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# The Amino-Terminal Residue of Sindbis Virus Glycoprotein E2 Influences Virus Maturation, Specific Infectivity for BHK Cells, and Virulence in Mice

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**The E2 glycoprotein of Sindbis virus is synthesized as a precursor, PE2, which is cleaved by furin or a furin-like host cell protease at a late stage of maturation. The four-residue PE2 cleavage signal conforms to the basic amino acid-X-basic-basic motif which is present in many other viral and cellular glycoproteins which are processed by the cellular enzyme(s). In this report, we present evidence that the amino acid which immediately follows the signal, the N-terminal residue of E2, can influence protease recognition, binding, and/or cleavage of PE2. Constructs encoding nine different amino acids at E2 position 1 (E2 1) were produced by site-directed mutagenesis of the full-length cDNA clone of our laboratory strain of Sindbis virus AR339 (pTRSB). Viruses derived from clones encoding Arg (TRSB), Asp, Ser, Phe, His, and Asn in a nonglycosylated form at E2 1 contained predominantly E2. Viruses encoding Ile, Leu, or Val at E2 1 contained the uncleaved form of PE2. The specific infectivity of TRSB (E2 Arg-1) for baby hamster kidney (BHK-21) cells was from 5- to greater than 100-fold higher than those of isogenic constructs with other residues at E2 1, suggesting that E2 Arg-1 represents a BHK-21 cell adaptive mutation in our laboratory strain. In newborn CD-1 mice, TRSB was more virulent than the PE2-containing viruses but less virulent than other PE2-cleaving viruses with alternative amino acids at E2 1. These results indicate that in TRSB, E2 Arg-1 increased the efficiency of virus-cell interactions in cultured BHK-21 cells but simultaneously decreased the ability of virus to mediate in vivo virus-cell interactions critical for the induction of disease. This suggests that the N terminus of E2 may participate in or be associated with virion domains which mediate these viral functions.**

Many viral glycoproteins are derived from larger precursors which are cleaved at specific sites during their maturation (63). Proteolytic processing can be required for the export of these proteins, for virus morphogenesis, and for the normal functional properties of the released virus particles (see reference 8 and references within for review). For many enveloped RNA viruses, including the alphaviruses (*Togaviridae* family), cleavage of the precursor is performed by a cellular protease(s) (24, 34, 61). Viral substrates for the host cell protease(s) contain a highly conserved sequence motif which functions as the signal for site-specific cleavage. This signal is similar in its arrangement of basic amino acids to that found in specific membrane and secretory proteins which are the natural cellular substrates for the enzyme(s) (17). Alphaviruses provide a useful system for analyzing the sequence requirements of the cleavage signal itself, and for investigating how the cleavage event influences specific steps in the normal viral replication cycle (15, 29, 46).

Alphaviruses are small, relatively simple, enveloped viruses (49). 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 (C) protein (2, 39). The nucleocapsid is contained within a host cell-derived lipid envelope (16). Two transmembranal, viral glycoproteins, E1 and E2, are exposed on the surface of the virion (44, 48). E1 and E2 are associated as heterodimers, which in turn are organized in groups of three to form the viral spikes (44). The viral spikes form an icosahedral array at the virus surface and are arranged with T=4 symmetry (11, 38, 57).

The structural proteins of alphaviruses are synthesized as a single polyprotein from which the glycoproteins PE2 (the E2 precursor) and E1 are cleaved while still nascent peptides (47). PE2 and E1 form heterodimers in the rough endoplasmic reticulum (44, 59, 64) and are processed and transported through the exocytic pathway of the host cell (10). At a late step in virus morphogenesis, PE2 is cleaved into E3 and E2 (21, 51). E3 is composed of the amino terminal 64 (Sindbis virus) or 66 (Semliki Forest virus) amino acids of PE2. E3 is lost to the medium from Sindbis virus-infected cells (62) but is retained in the Semliki Forest virus particle (13). E2 represents the remainder of PE2 and in mature virions, remains anchored in the membrane in association with E1.

