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Differential Processing of Sindbis Virus Glycoprotein PE2 in Cultured Vertebrate and Arthropod Cells

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A step in the maturation of Sindbis virus glycoproteins is the cleavage of the precursor glycoprotein PE2 into E3 and E2 by furin or a furin-like host cell protease. The results presented here suggest that PE2 cleavage is an obligatory event for Sindbis virus maturation in C6/36 cells and demonstrate that certain mutants display a cell-specific PE2 cleavage phenotype. We previously have described Sindbis virus variants which fail to cleave PE2 because of incorporation of a signal for N-linked glycosylation immediately adjacent to the PE2 cleavage site but are viable in BHK-21 cells by virtue of an additional mutation at E2 216 or E2 191 (TRSB-NE2G216 and TRSB-NE2T191, respectively) (H. W. Heidner, K. L. McKnight, N. L. Davis, and R. E. Johnston, J. Virol. 68:2683–2692, 1994). Other viable PE2 cleavage-defective mutants were constructed by substituting the parental residue at E2 position 1 (Arg), with Leu or Val (TRSB-E2L1 and TRSB-E2V1, respectively) (H. W. Heidner and R. E. Johnston, J. Virol. 68:8064–8070, 1994). When grown in BHK-21 cells, all four of these viruses replicated normally and incorporated PE2 in place of E2 in released virions. However, growth of TRSB-NE2G216 and TRSB-NE2T191 was severely restricted in cultured arthropod cells (C6/36 cells). Analysis of infected C6/36 cells by flow cytometry demonstrated that the restricted growth of TRSB-NE2G216 and TRSB-NE2T191 was not due to an impaired ability to initiate infection. In addition, TRSB-NE2G216 and TRSB-NE2T191 remained growth restricted in C6/36 cells following introduction of in vitro transcripts by electroporation. In contrast, the PE2 cleavage defect of TRSB-E2L1 and TRSB-E2V1 was cell type specific. In C6/36 cells, the majority of PE2 was converted to E2, and these viruses replicated normally in C6/36 cells. These results demonstrated a consistent link between expression of a PE2 cleavage defect and restricted growth in C6/36 cells and suggest that cleavage of PE2 is required for maturation of Sindbis virus late in infection of C6/36 cells.

Alphaviruses are enveloped, positive-strand RNA viruses, which cycle in nature by alternately infecting vertebrate and arthropod hosts. Sindbis virus is the type member of this genus (family *Togaviridae*) (28). In the natural infection cycle, the vertebrate host is usually a bird or mammal and the arthropod vector is a species of mosquito. Maintenance of this cycle is dependent upon viral replication in both hosts, and this requirement has undoubtedly influenced alphavirus evolution (31). As mutations arise, they would be subject to selection in cells of at least two evolutionarily diverged host species, and variants with impaired ability to bind, enter, replicate in, or exit from cells of either host would be selected against. Laboratory-derived alphavirus mutants which replicate normally in cells of one host but which are restricted for growth in cells of the other have been described (6, 9, 14, 20, 21). The bases for the host restrictions are probably related to fundamental differences in the intracellular environments of vertebrate and arthropod cells and/or to differences in species-specific virus-host cell interactions (13).

During Sindbis virus infection, the viral glycoproteins PE2 and E1 are synthesized as components of a single polyprotein. PE2 and E1 are cleaved from the polyprotein while still nascent peptides (27) and proceed to form heterodimers in the rough endoplasmic reticulum (23). In vertebrate cells, PE2-E1 heterodimers are then processed and transported in this form through the exocytic pathway of the host cell (7). At a late step in transport, PE2 is cleaved by furin or a furin-like host cell protease (4, 17, 32) into E3, which comprises the amino-terminal 64 amino acids of PE2, and E2, which remains anchored in the membrane in association with E1 (12, 29). During infection of invertebrate cells with Semliki Forest virus, a closely related alphavirus, the precursor glycoprotein may be cleaved into E3 and E2 at an early stage in transport, possibly prior to entry into the Golgi apparatus (18). In both cell types, alphavirus PE2 cleavage occurs at a site immediately following a conserved motif consisting of four residues ordered in a basic-X-basic-basic (bxbb) sequence (22, 30).

