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# Attenuation of Sindbis virus variants incorporating uncleaved PE2 glycoprotein is correlated with attachment to cell-surface heparan sulfate

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**Abstract**

Sindbis virus virions incorporating uncleaved precursor envelope protein PE2 bind efficiently to cell-surface heparan sulfate (HS) because the furin cleavage site (a consensus HS-binding domain) is retained in the mature virus particle. However, they are essentially nonviable. Resuscitating mutations selected in the E3 or E2 protein preserve the PE2 noncleaving phenotype and HS binding, but facilitate fusion, and thereby restore wild-type infectivity on cultured cells. Here, we have demonstrated that the resuscitated PE2 noncleaving virus was almost avirulent in vivo, but mutated during the infection. Mutants had increased virulence and cleavage of PE2, with reduced HS binding capacity. We hypothesize that HS binding leads to sequestration of PE2 noncleaving virus particles and suppression of serum viremia, thereby selecting for evolution of the virus into a PE2-cleaving, low HS-binding phenotype.


*Keywords:* Sindbis; Alphavirus; Pathogenesis; Virulence; Heparan sulfate; Cleavage; Attachment; Reversion; Mutation

## Introduction

Sindbis virus is the prototypic virus of the *Alphavirus* genus, family *Togaviridae* (reviewed by Johnston and Peters, 1996). This group of arthropod-borne RNA viruses includes several significant human pathogens and potential bioterrorism/biowarfare agents (Burgess et al., 2001; Smith et al., 1997). On the basis of the relative difficulty and risk of handling the more pathogenic alphaviruses, Sindbis virus has been studied extensively as a pathogenesis model. More recently, Sindbis virus vector systems have also been utilized in vaccine development (Gardner et al., 2000; Huang, 1996; Polo et al., 1999; Pugachev et al., 1995) and gene therapy (Tseng et al., 2002) applications. Consequently, there has been a resurgence of interest in characterizing Sindbis virus virulence/attenuation determinants.

Mature Sindbis virus particles contain an icosahedral nucleocapsid composed of capsid (C) monomers, cloaked in an envelope of membrane-anchored glycoprotein spike components, E1 and E2 (reviewed by Strauss and Strauss, 1994). The viral spike proteins facilitate attachment to cell surfaces, fusion, and entry. The consensus wild-type Sindbis virus strain, TR339, derived from an infectious clone that resurrects the sequence of the original natural AR339 isolate (Klimstra et al., 1998; McKnight et al., 1996; Klimstra et al., unpublished results), attaches initially to cells through a low affinity, primarily heparan sulfate (HS)-independent mechanism (Klimstra et al., 1998). In contrast, laboratory strains of Sindbis virus utilize cell-surface HS molecules as receptors to varying degrees (Byrnes and Griffin, 1998; Klimstra et al., 1998, 1999a). The HS-dependent cell-surface attachment phenotype is conferred by the acquisition of positive-charge amino acid mutations in the E2 attachment protein, rapidly selected during passage in cultured cells (Klimstra et al., 1998). Such mutations are believed either to form part of a conformation-dependent, nonlinear, HS-binding sequence or to promote the exposure of another such site (Klimstra et al., 1998).

We have previously demonstrated a second, distinct mechanism by which Sindbis virus can acquire the ability to bind to cell-surface HS (Klimstra et al., 1999a). During maturation of the virion, the precursor to the major E2 attachment protein, PE2, is cleaved by a host-encoded, furin-like protease (Strauss and Strauss, 1994). Significant-

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ly, the BXBB furin cleavage site (where B = basic, X = hydrophobic) in the PE2 protein also represents an inverted, consensus HS-binding domain (Cardin and Weintraub, 1989; Klimstra et al., 1999a). When the precursor PE2 protein is cleaved into the mature E2 glycoprotein, the E3 portion containing the BXBB site is lost from the virion, and therefore this site would not be expected to contribute to the virus–cell surface interaction. However, some laboratory strains of Sindbis virus retain a minor uncleaved PE2 component in the spike protein structures on the surface of the mature virion (Heidner and Johnston, 1994; Klimstra et al., 1999a). In this case, the PE2 cleavage phenotype of the virus is determined by the amino acid residue immediately downstream of the furin cleavage site, E2 position 1 (Heidner and Johnston, 1994; Heidner et al., 1994; Klimstra et al., 1999a), and the proportion of PE2 incorporated into the mature virion can be manipulated by mutating the amino acid at this position (Table 1). TR339 almost completely cleaves its PE2 glycoprotein (Klimstra et al., 1999a), whereas in the envelope of virus derived from the infectious clone of our laboratory Sindbis virus strain, designated TRSB, ~14% of the total E2 is in the PE2 form (Heidner and Johnston, 1994; Klimstra et al., 1999a). A mutation from Ser (TR339) to Arg (TRSB) at E2 position 1 is responsible for this cleavage difference (Klimstra et al., 1999a). The presence of PE2 has an enhancing effect on cell-surface attachment that is correlated with the relative PE2 content in the virus particles and dependent upon the presence of the BXBB domain (Klimstra et al., 1999a). Thus, the BXBB furin cleavage site in PE2 can mediate the binding of PE2-containing Sindbis viruses to cell-surface HS.

Previously, we generated a Sindbis virus mutant (TRSB-N) with an additional glycosylation site (Asn-Val-Thr) introduced by substituting Asn at E2 position 1 (Heidner and Johnston, 1994). Glycosylation at this site completely abrogates furin-mediated PE2 cleavage. However, TRSB-N virus particles, while attaching very efficiently to cell-surface HS through the BXBB site, are nonviable (Heidner and Johnston, 1994; Heidner et al., 1994) presumably because of inhibition of fusion or other uncoating processes (Smit et al., 2001). Second-site mutations in either E3 or E2 (e.g., E2 Gly 216) were selected, which maintain the PE2 noncleaving phenotype, but restore virus infectivity in vitro (Heidner et al., 1994). These mutations are referred to as "resuscitating" mutations. The E2 Gly 216 mutation does not alter the efficiency of virion binding to cells (Heidner et al., 1994), but improves the efficiency of uncoating and entry (Smit et al., 2001), resulting in an overall increase in infection efficiency in vitro.

We have previously observed that the consensus wild-type TR339 virus is significantly more virulent in neonatal mice than cell culture-adapted, HS-binding Sindbis virus strains with positive-charge mutations in E2 or partial PE2 incorporation (Klimstra et al., 1998, 1999a). This suggests that increased HS-binding and cell culture adaptation are inversely correlated with virulence in vivo. Similarly, while the resuscitated PE2 noncleaving mutants exhibit normal or nearly normal growth in BHK-21 (Heidner et al., 1994), Neuro-2A and primary mouse embryo fibroblast cells (Ryman and Johnston, unpublished data) are attenuated in mice (Heidner et al., 1994). Here, we have investigated the mechanism of attenuation of in vivo replication in the PE2 noncleaving viruses by analyzing virulent mutants selected during their replication within the host.

## Results

### *Construction and characterization of viruses*

The genotypes and phenotypes of the viruses used in this study are summarized in Table 1. pTR339 is a full-length cDNA clone constructed to encode the consensus wild-type Sindbis AR339 virus sequence (Klimstra et al., 1998; McKnight et al., 1996; Klimstra et al., unpublished results).

