Lethality of PE2 Incorporation into Sindbis Virus Can Be Suppressed by Second-Site Mutations in E3 and E2

HANS W. HEIDNER, KEVIN L. MCKNIGHT, NANCY L. DAVIS, AND ROBERT E. JOHNSTON*

Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, North Carolina 27599-7290

Received 12 October 1993/Accepted 5 January 1994

Sindbis virions contain two glycoproteins, E1 and E2. E2 is produced initially as a precursor, PE2, from which the amino-terminal 64 amino acids are cleaved by a cellular protease at a late stage in virion maturation. A mutation at E2 position 1 (Arg to Asn) was placed into Sindbis virus AR339 by site-directed mutagenesis of a full-length AR339 cDNA clone, pTRSB, to produce pTRSB-N. The mutation created a signal for N-linked glycosylation immediately adjacent to the PE2 cleavage signal. Virions derived from pTRSB-N were glycosylated at E2 position 1, and they quantitatively incorporated PE2 in place of E2. When pTRSB-N transcripts were electroporated into BHK-21 cells, TRSB-N particles were released with nearly normal efficiency; however, the specific infectivity of TRSB-N particles was very low. Analysis of seven infectious revertants of TRSB-N revealed that reversion was linked to (i) mutations that eliminated the signal for N-linked glycosylation and thus restored the PE2 cleavage phenotype or (ii) conservation of the PE2 cleavage defect combined with incorporation of suppressor mutations in E3 or E2. The genotype of each revertant was reconstructed in the genetic background of TRSB-N, and each reverting mutation also was replaced individually into the genetic background of wild-type virus (TRSB). Each PE2-containing revertant was attenuated in newborn CD-1 mice and replicated poorly in cultured mosquito cells (C6/36). Reverting mutations in the genetic background of TRSB did not reduce virulence in mice or growth in mosquito cells, suggesting that the phenotypes of attenuation in mice and reduced growth in mosquito cells were linked to failure of PE2 cleavage and not to the reverting mutations themselves.

The membrane glycoproteins comprising the spikes of enveloped viruses perform many important viral functions. Among these are attachment of virus to specific host cell receptors, fusion of the viral envelope with host cell membranes, budding from infected cells, and influencing pathogenicity in vivo. These functional properties of viral spikes are a direct consequence of the structure(s) of the individual spikes and their constituent components, which can vary depending upon their maturational state (9, 24, 33). The maturation process of enveloped RNA viruses often involves the proteolytic cleavage of a membrane glycoprotein precursor prior to release of virions from the cell. For most viruses of the orthomyxovirus, paramyxovirus, coronavirus, and retrovirus families, the fusogenic glycoproteins are synthesized as nonfusogenic precursors, which attain fusion competence at a late stage of viral maturation through a specific proteolytic event (see reference 67 for a review). A similar maturational event occurs during the morphogenesis of alphaviruses (family *Togaviridae*).

The alphavirus genome consists of a positive-sense, single-stranded RNA which is encapsidated within a T=4 nucleocapsid composed of 240 copies of capsid protein (3, 42). The nucleocapsid is contained within a host cell-derived lipid envelope (17). Two transmembranal viral glycoproteins, E1 and E2, are exposed on the surface of the virion (49, 54). E1 and E2 are associated as heterodimers, which in turn are organized in groups of three to form the viral spikes. The viral spikes are structured in an icosahedral form at the virus surface and are arranged with a T=4 symmetry (13, 41, 61).

The structural proteins of alphaviruses are translated as a single polyprotein from the 26S subgenomic mRNA (53).

During their synthesis, glycoproteins PE2, the precursor of E2, and E1 are cleaved from the common precursor as nascent peptides and inserted individually into the endoplasmic reticulum (see reference 55 for a review). PE2 and E1 associate in the endoplasmic reticulum (49, 63, 68) and are processed and transported in heterodimeric form (11, 38). Within either a trans- or post-Golgi compartment, PE2 is cleaved into E3 and E2 (23, 56). E3 is composed of the 64 (Sindbis virus) or 66 (Semliki Forest virus [SFV]) amino-terminal residues of PE2. E3 is retained in the SFV particle but is lost to the medium in Sindbis virus-infected cell cultures (14, 66). The larger cleavage product, E2, remains anchored in the membrane and associated with E1. PE2 cleavage is likely performed by a host cell protease (8, 40, 65) and occurs at a site immediately following a highly conserved canonical four-residue motif, Basic amino acid-X-Basic-Basic (5, 48). Cleavage of PE2 temporally coincides with the release of budded particles which contain structurally and functionally mature E2/E1 heterodimers. Mutants of SFV and Sindbis virus that fail to cleave PE2 but are still efficiently released from infected cells have been isolated (10, 50, 51). All of these viruses have defects in one or more biological functions, which can be linked to the presence of PE2 in virions.

In SFV, cleavage of PE2 was prevented by mutating the PE2 cleavage signal from Arg-His-Arg-Arg to Arg-His-Arg-Leu (29, 51). PE2-containing particles were efficiently released from transfected cells; however, these were not infectious for baby hamster kidney (BHK-21) cells because of impairment in functions of receptor binding and entry (51). However, when inoculated into 40-day-old mice, the particles displayed an attenuated phenotype but were not avirulent (15). This indicates that these particles were infectious to some extent in vivo or that infectious revertants were present in the virus preparations. Infectivity for BHK-21 cells could be restored by in vitro cleavage of PE2 in virions with trypsin or by exposing

* Corresponding author. Phone: (919) 966-3507. Fax: (919) 962-8103.

particles to pH 4.5 (51). Both treatments have demonstrable effects on heterodimer stability and virion structure (22, 29, 30, 63). Likewise, PE2-containing mutants of Sindbis virus, defective in functions of cell binding and penetration, have been produced by disruption of the cleavage signal through insertional mutagenesis (10). Consistent with these findings was the observation that particles of Sindbis virus AR339 released from RPE.40 cells, which are defective for the cellular protease thought to be responsible for PE2 cleavage, contained PE2/E1 heterodimers and were essentially noninfectious (65).

Interestingly, the link between PE2 cleavage and virion infectivity appears to differ among Sindbis virus strains. A PE2-containing mutant of the S.A.AR86 strain of Sindbis virus was isolated during selection for variants having a rapid-penetration phenotype (50). The PE2 processing defect in this mutant, S12, resulted from a single mutation at E2 amino acid 1 (E2 1), the +1 position relative to the cleavage site, in which a Ser-to-Asn substitution created a signal for N-linked glycosylation (Asn-X-Ser/Thr). In BHK-21 cells, S12 was as infectious as the PE2-cleaving parent (50) and both viruses grew to normal titers on RPE.40 cells (65). In contrast, S12 was markedly attenuated in newborn and adult CD-1 mice in comparison with the PE2-cleaving parent (50) and grew very poorly in cultured mosquito cells (46).

