cells. Modification of the standard plaque assay (35) was necessary to accommodate the number of the mutants under investigation. Virus stocks were thawed slowly on ice, and serial virus dilutions were made, on ice, into cold phosphate-buffered saline (PBS) deficient in MgCl₂ and CaCl₂ (PBS-D) containing 10% PBS, 10% glycerol, 4 μM phenol red, and 10 mM HEPES-HCl (pH 7.2). The 1% agarose (Sigma, St. Louis, Mo.) overlay was as usual with the addition of 10 mM HEPES-HCl (pH 7.2). The titer of mutant virus stocks was determined on C7-10 cells essentially as described previously (15) with the following changes. C7-10 cells were plated onto 25 cm² dishes into serum-free MEM containing Hanks salts supplemented with 10 mM morpholinepropanesulfonic acid (MOPS)-HCl (pH 7.2). Diluent was the same as that described for BHK cells above, except that HEPES-HCl was replaced with MOPS-HCl (pH 7.2).

Site-directed mutagenesis and reverse transcription (RT)-PCR analysis of mutant viruses. The TM mutants were all made under one of two methods, either by megaprimmer mutagenesis (39) or by QuickChange site-directed mutagenesis (Stratagene, La Jolla, Calif.). For the megaprimmer mutants, Y420 cDNA, as described above, was used as the template cDNA for all PCRs. For the Quick Change mutants, the template cDNA was pGem 3Z TE12, a generous gift of Richard Bitterli (University of Lausanne, Switzerland). In order to accommodate the structural genes of the Sindbis virus TE12 strain (21) and is thereby significantly smaller and more amenable to this method. Specific mutagenic primers were made for each of the TM mutants by deleting the desired regions as shown in Fig. 2B. The megaprimmer mutants were made under standard megaprimmer site-directed mutagenesis protocols using AmpliTaq DNA polymerase (Applied Biosystems, Foster City, Calif.) as described previously (14). The QuickChange mutants were made in two steps using PfU polymerase (Stratagene), a modification described by Wang and Malcolm (45). The initial PCR consisted of 200 ng of pGem 3Z TE12 DNA template, 5 μl of 10× PfU polymerase buffer (200 mM Tris-HCl [pH 8.8], 100 mM KCl, 100 mM [N]AcSO₄, 20 mM MgSO₄, 1% Triton X-100, and 1 mg of bovine serum albumin/ml), 100 μM (each) deoxyadenosine triphosphate, 200 μM of either the sense or antisense mutagenic primer, and 1 μl (2.5 U) of PfU DNA polymerase in a 50-μl final volume. The PCR conditions were 95°C for 1 min, 60°C for 2 min, and 68°C for 18 min; this cycle was repeated 15 times. After this first PCR step, 25 μl of each of the sense and antisense reactions were combined into one reaction, 1 μl of PfU polymerase was added, and the PCR program was repeated in full. After the desired mutations were made by either method and confirmed by sequencing, they were subcloned into the Y420 vector using the Bst XI and Bst HII (New England Biolabs, Beverly, Mass.) unique sites. After confirmation of the correct sequence throughout the insert, infectious RNA was transcribed in vitro using SP6 RNA polymerase (New England Biolabs) and introduced into cells by electroporation as described below and in reference 14.

To confirm that the desired deletions remained in the virus grown in cell culture, the RNA was extracted from the virus, reverse transcribed, and amplified by PCR (RT-PCR) as described above. Primer sequences used to verify all the desired deletions were, for the sense strand, 5’- GAC TTA CTA CCA TCG CCA TCC 3’, and for the antisense primer, 5’- CAA AGG TAT GCA CAA CTG G 3’. The products generated in this PCR are 277 bp in length for the wild-type template and were run on 4% agarose gels (NuSieve, FMC, Rockville, Maine) to confirm that the deletions were present. Virus extraction was a minimum of 10⁸ PFU of virus, pelleted at 50,000 rpm in a SW55Ti (Beckman Coulter, Fullerton Calif) rotor for 1 h. The pelleted virus was extracted as described previously (14). The RNA pellet was resuspended in 10 μl of diethyl pyrocarbonate-treated water and transcribed using MuLV RT (Applied Biosystems) under the conditions described by Hernandez (Hernandez et al.) (14). The primers designed for use in the RT reaction were compared with folded RNA structures to optimize RNA accessibility (23). Following the transcription, the cDNA pellet was resuspended in 20 μl of diethyl pyrocarbonate-treated water and added to a PCR master mix as described above and in the work of Hernandez et al. (14).

