An Amino Acid Change in the Exodomain of the E2 Protein of Sindbis Virus, Which Impairs the Release of Virus from Chicken Cells but Not From Mosquito Cells

Mei-Ling Li,* 1 Huey-Jane Liao,* 2 Lee D. Simon,† and Victor Stollar* 3

*Department of Molecular Genetics and Microbiology, UMDNJ–Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, New Jersey 08854-5635; and †Department of Genetics, Nelson Laboratory, Busch Campus, Rutgers University, Piscataway, New Jersey 08854

Received July 12, 1999; accepted August 16, 1999

INTRODUCTION

Sindbis virus contains three structural proteins, the capsid or C protein and two envelope proteins, E1 and E2. These are derived by processing of a polyprotein which is the translation product of the subgenomic 26S RNA (see Strauss and Strauss, 1994; Schlesinger and Schlesinger, 1996, for reviews). E2 is derived from a precursor protein, pE2, which associates with E1 in the rough endoplasmic reticulum (RER) to form a heterodimer. The pE2.E1 heterodimers are transported through the RER and the Golgi system; then, in a post-Golgi compartment, an N-terminal sequence is cleaved from pE2, leaving E2. Finally, the E2.E1 heterodimers are transported to the plasma membrane. At some stage the heterodimers trimerize to form the viral spikes. From the results of cross-linking experiments, Mulvey and Brown (1997) suggest that this occurs in the RER. Like most viral envelope proteins, E2 and E1 are acylated and glycosylated.

The E2 protein of Sindbis virus is 423 amino acids long, has a membrane-spanning anchor of about 26 amino acids, and has a 33-amino-acid endodomain or cytoplasmic tail. There is now strong evidence which indicates that the budding of alphaviruses from the cell surface requires and is driven by a specific interaction between the capsid protein and the cytoplasmic tail of the E2 protein (Lopez et al., 1994; Lee et al., 1996). In recent work, specific amino acids involved in the interaction between the cytoplasmic tail of E2 and the C protein have been identified (Skoging et al., 1996; Owen and Kuhn, 1997).

Some years ago we reported that the replication of our standard Sindbis virus (SVSTD) in Aedes albopictus mosquito cells was markedly inhibited when infected cells were deprived of methionine (Stollar, 1978). We then derived from SVSTD a viral mutant, SVLM21, which had a low methionine-resistant (LMR) phenotype; i.e., in contrast to SVSTD, it was able to replicate normally in methionine-deprived mosquito cells (Durbin and Stollar, 1985). SVLM21 subsequently proved instrumental in identifying the SV nonstructural (ns) protein, nsP1, as a methyltransferase, presumably the methyltransferase responsible for the methylation of the cap moiety of the viral mRNAs (Mi et al., 1989). The association of the alphavirus ns protein, nsP1, with a methyltransferase activity was later confirmed in work with Semliki forest virus (Lääkkonen et al., 1994).
To obtain SVLM21, we began with SVSTD and carried out 17 serial undiluted passages in methionine-deprived Ae. albopictus cells; the resulting stock was then plaque purified, also in Ae. albopictus cells. This virus, designated SVLM17, in Durbin and Stollar (1985), will now be referred to simply as SVLM17. SVLM17 grew well in mosquito cells and clearly had the LMR phenotype; however, it produced low yields of infectious virus from vertebrate cells. Typically the amount of infectious virus produced was about 100-fold less than that seen following a comparable infection with SVSTD.

Although our main interest at the time was to obtain a virus stock with the LMR phenotype, we also considered it desirable that this virus replicate well in both mosquito and vertebrate cells. Accordingly we passaged SVLM17 three times in BHK cells and then plaque purified it, also on BHK cells. The virus we obtained following the passage in BHK cells, SVLM17, retained the LMR phenotype, but was also able to replicate in vertebrate cells as efficiently as SVSTD (Durbin and Stollar, 1985). (The designation 21 in SVLM21 refers to a clone number, not the passage levels.)

Recently, we returned to the question of why SVLM17 replicated poorly in vertebrate cells. In this report, we show that the block to the replication of SVLM17 in chick embryo fibroblast (CEF) cultures is at a late stage in virus assembly and that it is accounted for by a change from Ala to Val at position 251 in the envelope protein E2. Our findings support the proposition that in addition to the interaction between the C protein and the cytoplasmic tail of E2, the ectodomain of E2 plays an important role in the late stages of viral assembly.

