Linkage of an alphavirus host-range restriction to the carbohydrate-processing phenotypes of the host cell

Karl W. Boehme,¹ Jacqueline C. Williams,¹ Robert E. Johnston² and Hans W. Heidner¹

¹Division of Life Sciences, University of Texas at San Antonio, 6900 North Loop 1604 West, San Antonio, TX 78249-0662, USA
²Department of Microbiology and Immunology, School of Medicine, University of North Carolina, Chapel Hill, NC 27599-7290, USA

The Sindbis virus mutant NE2G216 retains PE2 in place of E2 in its virion structure. NE2G216 is a host-range mutant that replicates with near-normal kinetics in vertebrate cells, but displays severely restricted growth in cultured mosquito cells (C6/36) due to defects in the virus maturation process. In this study we tested the hypothesis that the host-range phenotype of NE2G216 was linked to the differences in carbohydrate-processing phenotypes between vertebrate and arthropod cells. Arthropod cell-derived glycoproteins are distinguishable from those synthesized in vertebrate cells by the absence of complex- and hybrid-type N-linked oligosaccharides. To test our hypothesis we compared the growth of the wild-type virus, TRSB, NE2G216 and three PE2-containing, C6/36 cell-adapted variants, in vertebrate cells treated with 1-deoxymannojirimycin (1-dMM). 1-dMM inhibits the Golgi α-mannosidase I enzyme and limits oligosaccharide processing to high-mannose forms (Man₉,9GlcNAc₂). The growth of TRSB was not restricted by the action of 1-dMM; however, NE2G216 was restricted in a dose-dependent manner. In contrast, the growth of each PE2-containing, C6/36 cell-adapted mutant was enhanced by low concentrations of 1-dMM (up to 1500%) and was only slightly affected by the higher concentrations. These results demonstrate that virion maturation functions of NE2G216 are sensitive to the structure of cis-linked oligosaccharides, and indicate that the carbohydrate-processing phenotypes of the host cell can influence viral host-range and function as a selective pressure in alphavirus evolution.

Introduction

Alphaviruses are maintained in nature in a cycle that involves alternative infections of vertebrate and mosquito hosts. Alternating host replication very likely constrains the evolution of alphaviruses by selecting for only those viruses that are able to perform the multitude of functions required for productive replication within differentiated cells of both evolutionarily diverged host species. Repeated passage of alphaviruses within a single host-cell type often selects for host-range variants that are markedly less fit within cells of the alternative host (for reviews see Strauss & Strauss, 1994; Weaver et al., 1999). Although the viral mutations which are responsible for viral host-range phenotypes have been readily identified for many such viruses, the cellular basis for the host-range phenotype has been more difficult to define.

We have described a group of host-range variants of Sindbis virus AR339 which are growth-restricted in cultured mosquito cells (Heidner et al., 1994). Each of these variants harboured a mutation that prevented cleavage of the PE2 glycoprotein into E3 and E2, and each was physically distinguishable from the parental virus by the retention of PE2 in place of E2 in the virion structure. Preliminary inquiries into the cellular basis for the host-range phenotype expressed by these mutants have focused on a variant designated NE2G216 (Heidner et al., 1996). NE2G216 was derived from a non-viable parental virus designated TRSB-N (Heidner et al., 1994). TRSB-N is isogenic with the wild-type virus, TRSB, except for a single mutation in PE2 which altered the amino acid immediately adjacent to the site at which PE2 is cleaved (Asn for Arg at E2 residue 1). This substitution generated a signal for N-linked glycosylation which is utilized during virus replication (Heidner et al., 1994). Carbohydrate addition at this position is thought to prevent PE2 cleavage by restricting access to the cleavage site by the host-cell endoprotease, furin. TRSB-N virions mature normally in BHK-21 cells transfected with in vitro viral transcripts; however, they retain PE2 in place of E2 and are essentially non-infectious (Heidner et al., 1994; Paredes et al., 1998). NE2G216 was among a group of infectious, PE2-
containing revertants isolated from the supernatants of TRSB-N-transfected BHK-21 cells. NE2G216 replicates to near wild-type levels in vertebrate cells. In C6/36 cells, NE2G216 is normal in the processes of cell binding, entry and gene expression, but is specifically blocked in a late step of virus maturation. The replication defect of NE2G216 is not linked to the reduced temperature (28 °C) at which C6/36 cells are maintained (Heidner et al., 1996). The facts that NE2G216 contains mutations within a glycoprotein and is defective in a glycoprotein-mediated viral process suggests that its host-range phenotype is linked to a cellular phenotype(s) that differs between vertebrate and arthropod cells, and which must influence the structural and/or functional properties of glycoproteins.