Alphavirus particles which retain PE2 in place of E2 have been produced in cultured vertebrate cells, indicating that cleavage of PE2 is not an absolute requirement for viral morphogenesis. Such particles were obtained by passage of virus in a furin-defective Chinese hamster ovary cell line (33), by growth of virus in the presence of the ionophore monensin (19), by mutation or deletion of the bxbb signal (2, 26), or by selection or construction of specific mutations at E2 position 1 (E2 1), the residue immediately adjacent to the PE2 cleavage signal. These latter mutations created a signal for N-linked glycosylation (Asn-Val-Thr) at E2 1 (9, 25) or substituted a residue with a branched aliphatic side chain (Ile, Leu, or Val) in place of the parental amino acid (Arg) at this position (8). With a few exceptions (8, 25), these PE2-containing particles failed to infect other cells. However, the defect in replication in BHK-21 cells can be bypassed by electroporation of RNA transcripts of mutant cDNA clones, suggesting that the replicative defect occurred at an early event. In addition, second-

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TABLE 1. Genetic and phenotypic properties of TRSB and PE2-containing mutant viruses

Virus	E2 residue 1	N-linked glycosylation at E2 1	Additional mutations relative to TRSB	Plaque size on BHK-21 cells
TRSB	Arg	No	None	Large
Mutant viruses				
TRSB-NE2G216	Asn[a]	Yes	E2 216 Glu to Gly[b]	Small
TRSB-NE2T191	Asn[a]	Yes	E2 191 Pro to Thr[b]	Small
TRSB-E2L1	Leu	No	None	Medium
TRSB-E2V1	Val	No	None	Medium

[a] Substitution of Asn at E2 1 creates a signal for N-linked glycosylation (Asn-Val-Thr) immediately adjacent to the PE2 cleavage signal.
[b] This mutation suppresses the lethal effects of N-linked glycosylation at E2 1.

site suppressor mutations which restore viability of PE2-containing virions in vertebrate cells have been identified in the Sindbis virus and Venezuelan equine encephalomyelitis virus systems. Although these double mutants infected and replicated to near-normal titers in BHK-21 cells, their growth was severely restricted in mosquito cells (2, 9, 20). To address the possibility that arthropod host restriction was a general property of PE2-containing Sindbis viruses, the growth of the parental virus and representative mutants from two groups of PE2-containing viruses was compared in BHK-21 cells and in cells derived from *Aedes albopictus* mosquitoes (C6/36) (11).

The parental virus used in these studies, TRSB, was derived from a full-length cDNA clone (pTRSB) containing viral sequences from our laboratory strain of AR339 (SB) placed downstream of an SP6 phage promoter (16). The four PE2-containing mutants used in this study were constructed in the pTRSB background and are described briefly in Table 1. TRSB-NE2G216 and TRSB-N8R1 contain an additional N-linked glycan immediately adjacent to the PE2 cleavage signal as a result of an Arg-to-Asn substitution at E2 1. This mutation is lethal when placed individually in the genetic background of TRSB. Virions containing this mutation (TRSB-N) are efficiently released from cells electroporated with in vitro transcripts and bind to BHK-21 cells as efficiently as TRSB virions (unpublished data) but are essentially noninfectious (9). In this respect, TRSB-N is similar to a PE2-containing variant of Semliki Forest virus (ML), which was blocked at an early step in infection because of the inability of PE2-E1 heterodimers to dissociate in response to endosomal acidification (26, 32). TRSB-NE2G216 and TRSB-NE2T191 contain a single second-site reverting mutation at E2 residue 216 or 191, respectively, which suppresses the lethal effects of N-glycosylation at E2 1, presumably by destabilizing the PE2-E1 heterodimer. To remain consistent with the viral nomenclature used for the other mutants, TRSB-N8R1 has been renamed TRSB-NE2T191. TRSB-E2L1 and TRSB-E2V1 were constructed by replacing E2 Arg-1 (present in TRSB), with Leu or Val, respectively. All virus stocks used in this study were produced by electroporation of in vitro transcripts derived from the appropriate cDNA clone into BHK-21 cells as described previously (9, 15). The protein composition of these viruses grown in BHK-21 cells is presented in Fig. 1. Infected cells were metabolically labeled with [^{35}S]methionine, virus particles were purified by gradient centrifugation from infected-cell supernatants, and virion proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (8). Under these conditions, the majority of E2 comigrates with E1. Therefore, the PE2 cleavage phenotype of each virus is most accurately determined by the amount of PE2 in purified virions.