Table 1
Summary of genotypes and phenotypes of viruses used in this study[a]

| Virus strain | -1 B X B B +1 2 3[b] | % PE2[c] | Relative BHK infectivity[d] | Virulence[e] | HS-binding[f] |
|---|---|---|---|---|---|
| TR339 | G R S K R **S** V T | <10 % | low | +++ | - |
| TRSB-E2S1 | G R S K R **S** V T | <10 % | low | +++ | - |
| TRSB | G R S K R **R** V T | 10-20 % | high | ++ | + |
| TRSB-N | G R S K R **N** V T | >90 % | low | ND | +++ |
| TRSB-E2G216 | G R S K R **R** V T | 10-20 % | high | ++ | + |
| TRSB-NE2G216 | G R S K R **N** V T | >90 % | intermediate | +/- | +++ |

[a] Information taken from several sources including Heidner and Johnston (1994), Heidner et al. (1994), Klimstra et al. (1998, 1999a), and the current study.
[b] Boldface type denotes amino acids mutated from the TRSB-E2S1 virus genome sequence; shading denotes BXBB PE2 furin cleavage site/consensus HS binding site; underlining denotes potential glycosylation site, NXT.
[c] Percentage of PE2 as opposed to E2 incorporated into the envelope of the mature virion.
[d] Indicates relative infectivity of radio-labeled virus particles on BHK cells giving relative particle/PFU ratio.
[e] Virulence following subcutaneous inoculation of neonatal mice measured in terms of morbidity, mortality, and AST.
[f] Relative ability to bind HS determined by heparin-mediated inhibition of plaque formation, binding to and infectivity for HS- and GAG-deficient CHO cells or cells treated with heparinase.

pTRSB is a cDNA clone derived from a laboratory strain of Sindbis virus AR339 (McKnight et al., 1996). The wild-type phenotype is conferred by the exchange of Ser at E2 position 1 of pTR339 for Arg in pTRSB. pTR339 also differs from pTRSB at two other codons; nsp3 528 and E1 72 (Klimstra et al., 1998, 1999b). The virus clone with E2 Ser 1 in the TRSB background is designated pTRSB-E2S1. In this study, virus derived from clones with an E2 Asn 1 in the pTRSB background (pTRSB-N and pTRSB-NE2G216) is compared with pTRSB, pTRSB-E2S1, and pTR339-derived viruses. The substitution of Asn at E2 1 creates an Asn-Val-Thr glycosylation site, which prevents furin-mediated cleavage of the PE2 precursor protein into E2, such that the TRSB-N virus is essentially nonviable. TRSB-NE2G216 is a revertant of TRSB-N in which a second-site viability-resuscitating Glu to Gly mutation has been selected at E2 216. pTRSB-E2G216 contains only the E2 Gly 216 resuscitating mutation from pTRSB-NE2G216 cloned independently into the pTRSB sequence background.

### *Virulence of PE2 noncleaving Sindbis virus in neonatal mice*

Previous studies have demonstrated that although the resuscitated PE2 noncleaving viruses are not significantly restricted for growth in mammalian cells in vitro compared with TRSB, they are attenuated in vivo in terms of mortality rate and average survival time (AST; Heidner et al., 1994). To extend these observations, groups of neonatal mice, 12–24 h post-partum (p.p.), were inoculated subcutaneously with $10^3$ PFU of Sindbis virus strains TRSB, TRSB-E2S1, TR339, TRSB-E2G216, and TRSB-NE2G216, or mock-infected with PBS-1% donor calf serum (DCS). The percent survival of the virus-infected mice over the course of the experiment was determined (Fig. 1A). Confirming previous findings, TRSB, TRSB-E2S1, and TR339 viruses caused 100% mortality in neonatal mice with ASTs of 4.25 ± 0.88, 2.08 ± 0.49, and 2.16 ± 0.26 days, respectively. In comparison, TRSB-NE2G216, the resuscitated PE2 noncleaving virus, was significantly attenuated, with a mortality rate of 73% and an AST of 9.55 ± 3.33 days. Similar results were obtained in two repeats of this experiment. Interestingly, the survival times of individual neonatal mice infected with TRSB-NE2G216 were widely divergent, with deaths occurring between 3 and 18 days postinfection (p.i.). Daily weight change in TRSB-NE2G216-infected mice was used as a determinant of the clinical severity of infection (Fig. 1B). By individually identifying the TRSB-NE2G216-infected mice, it was possible to determine the severity of infection in each mouse and to predict the outcome of the infection several days before death. Overt clinical signs of disease varied from ataxia with rapid mortality through paresis with extended AST to a nonfatal flaccid hind-limb paralysis. TRSB-E2G216 virus, containing only the resuscitating mutation in the TRSB sequence background, was not significantly attenuated compared with the parental viruses.

### *Virus replication and dissemination in neonatal mice*

The pathogenesis of Sindbis virus infection in mice has been extensively studied. When inoculated subcutaneously into neonatal mice, consensus wild-type TR339 virus replicates in extraneural tissues, seeding a high titer serum viremia. Viremia facilitates neuroinvasion and replication occurs in the CNS tissues immediately before death (Klimstra et al., 1999b). Cell culture-adapted, HS-binding Sindbis virus strains were more attenuated, with restricted replication (Klimstra et al., 1999b; Trgovcich et al., 1996). To obtain correlates for virulence of TRSB, TRSB-E2G216 and TRSB-NE2G216, serum and brain tissue were collected from virus-infected neonates at 24 h intervals p.i., and infectious virus particles were titered (Figs. 2A, B). Replication of TRSB and TRSB-E2G216 varied little between infected animals reaching titers of $10^7$–$10^8$ PFU/ml in serum and $10^8$–$10^9$ PFU/g in brain before the death of the mouse, suggesting that infection severity does not differ significantly from one infected animal to another. In contrast, virus titers in serum and brain harvested from different TRSB-NE2G216-infected mice showed significant disparities on each day p.i. Virus titers correlated closely with the percent weight gain/loss and predicted severity of the infection (data not shown). Unlike TRSB-NE2G216 virus stocks, which exhibit a uniformly small-plaque phenotype compared with TRSB, the plaque size of virus titered from the infected mice was frequently mixed. These data are in keeping with the differences described in mortality rate, AST, and clinical signs of infection.

### *Pathological correlates of infection severity*

We have previously described interferon alpha/beta (IFN-α/β) induction and thymic involution as reliable indicators of a severe Sindbis virus infection. IFN-α/β induction parallels virus replication and severity of infection (Klimstra et al., 1999b; Trgovcich et al., 1996; Vilcek, 1964). Thymic involution is not associated with virus replication, but instead correlates with proinflammatory cytokine induction (Klimstra et al., 1999b; Trgovcich et al., 1997). Measurement of virus titer offers only a "snapshot" view of a process ongoing within the infected mouse and therefore IFN-α/β induction, and the severity of virus-mediated or cytokine-mediated lesions were used as more enduring correlates of virulence. Induced levels of IFN-α/β in both serum and brain were proportional to virus titer (Figs. 2C, D). The severity of both cytokine-mediated and virus-mediated lesions in TRSB-NE2G216-infected mice correlated with weight loss and clinical signs (data not shown). The correlation of all these criteria strongly suggested that the severity of disease caused by the same input virus in different animals was widely divergent.