One fundamental cellular process that differs significantly between cells of vertebrate and arthropod origin, and which is known to influence the conformational and functional properties of glycoproteins, is the enzymatic modification of N-linked oligosaccharides. In vertebrate cells, and in C6/36 cells, carbohydrate processing begins in the rough endoplasmic reticulum (RER) with the transfer of $\text{Glc}_3\text{Man}_4\text{GlcNAc}_2$-groups from $\text{Glc}_3\text{Man}_4\text{GlcNAc}_2\text{PP}$-dolichol to Asn residues contained in the proper context. This is followed by the rapid removal of terminal glucose residues and a variable number of mannose residues by glucosidase enzymes and mannosidase enzymes, respectively (Hsieh & Robbins, 1984; Kornfeld & Kornfeld, 1985). However, from this point the fates of these carbohydrate-processing intermediates differ in the two cell types. Vertebrate cells can convert the carbohydrate groups into complex and hybrid forms through the addition of N-acetylglucosamine, galactose, fucose and sialic acid (Kornfeld & Kornfeld, 1985). In contrast, the processing of N-linked oligosaccharides in arthropod cells is typically limited to the synthesis of low-mannose and high-mannose forms (Stollar et al., 1976; Butters & Hughes, 1981; Butters et al., 1981; März et al., 1995). Predictably, the composition of oligosaccharides bound to Sindbis virus glycoproteins E1 (2 sites) and E2 (2 sites) was shown to differ significantly between virions propagated in vertebrate or arthropod cells (Rice et al., 1981; Hsieh et al., 1983; Hsieh & Robbins, 1984; Mayne et al., 1985). When Sindbis virus was grown in BHK-21 cells, individual sites in E1 and E2 were linked exclusively to complex-type oligosaccharides. The remaining site in E2 was linked to a high-mannose oligosaccharide ($\text{Man}_{2-3}\text{GlcNAc}$), and the remaining site in E1 bound either a high-mannose or complex-type oligosaccharide (Hsieh et al., 1983; Mayne et al., 1985). When Sindbis virus was grown in C6/36 cells, individual sites on E1 and E2 bound either $\text{Man}_{2}\text{GlcNAc}$, $\text{Man}_{2}\text{GlcNAc}_2$, or $\text{Man}_{2}\text{GlcNAc}_3$ (Hsieh & Robbins, 1984).

In this study we tested the hypothesis that the host-range phenotype of NE2G216 was linked to the differences in the carbohydrate-processing phenotypes between vertebrate and arthropod cells. This hypothesis predicted that the maturation functions of the NE2G216 glycoproteins are dependent upon the structures of their cis-linked oligosaccharides, and that one or more critical functions are compromised if N-linked oligosaccharides cannot be processed beyond low- or high-mannose forms. It follows that the maturation defect experienced by NE2G216 in C6/36 cells is linked to the inability of these cells to synthesize complex- or hybrid-type N-linked oligosaccharides. To test this hypothesis, we compared the growth of TRSB, NE2G216 and several PE2-containing variants that were specifically adapted for growth on C6/36 cells, in a vertebrate cell line under conditions that restrict the ability of these cells to process N-linked oligosaccharides beyond high-mannose forms, and thus approximate, albeit not exactly, the carbohydrate-processing phenotypes of C6/36 cells.

### Methods

#### Viruses and cell lines.

The parental virus (TRSB), the noninfectious variant (TRSB-N) and infectious revertants of TRSB-N (TRSB-NE2G216 and TRSB-NE2R1; referred to in this report as NE2G216 and NE2R1, respectively), have been described previously (Heidner et al., 1994; McKnight et al., 1996).

BHK-21 cells were obtained from the ATCC. C6/36 cells were originally derived from mosquito larvae (Aedes albopictus) (Igarashi, 1978). Growth conditions for BHK-21 and C6/36 cells were as described (Heidner et al., 1994). The Chinese hamster ovary cell line Pro-5 was obtained from the ATCC. Pro-5 cells were maintained in alpha minimum essential medium (MEM) supplemented with 10% donor calf serum (DCS), 10% tryptose phosphate broth (TPB) and antibiotics (MEM-complete).