In vitro transcription and RNA transfection. The mutant and wild-type cDNA constructs were prepared for transcription and transcribed in vitro as described previously (14). In order to determine the phenotypes of the mutants in both vertebrate and invertebrate hosts, the RNA transcripts were electroporated into both BHK-21 and U4-4 cells. For the BHK cells, the electroporation was performed essentially as described by Liljestrom and Garoff (19). The BHK virus was heat-inactivated in a 37°C water bath for 24 h pretransfection, which overcame first. Since many of these mutants were found to be both heat and pH sensitive (see Results and Discussion below), 10 mM HEPES was added to the virus grown in BHK cells. The U4.4 cell transfection was performed as described previously (14) with the addition of 10 mM MOPS-HCl (pH 7.2) to the culture medium to maintain an optimum pH, and U4.4-grown virus was harvested 48 h posttransfection. Virus harvested from transfected cells was resuspended into PBS-D, chilled on ice, and flash frozen in liquid N₂. Virus aliquots were thawed only once after freezing.

Metabolic labeling, immunoprecipitation, and endoglycosidase H treatment of viral proteins. Anti-whole Sindbis virus wild type heat resistant (SVHR) antibody was produced in rabbits, and beads with purified immunoglobulin G were prepared using a protein A column (Hi Trap; Amersham Pharmacia Biotech, Piscataway, N.J.) and purified immunoglobulin G produced in rabbits. 100 μg of bromide-activated Sepharose beads (Sigma) were prepared and used as described previously (28) (Mulvey and Brown) and were stored in TNT lysis buffer (1% Triton X-100, 10 mM Tris [pH 7.4], 150 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride). BHK cells were transfected by electroporation as described above, and at 6.5 h posttransfection, 5 μl of fresh MEM containing 4 μg of actinomycin D (Act-D) (Calbiochem, San Diego, Calif.)/ml was added to 25 cm² dishes of cells (∼5 × 10⁶ cells) and incubated at 37°C for 1 h. The dishes were then washed twice with 5 ml of room temperature PBS before 5 ml of starvation medium (MEM deficient in methionine and cysteine, supplemented with 2 mM glutamine, 3% FBS) was added to the cells and returned to 37°C for 1 h. The transfected BHK cells were then washed twice with 10 ml of starvation condition supplemented with 10% FBS and incubated at 37°C for 1 h. The label was removed, and the cells were washed once with PBS containing 75 μg of cycloheximide/ml. A 45-min chase using MEM containing 10% cold Met/Cys (1 mM [each] Met/Cys) and 75 μg/ml cycloheximide followed the wash. Labeled proteins were processed for immunoprecipitation as detailed above and described previously (28). Two hundred microliters of anti-whole SVHR antibody beads were added to the supernatants, and the tubes were rocked overnight at 4°C and immunoprecipitated as described previously (28). Polyacrylamide gel electrophoresis (PAGE) analysis of the supernatant of the precipitation was done to confirm the efficiency of the antibody to remove virus protein. Processed beads were split into two equal aliquots; one aliquot was adjusted for volume with PBS, and the other aliquot was digested with endoglycosidase H. To prepare the proteins for digestion, denaturing buffer (5% sodium dodecyl sulfate [SDS], 10% β-mercaptoethanol) was added to the beads at a volume of 1/10 of the total volume of the beads. This denaturing solution was incubated at 100°C for 10 min. Following denaturation, a 1/10 volume of G5 buffer (0.5 M sodium citrate [pH 5.5]) and 0.5 μl (250 units) of endoglycosidase H were added to the beads, and the reaction was incubated at 37°C for 1 h.

