Role of a conserved tripeptide in the endodomain of Sindbis virus glycoprotein E2 in virus assembly and function

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Envelopment of Sindbis virus (SV) at the plasma membrane begins with the interaction of the E2 glycoprotein endodomain with a hydrophobic cleft in the surface of the pre-assembled nucleocapsid. The driving force for this budding event is thought to reside in this virus type-specific association at the surface of the cell. The specific amino acids involved in this interaction have not been identified; however, it has been proposed that a conserved motif (TPY) at aa 398–400 in the E2 tail plays a critical role in this interaction. This interaction has been examined with virus containing mutations at two positions in this conserved domain, T398A and Y400N. The viruses produced have very low infectivity (as determined by particle : p.f.u. ratios); however, there appears to be no defect in assembly, as the virus has wild-type density and electron microscopy shows assembled particles with no obvious aberrant structural changes. The loss of infectivity in the double mutant is accompanied by the loss of the ability to fuse cells after brief exposure to acid pH. These data support the idea that these residues are vital for production of infectious/functional virus; however, they are dispensable for assembly. These results, combined with other published observations, expand our understanding of the interaction of the E2 endodomain with the capsid protein.

INTRODUCTION

Sindbis virus (SV), the prototype of the alphaviruses, is a popular model system in which to study the assembly of a particular class of membrane-containing viruses, those that have icosahedral morphology (Strauss & Strauss, 1994). SV is a well-ordered, structurally complex virion composed of three structural proteins: capsid (C) and envelope proteins E1 and E2. There are 240 copies of each protein in a 1 : 1 : 1 stoichiometric arrangement in a mature virus particle. A mature SV virion is organized as nested \( T=4 \) icosahedrons with a host-derived lipid membrane bilayer sandwiched between the outer shell and the inner shell (nucleocapsid) (Paredes et al., 1993). E1 and E2 are organized as trimers of heterodimers and form 80 trimeric spikes on the surface of the virus. The integrity of the outer shell is maintained through lateral E1–E1 interactions on the virus surface (Anthony & Brown, 1991; Pletnev et al., 2001). The inner shell (nucleocapsid), also organized as a \( T=4 \) icosahedron (Coombs & Brown, 1987; Paredes et al., 1992, 1993), is an aggregate of the 49S viral RNA and C protein. The E2 protein contains a membrane-spanning domain and a cytoplasmic domain (endodomain/tail) (Hernandez et al., 2003; Liu & Brown, 1993b). The endodomain is a 33 aa domain that interacts specifically with the C protein during assembly (Cheng et al., 1995; Lee & Brown, 1994; Lee et al., 1996; Lopez et al., 1994; Owen & Kuhn, 1997; Wilkinson et al., 2005). This interaction connects the outer shell to the inner shell, an interaction that persists in the mature virus particle.

The assembly of the mature virion is a complex process involving multiple, specific protein–protein interactions. Initially, the virus structural proteins are translated from a 26S subgenomic RNA (NH\textsubscript{2}-C-pE2-6K-E1-COOH) (Liljestrom & Garoff, 1991). During translation, the C protein is cleaved autoproteolytically from the developing polyprotein chain. The C protein then assembles in the cytoplasm with the 49S viral RNA to form the nucleocapsid (Ferreira et al., 2003). The remaining proteins are integrated into the membrane of the endoplasmic reticulum (ER). In the ER, the polyprotein is processed by signalase at specific sites to produce pE2 and E1 and to release the intervening 6K protein (Liljestrom & Garoff, 1991). The role of 6K in virus maturation is unresolved. pE2 and E1 form trimers of heterodimers (Carleton et al., 1997; Mulvey & Brown, 1994, 1996), which are exported to the trans-Golgi network (TGN). In the TGN, pE2 is processed by furin protease to E2 and E3 (Nelson et al., 2005). At this point, E3 protein is released into the surrounding medium. The E2–E1 heterotrimeric complex is then exported to the plasma membrane, where the process of virus envelopment takes place (Brown et al., 1972).