#### Virus growth in vertebrate and arthropod cell lines.

The kinetics of virus growth were determined for TRSB and mutant viruses in C6/36 and Pro-5 cells. Infections were initiated by infection with free virus at an m.o.i. of 10 p.f.u. per cell. Infections of Pro-5 cells were performed on duplicate monolayers of cells grown in 60 mm Petri dishes ($2 \times 10^6$ cells per dish) and infection of C6/36 cells was done in suspension ($1 \times 10^7$ cells per infection). Virus was allowed to adsorb to cells for 30 min and unadsorbed virus was removed by repeated washes with PBS supplemented with 1% DCS and antibiotics. Cells were then grown in appropriate growth medium and placed at the appropriate growth temperatures (37 °C for Pro-5 cells and 28 °C for C6/36 cells). Supernatant samples were collected at regular intervals post-infection, clarified by microcentrifugation and stored at −70 °C until assayed. Infectious virus in each sample was quantified by plaque titration on BHK-21 cells. Virus titres are reported as the average of the duplicate samples.

C6/36 cells also were infected by electroporation of in vitro viral transcripts as described (Balasuriya et al., 1999), except that cells were used at a concentration of $5 \times 10^7$ cells/mL. Prior to electroporation, transcripts were analysed by agarose gel electrophoresis to check transcript quality, and to make visual estimates of transcript quantities. Based on the estimates made from visualization of gels, approximately 10 µg of RNA was used for each electroporation. Electroporations were performed in quadruplicate for each virus sample. Replicate samples were pooled into 50 ml of growth medium, gently mixed and distributed equally into ten 60 mm cell-culture dishes. At designated times post-electroporation, supernatant samples were collected from two dishes, clarified of cells by microcentrifugation and frozen at −70 °C. Cells from the two dishes were collected by gentle scraping, washed five times with ice cold PBS, resuspended in 1·0 ml PBS and frozen at −70 °C to lyse...
cells. Samples were then thawed, clarified of cellular debris and quantified by plaque assay on BHK-21 cells.

**Polyacrylamide gel analysis of [35S]methionine-labelled viral proteins.** Virions were metabolically labelled with [35S]methionine during growth in BHK-21, Pro-5 and C6/36 cells as described (Heidner et al., 1994). Radioiodinated virions were purified from cell supernatants by isolation on discontinuous potassium tartrate gradients (20–35%). Potassium tartrate solutions were made in TNE buffer (0·5 M Tris–HCl, pH 7·2, 0·1 M NaCl, 0·001 M EDTA). Banded virions were pelleted through sucrose cushions (20% in TNE) by ultracentrifugation. Radioiodinated virion preparations were quantified by liquid scintillation counting (LSC) and 150 000 c.p.m. of each was resolved in SDS–polyacrylamide (10% acrylamide) as described (Laemmli, 1970), and visualized by autoradiography.

**Analysis of virus production by density-gradient centrifugation.** Four 150 mm Petri dishes of Pro-5 cells were infected with NE2G216 at an m.o.i. of 10 p.f.u. per cell. At 4 h post-infection cells were changed into methionine-free MEM. Also at this time 1-deoxynojirimycin (1-dMM) was added to two dishes to a final concentration of 1 µM. At 8 h post-infection [35S]methionine was added to each dish to a final concentration of 10 µCi/ml. At 25 h post-infection, supernatants were harvested and clarified by low-speed spin. Clarified supernatants were overlaid onto 20% sucrose cushions and centrifuged at 24 000 r.p.m. for 3 h at 4 °C to pellet virions. Virus pellets were collected in 2 ml TNE and overlaid onto 15 ml continuous potassium tartrate gradients (20–35%). Samples were centrifuged at 24 000 r.p.m. for 18 h at 4 °C. Gradient fractions (0·5 ml) were collected from the bottom of each tube and 50 µl samples of each fraction were mixed with scintillation fluid and quantified by LSC.

**Specific infectivity of virions.** Pro-5 cells in 150 mm culture dishes were infected with NE2G216 at an m.o.i. of 10 p.f.u. per cell. Virions were metabolically labelled with [35S]methionine as described (Heidner et al., 1994). Virions also were radioiodinated in Pro-5 cells treated with 0·5 mM 1-dMM. At 16 h post-infection supernatants were harvested and clarified by low-speed spin. Supernatants were then passed through a 22 µm filter and centrifuged at 24 000 r.p.m. for 3 h at 4 °C to pellet virions. Supernatants were removed from tubes and replaced with 17 ml of TNE buffer and centrifuged as before. Virus pellets were collected in 100 µl TNE buffer and incorporated label was quantified by LSC. Infectious virus in each preparation was quantified by plaque assay on monolayers of BHK-21 cells. The specific infectivity of each virus preparation was calculated as p.f.u./c.p.m.