Gradient purification and particle/PUF ratio determination of TM mutant viruses. Subconfluent monolayers of BHK-21 cells in 75-cm² dishes were treated with 1 μg of Act-D/ml for 1 h. The Act-D was removed, and the cell monolayers were infected for 1 h. At 25°C with each of the U4.4-grown TM viruses at the highest multiplicity possible for each of the mutant viruses. In addition to the mutant infection, an infection of the Y420 parent virus using a corresponding multiplicity of infection was also done (multiplicity of infection = 0.01 to 0.80). Infected monolayers were incubated at 37°C for an additional 3 h and 45 min. The medium was removed, and the cell monolayers were washed twice with PBS-D and starved for Met/Cys in starvation medium at 37°C for 1 h. The medium was then removed and replaced with the above starvation medium containing 50 μCi of [³⁵S]Met/Cys protein labeling mix/ml. The monolayers were incubated at 37°C for 18 h. The virus supernatant was harvested and spun to equilibrium on 30 to 45% sucrose gradients in PBS-D buffer at 24,000 rpm in a Beckman SW-28 rotor overnight. The entire sucrose gradient was collected in 0.5-ml fractions, and 5 μl of each fraction was counted by scintillation spectrometry for detection of labeled virus. Once the virus fraction was determined, aliquots were removed to measure protein concentration using the Micro BCA protein assay reagent kit (Pierce, Rockford, Ill.) Titration of the same virus fraction was performed on BHK-21 cells as described previously (35) and above. The number of particles in a preparation of wild-type virus (SVHR) was determined under the electron microscope by the agar filtration protocol described by Kellenberger and Bitterli (16), and the particle count was correlated to the amount of protein.

Transmission electron microscopy. BHK cells were transfected with RNA transcribed from either Sindbis virus Y420 or individual deletion mutants as described above. At 25°C for 16 to 24 h posttransfection, the cell monolayers were scraped from the dishes and pelleted by low-speed centrifugation. Cell pellets were washed twice with PBS and fixed with 3% glutaraldehyde
in 0.1 M cacodylic acid buffer (pH 7.4) (Ladd Research Industries). After cells were washed three times with 0.1 M cacodylic acid, the cells were stained with 2% osmium tetroxide in cacodylic buffer for 1 h. Cells were then washed as before and embedded in 2% agarose. The agarose containing the cell sample was then prestained with 1% uranyl acetate (Polaron Instruments, Inc., Hatfield, Pa.) overnight at 4°C. The samples were washed and carried through ethanol dehydration. Infiltration was done using SPURR compound (LADD Research Industries). Blocks were then trimmed on an LKB NOVA Ultrotome (Leica Microsystems, Inc. Deerfield, Ill.). Ultra-thin sections were then obtained and were stained with 5% uranyl acetate in distilled water for 60 min and in Reynolds lead citrate (pH 12) (Mallinkrodt Baker Inc., Paris, Ky.) for 4 min. The samples were examined at 80 kV in a JEOL JEM 100S transmission electron microscope.

**FIG. 2.** Sequence of several alphavirus E2 TM domains (box) and flanking sequences (A) and the sequence of deletions (boldface) made in the domain (B). The transmembrane domains are characteristically hydrophobic but have no consensus sequence. SVHR, Sindbis wild type heat resistant; WEEV, western equine encephalitis virus; EEEV, eastern equine encephalitis virus; RRV, Ross River virus; SFV, Semliki Forest virus; ONNVG, O’nyong-nyong virus. In panel B, Deletion mutants are named for the number of amino acids remaining in the TM domain.