In this report, we have used a full-length cDNA clone of Sindbis virus AR339 to study the differential strain requirements for cleavage maturation of the PE2 precursor. A single mutation analogous to that identified in S12 was engineered into the clone by site-directed mutagenesis. The resulting substitution, Asn for Arg at E2 1, produced a signal for N-linked glycosylation, which was utilized and which proved to be lethal. PE2-containing particles were released with high efficiency following electroporation of transcripts from the mutant clone into BHK-21 cells; however, these were essentially noninfectious. Infectious PE2-containing virus could be rescued by a number of second-site suppressor mutations which mapped to the E3 or E2 gene. Each reverting mutation displayed a considerable degree of specificity for the original lesion at E2 1. Consistent with studies using the S12 mutant, PE2-containing AR339 revertants grew to nearly normal titers in RPE.40 cells, were markedly attenuated in newborn mice, and were restricted for growth in cultured mosquito cells.

MATERIALS AND METHODS

Viruses and cell lines. Sindbis virus AR339 was obtained originally from H. R. Bose (University of Texas at Austin). TRSB was derived from an infectious, full-length cDNA clone (pTRSB) containing viral sequences from our laboratory strain of AR339 (SB) placed downstream of an SP6 phage promoter (33a). Briefly, SB sequence fragments isolated from a cDNA library (43) were substituted for analogous fragments removed from the original Sindbis virus cDNA clone, Toto1101, described by Rice et al. (47). Sindbis virus S.A.AR86 was obtained through J. M. Dalrymple from J. Casals, Yale Arbovirus Research Unit. The passage history of this virus has been described elsewhere (50). The nomenclature identifying the viruses used in these studies is as follows. The prefix TRSB- was assigned to all mutant viruses constructed in either pTRSB or pTRSB-N (see the explanation below). Designations for biologically derived mutants lack this prefix. All virus stocks used in this study were grown in ATCC BHK-21 cells maintained as previously described (50). A parental line of Chinese hamster ovary cells (CHO-K1) and a *Pseudomonas* exotoxin A-resistant mutant strain (RPE.40) were kindly provided by J. M. Moehring and T. J. Moehring (University of Vermont)

(35). CHO-K1 and RPE.40 cells are auxotrophic for proline and were maintained in Eagle's minimal essential medium (MEM) (GIBCO-BRL) supplemented with 5% donor calf serum, penicillin (50 U/ml), and streptomycin (50 µg/ml). The invertebrate cell line, C6/36, was derived from *Aedes albopictus* cells by A. Igarashi (20) and was provided to us by D. T. Brown (University of Texas at Austin). C6/36 cells were maintained in MEM supplemented with 10% fetal bovine serum, 10% tryptose phosphate broth, and antibiotics as described above.

General recombinant DNA methods. DNA manipulations and plasmid DNA preparations were carried out essentially as described previously (31, 44). DNA fragments used in cloning procedures, and products from PCR procedures, were isolated in agarose gels and recovered with GeneClean (Bio 101, Inc., La Jolla, Calif.).

In vitro mutagenesis. Single codon substitutions were engineered into genetically defined cDNA clones by in vitro mutagenesis by the technique of Kunkel et al. (25). Mutagenesis was performed as described previously (44, 45), by using the M13-derived mutagenesis substrate vectors mp18SBGP and mp18SBGP-N and site-specific mutagenesis primers, each containing one or two mismatched nucleotides. mp18SBGP contains the PE2, 6K, and E1 gene sequences of SB. mp18SBGP-N is isogenic with mp18SBGP except for a single codon substitution (Asn for Arg) at E2 1. Individual phage clones were screened by sequence analysis of single-stranded phage DNA by the dideoxynucleotide chain-termination method (52). The unique restriction sites *Stu*I (nucleotide 8571) and *Bss*HII (nucleotide 9804) were utilized to generate restriction fragments from phage replicative-form DNA containing the desired substitutions. These were substituted for the analogous fragment removed from pTRSB. Each recombinant cDNA was sequenced across the entire replacement region by the *Taq* cycle sequencing procedure (United States Biochemical).

In vitro transcription and transfection of cells. BHK-21 cells were routinely transfected with in vitro transcripts for the production of virus stocks and for specific infectivity assays with Lipofectin reagent (GIBCO-BRL), as described previously (43, 44). Electroporation of in vitro transcripts into BHK-21 cells was performed as described earlier (28).

Specific infectivity assays were performed essentially as described by Rice et al. (47). For each construct to be tested, duplicate transcription mixes were prepared, one of which was supplemented with 5 µCi of ³⁵S-UTP. The quantity of incorporated label was measured for 1 µl of transcripts spotted onto DE81 ion exchange paper (Whatman) and quantitated on a liquid scintillation counter. Two microliters of unlabelled transcripts was transfected into duplicate 60-mm-diameter dishes of BHK-21 cells, and the cells were overlaid with agarose (0.9% in MEM complete) after a 20-min incubation. Plaques were visualized 30 to 48 h later by staining with neutral red, and the specific infectivity of transcripts was calculated as PFU per counts per minute.

Isolation and sequence analysis of revertant viruses. Individual plaques that formed in specific infectivity assays using transcripts from pTRSB-N were picked with a 1.0-cm³ tuberculin syringe, diluted into 1.0 ml of MEM (serum free), and placed onto BHK-21 cell monolayers in 25-cm² culture flasks. These plaque isolates were used as working stocks for the determination of biological revertant phenotypes.

Purification of virions and sequencing of virion RNA were done essentially as described previously (39, 50), except that sodium dodecyl sulfate (SDS)-disrupted virions were first digested with proteinase K (50 µg/ml) for 30 min at 37°C prior to extraction with phenol-chloroform (1:1 [vol/vol]) and pre-

cipitation in ethanol. Viral RNA was sequenced by the dideoxynucleotide chain-termination method, using modifications designed for sequencing RNA templates (6, 69). The oligonucleotide primers used for sequencing have been described previously (6, 50).

cDNA constructions of revertant genotypes by PCR. The genotype of each biological revertant was reconstructed in pTRSB-N. A reverse transcriptase-PCR procedure was used to obtain each reverting mutation in double-stranded DNA form. cDNA derived from virion RNA was amplified by PCR for 20 cycles with *Taq* DNA polymerase (Perkin-Elmer Cetus) in a model 480 thermocycler (Perkin-Elmer Cetus).

PCR fragments derived from revertants with mutations in E2 were digested with *Stu*I and *Bss*HII at unique restriction sites, generating fragments containing both the primary mutation at E2 1 and the reverting mutation. Fragments containing the reverting mutation in E3 were digested at the unique sites *Aat*II (nucleotide 7999) and *Stu*I. Restriction enzyme-digested PCR fragments containing the E2 reverting mutations were ligated into pTRSB from which the analogous *Stu*I-to-*Bss*HII fragment had been removed. Colonies were screened by sequencing transcripts (synthesized *in vitro* as described above) across the PE2 cleavage site, where the presence of E2 Asn-1 confirmed each PCR replacement. Restriction enzyme-digested PCR fragments containing the E3 mutation were ligated into pTRSB-N or pTRSB by using transcript sequencing to confirm proper replacement. Reverting mutations in E2 were placed into pTRSB by site-directed mutagenesis. Specific infectivities were determined for transcripts from each cDNA construct, and each *Stu*I-to-*Bss*HII or *Aat*II-to-*Stu*I replacement was sequenced in its entirety from virion RNA as described earlier.