RESULTS

Replication of SVLM17 in CEF and mosquito cells

Table 1 illustrates the host restriction of SVLM17. SVSTD, SVTOTO, and SVLM17 all produced high yields of infectious virus from mosquito cells, ranging from 5.8 to 9.8 \( \times 10^8 \) PFU/ml. On the other hand, whereas with both SVSTD and SVTOTO, the yield of infectious virus from CEF was somewhat higher than that from mosquito cells, in the case of SVLM17, the yield of virus from CEF was less than 1% of that from mosquito cells. Interestingly, however, the ability of SVLM17 to form plaques on CEF was not impaired (Table 2). Thus like both SVSTD and SVTOTO, SVLM17, when assayed directly by plaque formation, gave titers on CEF comparable to those measured on mosquito cells. This result suggests that although the release of SVLM17 from CEF into the liquid medium is greatly reduced, under the conditions used for the plaque assay, sufficient SVLM17 must still be able to spread from cell to cell in the CEF cultures to form plaques.

Localization of the block to replication of SVLM17 in CEF

In order to obtain information concerning the stage at which the replication of SVLM17 in CEF is blocked, we first examined the synthesis of the structural proteins and the processing of the precursor protein, pE2, to the envelope protein, E2. CEF were infected with either SVSTD or SVLM17, pulse-labeled with \(^{35}\)S)methionine, and then chased for up to 3 h. As shown in Fig. 1, comparable amounts of labeled viral proteins were made in both cultures; furthermore, the processing of pE2 to E2 in CEF cultures infected with SVSTD (lanes 2–8) and in CEF cultures infected with SVLM17 (lanes 10–16) was similar. Since the processing of pE2 to E2 occurs in a post-Golgi compartment, and since the envelope proteins are transported to the cell surface as a pE2/E2–E1 heterodimer, the SVLM17 envelope proteins synthesized in CEF must be transported normally through the endoplasmic reticulum and the Golgi system. This suggests that the block to the assembly and release of SVLM17 from CEF must occur at a late stage in virus maturation.

We then wished to learn whether in SVLM17-infected CEF the envelope proteins were able to reach the cell surface. This was determined by means of a fluorescent antibody technique. When SVLM17-infected CEF were permeabilized and then reacted with a polyclonal anti-E2 serum, intense fluorescence was observed in the cyto-

<table>
<thead>
<tr>
<th>Virus</th>
<th>Ae. albopictus (a)</th>
<th>CEF (b)</th>
<th>b/a</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVSTD</td>
<td>6.2 ( \times 10^8 )</td>
<td>9.7 ( \times 10^4 )</td>
<td>1.6</td>
</tr>
<tr>
<td>SVTOTO</td>
<td>4.4 ( \times 10^8 )</td>
<td>3.6 ( \times 10^4 )</td>
<td>8.2</td>
</tr>
<tr>
<td>SVLM17</td>
<td>8.1 ( \times 10^8 )</td>
<td>1.2 ( \times 10^4 )</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Note. Virus titers were assayed directly by plaque formation on primary CEF and on mosquito cell cultures as described under Materials and Methods.
plasm (Fig. 2B). When CEF were infected with either SVSTD or SV LM17 but were not permeabilized, fluorescence at the cell surface was observed in both cases (Figs. 2C and 2D, respectively). Thus we conclude that the SV LM17 pE2/E2–E1 heterodimers must reach the surface of infected CEF.

Next we examined SV STD- and SV LM17-infected cells by electron microscopy. In SV STD-infected CEF, nucleocapsids, or possible nucleocapsid precursors with electron transparent centers, were seen in the cytoplasm, and budding of viral particles was readily observed (Figs. 3C and 3D). In contrast, in SVLM17-infected cells (Fig. 3A), although nucleocapsid structures could be seen in the cytoplasm, they were not aligned under the plasma membrane, nor was budding of viral particles observed. In this particular section, there was an aggregation of nucleocapsids (arrows). The nucleocapsids with the less opaque centers are reminiscent of those observed in mouse brain during the early stage of infection with eastern equine encephalitis virus (Murphy, 1980). Figure 3B shows cells infected with SV TOTO/E2/A25IG, a mutant produced by site-directed mutagenesis, which has the SV LM17 phenotype (see below). No budding particles were seen; nucleocapsids or nucleocapsid precursors were seen in the cytoplasm but not beneath the plasma membrane.

Identification of the mutation responsible for the SV LM17 phenotype

Double-stranded cDNA was synthesized from SV LM17 viral RNA and digested with BamHI (nt 7334) and NsiI (nt 11452). The nt 7334–11452 fragment was then subcloned into pToto 7354 (this is a deleted form of pToto1101 having deletions from nt 1074 to 6528 and from nt 8038 to 8866) which had also been digested with BamHI and NsiI. Sequencing of the region containing the coding information for the viral structural proteins revealed a single mutation; at position 9382, there was a T in place of a C, changing Ala251 of E2 to Val.