**RESULTS**

**Production of Sindbis virus E2 TM domain mutants.** The topology of the Sindbis virus structural polyprotein as it is integrated into membranes of the endoplasmic reticulum results in the formation of six membrane-spanning domains which are potential targets for the introduction of deletion mutations (19) (Fig. 1). Three of these membrane-spanning domains have functions other than that of a membrane anchor which preclude their selection for mutational analysis. Domain 1 contains the sequence recognized by the signal recognition particle and is essential for targeting the polyprotein to the ER.

![Graph showing production of infectious virus by the TM deletion mutants in insect (hatched) and mammalian (solid) cells. The values shown represent the average for a minimum of three or maximum of five replicates. The error in these experiments is 10 to 20%.

**FIG. 3.**
Domain 3 is withdrawn from the membrane after export from the ER and contains sequences which recognize and bind to the nucleocapsid as envelopment takes place (17, 18, 30). Domain 5 has been demonstrated to play a role in the correct integration of the E1 protein into the membrane (19). Domains 2, 4, and 6 have not been demonstrated to have functions other than anchoring the protein into the membrane, and of these we chose domain 2, the E2 TM domain. We reasoned that introducing deletions into this domain, which is the second integrated into the membrane, might be most disruptive to virus assembly.

Figure 2A shows a sequence alignment of the E2 transmembrane domain sequences of a number of alphaviruses (38, 42). These domains do not display conserved amino acids, only a general hydrophobic nature and a relatively consistent length (26 to 28 amino acids). This analysis suggested that imbedded amino acid signals may not be present in this domain and that mutations in the TM α-helix that maintained the hydrophobic nature of the domain should be tolerated. We produced a series of deletions in the E2 transmembrane domain (as described in Materials and Methods) to determine the effect of progressive shortening of this domain on virus assembly, function, and stability. The differences in membrane thickness and composition of mammalian and insect cells present a unique opportunity to measure the requirements placed upon a membrane glycoprotein for proper assembly in biochemically diverse membrane environments.

We truncated the transmembrane domain of glycoprotein E2, incrementally, from its wild-type length of 26 amino acids to a minimum of 8 amino acids. The sequence of these deletions is presented in Fig. 2B. Mutants are named to reflect the number of amino acids remaining in the TM. The cDNA containing these mutations was transcribed into infectious RNA, and BHK-21 or A. albopictus cells were transfected by electroporation as described in Materials and Methods. Transfected cells were allowed to incubate for 48 h at 28°C for A. albopictus cells or 24 h at 37°C for BHK cells. At the end of the incubation period, the amount of virus produced by each mutant in either cell type was determined by plaque assay on BHK-21 cells as described in Materials and Methods. The virus titers of the TM mutants grown in BHK and Aedes cells are shown in Fig. 3.

The results of these experiments are complex. The data show that while the wild-type virus Y420 produces very high titers of virus in both host cell types, the deletion of a single amino acid (TM25) reduces virus production by 4 orders of magnitude and favors virus production in BHK cells. The mammalian cells infected with TM25 produce 10.5 times more virus than did infected insect cells. Reducing the length of the domain to 24 amino acids (TM24) increases virus production in both cell
types, but production is still well below wild-type levels and TM24 favors growth in insect cells. TM23 results in equivalent infectious virus production from both cell types. Reduction to 22 and 21 amino acids (TM22 and TM21, respectively) progressively increases virus production in mammalian cells but still favors growth in insect cells. Additional deletions to 20 and 19 amino acids (TM20 and TM19) increases virus production in mammalian cells, while growth in insect cells remains at the levels seen for TM22 and TM23. Deletion to TM18 results in wild-type levels of growth in both cell types. Additional deletions, producing TM17 and TM16, begin a progressive reduction in virus production from BHK cells accompanied by the dramatic retention of near-wild-type levels of growth in insect cells. The most dramatic difference is seen with TM16, which produces virus at a level 1,000-fold lower in BHK cells than production in Aedes cells. Reduction to TM14 and TM12 continues the trend of reduction in virus produced from the Aedes cells, although equivalent amounts of virus are produced in both cell types. These mutants disrupt the trend of differential virus production in the two cell lines seen with TM16 and TM17. Surprisingly, reduction to TM10 resulted in increased virus production in both cell types to levels only 100-fold below wild-type levels. The loss of 18 amino acids from the transmembrane domain (TM8) dramatically reduces virus production to only about 100 PFU/ml in both cell types. Sequencing of cDNA from the progeny virus produced by each of the TM mutants revealed that the original deletions installed in the TM mutants were recovered. No restoration of the wild-type sequence occurred in any of these viruses.