**Isolation and characterization of PE2-containing variants derived from C6/36 cells.** C6/36 cells were transfected with TRSB-N in vitro transcripts as described above. Supernatants were harvested at 12 and 20 h post-electroporation and plated onto monolayers of BHK-21 cells. Ten plaques were picked individually with a tuberculin syringe as described (Heidner et al., 1994), and passaged once in C6/36 cells for generation of virus stocks. Viral RNA was isolated from gradient-purified virions and used in an RT–PCR to amplify cDNAs representing the capsid-PE2 coding regions. Specific regions of each cDNA were sequenced using the Taq cycle sequencing kit (United States Biochemical). Candidate reverting mutations identified in three viruses (S2, S4 and S7) were reconstructed in the genetic background of TRSB-N, and two of the mutations (S2 and S4) also were reconstructed in the genetic background of NE2G216 using an RT–PCR-based cloning strategy. To construct viruses containing the S2 and S4 mutations oligonucleotide primers were designed such that the cDNA reaction products included the mutation of interest and also contained two restriction sites, AflII (nucleotide 7999) and Stul (nucleotide 8571), which flank the E3 mutations, and which are unique sites within the cDNA clones. The S7 mutation eliminated the Stul restriction site; therefore, in order to generate a clonal cDNA product containing this mutation a different negative-sense primer was designed which flanked the BssHII restriction site located within the 3′ region of the E2 gene (nucleotide 9804). Products of the RT–PCR reactions were digested with the appropriate restriction enzymes and ligated into the cDNA clones pTRSB-N and pNE2G216 from which the corresponding fragment had been removed. Recombinant plasmids were sequenced across the entire insert region.

**Results and Discussion**

**Virus growth in Pro-5 cells treated with 1-dMM**

Relationships between carbohydrate structure and viral glycoprotein functions have been addressed by studying virus replication in the presence of chemical inhibitors of carbohydrate processing [Elbein (1987) and references within]. Sindbis virus replication was shown to be severely restricted in the presence of chemical compounds such as deoxynojirimycin, castanospermine and bromoconduritol, which target glucosidase enzymes in the RER and prevent the removal of terminal glucose residues from asparagine-linked Glc3ManαGlcNAc2 groups (Datema et al., 1984; Schlesinger et al., 1985; McDowell et al., 1987). In contrast, enzyme inhibitors that limit carbohydrate processing to high-mannose forms (e.g. 1-dMM) had little or no effect on the replication of Sindbis or other enveloped viruses (Elbein et al., 1984; Bosch et al., 1985; Repp et al., 1985; Gruters et al., 1987; McDowell et al., 1987; Naim & Koblet, 1988). 1-dMM targets the Golgi α-mannosidase I enzyme and oligosaccharides formed in its presence are limited to high-mannose structures (Manα₃GlcNAc₂) (Fuhrmann et al., 1984; Bischoff et al., 1986).

The following experiments were performed to determine if the activity of 1-dMM would restrict the growth of NE2G216 in the permissive vertebrate cell line Pro-5. Pro-5 cells were infected with TRSB or NE2G216 at an m.o.i. of 10 p.f.u. per cell and maintained in growth medium alone or in growth medium supplemented with various concentrations of 1-dMM (0·1, 1·0 or 2·5 mM). Virus production under each condition was assayed over time. Uninfected Pro-5 cells maintained in the presence of 1-dMM exhibited no obvious signs of cytopathicity at any of the concentrations used. The presence of 1-dMM did not restrict the growth of TRSB even when present at high concentrations. In contrast, NE2G216 grew to high titres in the absence of 1-dMM, but its growth was restricted in a dose-dependent manner by addition of 1-dMM (Fig. 1).

The reduction of NE2G216 titres observed in these assays may be due to reduced virus yields; however, similar results would be obtained if virions produced under these conditions were markedly less infectious for BHK-21 cells. This issue was addressed in two experiments. First, NE2G216 virions grown in the presence or absence of 1-dMM (1·0 mM) were analysed by density-gradient ultracentrifugation (Fig. 2). The relative magnitudes of the virus-containing peaks were consistent with
AB

Fig. 1. Effects of 1-dMM on the growth kinetics of TRSB (A) and NE2G216 (B) in Pro-5 cells. Monolayers of Pro-5 cells were infected with each virus at an m.o.i. of 10 p.f.u. per cell. Following adsorption of virus, cells were washed repeatedly with PBS to remove free virus, and then overlaid with growth medium alone or with growth medium supplemented with increasing concentrations of 1-dMM. Final concentrations of 1-dMM are indicated within the graphs.