By substituting the sequence from position 7999 (AatII) to position 11749 (XhoI) of SV LM17 cDNA for the corresponding sequence of Toto1101, we generated pToto LM17/7999–11749. Infectious RNA transcripts were then made from this recombinant plasmid and used to produce virus in mosquito cells. Tables 3 and 4 show that SV TOTO/LM17/7999–11749, the virus which was obtained from the recombinant pToto, had the same...
phenotype as SVLM17; thus it produced a low yield of infectious virus from CEF, but showed no impairment when assayed by plaque formation on CEF.

Site-directed mutagenesis was performed to confirm that the amino acid change at position 251 of E2 was responsible for the phenotype of SVLM17. Thus, the C at position 9382 of pToto1101 was changed to a T, giving pTotoC9382T and subsequently the virus SV TOTO/E2/A251V, as seen in Tables 3 and 4, SV TOTO/E2/A251V had the same phenotype as SVLM17, confirming that the amino acid change at position 251 of E2 sufficed to produce the SVLM17 phenotype.

The change from an Ala residue to a Val residue is a conservative change. We therefore made two other similar changes at position 251 of E2. Mutations were made in pToto to change the Ala at this position in one case to a Gly (T9382G) and in the other case to a Leu (G9381C). In both cases, the release of virus was restricted in CEF (Table 3), but plaque formation was not impaired (Table 4). Thus the SVLM17 phenotype was retained. This result suggests that the presence of an Ala at position 251 of E2 is required for efficient maturation of SV in CEF and that it cannot be substituted for by another hydrophobic amino acid, or by Gly, which lacks a side chain.

**DISCUSSION**

The results which we have presented show that when CEF were infected with SVLM17, the viral structural proteins were made in normal amounts, pE2 was processed to E2, and the envelope proteins reached the cell surface. However, alignment of nucleocapsids with the plasma membrane was not observed, budding of viral particles did not occur, and the yield of virus in the medium was greatly reduced. Why the plaque-forming efficiency of SVLM17 on monolayers of CEF is not reduced...
remains unclear. A likely explanation is that although the amount of virus released from each infected cell in the SVLM17-infected CEF cultures is drastically reduced, the small amount of virus released from each cell is still sufficient to infect all the neighboring cells and thus to give rise to plaques. Interestingly, when Niesters and Strauss (1990) carried out a mutational analysis of the conserved 51 nucleotide sequence (nts 155–205) of Sindbis virus RNA, they observed that at least five different mutants which produced virus yields only 1% or less than that seen with the wild-type virus nevertheless produced normal-size plaques. Thus, although there is generally a correlation between virus yield and plaque size, this is not always the case.

The central question, however, is why the assembly and production of LVLM17 particles, which are dramatically inhibited in CEF, are apparently normal in mosquito cells. Although we cannot answer this question, this finding strongly suggests an important difference between the assembly of alphaviruses in mosquito cells and that in vertebrate cells. The most obvious difference reported so far relates to the site of assembly. Whereas in vertebrate cells virus budding occurs at the plasma membrane (Acheson and Tamm, 1967), in mosquito cells large numbers of viral particles are seen in cytoplasmic vesicles (Gliedman et al., 1975), suggesting that budding and assembly in mosquito cells occur mainly in association with cytoplasmic membranes rather than at the plasma membrane.

There are also significant biochemical differences between alphaviruses grown in mosquito cells and the same viruses grown in vertebrate cells. These differences relate to carbohydrate and lipid content and reflect the properties of the host cells. Thus, the complex N-linked glycans, which contain fucose and sialic acid and which are present on the envelope proteins of viruses grown in vertebrate cells, are lacking on the envelope proteins of viruses grown in mosquito cells. In the latter, these complex N-linked glycans are generally replaced by Man3 GlcNAc2 glycans, which are the most highly processed oligosaccharides in Aedes albopticus cells (Hsieh and Robbins, 1984). Similarly, whereas Semliki forest virus (SFV) grown in BHK cells contains phosphatidylethanolamine as the predominant phospholipid (42%), when SFV is grown in A. albopticus cells, the predominant phospholipid is phosphatidylethanolamine (62%) (Luukkonen et al., 1976). Whether these differences in the lipid composition or the glycosylating capability of the host cells might explain why SVLM17 is able to be assembled and released efficiently from mosquito cells but not from CEF remains unknown.