Polyacrylamide gel analysis of [³⁵S]methionine-labelled viral proteins. Viral proteins of TRSB-N, and of all the biological and cloned mutants, were radiolabelled with [³⁵S]methionine and visualized by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. BHK-21 monolayers were electroporated with transcripts from pTRSB-N or infected with viable virus at a multiplicity of infection of 10 PFU per cell. Cells were maintained in MEM complete for 5 h. Cells were then depleted of methionine for an additional 3 h by replacement of media with antibiotics and methionine-free MEM supplemented with 5% donor calf serum. At 8 h after electroporation or infection, [³⁵S]methionine was added to a final concentration of 20 μ Ci/ml, and cells were incubated at 37°C for an additional 12 h. Virus particles, purified from the culture supernatants, were analyzed on SDS-polyacrylamide gels (10% acrylamide) prepared as described previously (26). [¹⁴C]-methylated protein markers (Amersham) were included as molecular weight standards. Labelled proteins were visualized by fluorography (Amplify solution; Amersham).

Virulence studies in newborn CD-1 mice. Two to four litters of 1-day-old CD-1 mice (Charles River Breeding Laboratories Inc., Wilmington, Mass.) were infected with each virus by subcutaneous inoculation of 100 PFU of the virus in 50 μ l of phosphate-buffered saline (without Mg²⁺ or Ca²⁺) (PBS-D) containing 1% donor calf serum (PBS-D 1%). Two mice from each litter were inoculated with 50 μ l of PBS-D 1% only and served as noninfected controls. Mice were observed daily through 14 days postinoculation for calculations of percent mortality and average survival times of animals that died.

Viral growth in BHK-21, CHO-K1, RPE.40, and C6/36 cells. Growth curves were determined for selected viruses in BHK-21, CHO-K1, and RPE.40 cells. Duplicate 60-mm dishes were infected with each virus at a multiplicity of infection of 5 PFU

per cell. The virus was allowed to adsorb to cells for 30 min at 37°C. Monolayers were then washed twice with PBS-D 1%, overlaid with 4 ml of media, and incubated at 37°C. Samples were obtained at intervals postinfection and were quantitated by plaque assay on BHK-21 cells. In a separate experiment, peak titers for all viable viruses on each cell line were determined by plaque titration on BHK-21 cells.

Growth curves for all viable viruses also were determined in the C6/36 invertebrate cell line. In this case, the multiplicity of infection was 2 PFU per cell and incubation was at 28°C.

RESULTS

Construction and characterization of TRSB-N. pTRSB is a full-length cDNA clone containing the viral sequences of Sindbis virus AR339 placed downstream of an SP6 phage promoter. pTRSB was constructed by replacing all viral sequences of the Toto1101 cDNA clone (47) with those of our laboratory strain of Sindbis virus AR339 (SB) (33a). A single mutation, analogous to that identified in S.A.AR86-S12, was generated in the genetic background of Sindbis virus AR339 by site-directed mutagenesis of pTRSB. The mutant construct, designated pTRSB-N, substituted Asn (AAC) for the Arg codon at E2 1 (AGA). This substitution introduced a signal for N-linked glycosylation (Asn-Val-Thr) immediately adjacent to the site of PE2 cleavage. To determine whether the introduced mutation affected construct viability, transcripts derived from pTRSB and pTRSB-N were compared in a specific infectivity assay. The specific infectivity of pTRSB-N transcripts was reduced by a factor of approximately 350 below that of pTRSB (data not shown), and only occasionally did plaques appear on pTRSB-N assay dishes. This indicated that the introduced mutation was lethal.

The following experiment suggested that an early event was blocked by this mutation. BHK-21 cells were electroporated with pTRSB or pTRSB-N transcripts and radiolabelled with [³⁵S]methionine. Supernatants were harvested at 18 to 24 h postelectroporation to minimize amplification of possible revertants. Particles were isolated from the supernatants and analyzed by SDS-PAGE. TRSB particles were composed almost exclusively of E2 and E1; however, a small amount of PE2 was consistently present. Particles derived from pTRSB-N (TRSB-N) quantitatively substituted PE2 for E2 in virions (Fig. 1). In addition, PE2 from TRSB-N displayed decreased mobility in SDS-PAGE, suggesting that the introduced glycosylation signal at E2 1 was utilized (Fig. 1). Quantitation of labelled particles released from electroporated BHK-21 cells indicated that TRSB-N particles were released with greater than 90% efficiency relative to TRSB (100%); however, the specific infectivity of the TRSB-N particles on BHK-21 cells was reduced by a factor of 320 below that of TRSB particles (data not shown). Therefore, the effects of PE2 incorporation on replication and release of particles from cells were minimal but these PE2-containing particles were unable to initiate the infection of additional cells. We infer that the presence of PE2 in virions impaired an early event, since the block could be bypassed by direct introduction of RNA.

Isolation and sequence analysis of viable TRSB-N revertants. The rare appearance of plaques after transfection of pTRSB-N transcripts or infection with TRSB-N virions suggested that this block to infection occasionally was overcome. This could occur by low-frequency infection initiated by generally noninfectious, PE2-containing virions or by reversion of the TRSB-N phenotype. Seven individual plaques were isolated from agarose-overlaid BHK-21 cells transfected with

FIG. 1. SDS-PAGE analysis of glycoproteins present in wild-type virus (TRSB) and the noninfectious mutant (TRSB-N). BHK-21 cells were electroporated with *in vitro* transcripts, and virions were labelled metabolically with [35 S]methionine. Virions were purified from cell supernatants as described in the text. 14 C-methylated proteins were included as molecular weight markers (MWM; indicated on the left in thousands).

pTRSB-N transcripts to determine whether these viruses retained the genotype and/or PE2 cleavage phenotype of TRSB-N. These were cloned by plaque purification and passed once in BHK-21 cells for production of virus stocks. The PE2 cleavage phenotype of each isolate was determined by SDS-PAGE analysis of [35 S]methionine-labelled proteins from purified particles. Five of the viruses quantitatively incorporated PE2 into virions; and two viruses had reverted to the PE2 cleavage phenotype and incorporated E2 (Fig. 2).

RNA from each virus was initially sequenced across the E2 region containing the glycosylation signal at E2 1. A Thr-to-Pro mutation at E2 3 was identified in revertant N7R1, and

FIG. 2. SDS-PAGE analysis of wild-type virus (TRSB) and seven biological revertants of TRSB-N. Virions were metabolically labelled with [35 S]methionine and purified from infected-cell supernatants as described in the text. 14 C-methylated proteins were included as molecular weight markers (MWM; indicated on the left in thousands). Lane A, TRSB; lane B, N6R1; lane C, N7R1; lane D, N7R2; lane E, N7R3; lane F, N8R1; lane G, N8R2; lane H, N8R3. C, capsid protein.

revertant N7R2 had incorporated an Asn-to-Asp substitution at E2 1 (Table 1). In both viruses, disruption of the N-linked glycosylation signal at E2 1 correlated with reversion to the PE2 cleavage phenotype. Therefore, these mutations were presumed to be solely responsible for the revertant phenotypes of these viruses. N7R1 and N7R2 were designated group 1 revertants and were not sequenced further.