Relative infectivity of the TM mutants. Virus produced by the TM mutants was purified by equilibrium density gradient centrifugation on linear sucrose gradients as described in Materials and Methods. All of the TM mutants were found to produce virus which banded at a mean density of 1.20 gm/cm³. The purified virus was found to contain the typical content of polypeptides E1, E2, and C (data not shown). The relative infectivity (particle-to-PFU ratio) of the TM deletion mutants was determined as described in Materials and Methods. The result of this experiment is shown in Fig. 4A. The total number of particles (infectious and noninfectious) is presented in Fig. 4B. In general, the profile of particle-to-PFU ratio appears to be the inverse of the virus production profile shown in Fig. 3. The results show that all of the TM mutants produce large and similar amounts of virus particles, but the relative infectivities of these viruses differ greatly. For example, TM25 produces a low titer of virus from BHK cells, ca. 10⁵ PFU/ml (Fig. 3), but has a particle-to-PFU ratio of 10⁵ to 1 (Fig. 4A). Thus, TM25 produces about 10¹⁰ total particles (Fig. 4B). The wild-type Y420, by contrast, produces a titer of 10⁹ PFU/ml (Fig. 3) and has a particle-to-PFU ratio of about 10⁰ to 1 (Fig. 4A) or about 10¹¹ total particles/ml (Fig. 4B). It was not possible to determine the relative infectivity of TM8. The amount of virus produced by this mutant was so low (ca. 100 PFU/ml) that we could not accumulate enough material for analysis. Thus, each of the TM mutants which produce low titers (with exception of TM8) produces a significant amount of noninfectious material which purifies at virus density. This experiment suggests that the introduction of some deletions in the transmembrane domain of E2 has less effect on the process of particle assembly than it does on the ability of that virus to initiate productive infection. These data also suggest that low levels of virus production by some mutants are not due to reduced levels of protein synthesis or reduced ability to export protein to the cell surface.

Production of virus proteins and nucleocapsids by TM mutants. Several of the TM mutants were examined by electron microscopy of ultra-thin sections of infected cells. This assay was directed at determining if nucleocapsids were assembled in BHK cells infected with the mutants that displayed lowered titers. Electron micrographs of two of the mutants, TM8 and TM14, are shown in Fig. 5. In all cases examined, assembled nucleocapsids could be detected in the cell cytoplasm. Since capsid protein is released from the nascent polyprotein before the envelope protein domains are synthesized, this processing event was not expected to be affected by deletions in the E2 domain. Much evidence indicates that the assembly of nucleo-

FIG. 5. Electron micrographs of thin sections of cells infected with TM mutants. The arrows indicate the presence of assembled nucleocapsids. (A) Wild-type virus (Y420). (B) TM8. (C) TM14. Magnification bar, 100 nm.
capsids from capsid protein and progeny RNA takes place independently of glycoprotein synthesis and assembly (13, 43, 44).