Fig. 2. Analysis of NE2G216 virion production by density-gradient ultracentrifugation. NE2G216 virions were metabolically labelled with [35S]methionine during growth in Pro-5 cells in the presence or absence of 1-dMM (1.0 mM). Virions released under these conditions were analysed by density-gradient ultracentrifugation on continuous potassium tartrate gradients as described in Methods.

Fig. 3. Comparisons between titres of extracellular and cell-associated virions during the replication of TRSB and NE2G216 in C6/36 cells. C6/36 cells were electroporated with in vitro viral transcripts as described in the text. At the designated time-points post-electroporation, cell-free supernatants were collected for titration of extracellular virions, and cell pellets were collected, washed extensively and lysed for titration of cell-associated virions. Titres of extracellular virions are reported as p.f.u./ml of growth medium and titres of cell-associated virions are reported as p.f.u. per cell pellet.
Sindbis virus host-range mutant

Table 1. Genetic and phenotypic properties of wild-type and mutant viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mutations relative to TRSB</th>
<th>N-linked glycosylation at E2 residue 1</th>
<th>PE2 cleavage*</th>
<th>Growth in vertebrate cells†</th>
<th>Growth in mosquito cells†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>TRSB</td>
<td>None</td>
<td>No</td>
<td>Yes</td>
<td>Normal</td>
</tr>
<tr>
<td>Mutant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRSB-N</td>
<td>E2 residue 1 Arg to Asn‡</td>
<td>Yes</td>
<td>No</td>
<td>Defective cell entry</td>
<td>Defective cell entry + maturation</td>
</tr>
<tr>
<td>NE2G216</td>
<td>E2 residue 1 Arg to Asn‡</td>
<td>Yes</td>
<td>No</td>
<td>Near normal</td>
<td>Restricted</td>
</tr>
<tr>
<td>NE2G216</td>
<td>E2 residue 216 Glu to Gly§</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE6R1</td>
<td>E2 residue 1 Arg to Asn‡</td>
<td>Yes</td>
<td>No</td>
<td>Near normal</td>
<td>Restricted</td>
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<tr>
<td>NE6R1</td>
<td>E3 residue 25 Cys to Arg§</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* As determined by SDS–PAGE analysis of purified virions grown in BHK-21 cells.
† Virus growth was assessed in standard growth curves as described in Methods. In each assay virus growth was compared to that of TRSB (normal). For a phenotypic description of TRSB-N see Heidner et al. (1994). For phenotypic descriptions of NE2G216 and NE6R1 see Heidner et al. (1994, 1996).‡ Substitution of Asn at E2 residue 1 creates a signal for N-linked glycosylation immediately adjacent to the PE2 cleavage signal, and individually is lethal.§ This mutation suppresses the lethal effects of N-linked glycosylation at E2 residue 1.

the difference in virus titre between the supernatant samples prior to ultracentrifugation [1·2 × 10⁸ p.f.u./ml (no 1-dMM) and 2·5 × 10⁸ p.f.u./ml (1·0 mM 1-dMM)]. Results from this experiment suggest that the maturation of NE2G216 virions was severely restricted in the presence of 1-dMM. However, the possibility that NE2G216 virions grown under these conditions were structurally unstable in the potassium tartrate gradients could not be ruled out. In a second experiment, NE2G216 virions grown in the presence and absence of 1-dMM were isolated by a simple pelleting technique in which virions were not exposed to sucrose or potassium tartrate. NE2G216 virions derived from Pro-5 cells treated with 0·5 mM 1-dMM were found to be approximately 33% as infectious as virions derived from Pro-5 cells grown in normal growth medium. Together these experiments strongly suggest that the restricted growth of NE2G216 in the presence of 1-dMM resulted from defective virion maturation.

NE2G216 viruses derived from Pro-5 cells maintained in normal growth medium retained a stable small plaque phenotype on BHK-21 cells at all time-points; however, large plaque variants of NE2G216 arose in Pro-5 cells treated with 1-dMM, and such variants typically constituted a large proportion of virus produced beyond 18 h. Large plaque variants also arise during replication of NE2G216 in C6/36 cells, and genetic and molecular analyses demonstrated these viruses to be variants that had reverted to the PE2 cleavage phenotype through loss of the N-linked glycosylation signal at E2 1 (Heidner et al., 1996). Although this was not pursued further in this study, the generation of large plaque variants in these assays suggests that NE2G216 may experience similar selective pressures during growth in C6/36 cells and in Pro-5 cells treated with 1-dMM.