The five viruses that retained the PE2 cleavage defect also retained the glycosylation signal at E2 1. Sequencing across the entire E3 and E2 genes revealed that each virus had incorporated either one or two additional point mutations (Table 1). These viruses were designated group 2 revertants. Revertant N6R1 had a single mutation, Arg for Cys at E3 25, and provided the only example of a reverting mutation in E3. The mutations identified in revertants N8R1, N8R2, N7R3, and N8R3 all occurred within E2 (Table 1). Revertant N8R1 had a Thr-for-Pro substitution at E2 191, and revertant N8R2 had a His-for-Asn substitution at E2 239. Revertants N7R3 and N8R3 were double mutants. N7R3 had substitutions at E2 169 (Leu for His) and E2 198 (Met for Thr). The mutation at E2 198 eliminated one of the two natural N-linked glycosylation signals on E2 (at E2 196) (6). Predictably, this mutation increased the mobility of PE2 in SDS-PAGE compared with that for TRSB-N (Fig. 2). Mutations identified in N8R3 were Asp to Gly at E2 82 and Glu to Gly at E2 216.

To confirm the linkage between the candidate group 2 reverting mutations and the revertant phenotype, these mutations were reconstructed in the genetic background of pTRSB-N, as described in Materials and Methods. Transcripts derived from each clone were then tested for specific infectivity in BHK-21 cells. In every instance, replacement of the candidate mutations into the genetic background of the nonviable TRSB-N parent resulted in reversion to viability. In addition, the cloned revertants and cognate biological revertants shared identical PE2 cleavage phenotypes and glycoprotein migration patterns, as revealed by SDS-PAGE (data not shown).

Group 2 revertants N7R3 and N8R3 each contained two potentially reverting mutations. To determine whether both mutations were required for the revertant phenotype of N7R3 and N8R3, individual mutations were placed into pTRSB-N by site-directed mutagenesis. On the basis of the clustering of most group 2 mutations within an antigenically important region of E2 (see the description below), only the E2 198 (N7R3) and E2 216 (N8R3) mutations were replaced. Individually, each of these single mutations was sufficient to revert TRSB-N. Viruses derived from these constructs (TRSB-NE2M198 and TRSB-NE2G216) were viable by specific infectivity and shared the PE2 cleavage and gel migration phenotypes of their cognate biological mutants which contained two putative reverting mutations. Therefore, it appeared that the second mutations in N7R3 (E2 169) and in N8R3 (E2 82) were not necessary for the revertant phenotype of these viruses. With the exception of group 1 revertants, viruses derived from the cDNA clones were used in all subsequent analyses.

Construction of reverting mutations in the genetic background of TRSB. In addition to incorporating PE2 into virions, group 2 revertants displayed several other phenotypic differences from TRSB (see the description below). In order to unequivocally link individual mutations to these specific phenotypic differences, each reverting mutation also was analyzed individually in TRSB, a genetic context that was wild type at E2 1 (Table 1). Transcripts derived from these constructs were as infectious as those from pTRSB. In addition, all of these viruses incorporated E2 and E1 into virions and grew in BHK-21 cells to titers comparable to those of TRSB (data not shown).

TABLE 1. Genetic and phenotypic characteristics of biological and cloned viruses used in this study

Virus type, group, and clone	Mutation(s)	Glycosylation signal at E2 1-3	PE2 cleavage ^a
Parental			
TRSB	Wild type	Not present	Yes
TRSB-N	E2 1 Arg to Asn	Created	No
Biologically derived revertant ^b			
Group 1			
N7R1	E2 3 Thr to Pro	Lost	Yes
N7R2	E2 1 Asn to Asp	Lost	Yes
Group 2			
N6R1	E3 25 Cys to Arg	Intact	No
N7R3	E2 169 His to Leu, E2 198 Thr to Met	Intact	No
N8R1	E2 191 Pro to Thr	Intact	No
N8R2	E2 239 Asn to His	Intact	No
N8R3	E2 82 Asp to Gly, E2 216 Glu to Gly	Intact	No
Cloned			
Mutations constructed in pTRSB-N (E2 Asn-1)			
TRSB-N6R1	E3 25 Cys to Arg	Intact	No
TRSB-N7R3	E2 169 His to Leu, E2 198 Thr to Met	Intact	No
TRSB-N8R1	E2 191 Pro to Thr	Intact	No
TRSB-N8R2	E2 239 Asn to His	Intact	No
TRSB-N8R3	E2 82 Asp to Gly, E2 216 Glu to Gly	Intact	No
TRSB-NE2M198	E2 198 Thr to Met	Intact	No
TRSB-NE2G216	E2 216 Glu to Gly	Intact	No
Mutations constructed in pTRSB (E2 Arg-1)			
TRSB-E3R25	E3 25 Cys to Arg	Not present	Yes
TRSB-E2T191	E2 191 Pro to Thr	Not present	Yes
TRSB-E2M198	E2 198 Thr to Met	Not present	Yes
TRSB-E2G216	E2 216 Glu to Gly	Not present	Yes
TRSB-E2H239	E2 239 Lys to His	Not present	Yes

^a Determined by SDS-PAGE analysis of purified virions.^b Characteristics relative to TRSB-N.

Virulence of revertant viruses in newborn CD-1 mice. In the genetic background of Sindbis virus S.A.AR86, failure to cleave PE2 correlated with attenuation of the virus in newborn and adult CD-1 mice (50). To determine whether these phenotypes were linked in the genetic background of Sindbis virus AR339, the virulence phenotype of each viable virus was compared with that of TRSB. Virulence was assessed only in newborn CD-1 mice, as Sindbis virus AR339 is not virulent in adults. Viruses were categorized as virulent (causing 100% mortality) or attenuated (less than 100% mortality and/or increased average survival time). All control pups (PBS-D 1% inoculated) survived through the observation period and appeared normal. The two revertants that cleaved PE2 (group 1) were as virulent as TRSB. In addition, each of the five viruses containing only the reverting mutations in the genetic background of TRSB displayed virulence characteristics similar to those of TRSB (Table 2). All viruses that cleaved PE2 caused 100% mortality and displayed average survival time values ranging from 2.7 ± 0.4 days for N7R2 to 6.0 ± 1.3 days for TRSB-E2H239.

Virulence characteristics were markedly different for the viruses that failed to cleave PE2. The viable PE2-containing revertant viruses were attenuated in neonatal CD-1 mice, each causing less than 100% mortality and allowing significantly extended survival compared with that of animals succumbing to TRSB (Table 2). Viruses containing the same reverting

mutations placed into the genetic background of TRSB were not attenuated. Therefore, attenuation was linked only to the PE2 noncleavage phenotype of these viruses and not to the reverting mutations themselves.

Growth of TRSB and revertants in CHO-K1 and RPE.40 cells. PE2 cleavage can be inhibited by mutation as in TRSB-N. However, PE2 cleavage also can be inhibited by growth of TRSB on RPE.40 cells, a toxin-resistant CHO cell line defective in the furin or furin-like protease responsible for alphavirus PE2 cleavage (21). Both the parent cell line, CHO-K1, and RPE.40 cells release approximately equal numbers of particles following infection by Sindbis virus AR339; however, particles released from RPE.40 cells quantitatively incorporate PE2 and have much lower specific infectivity than CHO-K1-grown virus (65). Therefore, the availability of the RPE.40 cell line afforded the opportunity to determine whether the suppressor mutations, which restored viability to PE2-containing TRSB-N, also would confer viability on PE2-containing TRSB grown on RPE.40 cells.