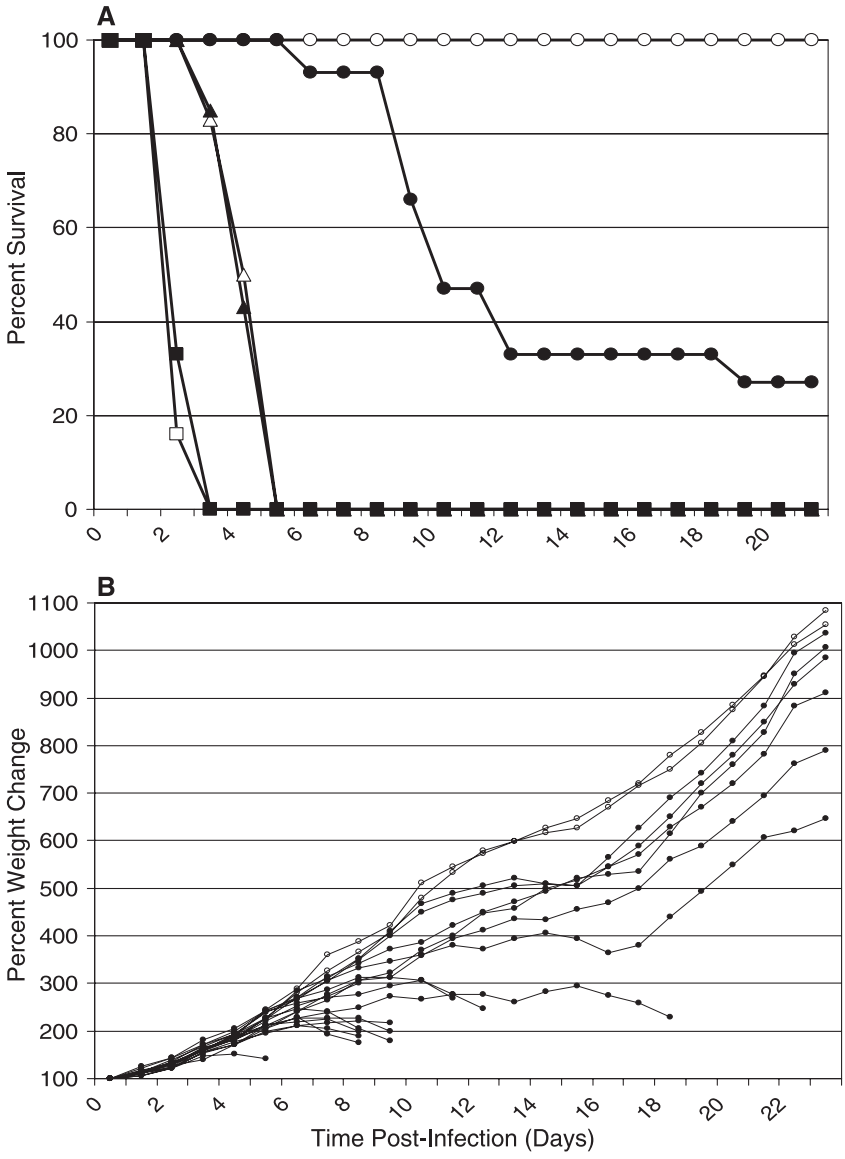


Fig. 1. Attenuation of virulence of PE2-containing TRSB-NE2G216 virus in neonatal mice. Neonatal mice, 12–24 h p.p., were infected subcutaneously in the ventral thorax with $10^3$ PFU of virus or mock-infected with virus diluent to generate a mortality curve for each virus (panel A): mock-infected (open circles), TRSB (closed triangles), TRSB-E2S1 (closed squares), TRSB-E2G216 (open triangles), TR339 (open squares), and TRSB-NE2G216 (closed circles). TRSB-NE2G216-infected mice were weighed daily and percent weight change relative to starting weight (1 day p.p.) was used as an indicator for severity of disease (panel B): TRSB-NE2G216-infected (closed circles) and mock-infected (open circles).

*Virulence of plaque isolates in neonatal mice*

The differences observed in the severity of clinical signs caused by infection with the TRSB-NE2G216 virus lead to the speculation that the virus was evolving into a more virulent form(s) within the mouse. Virus clones were isolated by plaque purification from serum or brain tissues of TRSB-NE2G216-infected mice 2, 3, 4, 5, 6, 8, or 10 days p.i., and amplified once on BHK-21 cells. The amplified plaque-purified variants were inoculated subcutaneously into neonatal mice at a dose of $10^3$ PFU. A range of virulence phenotypes were observed (Fig. 3 and Table 2), which could be divided into two groups: (1) attenuated viruses causing less than 100% mortality with ASTs of 6–9 days, and (2) virulent viruses causing 100% mortality with ASTs of <2–4 days. In most cases, the virulence of the plaque isolates correlated with the severity of the infection in the mouse from which the virus was selected (see below).

*Genotyping of plaque isolates*

Viral RNA was extracted from plaque-isolated, amplified virus particles and amplified by RT-PCR for se-

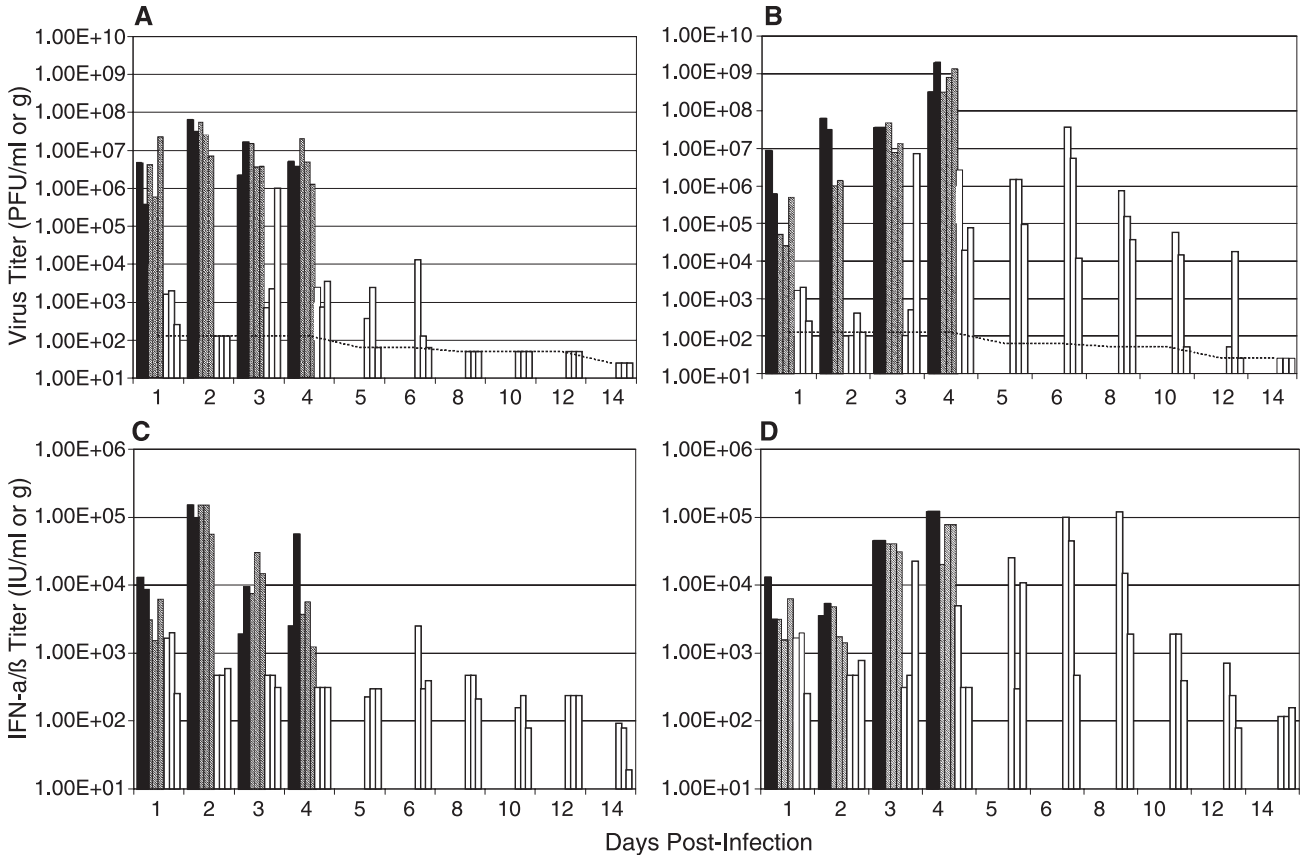


Fig. 2. Virus titers and corresponding IFN-α/β levels in serum and brain of virus-infected neonatal mice. Neonatal mice were inoculated subcutaneously with $10^3$ PFU virus and sacrificed at various times p.i. Virus titers in serum (panel A) and brain (panel B) were determined by BHK plaque assay. Levels of biologically active IFN-α/β in serum (panel C) and brain (panel D) were measured for the same mice. Results are plotted for individual mice infected with TRSB (solid black bars), TRSB-E2G216 (hatched bars), or TRSB-NE2G216 (solid white bars). The lower limit of detection is indicated (broken line).

quencing of the E2 gene and especially the furin cleavage sites at the E3/E2 junction in PE2. Five virus genotypes were present among virus clones isolated 3, 6, or 8 days p.i. (Table 2). Virus clones with the original genotype (E2 Asn 1/Thr 3) were isolated from samples with titers in the range of $62.5-1.2 \times 10^4$ PFU/ml or g.