Cleavage of PE2 occurs within a trans- or post-Golgi compartment and is likely performed by a host cell protease (7, 30, 61). The PE2 cleavage site immediately follows a highly conserved canonical motif consisting of four residues ordered in a basic-X-basic-basic (bxbb) sequence (43, 52). The residue following this sequence, at E2 position 1 (E2 1), has not previously been considered part of the cleavage signal. Serine is found at E2 1 in Semliki Forest virus (12), Ross River virus (3), Western equine encephalitis virus (14), Venezuelan equine encephalitis (VEE) virus (23), Ockelbo virus (50), and Sindbis virus S.A.AR86 (45) and in most laboratory strains derived from Sindbis virus AR339 (50). In O'Nyong-nyong virus, the E2 1 residue is Asn (27), and in Eastern equine encephalitis virus the E2 1 residue is Asp (1a). In our laboratory strain of Sindbis virus AR339 (SB), cleavage follows the sequence Arg-Ser-Lys-Arg, the last four residues of E3 (4). The N terminus of the mature E2 glycoprotein (E2 1) is Arg, as predicted by nucleotide sequencing (4) and as shown by microsequencing of purified E2 from virions (1).

In this study, a panel of viruses containing different amino

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acid substitutions at E2 1 was constructed and the phenotypic effects of these substitutions were determined. The results indicate that the amino-terminal residue of E2 influences the efficiency of PE2 cleavage, virion infectivity for baby hamster kidney (BHK-21) cells, and virulence in vivo.

## MATERIALS AND METHODS

**Viruses and cell lines.** Sindbis virus AR339 strain was obtained originally from H. R. Bose (University of Texas, Austin). The parental virus used in these studies, 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 (29a). The prefix TRSB was assigned to mutant viruses constructed in pTRSB. Biologically derived mutants lack this prefix. Two biologically derived mutants, N7R1 and N7R2, have been described previously (15). N7R1 contains an Asn substitution at E2 1 and a Thr-to-Pro substitution at E2 3. N7R2 contains an Asp substitution at E2 1. For consistency with the virus nomenclature used in this study, these viruses have been renamed E2N1 and E2D1, respectively. All virus stocks used in this study were produced by electroporation of in vitro transcripts from the cDNA clone into BHK-21 cells as described previously (15, 28). BHK-21 cells were maintained in supplemented Eagle's minimal essential medium (Gibco-BRL) (15). The construction and characterization of the full-length cDNA clone pTRSB-N also has been described before (15).

**In vitro mutagenesis.** Specific mutations were constructed in the genetic background of pTRSB by site-directed mutagenesis (25). Single codon substitutions were engineered into the E2 1 locus of pTRSB to produce viruses containing Ile (TRSB-E2I1), Val (TRSB-E2V1), Leu (TRSB-E2L1), Phe (TRSB-E2F1), or Ser (TRSB-E2S1). A Lys-to-Asn substitution at the E2 207 locus of pTRSB was made to produce TRSB-E2N207. Mutagenesis was performed as described previously (40, 41), using the M13-derived mutagenesis vector mp18SBGP and site-specific mutagenesis primers, each containing one or two mismatched nucleotides. mp18SBGP contains the PE2, 6K, and E1 gene sequences of SB. The screening of individual phage clones and replacement of restriction fragments from phage replicative-form DNA into pTRSB were conducted as described elsewhere (15). Each recombinant pTRSB was sequenced across the entire replacement region by the *Taq* cycle sequencing procedure (United States Biochemical).

**cDNA construction of revertant genotypes by PCR.** Mutations present in the biologically derived revertant viruses E2N1, E2D1, and E2H1 were identified by direct sequencing of virion RNA as described previously (4, 65). A reverse transcriptase-PCR procedure was used to obtain double-stranded DNA fragments containing these mutations. Briefly, viral RNA from each mutant was purified as described previously (15) and served as template for cDNA synthesis using reverse transcriptase and a complementary sense primer. This cDNA was amplified by PCR (20 cycles) using *Taq* DNA polymerase and a viral sense return primer. PCR fragments were digested with restriction enzymes, replaced into pTRSB, and sequenced (15).

**In vitro transcriptions and transfection of cells.** In vitro transcripts were routinely introduced into BHK-21 cells by electroporation for the production of virus stocks and for metabolic labelling of virus particles for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis as described elsewhere (15, 28). The specific infectivity was determined for in vitro transcripts derived from each construct (15). Briefly, transcripts were labelled in vitro with $^{35}$S-UTP, spotted onto DE-81 ion exchange paper (Whatman), and quantitated on a liquid scintillation counter. A duplicate sample of unlabelled transcripts was transfected into BHK-21 cells with Lipofectin reagent (Gibco-BRL), and the cells were overlaid with agarose. Plaques were visualized 30 to 48 h later by staining with neutral red. Specific infectivity values were calculated as PFU/counts per minute.