To assess yields of each virus in the two cell types following infection, growth experiments were performed on BHK-21 (37°C) and C6/36 (28°C) cells and samples were assayed on BHK-21 cells as described previously (9) (Fig. 2A and B). All viruses displayed similar growth kinetics in BHK-21 cells, and each achieved peak titers by 12 to 18 h postinfection. The peak titer for TRSB was four to eight times greater than that attained by any PE2-containing virus. In C6/36 cells, the mutants segregated into two groups on the basis of their kinetics of growth and peak titers. Replication of TRSB-E2L1 and TRSB-E2V1 in C6/36 cells was similar to that of the TRSB control, and the plaque morphology of progeny viruses remained stable over the course of the growth curve. In contrast, and consistent with prior reports (9), growth of TRSB-NE2G216 and TRSB-NE2T191 was severely restricted in these cells. At 12 h postinfection, when the titers of TRSB had reached their peak, the titers of TRSB-NE2G216 and TRSB-NE2T191 were approximately 4 $\log_{10}$ units lower.

The differential permissiveness of C6/36 cells for these two classes of PE2-containing viruses is the subject of this report. The decreased yields of TRSB-NE2G216 and TRSB-NE2T191 from C6/36 cells could result if these viruses were temperature sensitive (to the lower temperature) or were impaired in initiating infection (early events) or impaired in virus morphogenesis and/or in release (late events). The growth restriction in C6/36 cells was not due to temperature sensitivity, because these viruses grew to normal titers in BHK-21 cells maintained at 28°C (data not shown). Two experiments were performed to distinguish between the remaining possibilities. First, the relative ability of the five viruses to initiate infection of C6/36 cells was determined by flow cytometry, and second, growth experiments were performed on C6/36 cells following electroporation of in vitro viral transcripts. For flow cytometry, C6/36 cells

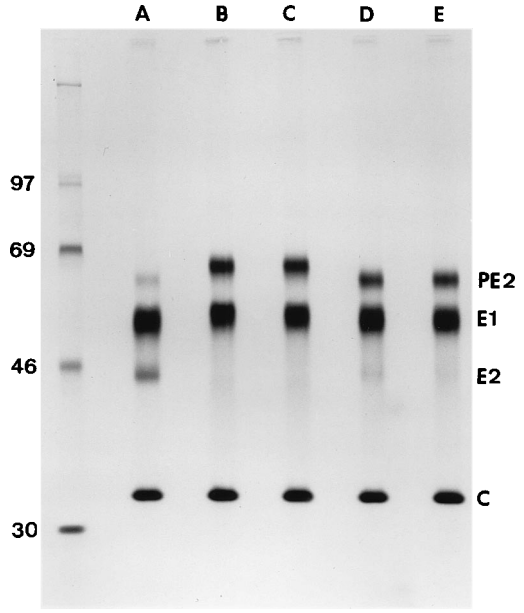

FIG. 1. SDS-PAGE analysis of TRSB and four PE2-containing mutants grown in BHK-21 cells. Viruses were metabolically labeled with [^{35}S]methionine and purified from infected BHK-21 cell supernatants as referred to in the text. Lanes: A, TRSB; B, TRSB-NE2T191; C, TRSB-NE2G216; D, TRSB-E2L1; E, TRSB-E2V1. ^{14}C-methylated proteins (far-left lane) were included as molecular weight standards. Molecular weights (in thousands) are shown on the left.