CHO-K1 and RPE.40 cells were infected with the cloned TRSB-N revertants and viruses containing the reverting mutations in the TRSB background. Samples of the supernatants were taken at 20 h postinfection, a time when maximum titer, as determined by preliminary experiments with representative mutants, was reached. Virus infectious for BHK-21 cells was quantitated by plaque assay (Table 3). Although the reverting

TABLE 2. Virulence of viruses in newborn CD-1 mice

Virus type and clone	No. of infected animals ^a	% Mortality	AST ^b (days)	Virulence phenotype
Wild-type TRSB	40	100	3.9 ± 0.9	Virulent ^c
Mutants incorporating PE2 into virions				
TRSB-N6R1	21	62	8.8 ± 2.9	Attenuated ^d
TRSB-N7R3	26	35	7.9 ± 1.5	Attenuated
TRSB-N8R1	21	28	9.7 ± 1.9	Attenuated
TRSB-N8R2	27	67	8.6 ± 2.6	Attenuated
TRSB-N8R3	35	51	6.6 ± 2.3	Attenuated
TRSB-NE2M198	35	46	7.6 ± 2.8	Attenuated
TRSB-NE2G216	34	44	8.7 ± 2.6	Attenuated
Mutants incorporating E2 into virions				
N7R1	22	100	2.8 ± 0.5	Virulent
N7R2	19	100	2.7 ± 0.4	Virulent
TRSB-E3R25	32	100	4.8 ± 1.0	Virulent
TRSB-E2T191	24	100	4.2 ± 0.8	Virulent
TRSB-E2M198	18	100	4.9 ± 0.7	Virulent
TRSB-E2G216	28	100	4.5 ± 0.7	Virulent
TRSB-E2H239	25	100	6.0 ± 1.3	Virulent

^a Total number of infected neonates from two to four litters.^b AST, average survival times of infected animals that died ± standard deviations.^c Virulent, causing 100% mortality in infected animals.^d Attenuated, causing <100% mortality and extended AST in infected animals.

mutations in the TRSB-N background allowed substantial production of BHK infectious virus in RPE.40 cells compared with production in CHO-K1, those same mutations in the TRSB background did not. Therefore, the reverting mutations appear to be specific for the TRSB-N lesion in PE2 cleavage rather than being able to compensate for a general failure to cleave PE2.

Growth kinetics in invertebrate cell lines. Comparisons between the S.A.AR86 parental strain and its PE2 cleavage-defective mutant, S12, demonstrated that replication of S.A.AR86 in cultured mosquito cells was greatly influenced by PE2 cleavage (46). Parental S.A.AR86 consistently grew to 250-fold or higher titers than S12. To determine whether this applied to the AR339 strain as well, the growth properties of TRSB and of each mutant virus were determined on C6/36

mosquito cells (Fig. 3). TRSB grew to high titers, as assayed on BHK-21 cells, reaching a plateau of greater than 10⁹ PFU/ml by 24 h postinfection. Production of infectious virus after infection with each PE2-containing revertant was markedly reduced, by 10¹ (TRSB-N8R3) to 10^{3.5} (TRSB-N7R3) fewer PFU than TRSB.

C6/36 cell growth curves for all viruses containing the reverting mutations in the genetic background of TRSB were essentially identical to that for TRSB (Fig. 3). Clearly, these mutations had no demonstrable effect on growth in the invertebrate cells when in the genetic background of a virus that cleaves PE2. Therefore, the decreased growth of the PE2-containing revertants in C6/36 cells was not attributable to the reverting mutations themselves but rather to the failure of these viruses to cleave PE2.

TABLE 3. Peak titers^a of viruses grown on CHO-K1 and RPE.40 cells

Virus type and clone	Titer (log ₁₀ PFU/ml)		PFU ratio (RPE.40/CHO-K1 × 100)
	CHO-K1	RPE.40	
Wild type			
TRSB	9.8	6.7	0.1
S.A.AR86	9.2	7.8	4.5
Mutations constructed in pTRSB-N (E2 Asn-1)			
TRSB-N6R1	8.8	8.6	66.7
TRSB-N7R3	8.6	7.7	13.7
TRSB-N8R1	9.2	7.8	3.3
TRSB-N8R2	8.9	7.9	9.2
TRSB-N8R3	9.1	8.5	25.0
Mutations constructed in pTRSB (E2 Arg-1)			
TRSB-E3R25	9.5	7.0	0.3
TRSB-E2M198	8.9	6.3	0.3
TRSB-E2T191	9.8	7.2	0.3
TRSB-E2H239	9.3	7.2	0.7
TRSB-E2G216	9.7	7.1	0.3

^a Virus titers were obtained at 20 h postinfection.

FIG. 3. Growth kinetics of TRSB and mutant viruses in C6/36 cells. (A) Growth kinetics of TRSB (+) and five PE2-containing revertants of TRSB-N: TRSB-N6R1 (Δ), TRSB-N8R1 (○), TRSB-N7R3 (---), TRSB-N8R3 (▲), and TRSB-N8R2 (●). (B) Growth kinetics of TRSB (+) and five viruses containing the reverting mutations in the genetic background of TRSB: TRSB-E3R25 (Δ), TRSB-E2T191 (○), TRSB-E2M198 (---), TRSB-E2G216 (▲), and TRSB-E2H239 (●). All viruses were derived from cDNA clones.

DISCUSSION

Many enveloped RNA viruses appear to regulate the functions mediated by their envelope glycoproteins through control of glycoprotein maturation by cleavage. The four-residue cleavage signal in TRSB is similar in its arrangement of basic amino acids to the canonical sequence motif (Basic-X-Basic-Basic) found in the glycoprotein cleavage sites of viruses in the flavivirus, retrovirus, orthomyxovirus, paramyxovirus, and coronavirus families (37). This motif is also found at the cleavage site of many prohormones (18). In alphaviruses, several spike functions appear to be linked to proteolytic cleavage immediately downstream of this site in the precursor glycoprotein PE2. The experiments reported here address several issues regarding the role of PE2 cleavage in the life cycle of Sindbis virus.

PE2 cleavage defects and virus genetic background. Mutations which prevent PE2 cleavage in SFV (51), Venezuelan equine encephalomyelitis virus (VEE) (7a), and the AR339 strain of Sindbis virus (65; these data) are lethal for the virus. Yet, in the genetic background of Sindbis virus S.A.AR86, N-linked glycosylation at E2 1 prevented PE2 cleavage and led to incorporation of PE2 in virions but did not affect replication

in BHK-21 cells (46, 50). The finding that second-site mutations can suppress the lethality of PE2 cleavage defects in Sindbis virus AR339 suggests that S.A.AR86 already contains, within its normal genetic background, sequences which are compatible with the presence of PE2 in virions.

Genetic reversion of the PE2 cleavage defect. Sequence analysis of TRSB-N revertants revealed that viability could be restored to PE2 cleavage-defective mutants in one of two ways. The first was demonstrated by group 1 revertants which eliminated the site for N-linked glycosylation at E2 1 through mutation of either the E2 1 or 3 position. Reversion in this case clearly correlated with restoration of the PE2 cleavage phenotype. The second was demonstrated by viable group 2 second-site revertants in E2 and E3 which retained the PE2 cleavage defect. None of the 22 coding differences between the structural genes of AR339 and S.A.AR86 (50) map precisely to residues identified in the group 2 revertants; however, one difference maps within the region of E2 where reverting mutations clustered (E2 191 to 239) and the single difference in E3 (E3 20) is close to the reverting mutation at E3 25.