**Specific infectivity assay for virions.** The relative abilities of TRSB and each mutant virus to initiate infection of BHK-21 cells were determined in a specific infectivity assay. [$^{35}$S]methionine-labelled virions were purified from infected BHK-21 cells as described below. Duplicate aliquots of each sample were precipitated with trichloroacetic acid and quantitated by liquid scintillation counting, and the values obtained were averaged. Values were adjusted for the additional E3 methionine present in non-PE2-cleaving viruses (26 total) and absent from PE2-cleaving viruses (25 total). Infectious particles were quantitated by a standard plaque assay on BHK-21 cell monolayers. The specific infectivity of each preparation was calculated as total PFU/counts per minute plated.

**Polyacrylamide gel analysis of [$^{35}$S]methionine-labelled viral proteins.** Virion proteins were radiolabelled with [$^{35}$S]methionine and resolved by SDS-PAGE. BHK-21 cell monolayers were infected with virus at a multiplicity of infection of 2 to 5 PFU per cell or electroporated with in vitro transcripts. Viral proteins were labelled with [$^{35}$S]methionine (final concentration, 10 μCi/ml) as described elsewhere (15). Labelled virions were purified from supernatants by sequential banding on discontinuous and continuous potassium tartrate gradients (20 to 35%) as described previously (37, 45). Labelled viral proteins were resolved by SDS-PAGE (10% acrylamide gels) under reducing conditions (50 mM 2-mercaptoethanol) (26) and visualized by autoradiography.

**Studies of virulence in newborn CD-1 mice.** Each infectious virus was assayed for virulence in litters of 1-day-old CD-1 mice (Charles River). Two to four litters of newborn mice (<24 h old) were infected with 100 PFU of virus in 50 μl of phosphate-buffered saline (PBS; without $Mg^{2+}$ or $Ca^{2+}$) plus 1% donor calf serum (PBS–D 1%) per mouse by subcutaneous inoculation. Two control mice from each litter were inoculated with 50 μl of PBS–D 1% only. Mice were observed daily for 14 days. Values were calculated for percent mortality and average survival times (AST) for the animals that died. Viruses causing 100% mortality with AST the same as or less than that for TRSB were considered virulent; those causing <100% mortality were considered attenuated.

## RESULTS

**Isolation and characterization of N7R4.** Site-directed mutagenesis of a Sindbis virus AR339 cDNA clone (pTRSB) was used previously to substitute Asn for Arg at E2 1 (15), to model a mutation found in an attenuated variant of Sindbis virus S.A.AR86 (45). In pTRSB, the mutation created a site for N-linked glycosylation (Asn-X-Thr). Full-length in vitro transcripts were introduced into BHK-21 cells by electroporation. Carbohydrate addition at E2 1 effectively prevented PE2 cleavage and resulted in the production of PE2-containing particles (TRSB-N) which were efficiently assembled and released but which were noninfectious. Eight infectious revertants of TRSB-N were isolated, and seven were characterized (15). Two of these revertants had mutations that abrogated the glycosylation signal and restored PE2 cleavage. Five of the revertants retained the PE2 cleavage defect and had incorporated a suppressor mutation(s) in either E3 or E2. Here, we present a genetic and phenotypic characterization of the eighth

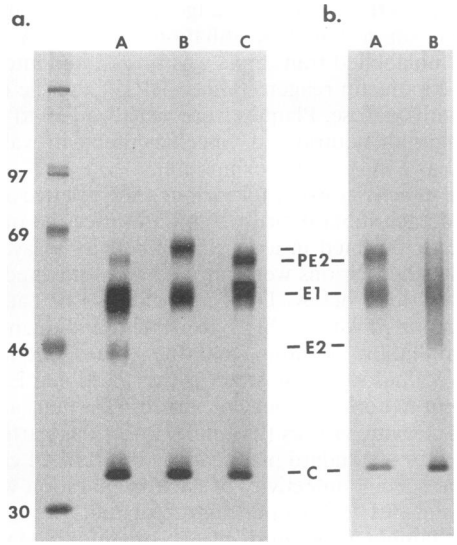


FIG. 1. SDS-PAGE analysis of purified viruses. Virions were metabolically labelled with [$^{35}$S]methionine and purified from infected BHK-21 cell supernatants as described in the text. $^{14}$C-methylated proteins (far left lane) marked (in thousands) on the left side of the gel were included as molecular weight markers. (Panel a) Lane A, TRSB; lane B, TRSB-N; lane C, TRSB-N7R4. (Panel b) Lane A, TRSB-E2I1; lane B, TRSB-E2N207. C, capsid protein.