Protein production by the TM mutants in BHK cells was analyzed by SDS-PAGE analysis of infected cell extracts. Virus proteins were partially purified prior to PAGE by immunoprecipitation as described in Materials and Methods. Proteins produced by wild-type virus (Y420) and several TM mutants are shown in Fig. 6A and B. The wild-type infection produces a typical protein profile consisting of virus membrane proteins E1, E2, and the precursor protein PE2. The B protein containing E3, E2, 6K, and E1 is also seen (Fig. 6A). The TM mutants which retain a large portion of the wild-type 26 amino acids, in particular TM14, TM16, and TM17, show a wild-type pattern of virus membrane protein production (Fig. 6A). The shorter TM mutants (TM10 and TM12) appear to produce E1, E2, and PE2 but also produce a larger protein migrating at a molecular mass of approximately 68 to 70 kDa (Fig. 6A). We reasoned that this might result from a failure to process one of the two signalase sites in the PE2, 6K, E1 polyprotein (see Fig. 1 and introduction). Due to the low resolution of the SDS-PAGE separation, it was not possible to discriminate between the two potential polyproteins (PE2-6K or 6K-E1). The gel piece containing the 70-kDa protein was provided to the mass spectrometry laboratory at the Mayo Clinic (Rochester, Minn.) for liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis. The protein was subjected to trypsin “in-gel” digestion, and upon mass-spectrometry analysis, we positively identified peptides from PE2 but not E1 or 6K. A possible explanation for failing to find the 6K peptides is that the four trypsin sites in 6K yield large peptides difficult to positively identify in mass spectrometry, and their hydrophobic nature may have prevented them from being recovered from the in-gel digest or from being effectively eluted from the C18 column used in the LC-MS/MS analysis. We inferred that the linear nature of the polyprotein (PE2-6K-E1) was maintained and that the 70-kDa protein resulted from a failure to cleave only the junction between PE2 and 6K. Preliminary experiments indicate that this misprocessed protein is retained in the ER in a form which is sensitive to endoglycosidase H. None of this larger protein was recovered in material banding at virus density.

Truncation of the transmembrane domain to eight amino acids blocked the formation of membrane proteins to almost undetectable levels while allowing the production of capsid protein (Fig. 6B) and nucleocapsids (Fig. 5B). The failure to produce the membrane proteins may result from the inability of the E2 protein to interact, correctly, with the ER membrane, preventing membrane-dependent translation of the PE2-6K-E1 polyprotein.

Heat stability of the TM mutants. The truncation of the transmembrane domain of E2 may place physical strain on the protein-protein associations which make up the icosahedral virus protein shells. This conformational strain could occur in the lateral associations of E2 with E1 or between the E2 endodomain and the virus nucleocapsid. To determine the stability of the TM mutants, we assessed their heat sensitivity following the protocols of Burge and Pfefferkorn (6, 7). The mutants, produced from both cell types, were exposed to various temperatures for a period of 5 min and then titered to determine loss of infectivity as described in Materials and Methods. The results of these experiments are shown in Fig. 7. For each mutant, the titer of virus after exposure to 37, 51, and 60°C is shown as a percentage of the titer at 25°C. These data clearly demonstrate that the size of the deletion and the cell type in which the mutant is grown profoundly affect heat sensitivity. The most dramatic effect of a deletion in the transmembrane domain is that seen in TM25 produced from BHK cells, which is much more resistant to heat treatment than is TM25 produced from A. albopictus cells. Insect-grown TM17, TM18, TM19, and TM20 also display decreased stability compared to the same virus grown in BHK cells. Insect-grown TM21 and TM17 are more heat sensitive than insect TM10, which contains a large deletion of 16 amino acids. Insect-grown TM14 is more sensitive to exposure to 51 and 60°C than BHK grown TM14, but virus from both sources is similarly resistant to exposure to 37°C, which is the reverse of the profile seen for TM16. It seems that the composition and physical properties of the membrane bilayer have a profound effect on virus stability.