It is at the plasma membrane that the virus assembles into its mature functional form. It is during the initiation of the...
process of envelopment that one of the critical events in virus assembly takes place: the association of the E2 endodomain with the preformed nucleocapsid (Ferreira et al., 2003; Owen & Kuhn, 1996, 1997). It has also been suggested that a preformed nucleocapsid is not necessary for assembly of alphaviruses (Forsell et al., 1996); however, preformed nucleocapsids attached to membranes are the preferred association, as shown by Ferreira et al. (2003). This association is a highly specific interaction between the 33 aa endodomain (aa 391–423) of E2 and the protein sequence in the hydrophobic cleft of the assembled nucleocapsid (C, aa 175–250) (Lee & Brown, 1994; Lee et al., 1996). The E2 tail is a multifunctional protein domain (Fig. 1); however, its primary function is to serve as the site of attachment for the C protein. The COOH-terminal portion of the E2 endodomain is initially buried in the membrane of the ER (Liu & Brown, 1993a). It is not known at which point in the secretory pathway this domain becomes exposed to the cytoplasm; however, exposure occurs after export from the ER and prior to arrival at the cell surface. We have suggested that a transient phosphorylation of either T398 or Y400 may play a role in tail extraction (Liu & Brown, 1993a). Once at the cell surface, interaction of the E2 endodomain with the nucleocapsid core is the first step in virus budding. Previous studies have provided insight into which amino acids play a role in tail extraction (Liu & Brown, 1993a). Once at the cell surface, interaction of the E2 endodomain with the nucleocapsid core is the first step in virus budding. Previous studies have provided insight into which amino acids play a critical role in this interaction (Gaedicke-Nitschko & Schlesinger, 1991; Lopez et al., 1994; Owen & Kuhn, 1996, 1997; Ryan et al., 1998; Weiss et al., 1994) (Table 1). The crystal structure of the C protein indicates that there is an aromatic interaction that could take place between two residues in the capsid, Y180 and W247, and one residue in the E2 tail, Y400, which is conserved throughout the alphaviruses (Lee et al., 1996; Skoging et al., 1996). Experiments from our laboratory support the conclusion that Y400 plays a critical role in this association (Liu et al., 1996). We have investigated the importance of Y400 in combination with another completely conserved residue, T398, in virus assembly and function. This mutant was previously characterized in our laboratory as a tool to study the role of phosphorylation at T398 and Y400 in virus production (Liu et al., 1996). We chose to revisit the properties of this mutant after the observation by Hernandez et al. (2000), which showed that shifting the location of Y400 in the endodomain caused a complete loss of virus production.

**METHODS**

**Cells, viruses and media.** Baby hamster kidney (BHK-21) cells were maintained in Eagle’s minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 5% tryptose phosphate broth and 2 mM glutamine as described previously (Renz & Brown, 1976). The cells were maintained at 37 °C under 5% CO₂. The wild-type SV used in these studies was produced from the SV clone Toto110 (Rice et al., 1987; Strauss et al., 1984). This clone contains a tyrosine at position 420 in the E2 protein that is a serine in Toto110. This substitution has no observable phenotype (Liu & Brown, 1993b). In this investigation, we employ a double mutant described previously (Liu et al., 1996).

**In vitro transcription, RNA transfection and plaque assay.** Full-length mutant and wild-type cDNAs were linearized by using the enzyme XhoI, treated with proteinase K, phenol-extracted and ethanol-precipitated. The templates were transcribed as described previously (Hernandez et al., 2003; Rice et al., 1987). The infectious RNAs were transected by electroporation as described by Liljestrom & Garoff (1991). Cells were pelleted and washed in RNase-free PBS (pH 7.0). Washed cells were resuspended at a concentration of 1 × 10⁶ cells ml⁻¹. RNA transcripts (20 μl) were added to 400 μl BHK-21 cells. The electroporation conditions were 1-5 kV, 25 μF and infinite resistance. Cells were pulsed once, allowed to sit for 10 min and then transferred into 10 ml MEM (no gentamicin) in 25 cm² tissue-culture flasks. Virus was harvested at 24 h post-transfection and flash-frozen in liquid nitrogen in 1 ml aliquots to be stored at −80 °C. To determine the titre of each virus, plaque assays were carried out as described previously (Renz & Brown, 1976).