revertant, N7R4, and of a panel of mutant viruses, each containing a different amino acid at E2 1.

The putative reverting mutation(s) in N7R4 was identified by sequencing N7R4 virion RNA across the E3 and E2 genes. Two mutations were identified: an Ile for Asn at E2 1, and an Asn for Lys at E2 207. Like one class of revertants (E2N1 and E2D1) described previously (15), the mutation at E2 1 eliminated the N-linked glycosylation signal at this site. This is reflected in the wild-type mobility of PE2 in SDS-PAGE (Fig. 1). However, unlike these revertants, N7R4 retained the PE2 cleavage defect and incorporated PE2 in place of E2 in virions. To confirm that one or both of these mutations were responsible for the revertant phenotype of N7R4, the genotype of N7R4 was reconstructed in pTRSB by a reverse transcriptase-PCR cloning procedure as described in Materials and Methods. Virus (TRSB-N7R4) derived from this clone (pTRSB-N7R4) recapitulated the PE2 glycosylation and cleavage phenotypes of N7R4 as determined by SDS-PAGE (data not shown).

The influence of the two individual mutations on PE2 cleavage and revertant phenotypes of N7R4 was determined by constructing each mutation singly in pTRSB by site-directed mutagenesis. Virus containing only the E2 Asn-207 mutation (TRSB-E2N207) cleaved PE2 and incorporated E2/E1 heterodimers into virions (Fig. 1). TRSB-E2N207 and TRSB produced large, comparably sized plaques on BHK-21 cells. Virus containing only the E2 Ile-1 mutation (TRSB-E2I1) failed to cleave PE2, and incorporated PE2/E1 heterodimers in virions (Fig. 1). TRSB-E2I1 produced extremely small plaques on BHK-21 cells. N7R4 and TRSB-N7R4 each produced plaques of intermediate size on BHK-21 cells. These results indicated that the E2 Ile-1 mutation alone was responsible for the PE2 cleavage defect of N7R4 and was sufficient for the reversion to viability as defined by the specific infectivity of transcripts containing this mutation. However, both mutations were required for expression of the intermediate-plaque-size phenotype of N7R4, suggesting that E2 Asn-207 provided an additional selective growth advantage on BHK-21 cells.

TABLE 1. Genotypic and phenotypic properties of TRSB and mutant viruses

| Virus type and clone | E2 1 | PE2 cleavage[a] |
|---|---|---|
| Viruses containing E2 Arg-1 | | |
| TRSB (parental) | Arginine | Yes |
| TRSB-E2N207 | Arginine[b] | Yes |
| E2 1 mutants | | |
| TRSB-E2N1 | Asparagine[c] | Yes |
| TRSB-E2D1 | Aspartic acid | Yes |
| TRSB-E2H1 | Histidine | Yes |
| TRSB-E2S1 | Serine | Yes |
| TRSB-E2F1 | Phenylalanine | Yes |
| TRSB-E2L1 | Leucine | No |
| TRSB-E2V1 | Valine | No |
| TRSB-E2I1 | Isoleucine | No |
| TRSB-N7R4 | Isoleucine[b] | No |

[a] As determined by SDS-PAGE analysis of purified virions.
[b] TRSB-E2N207 and TRSB-N7R4 also contain an E2 Asn-207 substitution.
[c] TRSB-E2N1 also contains an E2 Pro-3 substitution.

**Influence of the E2 1 residue on PE2 cleavage.** As demonstrated by TRSB-N, N-glycosylated Asn at E2 1 was not compatible with PE2 cleavage. However, PE2 cleavage occurred when the E2 1 residue was Asn in a nonglycosylated form (in E2N1 which has E2 Asn-1 plus E2 Pro-3), Asp (E2D1), or Arg (TRSB) (15). To address the possibility that amino acids other than Ile at E2 1 also were incompatible with PE2 cleavage, the PE2 cleavage phenotype was determined for seven additional viruses, each derived from a construct in pTRSB and each with a different amino acid at E2 1 (Table 1).