**DISCUSSION**

The introduction of deletion mutations into the transmembrane domain of a cloned vesicular stomatitis virus glycopro-
tein (G) allowed Adams and Rose (1) to determine the structural constraints placed upon the transmembrane domain for correct integration into cellular membranes and for transport to the cell surface. Expression of these DNAs showed that G proteins containing 18, 16, or 14 amino acids of the original transmembrane domain assumed a transmembrane configuration and were transported to the cell surface. G proteins containing only 12 or 8 amino acids of this domain also spanned intracellular membranes, but their transport was blocked within a Golgi-like region in the cell. The results presented above imply that in the production of intact Sindbis virus, the assembly and transport parameters may change. While a significant amount of TM10 protein is incorrectly processed, it is clear that the presence of only 10 amino acids in the transmembrane domain of Sindbis virus E2 allows for the correct integration, folding, and export of significant amounts of virus compared to the case with TM8 and TM12 (Fig. 3). It has been demonstrated that association with PE2 is necessary for the export of E1 to the cell surface (9). Deletions in the PE2 transmembrane domain do not prevent this function. The virus produced by the mutant TM10 is also very stable, with a relatively low particle-to-PFU ratio (Fig. 4A and 7).

The anomalous behavior of TM10 may be explained by the structure and assembly of the mature virus. The PE2 glycoprotein pairs with E1 in the endoplasmic reticulum after the early events in the folding of E1 have taken place (8, 9, 27, 28). The PE2-E1 heterodimers then form trimers, and after further folding of E1, the trimers of heterodimers are exported to the cell surface. At the plasma membrane, the heterotrimeric assemblies into an icosahedral lattice. Strauss et al. have shown that the hydrophobic domains of the Sindbis virus glycoproteins, E2 and E1, interact with one another during assembly (41). Our data suggest that a significant truncation in one of these transmembrane domains does not effect that interaction. It is possible that interactions in the ectodomains of E1 and E2 also contribute to the assembly and stability of the virus. It may be that the association of PE2/E2 with E1 holds the E2 protein in the developing spike trimer until binding of the E2 endodomain to the capsid protein locks the E2 protein in place as envelopment occurs. This model suggests that the host cell membrane is a scaffold for assembly of the virus glycoprotein and that once the icosahedral protein shell is formed, the structure of the membrane itself is inconsequential. This argument is supported by the relatively large amount of stable virus assembled by TM10, which in theory should not contain sufficient amino acids to span the membrane. In this regard we have shown previously that the icosahedral lattice of the virus can determine the shape of the membrane bilayer to the extent that if the icosahedral shell is opened, the membrane assumes the configuration of the protein shell (3). It has also been reported that alphaviruses which have had the membrane removed by detergent treatment retain infectivity (29).

Mutations with deletions of 12 amino acids or greater (TM12 and TM10) produce a large misprocessed protein composed of PE2 and 6K, indicating that the signalase-sensitive site located between these two proteins is not processed while a second site between 6K and E1 is processed. The site connecting PE2 and 6K may be unavailable because of misfolding of this domain in the ER. Alternatively, the truncation of the transmembrane domain may cause a population of the molecules to engage the ER membrane in a manner that places the
site on the cytoplasmic side of the ER membrane, making it inaccessible to the ER luminal protease.

The profile of infectious virus production by the TM mutants shown in Fig. 3 is surprising. We expected that virus production would progressively fall in mutants as deletions increased in size. Viruses with small deletions, such as the TM mutants in the 20- to 25-amino-acid range, were expected to retain wild-type properties. We found, instead, a precipitous drop in virus production with the deletion of a single amino acid followed by increasing and then falling infectious virus production. The removal of the single amino acid, E2 M379, might suggest that this particular amino acid and position in the transmembrane domain is critical for virus formation. We are testing this possibility by making other single deletions; it is significant, however, that in none of the other mutations is this amino acid restored to the transmembrane domain, including deletion mutant TM18, which restores virus production to wild-type levels.