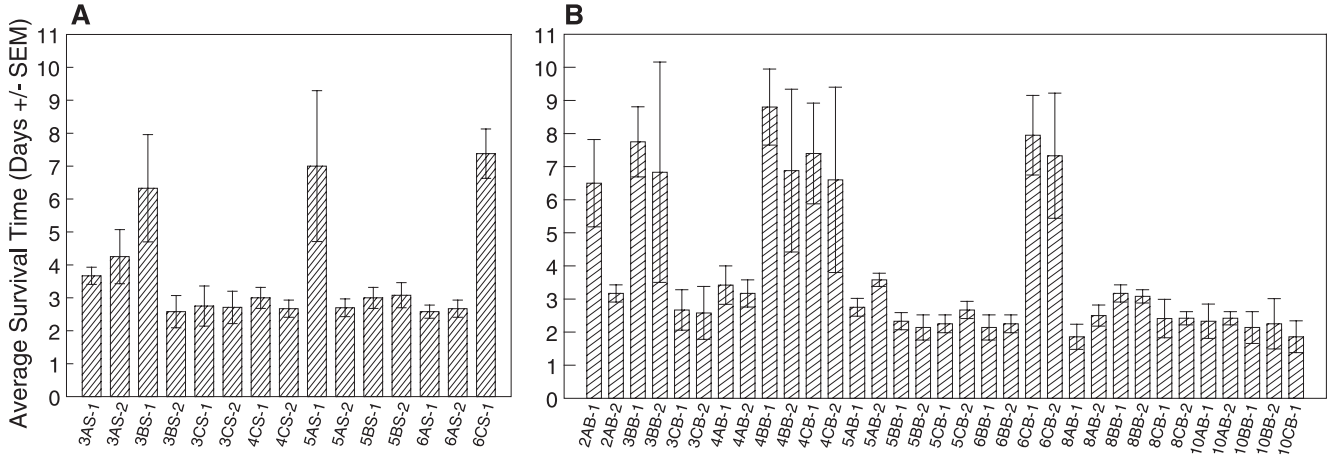


Fig. 3. Average survival times of neonatal mice infected with virus plaque isolated from TRSB-NE2G216-infected mice. Neonatal mice were inoculated subcutaneously with $10^3$ PFU of virus, plaque isolated from serum (panel A), or brain (panel B) of TRSB-NE2G216-infected mice. ASTs are shown in days ± standard error of the mean (SEM). The source of the plaque isolate is indicated by the sample name: e.g., sample "3AS-1" was isolated 3 days p.i. from mouse A serum and is the first of two such isolates; sample "2AB-1" was isolated 2 days p.i. from mouse A brain and is the first of two such samples.

Table 2
Source, genotype and phenotype of in vivo-selected mutant viruses

| E3/E2 Junction | Virus ID[a] | Time isolated | Source | Virus titer (PFU/ml or g)[b] | Mortality rate[c] | AST ± SD (days)[c] | BHK S.I.[d] | PE2 incorporation[e] |
|---|---|---|---|---|---|---|---|---|
| G R S K R N V T | 3BS-1 | 3 dpi | Serum | 700 | 100 % | 6.33 ± 1.63 | 1.0 | 0.259 ± 0.018 |
| (E2 Asn 1/Thr 3) | 3BB-1 | | Brain | 500 | 33 % | 7.75 ± 1.06 | | (~100%) |
| | 3BB-2 | | | | 50 % | 6.83 ± 3.33 | | |
| | 6CS-1 | 6 dpi | Serum | 62.5 | 67 % | 7.38 ± 0.75 | | |
| | 6CB-1 | | Brain | 1.17e4 | 43 % | 7.95 ± 1.20 | | |
| | 6CB-2 | | | | 100 % | 7.33 ± 1.89 | | |
| G R S K R **R** V T | 3AS-1 | 3 dpi | Serum | 700 | 100 % | 3.67 ± 0.26 | 8.4 | 0.053 ± 0.000 |
| (E2 Arg 1/Thr 3) | 3AS-2 | | | | 100 % | 4.25 ± 0.82 | | (~20%) |
| G R S K R N V **A** | 8BB-1 | 8 dpi | Brain | 1.50e5 | 100 % | 3.17 ± 0.26 | 0.5 | 0.052 ± 0.004 |
| (E2 Asn 1/Ala 3) | 8BB-2 | | | | 100% | 3.08 ± 0.20 | | (~20%) |
| G R S K R N V **I** | 3BS-2 | 3 dpi | Serum | 2.20e3 | 100 % | 2.58 ± 0.49 | 0.6 | 0.024 ± 0.011 |
| (E2 Asn 1/Ile 3) | 3CS-1 | | | 1.01e6 | 100 % | 2.75 ± 0.61 | | (~9%) |
| | 3CS-2 | | | | 100 % | 2.71 ± 0.49 | | |
| | 3CB-1 | | Brain | 7.20e6 | 100 % | 2.67 ± 0.61 | | |
| | 3CB-2 | | | | 100 % | 2.58 ± 0.80 | | |
| | 6AS-1 | 6 dpi | Serum | 1.31e4 | 100 % | 2.58 ± 0.20 | | |
| | 6AS-2 | | | | 100 % | 2.67 ± 0.26 | | |
| | 8CB-1 | 8 dpi | Brain | 3.70e4 | 100 % | 2.41 ± 0.58 | | |
| | 8CB-2 | | | | 100 % | 2.42 ± 0.20 | | |
| G R S K R **S** V T | 6BB-1 | 6 dpi | Brain | 5.50e6 | 100 % | 2.14 ± 0.38 | 0.4 | 0.023 ± 0.007 |
| (E2 Ser 1/Thr 3) | 6BB-2 | | | | 100 % | 2.25 ± 0.27 | | (~8%) |
| | 8AB-1 | 8 dpi | Brain | 7.50e5 | 100 % | 1.86 ± 0.38 | | |
| | 8AB-2 | | | | 100 % | 2.50 ± 0.32 | | |

[a] Denotes the source of each plaque isolate as described in Fig. 3.
[b] Titer of virus in the sample from which the mutant was isolated.
[c] Mortality rate and AST following subcutaneous inoculation of neonatal mice with $10^3$ PFU amplified plaque isolate.
[d] BHK-specific infectivity normalized to 1.0 for TRSB-NE2G216 (E2 Asn 1/Thr 3).
[e] Relative extent of uncleaved PE2 incorporation calculated by the formula PE2/(PE2 + E2 + E1 + C) such that a noncleaving virus with 100% PE2 incorporation has a value of ≥0.259 (7 methionine residues in PE2 of 27 total methionine residues).

Two virus genotypes that we have characterized previously, E2 Ser 1/Thr 3 (TRSB-E2S1) and E2 Arg 1/Thr 3 (TRSB), were isolated from samples titered at $7.5 \times 10^5$–$5.5 \times 10^6$ and 700 PFU/ml or g, respectively. In addition, two new virus genotypes, E2 Asn 1/Ala 3 and E2 Asn 1/Ile 3, were isolated from samples titered at $1.5 \times 10^5$ and $2.2 \times 10^3$–$7.2 \times 10^6$ PFU/ml or g, respectively. Neonatal mouse virulence co-segregated with genotype such that the order of virulence was E2 Ser 1/Thr 3 > E2 Asn 1/Ile 3 > E2 Asn 1/Ala 3 > E2 Arg 1/Thr 3 > E2 Asn 1/Thr 3. Every paired sample (two plaque isolates from the same sample) was identical except one. From mouse 3B (3 days p.i.), three clones of E2 Asn 1/Thr 3 (TRSB-NE2G216) and one clone of E2 Asn 1/Ile 3 were isolated. The presence of more than one virus genotype (and anticipated corresponding PE2 cleavage phenotype) in the same sample or the same mouse suggested that the selection process was ongoing. However, once a virulent virus clone was selected, it rapidly became the predominant genotype. Virus clones isolated from the CNS of mice 8 days p.i. were all of a more virulent genotype, E2 Ser 1/Thr 3, E2 Asn 1/Ala 3, or E2 Asn 1/Ile 3. Because the severity of infection and predicted outcome amongst these mice ranged from fatal to nonfatal, these data suggested that selection of a more virulent virus genotype may always occur eventually, but that the time of reversion may be critical in determining the outcome of the infection.

### *PE2 cleavage phenotypes and specific infectivities of plaque isolates*

The PE2 cleavage phenotypes and specific infectivities (i.e., the ratio of incorporated cpm to PFU) of the five virus genotypes isolated from TRSB-NE2G216-infected mice were compared (Table 2). A completely noncleaving virus (100% PE2) should give a ratio of PE2/(PE2 + E2 + E1 + C) of 0.259 (7 methionine residues in PE2 of 27 total methionine residues). Although the E2 Asn 1/Thr 3 virus had a PE2 noncleaving phenotype as expected (>0.259), the remaining three genotypes exhibited partial to complete PE2 cleavage in the order E2 Arg 1/Thr 3 ($0.053 \pm 0.000$) < E2 Asn 1/Ala 3 ($0.052 \pm 0.004$), E2 Asn 1/Ile 3 ($0.024 \pm 0.011$) < E2 Ser 1/Thr 3 ($0.023 \pm 0.007$). The specific infectivity of these viruses on BHK cells was directly proportional to the PE2 content.