The E2 191-to-239 region has been identified as an antigenically important domain in E2, most of which is exposed on the surface of virions. The E2a neutralization site of Sindbis virus maps to E2 190 (58, 59). The reverting mutation in N8R1 was identified at E2 191. The substitution at E2 216 was linked to reversion of N8R3, and this site maps directly to the E2b neutralization site at E2 209 to 216 (7, 34, 57). A transitional epitope that is normally inaccessible on native virions, but which becomes accessible to monoclonal antibodies following rearrangement of virions bound at the cell surface, maps to E2 residues 200 to 202 (12, 34). The reverting mutation identified in N7R3 (E2 198) is adjacent to this transitional epitope. The E2 239 reverting mutation in N8R2 lies outside the known limits of the major neutralization domain on Sindbis virions. However, neutralizing epitopes of VEE and Ross River virus have been identified in the vicinity of E2 239 (19, 62).

Previously, we reported the introduction of an N-linked glycosylation site at E2 codon 1 of another AR339-based clone, TR4000 (46). Transfection of RNA transcripts from this mutant clone (pTR4001-N) gave rise to a stock of PE2-containing, infectious virions, but only after several days of incubation. More careful characterization of this mutant clone, by quantitating the specific infectivity of RNA transcripts, demonstrated that like TRSB-N, the PE2 cleavage defect was, in fact, a lethal mutation. We conclude that the experiments done in the earlier study with TR4001-N were performed with virus containing one or more reverting mutations in addition to the PE2 cleavage defect.

Mechanism of second-site reversion of the cleavage defect. Changes in the overall architecture of the glycoprotein spike must form the structural basis for the loss of viability in TRSB-N and the restoration of viability by the reverting mutations. This is suggested by the identification of reverting mutations not only in E2 but also in E3 and, with the VEE system, in E1 (7a). The possible mechanisms by which the reverting mutations could restore viability include the following. The presence of the additional E3 amino acid residues and the carbohydrate side chain at E2 1 may cause a contortion of the spike into a dysfunctional configuration. A reverting mutation could restore function by recreating a functional spike conformation. A second possibility is that in the TRSB-N spike there is a direct interaction between the E3 domain of PE2 (and/or the carbohydrate moiety at E2 1) with a functional domain elsewhere on the spike. Such an interaction would sterically inhibit the activity of the functional domain, while mutations which could disrupt such an interaction would

potentially restore the spike to a functional state. In this scenario, the reverting mutation at E3 25 may lie within such an interacting E3 domain, or the Arg-for-Cys replacement could alter E3 structure by preventing disulfide bond formation through E3 residue 25. The putative interaction partner for E3 may be the major neutralization site on E2, the domain within which most of the reverting mutations lie. This domain is accessible by monoclonal antibodies on native, wild-type virions and hence is on the exterior of the spike (34). A third possibility is that the reverting mutations generate new functional domains on PE2-containing spikes, which perform at least some of the functions lost because of the presence of the E3 domain.

Replication step blocked by PE2 cleavage defects. PE2-containing mutants of SFV (51), and of Sindbis virus TR2001 (10), were impaired in functions of cell binding and of cell entry. Consistent with these observations, transcripts from pTRSB-N initiated a single round of infection resulting in the production of PE2 containing noninfectious particles. This strongly suggests that the TRSB-N particles fail to complete one or more of the early phases of normal infection. The two earliest events in the Sindbis virus infectious cycle occur on the topographic exterior of the cell: attachment to the receptor(s) and subsequent structural rearrangement of the glycoprotein spikes, as detected by monoclonal antibody binding to epitopes inaccessible on native virions (12, 34). This initial conformational change is followed by entry either by fusion of the envelope with the plasma membrane (1, 4) or by endocytosis (16, 32). PE2-containing virions may be excessively stable and thus unable to undergo a requisite rearrangement at the cell surface (12) or in endosomes after endocytosis (51). Experiments are in progress to determine the early event(s) affected in TRSB-N infection by utilizing comparisons between TRSB, TRSB-N, and PE2-containing second-site revertants of TRSB-N.

Effect of E2 residue 1 on efficiency of cleavage. The residue at the +1 position relative to the PE2 cleavage signal varies among alphaviruses (2, 5, 27, 50), and Sindbis virus AR339 can tolerate several residues at the +1 position relative to the cleavage site, including Arg (6), Ser (48), Asp (revertant N7R2), and Asn (revertant N7R1). Examination of SDS-PAGE profiles of virion glycoproteins containing these tolerated residues suggests that the residue at E2 1 can affect the efficiency of PE2 cleavage (Fig. 2 and data not shown). A Phe-to-Leu mutation at the residue occupying the analogous position in the F protein of Newcastle disease virus inhibited cleavage of this viral glycoprotein and was correlated with the loss of fusion activity and decreased virulence in animals (36).

Inhibition of PE2-containing revertants in mosquito cells. Infection with PE2-containing revertant viruses of Sindbis virus S.A.AR86 (46) and AR339 resulted in poor production of infectious virus in cultured mosquito cells. This likely reflects a difference(s) in the virus-host cell interactions affected by incorporation of PE2 during replication in BHK-21 cells as opposed to C6/36 cells. In the case of the S.A.AR86 S12 mutant, the block appeared to be at a late stage in infection (46), and the basis for the inhibition of AR339-derived strains is under active investigation. Alphaviruses must replicate in both vertebrate and invertebrate hosts to be maintained in nature. While mutations that prevent PE2 processing may be tolerated in the vertebrate host, these would be selected against in the mosquito vector.

In vivo attenuation of PE2-containing mutants. Incorporation of PE2 attenuated the virulence of S.A.AR86, and of each group 2 revertant of AR339, in newborn CD-1 mice. Thus, normal or nearly normal growth of these viruses in BHK-21

cells did not correlate with wild-type virulence in the mouse system. This may reflect the fact that each revertant was selected by growth in BHK-21 cells and that the effect of PE2 incorporation on replication in BHK-21 cells and in specific differentiated cell types *in vivo* may be fundamentally different. For instance, different putative Sindbis virus receptors have been identified on BHK-21 cells (64) and on mouse neuroblastoma cells (60). Therefore, it is possible that the reverting mutations have restored the ability of PE2-containing viruses to initiate infection of BHK-21 cells but not of important cell types in mice. Experiments are in progress to determine which cell and tissue tropism differences *in vivo* distinguish PE2-containing revertants from TRSB.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of Travis A. Knott, and thank J. M. Moehring and T. J. Moehring for their generous provision of the CHO-K1 and RPE.40 cell lines.

This work was supported by Public Health Service grants AI22186 and NS26681 from the National Institutes of Health. H.W.H. was supported by National Institutes of Health postdoctoral training grant AI07151.