The PE2 cleavage phenotype of each virus was determined by the amount of PE2 present in purified virions (Fig. 2). The amino acids Arg, Asp, Ser, Phe, His, and Asn (nonglycosy-

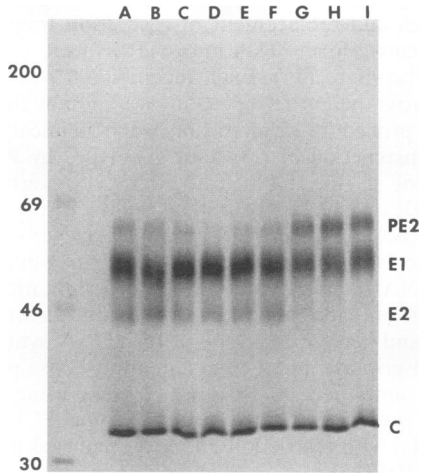


FIG. 2. SDS-PAGE analysis of wild-type virus (TRSB) and viable E2 1 mutants. Viruses were metabolically labelled with [$^{35}$S]methionine and purified from infected BHK-21 cell supernatants as described in Materials and Methods. $^{14}$C-methylated proteins (far left lane) marked (in thousands) on the left side of the gel were included as molecular weight markers. Lane A, TRSB; lane B, TRSB-E2N1; lane C, TRSB-E2D1; lane D, TRSB-E2S1; lane E, TRSB-E2H1; lane F, TRSB-E2F1; lane G, TRSB-E2L1; lane H, TRSB-E2I1; lane I, TRSB-E2V1.

TABLE 2. Specific infectivities of TRSB and mutant viruses for BHK-21 cells[a]

| Virus type and clone | E2 1 | Specific infectivity (%) |
|---|---|---|
| Viruses containing E2 Arg-1 | | |
| TRSB | Arginine | 100 |
| TRSB-E2N207 | Arginine[b] | 170 |
| E2 1 mutants (PE2 cleaving) | | |
| TRSB-E2N1 | Asparagine[c] | 3 |
| TRSB-E2D1 | Aspartic acid | <1 |
| TRSB-E2H1 | Histidine | 1 |
| TRSB-E2S1 | Serine | <1 |
| TRSB-E2F1 | Phenylalanine | 3 |
| E2 1 mutants (PE2 cleavage defective) | | |
| TRSB-E2L1 | Leucine | 21 |
| TRSB-E2V1 | Valine | 14 |
| TRSB-E2I1 | Isoleucine | 7 |
| TRSB-N7R4 | Isoleucine[b] | 56 |

[a] Viruses were labelled with [$^{35}$S]methionine and purified from infected BHK-21 cell supernatants as described in Materials and Methods. Virus preparation titers were determined by plaque assay on BHK-21 cell monolayers. Specific infectivity values were calculated as PFU/counts per minute and presented as a percentage of the specific infectivity for TRSB (100%). Values are the averages for two experiments.
[b] TRSB-E2N207 and TRSB-N7R4 also contain an E2 Asn-207 substitution.
[c] TRSB-E2N1 also contains an E2 Pro-3 substitution.

lated) at E2 1 specified the PE2 cleavage phenotype. Each virus incorporated predominantly E2/E1 heterodimers into virions, although a small amount of PE2 was present in each (Fig. 2). Extremely inefficient cleavage of PE2 was apparent in viruses containing Ile, Val, or Leu at E2 1, and these viruses incorporated PE2/E1 heterodimers almost exclusively (Fig. 2). Clearly, certain amino acids at E2 1, specifically those with branched aliphatic side chains, can prevent protease recognition, binding, and/or activity at the PE2 cleavage signal. In contrast to TRSB-N particles, which were essentially noninfectious, these PE2-containing viruses were viable in the absence of resuscitating mutations.

**Specific infectivity of TRSB and E2 1 mutant viruses.** With the exception of the pTRSB-N mutation, none of the amino acids substituted at E2 1 was lethal, as determined by the specific infectivity of in vitro transcripts (data not shown). However, the E2 1 substitutions did affect the biological properties of the released virions. The specific infectivity of each viable virus was determined as an average of two independent experiments and is presented as a percentage of the TRSB specific infectivity (100%) (Table 2).