Intracellular maturation of NE2G216 in C6/36 cells

The maturation of Sindbis virus in various mosquito cell lines has been studied at the ultrastructural level (Gliedman et al., 1975; Miller & Brown, 1992). The predominant mechanism of virus maturation in C6/36 cells was shown to involve virus budding into intracellular vesicles, which then proceeded to release their virus contents to the cell exterior by fusion with the plasma membrane (Miller & Brown, 1992). These authors also described virus budding at the plasma membrane of C6/36 cells but noted that this form of virus maturation was much less extensive than budding at intracellular sites. It also has been shown that limiting carbohydrate processing to high-mannose forms by the addition of 1-dMM induced Sindbis virus to bud internally in BHK-21 cells (McDowell et al., 1987). These authors suggested that mannos trimming may be important for determining the cellular destination of viral glycoproteins, and drew a parallel between the intracellular virus budding that occurs in Sindbis virus-infected C6/36 cells and in BHK-21 cells treated with 1-dMM.

Our results suggest that the maturation defect of NE2G216 in C6/36 cells is linked to the carbohydrate-processing
phenotype of these cells. However, the precise glycoprotein function(s) that are deficient in these cells is not known, nor is it known if the functional deficit(s) have similar effects on intracellular virion maturation and virion maturation at the plasma membrane. Defective virion maturation could result from defects in a number of processes including: inefficient glycoprotein heterodimer formation, inefficient heterodimer transport to sites of virion budding, inefficient envelopment of nucleocapsids at intracellular sites, retention of intracellularly matured virions and/or defective virion budding at the plasma membrane. As an initial examination of these possibilities C6/36 cells were electroporated with in vitro transcripts of TRSB or NE2G216 and the titres of virus released into the medium, and virus which remained cell-associated were measured separately (Fig. 3). These infections were initiated by transfection instead of infection with free virus in order to eliminate the contaminating effects of free virions, and to restrict our analysis to virus maturation events. The titres of infectious, cell-associated virus were approximately equal for TRSB and NE2G216; however, the titre of infectious NE2G216 virions released into the medium was reduced by 2–4 log$_{10}$ at all time-points compared to TRSB.

The finding that intracellular maturation of NE2G216 appeared to proceed normally in C6/36 cells indicated that the processes of heterodimer formation and heterodimer transport to the site(s) of intracellular virus maturation were not severely affected in these cells. The decreased titres of NE2G216 in the extracellular media may be due to inefficient release of these intracellularly matured virions. It also is possible that the viral glycoproteins of NE2G216 are transported normally to the surface of infected C6/36 cells but that they are not functional for participating in virus assembly at this site. This phenotype has been described for several other alphavirus mutants including two Sindbis virus host-range mutants designated S12 and SV$_{\text{ap}}$ (Russell et al., 1989). S12 is a variant of Sindbis virus strain S.A.A.R86 which, like NE2G216, contains a mutation which generated an additional signal for N-linked oligosaccharide addition at E2 1 (Russell et al., 1989). SV$_{\text{ap}}$ is a variant of Sindbis virus strain AR339, which was isolated from a culture of persistently infected Aedes albopictus cells (Durbin & Stollar, 1984). The SV$_{\text{ap}}$ mutant incorporated an additional N-linked oligosaccharide within E2 and was shown to be restricted for growth in both BHK and CEF cell lines (Durbin & Stollar, 1984, 1986). Ultrastructural analysis of TRSB- and NE2G216-infected C6/36 cells using transmission electron microscopy will be helpful for addressing these issues.