REFERENCES

1. Cassell, S., J. Edwards, and D. T. Brown. 1984. Effects of lysosomotropic weak bases on infection of BHK-21 cells by Sindbis virus. *J. Virol.* **52**:857-864.
2. Chang, G. J., and D. W. Trent. 1987. Nucleotide sequence of the genome region encoding the 26S mRNA of Eastern equine encephalomyelitis virus and the deduced amino acid sequence of the viral structural proteins. *J. Gen. Virol.* **68**:2129-2142.
3. Choi, H. K., L. Tong, W. Minor, P. Duma, U. Boege, M. G. Rossmann, and G. Wengler. 1991. Structure of Sindbis virus core protein reveals a chymotrypsin-like serine proteinase and the organization of the virion. *Nature (London)* **354**:37-43.
4. Coombs, K., E. Mann, J. Edwards, and D. T. Brown. 1981. Effects of chloroquine and cytochalasin B on the infection of cells by Sindbis virus and vesicular stomatitis virus. *J. Virol.* **37**:1060-1065.
5. Dalgarno, L., C. M. Rice, and J. H. Strauss. 1983. Ross River virus 26S RNA: complete nucleotide sequence and deduced sequence of the encoded structural proteins. *Virology* **129**:170-187.
6. Davis, N. L., F. J. Fuller, W. G. Dougherty, R. A. Olmsted, and R. E. Johnston. 1986. A single nucleotide change in the E2 glycoprotein gene of Sindbis virus affects penetration rate in cell culture and virulence in neonatal mice. *Proc. Natl. Acad. Sci. USA* **83**:6771-6775.
7. Davis, N. L., D. F. Pence, W. M. Meyer, A. L. Schmaljohn, and R. E. Johnston. 1987. Alternative forms of a strain-specific neutralizing antigenic site on the Sindbis virus E2 glycoprotein. *Virology* **161**:101-108.
- 7a. Davis, N. L., J. F. Smith, and R. E. Johnston. Unpublished data.
8. De Curtis, I., and K. Simons. 1988. Dissection of Semliki Forest virus glycoprotein delivery from the trans-Golgi network to the cell surface in permeabilized BHK cells. *Proc. Natl. Acad. Sci. USA* **85**:8052-8056.
9. Doms, R. W., R. A. Lamb, J. K. Rose, and A. Helenius. 1993. Folding and assembly of viral membrane proteins. *Virology* **193**:545-562.
10. Dubuisson, J., and C. M. Rice. 1993. Sindbis virus attachment: isolation and characterization of mutants with impaired binding to vertebrate cells. *J. Virol.* **67**:3363-3374.
11. Erwin, C., and D. T. Brown. 1980. Intracellular distribution of Sindbis virus membrane proteins in BHK-21 cells infected with wild-type and maturation-defective mutants. *J. Virol.* **36**:775-786.
12. Flynn, D. C., W. J. Meyer, J. M. Mackenzie, Jr., and R. E. Johnston. 1990. A conformational change in Sindbis virus glycoproteins E1 and E2 is detected at the plasma membrane as a consequence of early virus-cell interaction. *J. Virol.* **64**:3643-3653.
13. Fuller, S. 1987. The T=4 envelope of Sindbis virus is organized by interactions with a complementary T=3 capsid. *Cell* **48**:923-934.
14. Garoff, H., K. Simons, and O. Renkonen. 1974. Isolation and