TRSB and TRSB-E2N207 virions, which both contain Arg at E2 1, displayed the highest specific infectivity for BHK-21 cells. Surprisingly, the specific infectivities of all other PE2-cleaving viruses were dramatically reduced (<1 to 3% relative to that of TRSB). Similarly, the specific infectivity of each PE2-containing virus also was lower than that of TRSB. The specific infectivity of TRSB-E2I1 was only 7% that of TRSB. However, the specific infectivity of TRSB-N7R4, which also contains the E2 Asn-207 mutation, increased to 56% that of TRSB. The specific infectivities of TRSB-E2L1 and TRSB-E2V1 (PE2 cleavage defective) were consistently higher than those for all PE2-cleaving viruses except those with E2 Arg-1. Therefore, a direct relationship between the efficiency of PE2 cleavage and specific infectivity in BHK-21 cells was not observed. Instead, these results suggested that the differences in specific infectivity were directly related to the influence of the amino-terminal residue of E2. This region of E2 likely participates in one or more spike functions related to infectivity in BHK-21 cells, even when in the context of PE2.

**Virulence of TRSB and E2 1 mutant viruses in mice.** Each of the viruses containing alternative amino acids at E2 1 was inoculated into newborn mice to determine if differences in specific infectivity on BHK-21 cells or PE2 cleavage phenotype correlated with differences in virulence in mice.

Consistent with our previous studies, the PE2 cleavage defective and attenuation phenotypes were directly linked. Each PE2-containing virus was attenuated, causing between 59 and 93% mortality and ASTs ranging from 5.2 to 9.5 days (Table 3). The values for TRSB-E2L1 and TRSB-E2V1 were similar to those obtained previously for the PE2-containing revertants of TRSB-N (15). TRSB-E2I1, although causing less than 100% mortality, was the most virulent of this group, and the AST for those animals succumbing to infection (5.4 days) was markedly shorter than for any other PE2 cleavage-defec-

TABLE 3. Virulence of TRSB and mutant viruses in newborn mice

| Virus type and clone | No. of litters (no. of animals) | % Mortality | Range[a] | AST (days)[b] | Virulence[c] |
|---|---|---|---|---|---|
| Viruses containing E2 Arg-1 | | | | | |
| TRSB | 3 (31) | 100 | NA | 4.1 ± 0.6 | Virulent |
| TRSB-E2N207 | 2 (22) | 100 | NA | 4.6 ± 0.7 | Virulent |
| E2 1 mutants (PE2 cleaving) | | | | | |
| TRSB-E2N1 | 3 (27) | 100 | NA | 2.8 ± 0.4 | Virulent |
| TRSB-E2D1 | 3 (29) | 100 | NA | 2.6 ± 0.5 | Virulent |
| TRSB-E2H1 | 4 (38) | 100 | NA | 2.7 ± 0.7 | Virulent |
| TRSB-E2S1 | 3 (34) | 100 | NA | 2.3 ± 0.4 | Virulent |
| TRSB-E2F1 | 3 (30) | 100 | NA | 2.8 ± 0.4 | Virulent |
| E2 1 mutants (PE2 cleavage defective) | | | | | |
| TRSB-E2L1 | 4 (38) | 61 | 20–89 | 8.4 ± 3.0 | Attenuated |
| TRSB-E2V1 | 4 (44) | 61 | 40–77 | 8.0 ± 2.8 | Attenuated |
| TRSB-E2I1 | 4 (42) | 93 | 83–100 | 5.2 ± 3.0 | Attenuated |
| TRSB-N7R4 | 2 (29) | 59 | 45–67 | 9.5 ± 2.4 | Attenuated |

[a] Range indicates percent mortality values for litters having the lowest and highest percent mortality rates. NA, not applicable.
[b] AST ± standard deviations for animals that died.
[c] Virulent, causing 100% mortality of infected animals. Attenuated, causing <100% mortality.

tive virus. When combined with the E2 Asn-207 mutation, an E2 Ile-1-containing virus (TRSB-N7R4) displayed virulence characteristics similar to those of other attenuated PE2-containing viruses (59% mortality and an AST of approximately 9.5 days).