**Mutagenesis and RT-PCR.** Mutagenesis was done as described previously (Liu et al., 1996). Briefly, using the megaprimer method of PCR mutagenesis, the mutations were inserted at the correct positions (T398 and Y400) by using AmpliTaq polymerase (Applied Biosystems) and specific PCR conditions. To analyse the mutant by RT-PCR, virus from transfections was first pelleted at 50 000 r.p.m. for 1 h in a Beckman SW55Ti rotor. The pellet was then incubated in 100 μl Tris/EDTA and 100 μl 2× lysis buffer [100 mM Tris/HCl (pH 7.0), 20 mM EDTA, 1% SDS] for 20 min at 37 °C (vortexing every 5 min). The lysed virus was then extracted sequentially with phenol (twice), phenol/chloroform (once) and chloroform (once). The extracted RNA was then precipitated in RNase-free ethanol overnight at −80 °C. The RNA was then pelleted and resuspended in 10 μl DEPC-treated water. RNA was transcribed by using murine leukemia virus (MuLV) reverse transcriptase (Applied Biosystems) under the following conditions. The reaction contained 10 μl PCR buffer [10 mM Tris/HCl (pH 8.3), 50 mM KCl and 5 mM MgCl₂], 20 U SuperRNasin (Ambion), 200 μM each dNTP and 0.5 μM reverse primer and 50 μM MuLV reverse transcriptase in a final volume of 20 μl. Reverse transcription was performed at 42 °C for 2 min and 99 °C for 5 min. After the transcription reaction, the volume of the reaction mixture was increased to 100 μl and the concentrations of the dNTPs and MgCl₂ were adjusted for the increased volume. Forward primer was added to a final concentration of 2.0 μM and an additional 1.0 μM reverse primer was added. Taq DNA polymerase was added to a final concentration of 2.5 U per 100 μl reaction mixture. The primers used to sequence through the E2 endodomain and C protein were the following: C, 5′-GGGGGTGCTGCTTAATTGCTTCCC-3′; endodomain, 5′-CAAAAGTATGCAACTGGG-3′.

**Metabolic labelling of infected cells.** Subconfluent monolayers of BHK-21 cells were first treated with 5 ml medium containing 4 μg actinomycin-D (Calbiochem) for 1 h. Cells were then infected with both wild-type and T398A/Y400N virus at an m.o.i. of 1. Virus was diluted in 1× PBS-D (PBS without calcium or magnesium) containing 3% FBS. Infection was carried out at room temperature.

**Fig. 1.** Functional map of the E2 endodomain. T398 and Y400 are putative phosphorylation sites.
### Table 1. Summary of previous investigations on the role of the E2 endodomain in virus assembly

<table>
<thead>
<tr>
<th>Position</th>
<th>Mutation</th>
<th>Effect</th>
<th>Compensatory</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>K391</td>
<td>Deletion of K391</td>
<td>No virus produced in BHK-21 cells; wild-type virus levels produced in insect cells</td>
<td>None</td>
<td>Hernandez <em>et al.</em> (2000)</td>
</tr>
<tr>
<td>T398/Y400</td>
<td>T398A/Y400N</td>
<td>Originally no virus produced; blocked in phosphorylation; no transport defects; no synthesis defects</td>
<td>Revertants observed at 400, but not 398; also had deletion of 402–406, substitution of L402 with V</td>
<td>Liu <em>et al.</em> (1996)</td>
</tr>
<tr>
<td>P399</td>
<td>P399G</td>
<td>Released lower than normal levels of virus; produced multi-cored particles; more heat-stable than wild type</td>
<td>None reported</td>
<td>Ivanova &amp; Schlesinger (1993)</td>
</tr>
<tr>
<td>A401</td>
<td>A401I, A401K</td>
<td>Released lower levels of particles; multi-cored particles; more heat-stable</td>
<td>None reported</td>
<td>Ivanova &amp; Schlesinger (1993)</td>
</tr>
<tr>
<td>P404</td>
<td>P404G</td>
<td>Released lower levels of particles; multi-cored particles; more heat-stable</td>
<td>S182N in capsid (no real effect alone), T398M in E2 tail (not studied)</td>
<td>Ivanova &amp; Schlesinger (1993); Ryan <em>et al.</em> (1998)</td>
</tr>
<tr>
<td>Y400</td>
<td>Y400F</td>
<td>Reduced particle formation by 3–4 logs</td>
<td>None reported</td>
<td>Gaedigk-Nitschko &amp; Schlesinger (1991)</td>
</tr>
<tr>
<td>L402</td>
<td>Y, C, T, G, N, D, R</td>
<td>Y: medium plaques and 2-log decrease C, T: small plaques and 4-log decrease G, N, D: very small plaques and 5-log decrease R: dead; no processing, transport or translocation defects</td>
<td>None reported</td>
<td>Owen &amp; Kuhn (1997)</td>
</tr>
<tr>
<td>C416</td>
<td>C416A</td>
<td>Produced virus slower than wild type; produced multi-cored particles and site of palmitoylation</td>
<td>None reported</td>
<td>Ivanova &amp; Schesinger (1993)</td>
</tr>
<tr>
<td>C417</td>
<td>C417A</td>
<td>Highly defective in virus release; produced multi-cored particles and site of palmitoylation; defective in processing pE2–6K protein</td>
<td>T256M in capsid (alone no effect), S411L in E2 tail (not studied)</td>
<td>Ivanova &amp; Schlesinger (1993); Ryan <em>et al.</em> (1998)</td>
</tr>
<tr>
<td>S420</td>
<td>S420C</td>
<td>Affected pE2–6K cleavage</td>
<td>None reported</td>
<td>Gaedigk-Nitschko &amp; Schlesinger (1991)</td>
</tr>
</tbody>
</table>
After 1 h, the medium was removed and 5 ml fresh medium was added to the cells; the cells were then incubated for 5 h at 37 °C. Following the 5 h incubation, the cells were starved of cysteine and methionine for 1 h. Finally, the monolayers were labelled overnight with a [35S]methionine–cysteine protein-labeling mixture at a concentration of 50 μCi (1.85 MBq) ml⁻¹.