- characterization of the membrane polypeptides of Semliki Forest virus. *Virology* **61**:493–504.
15. Glasgow, G. M., B. J. Sheahan, G. J. Atkins, J. M. Wahlberg, A. Salminen, and P. Liljeström. 1991. Two mutations in the envelope glycoprotein E2 of Semliki Forest virus affecting the maturation and entry patterns of the virus alter pathogenicity for mice. *Virology* **185**:741–748.
 16. Helenius, A., J. Kartenbeck, K. Simons, and E. Fries. 1980. On the entry of Semliki Forest virus into BHK-21 cells. *J. Cell. Biol.* **84**:404–420.
 17. Hirschberg, C. B., and P. W. Robbins. 1974. The glycolipids and phospholipids of Sindbis virus and their relation to the lipids of the host cell plasma membrane. *Virology* **61**:602–608.
 18. Hosaka, M., M. Nagahama, W. S. Kim, T. Watanabe, K. Hatsu-zawa, J. Ikemizu, K. Murakami, and K. Nakayama. 1991. Arg-X-Lys/Arg-Arg motif as a signal for precursor cleavage catalyzed by furin within the constitutive secretory pathway. *J. Biol. Chem.* **266**:12127–12130.
 19. Hunt, A. R., A. J. Johnson, and J. T. Roehrig. 1990. Synthetic peptides of Venezuelan equine encephalomyelitis virus E2 glycoprotein. I. Immunogenic analysis and identification of a protective peptide. *Virology* **179**:701–711.
 20. Igarashi, A. 1978. Isolation of a Singh *Aedes albopictus* cell clone sensitive to Dengue and Chikungunya viruses. *J. Gen. Virol.* **40**:531–544.
 21. Inocencio, N. M., J. M. Moehring, and T. J. Moehring. 1993. A mutant CHO-K1 strain with resistance to *Pseudomonas* exotoxin A is unable to process the precursor fusion glycoprotein of Newcastle disease virus. *J. Virol.* **67**:593–595.
 22. Jain, S. K., S. Decandido, and M. Kielian. 1991. Processing of the p62 envelope precursor protein of Semliki Forest virus. *J. Biol. Chem.* **266**:5756–5761.
 23. Jones, K. J., M. R. F. Waite, and H. R. Bose. 1974. Cleavage of a viral envelope precursor during the morphogenesis of Sindbis virus. *J. Virol.* **13**:809–817.
 24. Kielian, M., and S. Jungerwirth. 1990. Mechanisms of enveloped virus entry into cells. *Mol. Biol. Med.* **7**:17–31.
 25. Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**:367–382.
 26. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
 27. Levinson, R. S., J. H. Strauss, and E. G. Strauss. 1990. Complete sequence of the genomic RNA of O'Nyong-Nyong virus and its use in the construction of alphavirus phylogenetic trees. *Virology* **175**:110–123.
 28. Liljeström, P., S. Lusa, D. Huylebroeck, and H. Garoff. 1991. In vitro mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular-weight membrane protein modulates virus release. *J. Virol.* **65**:4107–4113.
 29. Lobigs, M., and H. Garoff. 1990. Fusion function of the Semliki Forest virus spike is activated by proteolytic cleavage of the envelope glycoprotein p62. *J. Virol.* **64**:1233–1240.
 30. Lobigs, M., J. M. Wahlberg, and H. Garoff. 1990. Spike protein oligomerization control of Semliki Forest virus fusion. *J. Virol.* **64**:5214–5218.
 31. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 32. Marsh, M., E. Bolzau, and A. Helenius. 1983. Penetration of Semliki Forest virus from acidic prelysosomal vacuoles. *Cell* **32**:931–940.
 33. Marsh, M., and A. Helenius. 1989. Virus entry into animal cells. *Adv. Virus Res.* **36**:107–151.
 - 33a. McKnight, K. L., et al. Unpublished data.
 34. Meyer, W. J., and R. E. Johnston. 1993. Structural rearrangement of infecting Sindbis virions at the cell surface: mapping of newly accessible epitopes. *J. Virol.* **67**:5117–5125.
 35. Moehring, J. M., and T. J. Moehring. 1983. Strains of CHO-K1 cells resistant to *Pseudomonas* exotoxin A and cross-resistant to diphtheria toxin and viruses. *Infect. Immun.* **41**:998–1009.
 36. Morrison, T., C. McQuain, T. Sergel, L. McGinnes, and J. Reitter. 1993. The role of the amino terminus of F₁ of the Newcastle disease virus fusion protein in cleavage and fusion. *Virology* **193**:997–1000.
 37. Nagai, Y. 1993. Protease-dependent virus tropism and pathogenicity. *Trends Microbiol.* **1**:81–87.
 38. Naim, H. Y., and H. Koblet. 1990. The cleavage of p62, the precursor of E2 and E3, is an early and continuous event in Semliki Forest virus-infected *Aedes albopictus* cells. *Arch. Virol.* **110**:221–237.
 39. Olmsted, R. A., W. J. Meyer, and R. E. Johnston. 1986. Characterization of Sindbis virus epitopes important for penetration in cell culture and pathogenesis in animals. *Virology* **148**:245–254.
 40. Orci, L., M. Ravazzola, M. Amherdt, A. Madsen, A. Perrelet, J. D. Vassalli, and R. G. W. Anderson. 1986. Conversion of proinsulin to insulin occurs coordinately with acidification of maturing secretory vesicles. *J. Cell Biol.* **103**:2273–2281.
 41. Paredes, A. M., D. T. Brown, R. Rothnagel, W. Chiu, R. J. Schoepp, R. E. Johnston, and B. V. Prasad. 1993. Three-dimensional structure of a membrane-containing virus. *Proc. Natl. Acad. Sci. USA* **90**:9095–9099.
 42. Paredes, A. M., M. N. Simon, and D. T. Brown. 1992. The mass of the Sindbis virus nucleocapsid suggests it has T=4 icosahedral symmetry. *Virology* **187**:329–332.
 43. Polo, J. M., N. L. Davis, C. M. Rice, H. V. Huang, and R. E. Johnston. 1988. Molecular analysis of Sindbis virus pathogenesis in neonatal mice by using virus recombinants constructed in vitro. *J. Virol.* **62**:2124–2133.
 44. Polo, J. M., and R. E. Johnston. 1990. Attenuating mutations in glycoproteins E1 and E2 of Sindbis virus produce a highly attenuated strain when combined in vitro. *J. Virol.* **64**:4438–4444.
 45. Polo, J. M., and R. E. Johnston. 1991. Mutational analysis of a virulence locus in the E2 glycoprotein gene of Sindbis virus. *J. Virol.* **65**:6358–6361.
 46. Presley, J. F., J. M. Polo, R. E. Johnston, and D. T. Brown. 1991. Proteolytic processing of the Sindbis virus membrane protein precursor PE2 is nonessential for growth in vertebrate cells but is required for efficient growth in invertebrate cells. *J. Virol.* **65**:1905–1909.
 47. Rice, C. M., R. Levis, J. H. Strauss, and H. V. Huang. 1987. Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. *J. Virol.* **61**:3809–3819.
 48. Rice, C. M., and J. H. Strauss. 1981. Nucleotide sequence of the 26S mRNA of Sindbis virus and deduced sequence of the encoded virus structural proteins. *Proc. Natl. Acad. Sci. USA* **78**:2062–2066.
 49. Rice, C. M., and J. H. Strauss. 1982. Association of Sindbis virion glycoproteins and their precursors. *J. Mol. Biol.* **154**:325–348.
 50. Russell, D. L., J. M. Dalrymple, and R. E. Johnston. 1989. Sindbis virus mutations which coordinately affect glycoprotein processing, penetration, and virulence in mice. *J. Virol.* **63**:1619–1629.
 51. Salminen, A., J. M. Wahlberg, M. Lobigs, P. Liljeström, and H. Garoff. 1992. Membrane fusion process of Semliki Forest virus II: cleavage-dependent reorganization of the spike protein complex controls virus entry. *J. Cell Biol.* **116**:349–357.
 52. Sanger, R., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 53. Schlesinger, M. J., and S. Schlesinger. 1973. Large-molecular-weight precursors of Sindbis virus proteins. *J. Virol.* **11**:1013–1016.
 54. Schlesinger, M. J., S. Schlesinger, and B. W. Burge. 1972. Identification of a second glycoprotein in Sindbis virus. *Virology* **47**:539–541.
 55. Schlesinger, S., and M. J. Schlesinger (ed.). 1986. The Togaviridae and Flaviviridae. Plenum Publishing Corp., New York.
 56. Smith, J. F., and D. T. Brown. 1977. Envelopment of Sindbis virus: synthesis and organization of proteins in cells infected with wild-type and maturation-defective mutants. *J. Virol.* **22**:662–678.
 57. Stec, D. S., A. Waddell, C. S. Schmaljohn, G. A. Cole, and A. L. Schmaljohn. 1986. Antibody-selected variation and reversion in Sindbis virus neutralization epitopes. *J. Virol.* **57**:715–720.
 58. Strauss, E. G., A. L. Schmaljohn, D. E. Griffin, and J. H. Strauss. 1987. Structure-function relationships in the glycoproteins of

- alphavirus. UCLA Symp. Mol. Cell. Biol. **54**:365–378.
59. **Strauss, E. G., D. S. Stec, A. L. Schmaljohn, and J. H. Strauss.** 1991. Identification of antigenically important domains in the glycoproteins of Sindbis virus by analysis of antibody escape variants. *J. Virol.* **65**:4654–4664.
 60. **Ubol, S., and D. E. Griffin.** 1991. Identification of a putative alphavirus receptor on mouse neural cells. *J. Virol.* **65**:6913–6921.
 61. **Von Bonsdorff, C. H., and S. C. Harrison.** 1975. Sindbis virus glycoproteins form a regular icosahedral lattice. *J. Virol.* **16**:141–145.
 62. **Vrati, S., C. A. Fernon, L. Dalgarno, and R. C. Weir.** 1988. Location of a major antigenic site involved in Ross River virus neutralization. *Virology* **162**:346–353.
 63. **Wahlberg, J. M., W. A. M. Boere, and H. Garoff.** 1989. The heterodimeric association between the membrane proteins of Semliki Forest virus changes its sensitivity to low pH during virus maturation. *J. Virol.* **63**:4991–4997.
 64. **Wang, K.-S., R. J. Kuhn, E. G. Strauss, S. Ou, and J. H. Strauss.** 1992. High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. *J. Virol.* **66**:4992–5001.
 65. **Watson, D. G., J. M. Moehring, and T. J. Moehring.** 1991. A mutant CHO-K1 strain with resistance to *Pseudomonas* exotoxin A and alphaviruses fails to cleave Sindbis virus glycoprotein PE2. *J. Virol.* **65**:2332–2339.
 66. **Welch, W. J., and B. M. Sefton.** 1979. Two small virus-specific polypeptides are produced during infection with Sindbis virus. *J. Virol.* **29**:1186–1195.
 67. **White, J. M.** 1990. Viral and cellular membrane fusion proteins. *Annu. Rev. Physiol.* **52**:675–697.
 68. **Ziemiacki, A., H. Garoff, and K. Simons.** 1980. Formation of the Semliki Forest virus membrane glycoprotein complexes in the infected cell. *J. Gen. Virol.* **50**:111–123.
 69. **Zimmern, D., and P. Kaesberg.** 1978. 3'-terminal nucleotide sequence of encephalomyocarditis virus RNA determined by reverse transcriptase and chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **75**:4257–4261.