Removal of amino acids from the PE2/E1 transmembrane domains affects the production of infectious virus from the two host cell types. For some deletion mutants virus production is favored in mammalian cells, while for others production is optimal in insect cells (viruses produced from the two cell types produce equal numbers of plaques in either cell type [data not shown]). This effect is most dramatic with the mutant TM16, where virus production in the mammalian cells is nearly 3 orders of magnitude less than in insect cells. Since the mutant protein sequences are identical in both cell types, it is clear that some host-contributed component is responsible for the difference. The most obvious of these is the membrane bilayer, which is a product of the cell in which the virus is grown. As one may expect, many differences exist between the membranes of the phylogenetically unrelated insect and mammalian cells. One of the most dramatic differences is that insects have no pathway for the production of cholesterol, and their membranes are free of cholesterol (10, 11). Mammalian cells by contrast incorporate cholesterol into membranes of the endoplasmic reticulum, which is concentrated in the secretory pathway in an increasing gradient as the membranes approach the cell surface (4). Alterations imparted to the proteins as a result of the deletions present in the transmembrane domain of PE2/E2 may alter the ability of the protein to interact with membranes of different compositions.

The cyclical nature of the trend of infectious virus produced from TM12 to TM25 (Fig. 3) suggests a periodicity in the effect of the deletion size on the production of infectious virus. The complementary periodicity displayed in the particle-to-PFU ratio (Fig. 4A) suggests that low levels of relative infectivity reflect increased production of noninfectious virus (Fig. 4B). This effect may be related to the constraints placed upon the virus as assembly takes place. The Sindbis virion is composed of two geometrically identical protein shells, between which is situated a membrane bilayer (32). The two shells are locked to one another by the E2 protein, which penetrates the membrane bilayer and binds into a hydrophobic cleft in the internally situated capsid protein (17, 18). The rigid nature of these associations suggests that the E2 endodomain must emerge from the membrane in an orientation allowing for a “correct” interaction with capsid. The E2 transmembrane domain is predicted to assume an α-helical conformation within the membrane. Amino acids in this type of helical structure have a defined and regular organization in which each successive amino acid is found in a 100° rotation from its neighbor. Assuming that the organization of the glycoprotein icosahedral shell determines the orientation of the helix at the luminal junction of the membrane bilayer, each deletion made in the transmembrane domain would alter the orientation of the E2 protein endodomain as it emerges from the membrane bilayer into the cell cytoplasm. As deletions are made, some conformations of the endodomain may favor the production of virions which are stable and infectious, while other deletions, resulting in different endodomain conformations, may allow for virus assembly but produce associations of E2 with C which render these particles noninfectious. In this regard we have previously shown that a mutation in the hydrophobic cleft of the capsid protein results in the production of virus with very low infectivity (17). The reason for the low relative infectivity of some of the mutants is not clear. TM25, which has a high particle-to-PFU ratio, produces cell-cell fusion from without after brief exposure to low pH as efficiently as wild-type virus when equal numbers of particles are employed (data not shown). This result suggests that the noninfectious particles attach to cells and that some membrane protein functions (those related to low-pH-mediated fusion) are retained. We are attempting to produce compensatory mutations in the virus structural proteins of the TM mutants which will improve infectivity and stability while retaining the deletion. These mutants may allow identification of the domains of protein interaction which are affected by the truncations.

The mutations produced in this study provide new tools for the examination of the role that glycoprotein transmembrane domains play in the process of virus assembly and how the composition and physical properties of the membrane affect that process. The properties of the individual mutations described above are under investigation. It is particularly intriguing that the transmembrane domain of alphaviruses is conserved at a length of 26 amino acids when 18 amino acids seems to be adequate for wild-type levels of virus production in both insect and mammalian cells. It is generally accepted that these viruses are insect viruses which have expanded their host range to include vertebrates (40). We are examining the proliferation of these mutants in model mammals and insects to determine if the length of the transmembrane domain plays a role in tissue tropism and distribution in the alternate hosts.

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REFERENCES

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