**Purification and particle: p.f.u. ratio determination of T398A/Y400N.** Transfections were carried out as described above. The supernatants from transfected monolayers were harvested at 20–24 h post-infection and layered over a sedimentation gradient of 15% tartrate (11 ml) over a 35% cushion of tartrate (6 ml) (in 1× PBS-D). The labelled virus was centrifuged overnight at 24,000 r.p.m. in a Beckman SW28 rotor. A band was observed for both the wild-type and mutant virus; the refractive index was taken for each of the samples. These bands were analysed by plaque assay to determine the titre of the purified virus. Purified virus protein concentrations were determined by Micro BCA analysis (Pierce). The same fractions were titrated on BHK-21 cells as described above. The number of particles in a preparation of wild-type virus was determined by using electron microscopy by the agar-filtration protocol described by Kellenberger & Bitterli (1976) and the particle count was correlated to the protein concentration as determined by BCA (Hernandez et al., 2003). These calculations were used to determine the particle:p.f.u. ratio for the mutant and wild type. Virus collected from sedimentation gradients was also layered over a tartrate density gradient (15–30%) and centrifuged for 2 h at 26,000 r.p.m. in a Beckman SW28 rotor. Virus bands were collected and analysed by electron microscopy for aberrant particle formation.

**Negative staining.** Virus collected from potassium tartrate density gradients was attached to carbon-coated grids, washed three times with sterile H₂O and negatively stained with 1% uranyl acetate. The grids were viewed under a JEOL 100S transmission electron microscope.

**Low pH-mediated virus–cell fusion.** Fusion experiments were carried out as described previously (Edwards & Brown, 1986; Edwards et al., 1983). BHK-21 cells were split into 12-well plates to perform the low pH-mediated fusion from without. Plates were preincubated on an ice/water bath for 15–20 min in order to reach 4 °C. Either T398A/Y400N or wild-type virus was attached to cell monolayers for 15 min at 4 °C. After 15 min, the inoculum was removed, the wells were washed with 1× PBS-D and then treated with fusion medium (pH 5.3) for 5 min at room temperature. The fusion medium was washed off and the cells were then treated with fusion medium (pH 7.4) for 5 min at room temperature. The pH 7.4 fusion was then washed off, growth medium (1× MEM) was added and the plates were incubated for 1 h at 37 °C. After 1 h, the cell monolayers were analysed for fusion and photographed.