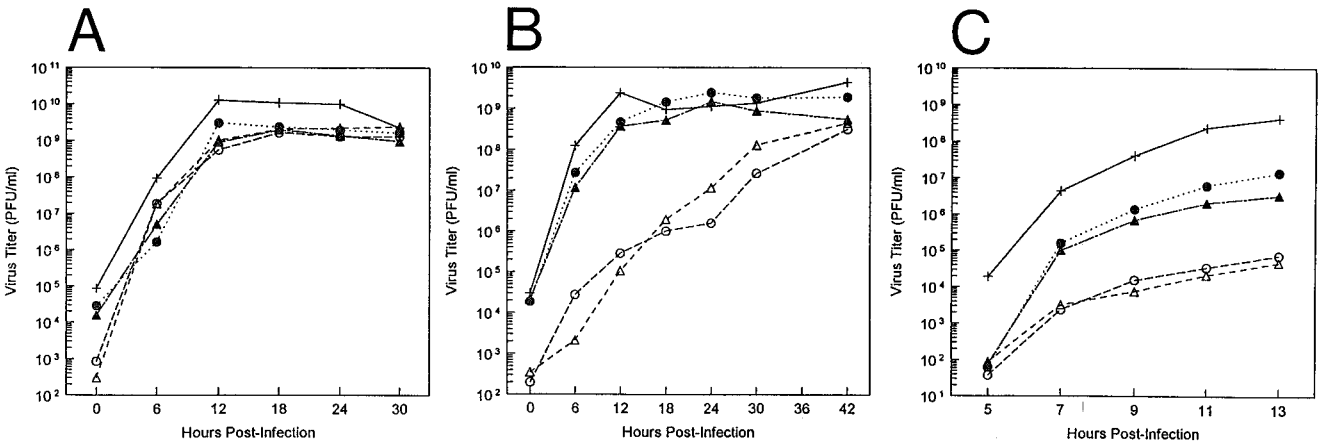

FIG. 2. Growth kinetics of TRSB and each PE2-containing mutant following infection of BHK-21 cells (A), infection of C6/36 cells (B), and electroporation of C6/36 cells (C) with in vitro viral transcripts. Symbols: +, TRSB; △, TRSB-NE2G216; ○, TRSB-NE2T191; ●, TRSB-E2L1; ▲, TRSB-E2V1.

were harvested from culture flasks and infected in suspension at a multiplicity of infection of 1.0 PFU per cell. Cells were placed at 28°C for 1 h, after which time they were gently pelleted, resuspended in medium, and plated into 60-mm-diameter petri dishes. At 6 h postinfection, the cells were harvested from the dishes, washed twice with phosphate-buffered saline, fixed in 1.0% paraformaldehyde, and permeabilized with methanol as described previously (1). Virus-infected cells were detected by indirect immunofluorescent staining of intracellular viral antigens. Briefly, cells were reacted with the positive-staining monoclonal antibody R9 (specific for E1) or with the negative control monoclonal antibody 290 (specific for VP7 of bluetongue virus) and then with a fluorescein isothiocyanate-conjugated secondary antibody. The percentage of positive fluorescent cells was then determined by flow cytometry. TRSB, TRSB-E2L1, and TRSB-E2V1 were equally efficient in initiating infection of C6/36 cells; however, TRSB-NE2G216 and TRSB-NE2T191 were approximately two to four times less efficient than TRSB (Table 2). These relatively small differences in infection efficiency would not be expected to account for the 3 to 4 $\log_{10}$ unit reductions in virus yields obtained in the growth curves described earlier. When the early events of infection were bypassed by introducing in vitro transcripts directly into the cytoplasm of C6/36 cells by electroporation, the virus yields at early time points (chosen to measure virus release from electroporated cells only) for TRSB-E2L1 and TRSB-E2V1 were reduced relative to TRSB but again were much higher than those for TRSB-NE2G216 and TRSB-NE2T191. As was observed following infection with free virus, the yields of TRSB-NE2G216 and TRSB-NE2T191 from electroporated C6/36 cells were reduced by approximately 4 $\log_{10}$ units relative to TRSB (Fig. 2C). Together, these results suggest that the primary influence of the PE2 cleavage defect on viral replication in C6/36 cells is exerted on one or more late processes in the infection cycle.