### *HS-binding phenotypes*

We have previously demonstrated that viruses incorporating a proportion of PE2 bind to the cell surface via HS-

mediated attachment. In order to determine the effect of the resuscitating mutation on HS-dependent infection, virus infectivity was evaluated on wild-type CHO K1 cells and CHO mutants defective in GAG synthesis (pgsA-745) (Esko et al., 1985; Klimstra et al., 1998). On the basis of variable cytopathic effect on these CHO-derived cell lines, double-promoter viruses expressing green fluorescent protein (GFP) were used in a fluorescent center assay to evaluate infectivity (Fig. 4). Although the PE2 noncleaving virus, TRSB-N, is known to bind efficiently to wild-type CHO K1 cells via an HS-dependent mechanism, as expected, the virus had extremely limited infectivity. The resuscitating E2 Gly 216 mutation in TRSB-NE2G216 restored infectivity for CHO K1 cells, although HS-dependent cell-surface attachment remained unaffected (data not shown). The resuscitating mutation alone in the TRSB background (TRSB-E2G216) did not confer an attachment/infectivity advantage over TRSB.

The HS-dependence of the plaque-isolated revertants selected in vivo was assessed in infectivity assays. GFP-expressing double promoter viruses encoding E2 Ser 1/Thr 3 (E2S1-3′GFP), E2 Arg 1/Thr 3 (TRSB-3′GFP), and E2 Arg 1/Thr 3 with E2 Gly 216 (TRSB-E2G216-3′GFP) were already available. An RT-PCR amplified fragment was cloned into the E2S1-3′GFP double-promoter virus between the *Stu*I and *Bss*HII restriction sites, thereby replacing the E3/E2 protein junction of PE2 and introducing the E2 Ser 1, E2 Ala 3, E2 Ile 3 mutations identified in the plaque-isolated revertants in combination with E2 Gly 216. The relative numbers of GFP-positive infected cells on CHO K1 and GAG-deficient pgsA-745 cells were analyzed (Fig. 4). Decreased efficiency of HS-binding correlated with increased virulence and with increased extent of PE2 cleavage.

## Discussion

In this study, we have characterized the pathogenesis of the resuscitated PE2 noncleaving TRSB-NE2G216 Sindbis virus variant, to further investigate the effects of HS-mediated attachment on virulence in vivo. Enhanced infectivity mediated by binding of the BXBB furin cleavage site to cell-surface HS appears to be a delicate balance between increased attachment efficiency and decreased fusion competence. Thus, partial PE2 noncleaving viruses such as TRSB are more infectious in vitro than consensus wild-type TR339 virus (which almost completely cleaves PE2), although the PE2 noncleaving virus, TRSB-N, is nonviable. In vitro, we believe that the attenuating factor is the inability of the virus to fuse with host cell membranes after binding to the cell surface (Smit et al., 2001). The resuscitating mutation at E2 position 216 and other resuscitating mutations identified by Heidner et al. (1994) restore the ability of the PE2 noncleaving virus to replicate in mammalian cells. TRSB-NE2G216 virus binds efficient-

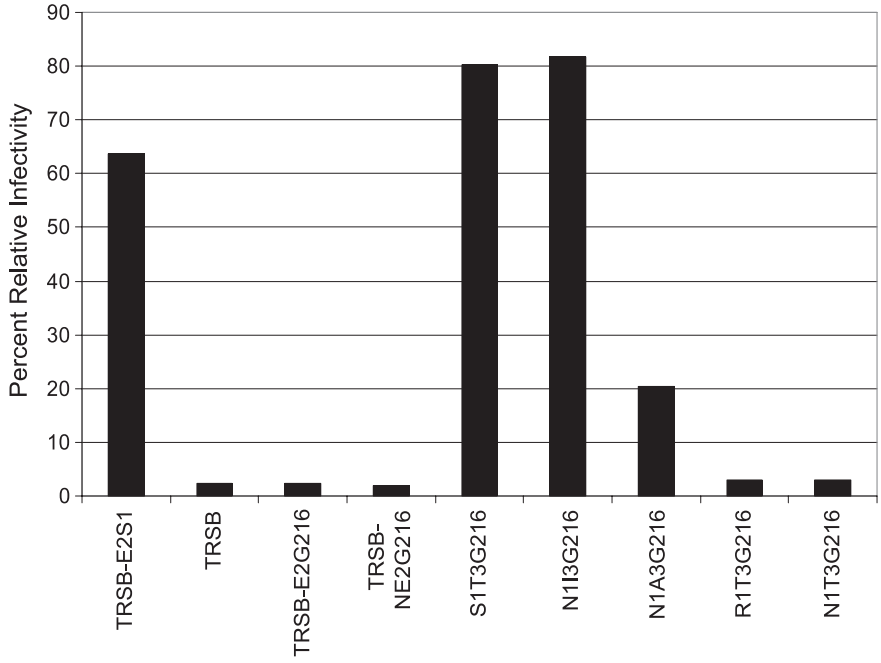


Fig. 4. HS-binding phenotypes of TRSB-NE2G216 and plaque-isolated viruses. The infectivities of TRSB-E2S1, TRSB, TRSB-E2G216, and TRSB-NE2G216 GFP-expressing double-promoter viruses for wild-type CHO-K1 cells and CHO cell mutants deficient in GAGs (pgsD-745) were compared. Monolayers of each cell type were infected with 100–200 PFU followed by three washes with PBS-1% DCS, medium replacement, and incubation for 8–12 h. Cells expressing GFP were enumerated by fluorescence microscopy. The relative infectivity was calculated as follows: (the average number of pgsD-745 cells infected/the average number of CHO K1 cells infected) × 100%. Data represent the average of three infections and were reproduced in two independent assays.

ly to cell-surface HS and is also fusion competent. Therefore, the BHK cell-specific infectivity of TRSB-NE2G216 is greater than that of TR339 and similar to that of TRSB. However, in vivo, the resuscitated TRSB-NE2G216 virus remains significantly attenuated compared with either TR339 or TRSB viruses.

*Pathogenesis of resuscitated PE2 noncleaving virus*

In neonatal mice, Sindbis virus strains cause a range of disease phenotypes. Replication of a given virus strain may be quantitatively, but not qualitatively, distinct, leading to a very different innate immune response that apparently determines the outcome of the infection. At one end of the spectrum, consensus wild-type TR339 virus is highly virulent in neonatal mice when administered by subcutaneous inoculation (Klimstra et al., 1999b). Increased mortality and decreased AST were correlated with higher levels of virus replication and increased magnitude of induction of proinflammatory cytokines and systemic stress response mediators, reminiscent of a systemic inflammatory response syndrome (SIRS). This is observed in the absence of classical encephalitis. Severe thymic involution is consistent with the high levels of tumor necrosis factor alpha (TNF-α) or corticosterone. Increasing divergence from the consensus sequence with adaptation to growth in cultured cells occurs coincident with attenuation of virulence, diminished SIRS-like disease signs, and appearance of encephalitic pathology (Klimstra et al., 1999b).

Overall, the disease caused by TRSB-NE2G216 virus in neonates is significantly attenuated compared to TR339 and TRSB viruses, with 50–75% mortality and an extended AST. However, the range of disease severities exhibited by TRSB-NE2G216-infected mice is very diverse. We have compared and correlated several virulence factors to predict the outcome of a given infection. Clinical signs, titers, and pathological correlates all suggest that infection with the PE2 noncleaving virus, TRSB-NE2G216, is not consistent. Severity appears to range from rapidly fatal with extensive virus replication, cytokine induction and thymic involution (similar to TR339), to attenuated with restricted virus replication, little or no cytokine-mediated pathology and mild encephalitic sequelae.