Each of the PE2-cleaving viruses caused 100% mortality. The ASTs for viruses having an amino acid other than Arg at E2 1 (2.3 to 2.8 days) were significantly lower than those for E2 Arg-1-containing viruses (TRSB and TRSB-E2N207; by *t* test, $P < 0.001$ for each). The inoculating dose for each virus in this experiment was based on PFU assayed in BHK-21 cells. However, the actual number of virus particles in each animal inoculum varied in proportion to the specific infectivity of each virus in BHK-21 cells. Therefore, the decreased AST associated with each PE2-cleaving, position 1 mutant may actually reflect the effect of a higher dose in terms of virus particles. To address this possibility, four litters of newborn CD-1 mice (34 animals) were inoculated subcutaneously with an average of 1.0 PFU per mouse of TRSB-E2S1, an infecting dose designed to approximate, in numbers of virus particles, a dose of 100 PFU of TRSB. These infections resulted in 100% mortality and an AST of $2.8 \pm 0.4$ days. The difference in AST between 1 BHK-21 cell PFU of TRSB-E2S1 and 100 PFU of TRSB was statistically significant (by *t* test, $P < 0.001$). This indicates that the reduced ASTs for PE2-cleaving, position 1 mutants reflect actual differences in virulence and strongly suggests that the virulence of Sindbis virus AR339 can be influenced directly by the amino-terminal residue of E2.

## DISCUSSION

The results presented in this report provide additional information about the amino acid sequence motif constituting the PE2 cleavage signal, biological functions of the amino terminus of E2, and the relationship of cell culture adaptation to reduction of virulence in vivo.

**Nature of the PE2 cleavage signal.** The bxbb amino acid motif immediately upstream of the PE2 cleavage site is the major constituent of the cleavage signal. Cleavage of PE2 from Semliki Forest virus was prevented by mutating the amino acid at the −1 position relative to the cleavage site (29, 46). Insertional mutagenesis of the bxbb signal in Sindbis virus resulted in the production of PE2-containing virions (9). In addition, PE2-containing virions were produced by passage of Sindbis virus in a cell line (RPE.40) that is defective in a protease which recognizes the bxbb motif and is responsible for PE2 cleavage (30, 31, 61). It has now been shown that substitution of amino acids with branched aliphatic side chains (Ile, Leu, or Val) at the +1 position relative to the bxbb motif also prevents PE2 cleavage.

The mechanism by which these particular substitutions prevent cleavage is unknown. A global effect on PE2 conformation, whereby folding of the precursor containing Ile, Leu, or Val at E2 1 occluded the bxbb signal motif, cannot be ruled out. However, it is unlikely that the hydrophobicity of these residues, or their potential for sterically hindering access to the bxbb motif locally, accounts for their inhibition of PE2 cleavage. Another hydrophobic substitution, E2 Phe-1, was compatible with efficient PE2 cleavage. Also, Phe, Arg, and His residues, which were permissive for PE2 cleavage, are equal to or greater in size than Ile, Leu, and Val.

Alternatively, the E2 1 residue may contribute to the cleavage signal itself, which would suggest that Ile, Leu, or Val at this locus prevents effective recognition of the bxbb signal by the cellular protease. Likewise, other substitutions at E2 1, such as Ser and His, increase the efficiency of PE2 cleavage relative to that of E2 Arg-1 in TRSB. Enzymatic activity of the cellular protease(s) can be inhibited by analogous amino acid substitutions in the context of a variety of viral and cellular substrates. In Newcastle disease virus (*Paramyxoviridae* family), processing of the precursor $F_0$ to $F_1$ and $F_2$ was inhibited by a Phe-to-Leu substitution at the site analogous to E2 1 (32), and Leu occupies this locus in many avirulent strains of Newcastle disease virus which contain an uncleaved $F_0$ protein (54, 55). Cleavage of the cellular precursor polypeptide proalbumin was prevented if Glu at the +1 position relative to the cleavage signal was replaced by Leu, Val, or Ile (35). These findings support the hypothesis that the +1 residue relative to the cleavage site contributes to the signal itself and that amino acids with branched aliphatic side chains are not compatible with protease recognition, binding, and/or cleavage.