Viral titers in TRSB-NE2G216- and TRSB-NE2T191-infected C6/36 cell supernatants did increase beyond 12 h, but this increase was associated with the appearance of large-plaque variants. The proportion of viruses in the population having a large-plaque phenotype increased with time, and such variants formed the vast majority of virus present by 42 h postinfection. To determine whether large-plaque variants of TRSB-NE2G216 and TRSB-NE2T191 reflected adaptation to C6/36 cells and to determine the genetic basis for such an adaptation, four large-plaque variants from each parental virus were isolated at 66 h postinfection and plaque purified once on BHK-21 cells as described previously (9). For each variant, viral RNA was purified, amplified by reverse transcriptase PCR, and sequenced across the PE2 cleavage site by cycle sequencing (United States Biochemical) (9). This analysis revealed that each large-plaque variant had incorporated a single mutation, at either E2 residue 1 or E2 residue 3 (Table 3).

TABLE 2. Flow cytometry detection of intracellular viral antigens in C6/36 cells similarly infected by TRSB and each PE2-containing mutant

Virus	Virus-infected cells[a] (% of total)	% Positive relative to TRSB
TRSB	18	100
TRSB-NE2G216	5	28
TRSB-NE2T191	7	39
TRSB-E2L1	20	111
TRSB-E2V1	20	111

[a] Fixed and permeabilized cells were stained with monoclonal antibody R9, specific for E1.

TABLE 3. Sequence analysis of TRSB-NE2G216 and TRSB-NE2T191 large-plaque variants selected on C6/36 cells

Parental virus	Variant no.	E2 residues 1 to 3[a]	Heterodimer constituents[b]
TRSB-NE2G216		Asn-Val-Thr	PE2-E1
	1	Asn-Val-Ala	E2-E1
	2	Asn-Val-Ala	E2-E1
	3	Asn-Val-Pro	E2-E1
	4	Asn-Val-Pro	E2-E1
TRSB-NE2T191		Asn-Val-Thr	PE2-E1
	1	Lys-Val-Thr	E2-E1
	2	Asn-Val-Asn	E2-E1
	3	Lys-Val-Thr	E2-E1
	4	Lys-Val-Thr	E2-E1

[a] E2 residues 1 to 3 in parental viruses comprise the N-linked glycosylation signal immediately following the PE2 cleavage signal. Residues which are changed from parental sequence by mutation of single nucleotides are underlined.

[b] As determined by SDS-PAGE analysis of purified, radiolabelled virions.

Each of these mutations eliminated the N-linked glycosylation signal at E2 1. Analysis of purified, radiolabelled virions of each large-plaque variant by SDS-PAGE demonstrated that each had reverted to the PE2 cleavage phenotype (data not shown). This finding is consistent with previous studies with a PE2-containing mutant of the Sindbis virus-like isolate S.A.AR86 (S12) (20). Like TRSB-NE2G216 and TRSB-NE2T191, growth of S12 is restricted in C6/36 cells and passage of S12 in these cells rapidly selected for large-plaque variants. Genetic analysis of these variants demonstrated that the large-plaque phenotype was the result of mutations which restored PE2 cleavage. In prior studies with TRSB-N (the noninfectious PE2-containing parent of TRSB-NE2G216 and TRSB-NE2T191), adaptation to growth on BHK-21 cells was most commonly linked to second-site suppressor mutations arising in E3 or E2 (9). Infectivity of these second-site revertants was not associated with restoration of the PE2 cleavage phenotype. In contrast, none of the eight variants which had adapted for growth on C6/36 cells retained the PE2 cleavage defect of the parent virus. These results suggest that in the genetic context of TRSB-NE2G216 and TRSB-NE2T191, substitution of PE2 in place of E2 interfered with one or more steps in viral morphogenesis in C6/36 cells and that this interference could be overcome most readily, if not exclusively, by mutations which restored PE2 cleavage.