*Reversion to virulence in vivo*

The above observations strongly suggested that a dynamic process of virus adaptation to the host was occurring during the infection. By isolating individual virus clones from the TRSB-NE2G216-infected mice, we were able to analyze this selection process at the molecular level. In addition to the original virus genotype, four predominant mutations were selected. Two of these were characterized previously, E2 Ser 1/Thr 3 (TRSB-E2S1) and E2 Arg 1/Thr 3 (TRSB), and two were unique, E2 Asn 1/Ala 3 and E2 Asn 1/Ile 3. Determination of the PE2 cleavage phenotype of the revertant viruses revealed that the reverting mutations increased cleavage of the PE2 precursor protein at the E3/E2 junction by ablating the Asn-Val-Thr glycosylation site at E2 position 1. This was achieved by mutating either the Asn at E2 position 1 to Arg or Ser, or the Thr at E2 position 3 to Ile or Ala while retaining the E2 Asn 1. The resuscitating mutation at E2 position 216 was retained in each revertant. This was not surprising as this mutation did not attenuate the virus and only appears to have a phenotype in combination with the E2 Asn 1 mutation. The reverting mutations selected also enhanced the virulence of each virus in vivo. All of the revertant viruses caused 100% mortality in neonatal mice with slightly different ASTs such that the order of virulence was E2 Ser 1/Thr 3 > E2 Asn 1/Ile 3 > E2 Asn 1/Ala 3 > E2 Arg 1/Thr 3 > E2 Asn 1/Thr 3. More than one mutation was not selected in the same animal, suggesting that once selected, the PE2 cleaving virus replicates and disseminates rapidly becoming the predominant genotype. The rapidity with which this selection occurs in vivo likely determines the outcome of infection.

*Reversion to non-HS-binding phenotype in vivo*

Although an expanding group of viruses is being shown to bind to cell-surface HS in vitro, only a limited number of studies have examined the influence of HS-mediated attachment on infection in vivo including studies of HSV (WuDunn and Spear, 1989), FMDV (Sa-Carvalho et al., 1997), VEEV (Bernard et al., 2000), Sindbis (Byrnes and Griffin, 2000; Klimstra et al., 1998), and classical swine fever virus (Hulst et al., 2001). We have demonstrated that Sindbis virus acquires the ability to bind HS as an adaptation to growth in cultured cells (Klimstra et al., 1998). We (Bernard et al., 2000; Klimstra et al., 1998, 1999a) and others (Byrnes and Griffin, 1998, 2000) have proposed that the ability of alphaviruses to bind HS results in attenuation in vivo because of a decreased ability to spread from the site of inoculation and seed a sustained serum viremia. Heparin-binding proteins are rapidly sequestered by tissue HS, particularly by the highly sulfated HS found in the liver (Bernfield et al., 1992; Lyon et al., 1994). By comparison, wild-type non-HS-binding viruses would be expected to seed a higher titer viremia, clear more slowly from the blood, and likely cause higher mortality (Bernard et al., 2000; Byrnes and Griffin, 2000; Klimstra et al., 1998). Similar conclusions have been made for other viruses including FMDV (Sa-Carvalho et al., 1997) and classical swine fever virus (Hulst et al., 2001). Byrnes and Griffin (2000) have directly linked the rapid clearance of certain attenuated Sindbis virus strains from the bloodstream with the HS-binding phenotype and demonstrated that there are strong selection pressures in vivo against cell culture-adaptive, positive-charge amino acid mutations, and for the ability to disseminate efficiently throughout the host in the circulatory system.

We have demonstrated in this study and previously that the resuscitated PE2 noncleaving viruses are not significantly restricted in their ability to infect and propagate within mammalian cells in vitro (Heidner et al., 1994), although their replication remains defective in arthropod cells because of a block in egress (Boehme et al., 2000; Heidner et al., 1996). However, although TRSB-NE2G216 virus binds better to and is more infectious for mammalian cells than the consensus wild-type TR339 virus, the virus spontaneously reverted to a PE2-cleaving, low/non-HS-binding phenotype during infection of neonatal mice by ablation of the glycosylation site at E2 position 1. We believe this is, at least in part, due to a strong positive selection pressure for the ability to spread in vivo. The reversion event appeared to be relatively inefficient and occurred at different times p.i. resulting in less than 100% mortality and divergent survival times. These data further suggest that while the presence of PE2 is necessary to protect the virus from premature fusion with the infected host cell endoplasmic reticulum during egress (Carleton et al., 1997), the PE2 precursor protein must be cleaved during maturation to facilitate dissemination of the virus within the host. The virus is able to mutate rapidly in the infected animal to maximize its ability to replicate, seed a high titer serum viremia, and disseminate throughout the host. This capability is of critical importance in the lifecycle of any arthropod-borne virus in nature, to facilitate transmission via a blood meal to the mosquito vector.

While these data provide a strong correlation between incorporation of PE2 in the virion, the ability to bind to HS and attenuation of the virus in vivo, viruses with Asn at E2 position 1 also differ from PE2-cleaving viruses by the retention of E3 in the virion and by the possession of an extra glycosylation site. Mutations to Leu or Val at E2 position 1 have also been shown to block PE2 cleavage and attenuate virulence of Sindbis virus (Heidner and Johnston, 1994; Klimstra et al., 1999a), suggesting that addition of a glycosylation site is not solely responsible for reduced virulence. We have recently demonstrated, however, that carbohydrate modifications on the virion surface in mosquito-derived Sindbis virus mediate attachment to cell-surface C-type lectin molecules, dendritic cell (DC-SIGN), and liver/lymph node (L-SIGN)-specific ICAM-3 grabbing non-integrins (Klimstra et al., 2003). It is also conceivable that the retention of E3 in the mature particle may contribute to the attenuated phenotype by as yet unidentified mechanisms.

### *Relevance to other virus families*

In the last 5 years, HS binding has been implicated in the in vitro infection of cells by laboratory strains of a large number of viruses. However, with the exception of herpesviruses (WuDunn and Spear, 1989), a role for HS interaction has not been established with natural virus isolates. For several of these viruses, including Sindbis virus (Klimstra et al., 1998), VEEV (Bernard et al., 2000), Murray Valley encephalitis virus (Lee and Lobigs, 2000, 2002), FMDV (Jackson et al., 1996; Sa-Carvalho et al., 1997), and classical swine fever virus (Hulst et al., 2000, 2001), the ability to bind cell-surface HS has been linked with cell culture adaptation and attenuation in vivo.

The surface glycoproteins of many enveloped viruses, including retroviruses (Hallenberger et al., 1992), paramyxoviruses (Bolt and Pedersen, 1998; Bolt et al., 2000; Fujii et al., 1999), Borna disease virus (Gonzalez-Dunia et al., 1998), filoviruses (Feldmann et al., 1999), orthomyoviruses (Jankovics, 1996), and flaviviruses (Chambers et al., 1990), are initially synthesized as inactive precursors, and proteolytic cleavage is often required for maturation and fully functional activity. In several virus families, this processing step is carried out by cellular proprotein convertases, most commonly furin, a component of the constitutive secretory pathway of many different types of cells. Therefore, many of these viruses possess potential HS-binding domains. Therefore, this study has wide applicability, particularly amongst viruses that are dependent upon a serum viremia for dissemination.

## Materials and methods

### *Cell culture*

Baby hamster kidney cells (BHK-21: ATCC CCL-10) were maintained in alpha minimal essential medium (αMEM), supplemented with 10% donor calf serum (DCS), 2.9 mg/ml tryptose phosphate, 0.29 mg/ml L-glutamine, 100 U/ml penicillin, and 0.05 mg/ml streptomycin (37 °C; 5% $CO_2$). Chinese hamster ovary (CHO K1; ATCC CRL-61) and CHO mutant (psgA-745; ATCC CRL-2242) cells were maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum.