**RESULTS**

**Construction of SV E2 endodomain mutants**

X-ray crystallographic analysis of expressed C protein has revealed a hydrophobic cleft in the surface of the C protein (Choi et al., 1991). The cleft is bordered by two aromatic residues, one on either side: Y180 and W247 (Lee et al., 1996). Experiments in which capsid residue Y180 was converted to S identified Y180 as being critical for attachment of the capsid to the E2 tail and for function of the mature virion (Lee & Brown, 1994; Lee et al., 1994). These observations led to the hypothesis that these residues participate in an aromatic ring interaction with Y400 of the SV E2 tail (Skoging et al., 1996). Y400 is part of a sequence of 3 aa that are conserved among all the alphaviruses. We have shown previously that Y400, but not T398, is required for virus production (Liu & Brown, 1993a). To further examine the role of these amino acids in the interaction of the endodomain with the C protein, a mutant was constructed at E2 Y400 that also contained a point mutation at position T398. This double mutant was constructed to examine (i) the requirement for the aromatic association of E2 Y400 with C Y180 and W247, and (ii) the role that phosphorylation of E2 T398 or E2 Y400 plays in virus production. The double mutant T398A/Y400N prevents phosphorylation at either position and precludes any aromatic interaction. In a previous study, a point mutation at E2 Y400F caused a decrease in infectious virus formation of three to four orders of magnitude (Gaedigk-Nitschko & Schlesinger, 1991).

**Growth of the mutant T398A/Y400N in BHK-21 cells**

To determine the phenotype of the double mutant (T398A/Y400N), viral RNA was synthesized by in vitro transcription and the infectious RNA was introduced into BHK-21 cells via electroporation as described in Methods. Cells were transfected with either wild-type or mutant RNA and the culture medium was harvested 18–24 h post-transfection, when cytopathic effect was evident. Medium was flash-frozen in liquid nitrogen and stored at −80 °C prior to analysis. Plaque assay was used to assay the medium for the presence of infectious virus as described in Methods. Growth of the mutant virus is shown in Fig. 2. Compared to the wild-type parental virus, the double mutant showed a significant reduction (three to four orders of magnitude) in infectious virus production in BHK-21 cells. A low but significant amount of infectious virus was produced consistently from this double mutant, indicating that the proposed aromatic interaction between Y400 and the capsid residues Y180 and W247 is not absolutely required for the production of infectious virus. Analysis by RT-PCR through the E2 endodomain has shown that virus produced from

![Fig. 2. Production of infectious virus by cells transfected with RNA of the double mutant E2 T398A/Y400N or wild type (WT). Virus titres were determined on BHK-21 cells as described in Methods.](image-url)
T398A/Y400N retained the mutations at both positions. In addition, these data also indicate that, whilst phosphorylation of the E2 tail occurs, it is not absolutely required for production of infectious virus, as thought previously (Liu & Brown, 1993a), as both potential phosphorylation sites (T398 and Y400) are lost in the double mutant.

**Infectivity and particle assembly of mutant E2 T398A/Y400N**

The mutant E2 T398A/Y400N results in decreased infectious virus particle formation. It is possible that these mutations might alter the amount of infectious virus produced without altering the total number of particles produced, resulting in the formation of non-infectious particles. To determine whether the effect of the mutation T398A/Y400N was to produce non-infectious virus or whether particle assembly itself was inhibited, we determined the relative infectivity (particle: p.f.u. ratio) of the double mutant. Virus was purified by density-gradient centrifugation as described in Methods. The density of the collected virus bands is shown in Fig. 3 and the virus produced by the mutant was found to band at the same density as wild-type virus. BCA analysis of the purified virus proteins indicated that T398A/Y400N produced a slightly lesser amount of virus protein compared with the wild-type virus (Fig. 3). Purified virus collected from the gradient was also analysed by plaque assay. The particle: p.f.u. ratio was determined by using the obtained protein concentration and the titre of the purified virus. The virus produced by the double mutant was found to be significantly less infectious than that produced by the wild-type virus. The particle: p.f.u. ratio for wild-type virus was 6·25 : 1, whereas that for the double mutant was found to be 4·8 × 10^4 : 1. These data support the hypothesis that the mutations present in T398A/Y400N do not prevent virus assembly, but are important for virus infectivity. In order to determine whether the protein produced by T398A/Y400N observed in the BCA assay is actually incorporated into typical virus particles, twice-purified T398A/Y400N mutant virus was examined by negative-stain electron microscopy (Fig. 4). Virus particles did not appear to be empty, nor did they appear to have any aberrant structural properties compared with wild-type virus preparations.