Characterization of PE2-containing variants derived from C6/36 cells

The association between defective PE2 cleavage and restricted growth in cultured mosquito cells has been described for other PE2-containing alphaviruses such as Sindbis virus S.A.A.R86 (mutant S12) (Russell et al., 1989; Presley et al., 1991) and Venezuelan equine encephalitis virus (mutant V3526) (Davis et al., 1995). PE2-containing variants of Semliki Forest virus have been isolated but their growth characteristics in arthropod cells were not described (Salminen et al., 1992; Tubulekas & Liljestrom, 1998). These studies suggest a
Table 2. Genetic and phenotypic properties of C6/36 cell-derived variants of TRSB-N and related viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Plaque morphology*</th>
<th>Mutations relative to TRSB-N</th>
<th>PE2 cleavage phenotype†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRSB-N</td>
<td>None ‡</td>
<td>None</td>
<td>Cleavage defective</td>
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<tr>
<td>Biologically derived revertants</td>
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<td></td>
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<tr>
<td>L1</td>
<td>Large</td>
<td>E2 residue 1 Asn to Asp</td>
<td>Cleavage competent</td>
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<tr>
<td>L2</td>
<td>Large</td>
<td>E2 residue 3 Thr to Ala</td>
<td>Cleavage competent</td>
</tr>
<tr>
<td>S3</td>
<td>Small</td>
<td>E2 residue 3 Thr to Pro</td>
<td>Cleavage competent</td>
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<tr>
<td>S5</td>
<td>Small</td>
<td>E3 residue 40 Asn to Asp</td>
<td>Cleavage competent</td>
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<tr>
<td></td>
<td></td>
<td>E2 residue 3 Thr to Lys</td>
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<td></td>
<td>Val–Ser Insertion between E2 residues 3 and 4</td>
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<tr>
<td>S1</td>
<td>Small</td>
<td>E3 residue 46 Tyr to His</td>
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<td>Small</td>
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<td>Small</td>
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<td>S6</td>
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<td>S4</td>
<td>Small</td>
<td>E3 residue 34 Leu to His</td>
<td>Cleavage defective</td>
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<td>Cleavage defective</td>
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<td>N-S7</td>
<td>Small</td>
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<tr>
<td>216-S2</td>
<td>Pinpoint</td>
<td>E2 residue 216 Glu to Gly</td>
<td>Not determined</td>
</tr>
<tr>
<td>216-S4</td>
<td>Pinpoint</td>
<td>E2 residue 216 Glu to Gly</td>
<td>E3 residue 34 Leu to His</td>
</tr>
</tbody>
</table>

* Plaque sizes were compared to those produced by the wild-type virus TRSB (large).
† As determined by SDS–PAGE analysis of purified virions grown in BHK-21 cells.
‡ TRSB-N is a non-infectious virus that is incapable of plaque production.

causal relationship between the failure to cleave PE2 and restricted virus maturation in cultured mosquito cells. As for NE2G216, efficient growth of the S12 and V3526 variants was linked to the incorporation of single site mutations arising during replication of the parental viruses in cultured vertebrate cells. The mutation present in S12 is described above, and the mutation identified in V3526 mapped to E1 residue 253 (Russell et al., 1989; Davis et al., 1995). The inability of these PE2-containing alphaviruses to replicate efficiently in cultured mosquito cells indicates that the adaptive mutation present within each virus is specific for the environment of the vertebrate cell, and suggests that other mutations may similarly adapt PE2-containing alphaviruses to growth in the environment of the cultured mosquito cell.

In a study separate from those described here we isolated a group of infectious viruses from the supernatants of C6/36 cells transfected with TRSB-N in vitro transcripts (Table 2). Two of these variants (L1 and L2) produced large plaques on BHK-21 cells and the remaining eight viruses (S1–S8) produced small plaques on BHK-21 cells. To identify the mutations responsible for reversion of TRSB-N in C6/36 cells each virus was sequenced across regions encoding the major neutralization domain of E2, the PE2 cleavage site and all of E3. Candidate reverting mutations were identified in each virus. These mutations were of two types: one which eliminated the N-linked glycosylation signal adjacent to the PE2 cleavage site, and a second which clustered within a defined region of E3 (Table 2). Viruses that contained mutations which eliminated the N-linked carbohydrate at E2 1 (L1, L2, S3 and S5) had reverted to a PE2 cleavage-competent phenotype. Interestingly, the S3 and S5 variants possessed a small plaque phenotype. The S5 variant contained an insertion of two amino acids near the N terminus of E2. This insertion may have a negative effect on the replication of the S5 variant, which may account for the small plaque phenotype of this virus. The S3 variant also may contain mutation(s) in addition to that which was identified at E2 residue 3 (Thr to Pro), and which may be responsible for the small plaque phenotype of this virus. Only small regions of the virus genome were sequenced for each variant and it is possible that additional mutations lie outside these regions. In addition, we have reported findings on another Sindbis virus mutant that contained a Thr to Pro

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substitution at E2 residue 3, and this virus, TRSB-N7R1, possessed a large plaque phenotype in BHK-21 cells (Heidner et al., 1994). The viruses that contained individual mutations within E3 (S1, S2, S4, S6, S7 and S8) retained PE2 within their virion structure (Fig. 4). To confirm that the mutations which clustered within E3 were responsible for reverting TRSB-N in C6/36 cells, the E3 mutations present within representative viruses (S2, S4 and S7) were constructed individually in the genetic background of TRSB-N, generating viruses designated N-S2, N-S4 and N-S7, respectively. The individual mutations identified in the S2 and S4 variants also were constructed in the genetic background of NE2G216, generating viruses designated 216-S2 and 216-S4, respectively.