### *Viruses*

Virus stocks were generated from cDNA clones. Construction of the pTR339, pTRSB, pTRSB-E2S1, pTRSB-N, pTRSB-E2G216, pTRSB-NE2G216 plasmids has been described previously (Heidner and Johnston, 1994; Heidner et al., 1994; Klimstra et al., 1998; McKnight et al., 1996). Viruses expressing GFP from a second 26S subgenomic promoter were constructed by introducing the *mut2-gfp* gene (kindly provided by Dr. Stanley Falkow, Stanford University) into the Sindbis virus double 26S promoter vector, pTE3′2J, a generous gift from Dr. Charles Rice (Rockefeller University). The *Bsi*WI–*Xho*I fragment of pTE3′ 2J-GFP was cloned into pTRSB. This construct contains a second copy of the 26S subgenomic promoter driving expression of the *gfp* gene downstream of the authentic 26S promoter, but is otherwise isogenic with pTRSB (pTRSB-3′ GFP). By exchanging a *Stu*I–*Bss*HII restriction fragment

flanking the E2 gene, GFP-expressing double-promoter viruses coated in the E2 protein variants were generated. All genetic manipulations were confirmed by DNA sequencing at the UNC-CH Automated DNA Sequencing Facility using a model 373A DNA sequencer and the Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). Infectious viral RNA was transcribed in vitro from *Xho*I-linearized plasmid DNA template and transfected into BHK-21 cells by electroporation. Virus particles were harvested from the supernatant after 18–20 h, clarified by centrifugation, and stored at −70 °C in single-use aliquots. Virus stocks were titered by standard BHK-21 cell plaque assay and titers were expressed as PFU/ml. For mouse inoculations, a thawed virus stock aliquot was diluted in low-endotoxin, phosphate-buffered saline supplemented with 1% DCS (PBS-1% DCS). Single-use aliquots of diluted virus were stored at −70 °C and the titer of one thawed aliquot was confirmed by BHK plaque assay.

*Mouse procedures*

Timed pregnant CD-1 mice (13–15 days gestation) were obtained (Charles River). Mouse pups born within a 12-h period were pooled and randomized as 10–12 pup litters. Virus inocula containing $10^3$ PFU virus in 50 μl ($2 \times 10^4$ PFU/ml) were administered subcutaneously in the ventral thorax using a 27-gauge needle and 1-ml hypodermic syringe. Mock-infected mice received 50 μl of PBS-1% DCS by the same route. Virus-infected and corresponding mock-infected mice were observed at 12-h intervals, scored for degree of sickness, and weighed at 24-h intervals where appropriate. Individual mouse pups were identified by India ink ''tattooing'' of the hindquarters. AST and percent mortality were calculated.

*Pathogenesis studies*

At predetermined intervals, p.i. groups of three mice per treatment were randomly selected from different litters. The thoracic cavity of each mouse was opened under Metofane-anesthesia (Schering-Plough) and blood was collected by cardiac puncture. Serum was separated from whole blood using microtainer tubes (Becton-Dickinson), aliquoted, and stored at −70 °C. Each mouse was perfused with PBS-1% DCS for 10 min at a rate of 1.5 ml/min. Brain tissues were homogenized in PBS-1% DCS (33% suspension) by one freeze-thaw and mechanical disruption, and clarified by centrifugation (13 000 × *g*, 15 min, 4 °C). The supernatant was assayed for virus by standard plaque assay or for IFN-α/β as described below.

*IFN-α/β assay*

Serum IFN-α/β was titered by standard biological assay on L929 cells as described previously (Trgovcich et al., 1996), using a commercially prepared IFN-α/β standard (Access Biomolecular) and encephalomyocarditis virus (EMCV) as the indicator virus. The endpoint was defined as the dilution of IFN-α/β required to protect 50% of the cells from EMCV-induced cpe and the level of IFN-α/β was expressed as international units (IU)/ml or g.

*Histopathology*

Three mice per treatment were randomly selected from different litters. Under anesthesia, each mouse was perfused with 4% paraformaldehyde (PFA) in PBS (pH 7.4) as described above. Whole PFA-perfused mice were further fixed in 4% PFA (pH 7.4) for 1 week when tissues were paraffin-embedded and sectioned. Hematoxylin and eosin (H&E) stained sections were viewed by light microscopy.

*Revertant viruses*

Individual virus plaques formed on BHK cells were isolated using sterile plastic 1-ml Pasteur pipettes and resuspended in supplemented αMEM. The virus from each plaque was amplified by one round of replication in BHK cells infected at high moi. Viral RNA was extracted from virus particles in the supernatant using Ultraspec reagent (Biotecx), and the E2 gene was amplified by RT-PCR. PCR products were sequenced as described above. PCR fragments were digested with *Stu*I and *Bss*HII and cloned directly into the GFP-expressing double-promoter virus infectious clone template to generate mutant virus.

*Fluorescent center assay*

Fluorescent center assays were performed essentially as described by Klimstra et al. (1998). CHO K1 and CHO mutant (pgsA-745) cells seeded in 24-well plates were infected for 1 h at 37 °C with GFP-expressing double-promoter viruses serially diluted in 200 μl PBS-1% DCS. Monolayers were washed twice with PBS-1% DCS and overlaid with 1% immunodiffusion agarose (ICN)-containing growth medium. Approximately 24 h p.i., plaques of virus-infected cells expressing GFP were enumerated under a Nikon fluorescence microscope using a fluorescein isothiocyanate filter block.

*Specific infectivity assays*

Radiolabeled virus was prepared by incubation of virus-infected BHK cell monolayers with 10 μCi/ml [$^{35}$S]-methionine (Amersham), purified on a discontinuous sucrose gradient and concentrated by pelleting through sucrose (Heidner and Johnston, 1994; Klimstra et al., 1998). The BHK cell-specific infectivity (PFU per count per minute

[cpm]) was calculated for each virus preparation by plaque assay on BHK cells (Klimstra et al., 1998).

*Polyacrylamide gel analysis of [$^{35}$S]-methionine-labeled viral proteins*

Individual structural proteins from [$^{35}$S]-methionine-labeled virus particles were resolved by SDS-PAGE (10% acrylamide) under reducing conditions (50 mM 2-mercaptoethanol) (Klimstra et al., 1999a). The radiolabeled protein bands were visualized with a Storm model PhosphorImager and ImageQuant software (Molecular Dynamics). The relative PE2 cleavage efficiency was determined by dividing the image densities of bands corresponding to PE2 by the total image density of PE2, E1, E2, and C proteins. The results slightly underestimate the difference between cleaving and noncleaving viruses due to the loss of E3, which contains one methionine (a total of 27 in PE2), from the PE2-cleaving virus lanes.

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## References

Bernard, K.A., Klimstra, W.B., Johnston, R.E., 2000. Mutations in the E2 glycoprotein of Venezuelan equine encephalitis virus confer heparan sulfate interaction, low morbidity, and rapid clearance from blood of mice. Virology 276, 93–103.

Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M.T., Spring, J., Gallo, R.L., Lose, E.J., 1992. Biology of the syndecans: a family of transmembrane heparan sulfate proteoglycans. Annu. Rev. Cell Biol. 8, 365–393.

Boehme, K.W., Williams, J.C., Johnston, R.E., Heidner, H.W., 2000. Linkage of an alphavirus host-range restriction to the carbohydrate-processing phenotypes of the host cell. J. Gen. Virol. 81, 161–170.

Bolt, G., Pedersen, I.R., 1998. The role of subtilisin-like proprotein convertases for cleavage of the measles virus fusion glycoprotein in different cell types. Virology 252, 387–398.

Bolt, G., Pedersen, L.O., Birkeslund, H.H., 2000. Cleavage of the respiratory syncytial virus fusion protein is required for its surface expression: role of furin. Virus Res. 68, 25–33.

Burgess, T.H., Steele, K.E., Schoneboom, B.A., Grieder, F.B., 2001. Clinicopathologic features of viral agents of potential use by bioterrorists. Clin. Lab. Med. 21, 475–493 (viii).

Byrnes, A.P., Griffin, D.E., 1998. Binding of Sindbis virus to cell surface heparan sulfate. J. Virol. 72, 7349–7356.

Byrnes, A.P., Griffin, D.E., 2000. Large-plaque mutants of Sindbis virus show reduced binding to heparan sulfate, heightened viremia, and slower clearance from the circulation. J. Virol. 74, 644–651.

Cardin, A.D., Weintraub, H.J.R., 1989. Molecular modeling of protein–glycosaminoglycan interactions. Arteriosclerosis 9, 21–32.

Carleton, M., Lee, H., Mulvey, M., Brown, D.T., 1997. Role of glycoprotein PE2 in formation and maturation of the Sindbis virus spike. J. Virol. 71, 1558–1566.

Chambers, T.J., Hahn, C.S., Galler, R., Rice, C.M., 1990. Flavivirus genome organization, expression, and replication. Annu. Rev. Microbiol. 44, 649–688.

Esko, J.D., Stewart, T.E., Taylor, W.H., 1985. Animal cell mutants defective in glycosaminoglycan biosynthesis. Proc. Natl. Acad. Sci. U.S.A. 82, 3197–3201.