**Compensating mutations in the C protein**

RNA viruses are known for their propensity for acquiring mutations (Drake & Holland, 1999). It is a common occurrence in RNA viruses that the effects of a mutation made in one residue or protein domain are offset by a compensating mutation at another position in the genome. These compensating mutations are frequently found in the protein with which the mutated protein interacts during virus assembly (Lopez et al., 1994). Compensating mutations provide important information on protein–protein interactions essential for virus assembly and function. It has been hypothesized that the SV E2 protein interacts through its endodomain with a hydrophobic cleft in the surface of the C protein (Lee & Brown, 1994; Lee et al., 1996). In order to determine whether the observed virus production in T398A/Y400N is due to a compensating mutation in or around the cleft of the C protein, RT-PCR was performed on RNA extracted from virus. RT-PCR was limited to the region of the capsid believed to interact with E2, the hydrophobic cleft (aa 160–264). As described in Methods, virus harvested from a transfection was pelleted at 50 000 r.p.m. and subjected to multiple extractions to remove virus proteins. Virus RNA was then reverse-transcribed and the resulting DNA was amplified by PCR. The DNA was sequenced and the resulting sequence was compared with that of the wild type through the C protein. This examination of the

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**Fig. 3.** Production of mutant virus of wild-type virus density by E2 T398A/Y400N. Labelled wild-type and mutant virus was purified and analysed as described in Methods. The refractive index for gradients containing the wild type (◯) or E2 T398A/Y400N (■) was measured by using a refractometer and the distribution of radiolabelled protein was determined by scintillation spectrometry.

**Fig. 4.** Electron microscopy of virus produced by mutant E2 T398A/Y400N. Negative staining of virus preparations was carried out as described in Methods. Density gradient-purified wild-type and mutant virus preparations were examined, although only the mutant is shown.
hydrophobic-cleft region between residues 160 and 264 revealed no mutations in this region of the C protein. This was an unexpected result, because this mutant has such a deleterious effect on the production of infectious virus; however, repeated analyses of virus produced by this mutant were consistent. The mutations were maintained in the tail, and compensating mutations were not found in the capsid or in other regions of the E2 tail. This suggests that the virus can tolerate the T398A and Y400N mutations during the assembly process, but not during the infection cycle.

**Fusion of cells by mutant E2 T398A/Y400N**

The glycoproteins of SV have the ability to produce cell–cell fusion after brief, transient exposure to acid pH (Edwards & Brown, 1986; Edwards et al., 1983; Mann et al., 1983; Paredes et al., 2004). It is possible that the mutations produced in the endodomain of E2, which reduce virus infectivity, also reduce the ability to mediate cell fusion.

To examine the ability of the double mutant to produce cell fusion, mutant or wild-type virus was attached to BHK-21 cells in different amounts to determine whether the ability to fuse cells correlated with the total number of particles or the number of infectious particles absorbed (Table 2). The low titres obtained for the mutant virus restricted the amount of virus that could be employed in this assay. Fifteen minutes at 4°C was allowed for absorption of virus and then the monolayers were washed into fusion medium (pH 5-3) at room temperature for 5 min, after which the low-pH medium was replaced with medium at pH 7-2 for 1 h. At the end of the incubation, the cells were examined under a phase-contrast microscope and the percentage of cells fused was determined (Table 2). The amount of infectious virus employed in this experiment was low, limited by the concentration of the double mutant. The amount of fusion obtained appeared to correlate with the amount of infectious virus. Ten times the number of mutant virus particles as wild-type particles resulted in equivalent m.o.i.s and produced the same amount of fusion (Table 2). Increasing the number of infectious virus particles increased the percentage of cells fused. This result indicated that the defect in the double mutant that reduced virus infectivity also reduced its ability to fuse cells after brief exposure to acid pH.