**Influence of the E2 amino terminus on alphavirus spike function.** Phenotypic comparisons between TRSB and the E2 1 mutant viruses which are PE2 cleavage competent suggest that the amino-terminal residue of E2 influences and/or participates in one or more functions of the glycoprotein spike. TRSB virions (E2 Arg-1) were approximately 30- to 250-fold more infectious in BHK-21 cells than viruses containing any of the other E2 1 amino acids. This suggests that E2 Arg-1 provides a selective advantage to the infecting virion in these particular cells. In most strains of Sindbis viruses and closely related Sindbis-like viruses, the E2 1 amino acid is Ser (5, 45, 50). E2 Arg-1, present in our laboratory strain of Sindbis virus AR339 (SB), is an exception and may have been selected during passage of SB on BHK-21 cells. The high specific infectivity of TRSB virions may reflect enhanced binding to BHK-21 cell receptors or more efficient internalization and uncoating. Several reports support the hypothesis that the amino terminus of E2 participates in these events. Tandem mutations at E2 3 and E2 4 were linked to rapid penetration of BHK-21 cells in the related alphavirus VEE virus (6, 20). The rapid penetration phenotype of a Sindbis virus S.A.AR86 mutant, S12, also was linked to a substitution at E2 1 which prevented PE2 cleavage (45). Dubuisson and Rice (9) demonstrated that insertions near the PE2 cleavage site of Sindbis virus produced mutants which were defective for binding to BHK-21 cells and chicken embryo fibroblasts and which also exhibited slower penetration kinetics. These results implicate domains composed of or including the N terminus of E2 as participating in alphavirus binding and penetration.

Domains on the alphavirus glycoprotein spike which participate in receptor interactions have been identified indirectly as a result of using anti-idiotype approaches to identify cellular receptors for Sindbis virus (56, 60). One of the monoclonal antibodies used in these studies binds to the E2a neutralization site and yields monoclonal antibody escape mutations at E2 214 (53). Two observations suggest an association of the amino terminus of E2 with the alphavirus major neutralization domain (E2 181 to 216 in Sindbis virus [53], E2 180 to 210 in VEE virus [19], and E2 216 to 251 in Ross River virus [22, 58]). Peptides representing the amino terminus of VEE virus E2 elicit protective antibodies which compete for binding to virions with monoclonal antibodies directed to the major neutralizing domain (18). In addition, second-site mutations in Sindbis virus E2, which increase the specific infectivity of cleavage-defective viruses for BHK-21 cells, all map between E2 residues 191 and 239 (15). These observations are consistent with the phenotypes of mutations at or near the E2 amino terminus and suggest that it may participate in or be associated with virion domains important for receptor binding and other early events in infection. Failure to cleave PE2, therefore, may restrict the normal function of the E2 amino terminus.

The amino acid at E2 1 also influenced virulence in newborn mice, either directly as the amino-terminal residue of E2 or by preventing PE2 cleavage. In those viruses whose E2 1 substitution inhibited PE2 cleavage, virulence was reduced. This is consistent with the previous finding that a PE2 cleavage-defective phenotype was correlated with decreased virulence in animals (15, 42, 45). The surprising result was that mutants with E2 1 substitutions which allowed PE2 cleavage were significantly more virulent than virus derived from the TRSB clone. This may reflect the increased efficiency of PE2 cleavage observed with most of these substitutions. However, nonglycosylated E2 Asn-1 was approximately as efficient as E2 Arg-1 in terms of PE2 cleavage, yet virus with the former mutation was significantly more virulent than that with the latter in neonatal mice. Alternatively, the increase in virulence may be a consequence of the seemingly inverse relationship between efficiency of growth on cultured cells and virulence in vivo (see reference 33 for a review). Compared with the E2 Ser-1 virus, which has the consensus amino acid at this locus, the E2 Arg-1 virus had a higher specific infectivity for BHK-21 cells and allowed extended survival in mice. A similar inverse relationship is evident in a comparison of virus containing E2 Ile-1 and virus containing E2 Asn-207 in the E2 Ile-1 background. Mutations which increase the efficiency of virus-cell interactions with BHK-21 cells in culture may simultaneously decrease the ability of these viruses to mediate in vivo virus-cell interactions critical for the induction of disease. These data are consistent with previous studies of Sindbis virus and VEE virus which demonstrate that selection for efficient growth on BHK-21 cells strongly coselects for attenuation in vivo (20, 36, 45).

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