Feldmann, H., Volchkov, V.E., Volchkova, V.A., Klenk, H.D., 1999. The glycoproteins of Marburg and Ebola virus and their potential roles in pathogenesis. Arch. Virol., Suppl. 15, 159–169.

Fujii, Y., Sakaguchi, T., Kiyotani, K., Yoshida, T., 1999. Comparison of substrate specificities against the fusion glycoprotein of virulent Newcastle disease virus between a chick embryo fibroblast processing protease and mammalian subtilisin-like proteases. Microbiol. Immunol. 43, 133–140.

Gardner, J.P., Frolov, I., Perri, S., Ji, Y., MacKichan, M.L., Zur, M.J., Chen, M., Belli, B.A., Driver, D.A., Sherrill, S., Greer, C.E., Otten, G.R., Barnett, S.W., Liu, M.A., Dubensky, T.W., Polo, J.M., 2000. Infection of human dendritic cells by a Sindbis virus replicon vector is determined by a single amino acid substitution in the E2 glycoprotein. J. Virol. 74, 11849–11857.

Gonzalez-Dunia, D., Cubitt, B., de la Torre, J.C., 1998. Mechanism of Borna disease virus entry into cells. J. Virol. 72, 783–788.

Hallenberger, S., Bosch, V., Angliker, H., Shaw, E., Klenk, H.D., Garten, W., 1992. Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. Nature 360, 358–361.

Heidner, H.W., Johnston, R.E., 1994. The amino-terminal residue of Sindbis virus glycoprotein E2 influences virus maturation, specific infectivity for BHK cells, and virulence in mice. J. Virol. 68, 8064–8070.

Heidner, H.W., McKnight, K.L., Davis, N.L., Johnston, R.E., 1994. Lethality of PE2 incorporation into Sindbis virus can be suppressed by second-site mutations in E3 and E2. J. Virol. 68, 2683–2692.

Heidner, H.W., Knott, T.A., Johnston, R.E., 1996. Differential processing of Sindbis virus glycoprotein PE2 in cultured vertebrate and arthropod cells. J. Virol. 70, 2069–2073.

Huang, H.V., 1996. Sindbis virus vectors for expression in animal cells. Curr. Opin. Biotechnol. 7, 531–535.

Hulst, M.M., van Gennip, H.G., Moormann, R.J., 2000. Passage of classical swine fever virus in cultured swine kidney cells selects virus variants that bind to heparan sulfate due to a single amino acid change in envelope protein E(rns). J. Virol. 74, 9553–9561.

Hulst, M.M., van Gennip, H.G., Vlot, A.C., Schooten, E., de Smit, A.J., Moormann, R.J., 2001. Interaction of classical swine fever virus with membrane-associated heparan sulfate: role for virus replication in vivo and virulence. J. Virol. 75, 9585–9595.

Jackson, T., Ellard, F.M., Abu Ghazaleh, R., Brookes, S.M., Blakemore, W.E., Corteyn, A.H., Stuart, D.I., Newman, J.W.I., King, A.M.Q., 1996. Efficient infection of cells in culture by type O foot and mouth disease virus requires binding to cell surface heparan sulfate. J. Virol. 70, 5282–5287.

Jankovics, I., 1996. The influenza viruses: past, present and future. Lege Artis Med. 6, 62–68.

Johnston, R.E., Peters, C.J., 1996. Alphaviruses. In: Fields, B.N., Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, vol. 1. Lippincott-Raven, Philadelphia, pp. 843–898.

Klimstra, W.B., Ryman, K.D., Johnston, R.E., 1998. Adaptation of Sindbis virus to BHK cells selects for use of heparan sulfate as an attachment receptor. J. Virol. 72, 7357–7366.

Klimstra, W.B., Heidner, H., Johnston, R.E., 1999a. The furin protease cleavage recognition sequence of Sindbis virus PE2 can mediate virion attachment to cell surface heparan sulfate. J. Virol. 73, 6299–6306.

Klimstra, W.B., Ryman, K.D., Bernard, K.A., Nguyen, K.B., Biron, C.A., Johnston, R.E., 1999b. Infection of neonatal mice with Sindbis virus results in a systemic inflammatory response syndrome. J. Virol. 73, 10387–10398.

Klimstra, W.B., Nangle, E.M., Smith, M.S., Yurochko, A.D., Ryman, K.D., 2003. DC-SIGN and L-SIGN can act as attachment receptors for alphaviruses and distinguish between mosquito cell- and mammalian cell-derived viruses. J. Virol. 77, 12022–12032.

Lee, E., Lobigs, M., 2000. Substitutions at the putative receptor-binding site of an encephalitic flavivirus alter virulence and host cell tropism and reveal a role for glycosaminoglycans in entry. J. Virol. 74, 8867–8875.

Lee, E., Lobigs, M., 2002. Mechanism of virulence attenuation of glycosaminoglycan-binding variants of Japanese encephalitis virus and Murray Valley encephalitis virus. J. Virol. 76, 4901–4911.

Lyon, M., Deakin, J.A., Gallagher, J.T., 1994. Liver heparan sulfate structure. A novel molecular design. J. Biol. Chem. 269, 11208–11215.

McKnight, K.L., Simpson, D.A., Lin, S.-C., Knott, T.A., Polo, J.M., Pence, D.F., Johannsen, D.B., Heidner, H.W., Davis, N.L., Johnston, R.E., 1996. Deduced consensus sequence of Sindbis virus strain AR339: mutations contained in laboratory strains which affect cell culture and in vivo phenotypes. J. Virol. 70, 1981–1989.

Polo, J.M., Belli, B.A., Driver, D.A., Frolov, I., Sherrill, S., Hariharan, M.J., Townsend, K., Perri, S., Mento, S.J., Jolly, D.J., Chang, S.M., Schlesinger, S., Dubensky Jr., T.W., 1999. Stable alphavirus packaging cell lines for Sindbis virus and Semliki Forest virus-derived vectors. Proc. Natl. Acad. Sci. U.S.A. 96, 4598–4603.

Pugachev, K.V., Mason, P.W., Shope, R.E., Frey, T.K., 1995. Double-subgenomic Sindbis virus recombinants expressing immunogenic proteins of Japanese encephalitis virus induce significant protection in mice against lethal JEV infection. Virology 212, 587–594.

Sa-Carvalho, D., Reider, E., Baxt, B., Rodarte, R., Tanuri, A., Mason, P., 1997. Tissue culture adaptation of foot-and-mouth disease virus selects viruses that bind to heparin and are attenuated in cattle. J. Virol. 71, 5115–5123.

Smit, J.M., Klimstra, W.B., Ryman, K.D., Bittman, R., Johnston, R.E., Wilschut, J., 2001. PE2 cleavage mutants of Sindbis virus: correlation between viral infectivity and pH-dependent membrane fusion activation of the spike heterodimer. J. Virol. 75, 11196–11204.

Smith, J.F., Davis, K., Hart, M.K., Ludwig, G.V., McClain, D.J., Parker, M.D., Pratt, W.D., 1997. Viral encephalitides. In: Sidell, F.R., Takafuji, E.T., Franz, D.R. (Eds.), Medical Aspects of Chemical and Biological Warfare. Office of The Surgeon General, Department of the Army, WA, USA, p. 561.

Strauss, J.H., Strauss, E.G., 1994. The alphaviruses: gene expression, replication, and evolution. Microbiol. Rev. 58, 491–562.

Trgovcich, J., Aronson, J.F., Johnston, R.E., 1996. Fatal Sindbis virus infection of neonatal mice in the absence of encephalitis. Virology 224, 73–83.

Trgovcich, J., Ryman, K., Extrom, P., Eldridge, J.C., Aronson, J.F., Johnston, R.E., 1997. Sindbis virus infection of neonatal mice results in a severe stress response. Virology 227, 234–238.

Tseng, J.C., Levin, B., Hirano, T., Yee, H., Pampeno, C., Meruelo, D., 2002. In vivo antitumor activity of Sindbis viral vectors. J. Natl. Cancer Inst. 94, 1790–1802.

Vilcek, J., 1964. Production of interferon by newborn and adult mice infected with Sindbis virus. Virology 22, 651–652.

WuDunn, D., Spear, P.G., 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. 63, 52–58.