Each of the E3 mutations was independently capable of reverting TRSB-N in C6/36 cells (Fig. 5). Since TRSB-N is incapable of mediating efficient cell entry or maturation in C6/36 cells (K. W. Boehme & H. W. Heidner unpublished observations), these E3 mutations are independently able to suppress both of these parental replication defects. An additional virus, N6R1, was included in these assays (Table 1). N6R1 is a PE2-containing revertant of TRSB-N which like NE2G216 was isolated from transfected BHK-21 cells, and was previously shown to be restricted in C6/36 cells (Heidner et al., 1994). The suppressor mutation in N6R1 is located at E3 residue 25 (Arg for Cys), which lies just outside of the region in which the suppressor mutations identified in the S2, S4 and S7 viruses clustered. The restricted growth of N6R1 in these assays suggests that E3 mutations capable of reverting TRSB-N in this cell type may be restricted to the domain tentatively defined by mutations identified in the S2, S4 and S7 variants (E3 residues 34–46). Yields of 216-S2 and 216-S4 were only slightly increased over those of NE2G216 in C6/36 cells (data not shown), which suggests that the suppressor function of the E3 mutations may be specific for the TRSB-N genotype, and that their suppressor activity is somehow influenced by the residue at E2 216.

If the suppressor mutation(s) identified in these newly isolated revertants function by adapting these viruses to the carbohydrate structures synthesized in C6/36 cells, then we predicted that the titres of N-S2, N-S4 and N-S7 viruses grown in Pro-5 cells treated with 1-dMM would equal or exceed their titres in Pro-5 cells grown under normal conditions. The growth of TRSB, NE2G216, N-S2, N-S4 and N-S7 was compared in Pro-5 cells treated with varying concentrations of 1-dMM (0–1 mM). Cells were infected with each virus, washed repeatedly to remove unbound virions, and supernatant samples were then collected at 12 and 24 h post-infection. Infectious virus in each sample was quantified by plaque assay on BHK-21 cells (Fig. 6). TRSB titres increased slightly in the presence of 1-dMM while NE2G216 titres were reduced in a dose-dependent fashion to the point where virus titres obtained in the presence of 1 mM 1-dMM were only...
Concluding remarks

The results presented here provide biochemical and genetic evidence supporting the hypothesis that the host-range phenotype of NE2G216 is linked to the differences in the carbohydrate-processing phenotypes of vertebrate and mosquito cells. To the best of our knowledge this represents the first experimental evidence that the carbohydrate-processing phenotypes of the host cell can form the basis for an alphavirus host-range restriction. The structure of cis-linked oligosaccharides is known to influence the folding and conformational properties of glycoproteins (Doms et al., 1993; Fiedler & Simons, 1995); therefore, we assume that the maturation defect experienced by NE2G216 in C6/36 cells is somehow linked to carbohydrate-induced structural changes in one or both viral glycoproteins which in turn affects one or more maturation-related glycoprotein functions. It follows that the reverting mutations identified in E3 reverse or somehow compensate for these structural changes and thus restore the critical maturation function.

In nature, alphaviruses replicate within a wide range of mammalian and avian species and the maintenance cycle consists of alternating infections of vertebrate and mosquito hosts. These aspects of alphavirus biology require that the viral glycoproteins maintain all critical functions independently of the different carbohydrate structures synthesized in cells of the vertebrate and mosquito host. As would be expected, alphavirus replication is not significantly affected by the subtle differences in carbohydrate-processing phenotypes between different vertebrate cell lines, (Keegstra et al., 1975; Burke & Keegstra, 1979; Hsieh et al., 1983; Mayne et al., 1985), or by the more significant differences that exist between vertebrate and mosquito cells. Our results suggest that the carbohydrate-processing phenotypes of the host cell are a constraining force on alphavirus evolution, and should viruses such as NE2G216 arise in nature, they would likely be strongly selected against in the arthropod segment of the maintenance cycle. However, as alphavirus phenotypes determined in cultured mosquito cells are not necessarily recapitulated in vivo (Stollar & Hardy, 1984), efforts are under way to determine if NE2G216 also is growth restricted within living Aedes albopictus mosquitoes.

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References


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