Not all PE2-containing Sindbis viruses were restricted in mosquito cells. Growth of TRSB-E2L1 and TRSB-E2V1 in C6/36 cells was similar to that of TRSB and distinctly unlike that of other PE2-containing viruses. Therefore, the possibility that PE2 processing was occurring in the TRSB-E2L1- and TRSB-E2V1-infected C6/36 cells was investigated. Viruses were radiolabelled with [^{35}S]methionine and purified from infected BHK-21 and C6/36 cell supernatants as described previously (9). Supernatants were harvested at 24 h to minimize amplification of C6/36 cell-adapted variants. The yields of TRSB-NE2G216 and TRSB-NE2T191 in the C6/36 cell supernatants were too low to allow analysis by this procedure; therefore, the PE2 cleavage phenotype of these viruses grown in arthropod cells was not determined. Sufficient quantities of TRSB, TRSB-E2L1, and TRSB-E2V1 were readily obtained from both cell lines. The protein constituents of these virions were compared by SDS-PAGE analysis (Fig. 3). Virions derived from BHK-21 cells contained a high proportion of PE2, as expected. However, the proportion of PE2 was much reduced in TRSB-E2L1 and TRSB-E2V1 virions grown in C6/36 cells, and the PE2 content of these virions appeared similar to that present in TRSB virions grown in BHK-21 cells. This result demonstrated that PE2 from TRSB-E2L1 and TRSB-E2V1 was indeed processed differently in the two cell types. The essentially normal growth of TRSB-E2L1 and TRSB-E2V1 in C6/36 cells probably reflected the fact that these viruses were not PE2 cleavage defective in these cells. The small amount of PE2 consistently present in TRSB virions grown in BHK-21 cells was nearly absent from TRSB virions grown in C6/36 cells, demonstrating that wild-type PE2 also was more efficiently cleaved in the arthropod cells.

The basis for the differential processing of PE2 in BHK-21 and C6/36 cells has not yet been determined, but at least three possibilities can be proposed. In vertebrate cells, PE2 cleavage is mediated by furin or a furin-like protease (4, 17, 32). Although this has not yet been examined in mosquito cells, several subtilisin-like proteases which displayed cleavage specificities similar to mammalian furin have been identified in *Drosophila melanogaster* (3, 24). Cleavage of PE2 from TRSB-E2L1 and TRSB-E2V1 in C6/36 cells but not in BHK-21 cells might reflect a broader sequence specificity of these insect furin-like enzymes for their protein substrates. Specifically, the insect furin-like enzyme may recognize and process substrates having Leu or Val residues following the core bxbb recognition signal, whereas their mammalian counterparts may not. A second possibility is that PE2 cleavage in C6/36 cells is mediated by a subtilisin-like protease, possessing the required sequence specificity to cleave the mutant forms of PE2, for which there is no mammalian counterpart.