**DISCUSSION**

Previous studies have suggested that the E2 endodomain interacts specifically with a hydrophobic pocket in the surface of the SV C protein (Lee & Brown, 1994; Lee et al., 1996; Owen & Kuhn, 1997; Wilkinson et al., 2005). This interaction is critical for the assembly of mature virus particles and represents the initial stage of the budding process by which mature SV particles exit an infected cell. The endodomain of E2 is a 33 aa domain containing the sequence KARRECLTPYALAPNAVPTSLALLCCVRSANA (391–423). The domain itself has multiple functions, the primary function being attachment to the capsid to initiate the budding process. Secondary functions of this domain include producing the signal sequence for insertion of the 6K protein into the membrane of the ER prior to processing of the polyprotein (Liljestrom & Garoff, 1991). The 33 aa endodomain also contains the signalase cleavage site for separation of pE2 from the 6K protein (Liljestrom & Garoff, 1991).

One region of the E2 tail that has been well studied and proposed to be involved in binding to the C protein is the ‘TPY’ domain, aa 398–400 (Skoging et al., 1996). This domain is conserved throughout the alphaviruses and has been proposed to be involved in several different ways with the binding to the C protein. It has been suggested that Y400 is involved in an aromatic interaction with two residues in the C protein, Y180 and W247 (Skoging et al., 1996). It was proposed that this interaction stabilized the protein–protein interaction and allowed for the budding process to continue to completion (formation of mature viruses). It was also proposed that the ‘TPY’ domain was a site of phosphorylation during assembly and that phosphorylation and dephosphorylation were both required in order to form mature virus particles (Liu & Brown, 1993a). The data presented here suggest that the ‘TPY’ sequence is not essential for the formation of virus particles, but is essential for virus function. The ability of the double mutant to assemble virions suggests that aromatic interactions and phosphorylation events are not essential for this process. A revertant of the double mutant restored the Y at position 400, but left the A at position 398 (Liu et al., 1996), suggesting that the component essential for infectivity is the Y at position 400. In a previous study, we found that this double mutant did not produce detectable amounts of virus particles (Liu & Brown, 1993a). In this study, the RNA produced from the original clone transfected into BHK-21 cells does produce virus particles. The reason for this difference is not known, but is probably related to changes in the BHK-21 cell line in the intervening 12 years, which also includes a geographical change in the location of the laboratory. We have shown that a change in the host cell can alter the phenotype of a particular mutation (Hernandez et al., 2000, 2003, 2005; Nelson et al., 2005). The mutant is still inhibited severely in its ability to produce virions.

The mutations that we have produced in the E2 endodomain result in residues at positions 398 and 400 that are less bulky than those in the wild-type sequence. The change in

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**Table 2. Low pH-mediated fusion of cells by mutant and wild-type SV**

Cells were exposed to virus and fused as described in Methods.

<table>
<thead>
<tr>
<th>Virus</th>
<th>m.o.i.</th>
<th>Particles per cell</th>
<th>Cells fused (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.3</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>E2 T398A/Y400N</td>
<td>0.3</td>
<td>210</td>
<td>5</td>
</tr>
<tr>
<td>Wild type</td>
<td>17</td>
<td>765</td>
<td>95</td>
</tr>
<tr>
<td>E2 T398A/Y400N</td>
<td>0.18</td>
<td>126</td>
<td>1</td>
</tr>
</tbody>
</table>
atomic density probably affects the specific nature of this critical association, resulting in the reduced infectivity of the virus produced. Previous studies have demonstrated that there are other regions of the endodomain involved in this interaction (Lee et al., 1996; Lopez et al., 1994; Owen & Kuhn, 1997; Wilkinson et al., 2005) and that these interactions may be more critical to the process of assembly than the ‘TPY’ domain. Because of the changes in the T398A/Y400N mutant, the capsid protein may be bound less tightly to the endodomain and this association may be critical for release of the RNA.

The data presented above suggest that alterations at position Y400 result in production of virus particles that have low infectivity. It is noteworthy that other research has demonstrated that the position of the conserved Y in the ‘TPY’ sequence also plays a critical role in the assembly process (Hernandez et al., 2000). That research has shown that a distance of 9–10 amino acids from the inner surface of the membrane to the conserved Y is required for the production of virus particles. A single deletion at the membrane interface resulted in the inability to assemble virus and blocked the attachment of capsids to the endodomain (Hernandez et al., 2000). Other insertions or deletions in the region between the membrane and Y400 resulted in the loss of virus production and the production of structurally aberrant virions (Hernandez et al., 2005). The number of amino acids in the endodomain between the membrane and Y400 seems to be more critical for virus assembly, whilst Y at 400 seems to be critical for the infectivity of assembled particles.

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