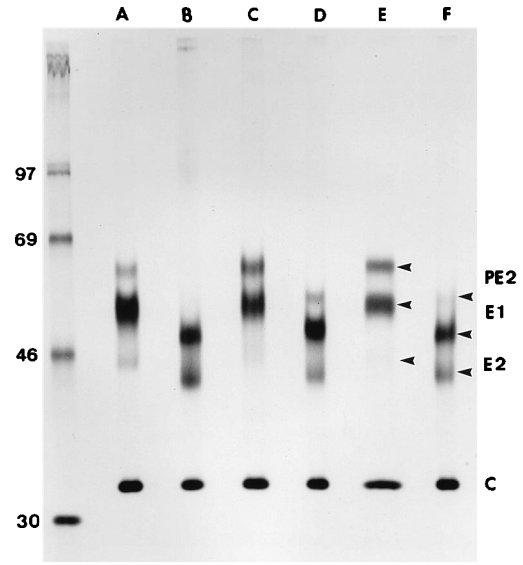

FIG. 3. SDS-PAGE analysis of TRSB, TRSB-E2L1, and TRSB-E2V1 isolated from BHK-21 and C6/36 cells. Virions were radiolabelled with [^{35}S]methionine and purified from infected cell culture supernatants as referred to in the text. Lanes: A, TRSB (BHK-21); B, TRSB (C6/36); C, TRSB-E2L1 (BHK-21); D, TRSB-E2L1 (C6/36); E, TRSB-E2V1 (BHK-21); F, TRSB-E2V1 (C6/36). ^{14}C-methylated proteins (far-left lane) were included as molecular weight standards. The position of each glycoprotein is shown by arrowheads. Molecular weights (in thousands) are shown on the left.

A third possibility is that cleavage is mediated by similar furin-like proteases but that access to the mutant PE2 cleavage sites is different in the two cell types. At least two posttranslational events differ between BHK-21 and C6/36 cells that could differentially affect the conformation and subsequent cleavage of PE2. One difference involves the site within the cell where PE2 encounters the furin-like enzyme. In vertebrate cells, this occurs within a trans- or post-Golgi compartment after PE2 has formed heterodimers with E1 and the carbohydrate groups on both glycoproteins have been fully processed. PE2 cleavage reportedly occurs much earlier in the export pathway in C6/36 cells infected by Semliki Forest virus, a condition under which carbohydrate groups would not be fully processed (18). The effects of Leu and Val substitution at E2 residue 1 on the conformation of PE2 and/or PE2/E1 heterodimers may be quite different at early and late stages of glycoprotein transport. Thus, the PE2 cleavage site may be accessible to the processing enzyme early but not late in the transport pathway, explaining why PE2 is cleaved in C6/36 cells but not in BHK-21 cells. Alternatively, carbohydrate addition and subsequent modifications strongly influence the oligomerization, conformation, and export of viral glycoproteins (5). Although the same sites on PE2 and E1 are utilized for carbohydrate addition in BHK-21 and C6/36 cells, the carbohydrate groups are more extensively modified in BHK-21 cells (10). These differences in carbohydrate modification, as reflected in the dissimilar SDS-PAGE migration profiles of PE2

and E1 derived from the two cell lines (Fig. 3), may differentially affect access to the TRSB-E2L1 and TRSB-E2V1 PE2 cleavage signals in the two cell lines.

These studies reveal a consistent link between the failure to cleave PE2 and restricted viral growth in arthropod cells. Each mutant virus used contained PE2 when derived from BHK-21 cells. However, viruses which displayed normal growth in C6/36 cells either were already PE2 cleavage competent in these cells (TRSB-E2L1 and TRSB-E2V1) or had incorporated a mutation which restored the PE2 cleavage phenotype (large-plaque variants of TRSB-NE2G216 and TRSB-NE2T191). The molecular basis of the arthropod host restriction has not been determined; however, the restriction probably results from the PE2 cleavage defect adversely affecting critical virus-host cell interactions necessary for morphogenesis and/or release. That the PE2 cleavage defect has little effect on intracellular virus replication and morphogenesis in BHK-21 cells may indicate that analogous interactions may not be required in vertebrate cells. These viruses will be useful tools for elucidating differences between vertebrate and arthropod cells with respect to alphavirus replication, glycoprotein processing, and maturation and should provide insight into how alphaviruses have adapted for growth in cells of two such evolutionarily diverged host species.

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