Other mutants of Sindbis virus that have (1) an amino acid change in the ectodomain of E2 and (2) a block at a late stage in assembly and/or a defect or alteration in the budding process are listed in Table 5. SVAP15/21, like SVLM17, is restricted in vertebrate cells and in addition is temperature-sensitive. Furthermore, the SVAP15/21 nucleocapsids align with the plasma membrane, an indication that the nucleocapsid (NC) is interacting with the cytoplasmic tail of E2. The phenotype of SVAP15/21 has been shown to result from the generation of a new glycosylation site at Asn275 of E2 (Durbin and Stollar, 1984, 1986).

Although the temperature-sensitive mutant of Sindbis virus, ts-20, fails to process pE2 to E2 under nonpermissive conditions, the viral NCs do become aligned beneath the plasma membrane (Brown and Smith, 1975) and released efficiently from mosquito cells but not from CEF. Interestingly, the amino acid changes responsible for the phenotypes of SVLM17, SVAP15/20, ts-20, and ts-103 are all present in a fairly small region of the ectodomain of E2 between positions 251 and 344. At none of these positions, however, are the amino acids highly con-

### TABLE 3

<table>
<thead>
<tr>
<th>Virus</th>
<th>Ae. albopictus (a)</th>
<th>CEF (b)</th>
<th>b/a</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVLM17</td>
<td>9.0 x 10^1</td>
<td>6.3 x 10^9</td>
<td>0.7</td>
</tr>
<tr>
<td>SVTOTO</td>
<td>8.4 x 10^1</td>
<td>8.8 x 10^9</td>
<td>1.0</td>
</tr>
<tr>
<td>SVTOTO/LM17</td>
<td>6.4 x 10^1</td>
<td>5.3 x 10^9</td>
<td>8.3 x 10^-1</td>
</tr>
<tr>
<td>SVTOTO/201</td>
<td>9.1 x 10^1</td>
<td>8.5 x 10^9</td>
<td>9.3 x 10^-4</td>
</tr>
<tr>
<td>SVTOTO/201G1</td>
<td>1.4 x 10^-3</td>
<td>2.8 x 10^9</td>
<td>2.0 x 10^-1</td>
</tr>
<tr>
<td>SVTOTO/201L</td>
<td>1.2 x 10^-4</td>
<td>3.5 x 10^8</td>
<td>2.4 x 10^-4</td>
</tr>
<tr>
<td>SVTOTO/201G1L</td>
<td>1.0 x 10^4</td>
<td>5.5 x 10^5</td>
<td>5.5 x 10^-4</td>
</tr>
</tbody>
</table>

Note. Confluent primary CEF or Ae. albopictus cells were infected at an m.o.i. of one PFU/cell and incubated at 34°C for 24 h. Medium was then harvested and titrated for infectious virus by plaque formation on primary CEF.

### TABLE 4

<table>
<thead>
<tr>
<th>Virus</th>
<th>Ae. albopictus (a)</th>
<th>CEF (b)</th>
<th>b/a</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVLM17</td>
<td>6.5 x 10^8</td>
<td>1.5 x 10^9</td>
<td>2.3</td>
</tr>
<tr>
<td>SVTOTO</td>
<td>1.4 x 10^8</td>
<td>1.0 x 10^9</td>
<td>0.7</td>
</tr>
<tr>
<td>SVTOTO</td>
<td>1.0 x 10^9</td>
<td>1.4 x 10^9</td>
<td>1.4</td>
</tr>
<tr>
<td>SVTOTO/201</td>
<td>5.2 x 10^1</td>
<td>1.4 x 10^10</td>
<td>0.3</td>
</tr>
<tr>
<td>SVTOTO/201G1</td>
<td>1.6 x 10^-10</td>
<td>1.2 x 10^10</td>
<td>0.7</td>
</tr>
<tr>
<td>SVTOTO/201L</td>
<td>1.1 x 10^9</td>
<td>1.2 x 10^9</td>
<td>1.1</td>
</tr>
<tr>
<td>SVTOTO/201G1L</td>
<td>9.3 x 10^-3</td>
<td>1.3 x 10^9</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Note. Virus titers were assayed directly by plaque formation on primary CEF and on mosquito cell cultures as described under Materials and Methods.
served. At the site corresponding to position 251 of the SV E2 protein where the amino acid change responsible for the host restriction of SVLM17 occurs, Semliki forest and Ross river viruses also have an Ala. However, O’nyong-nyong, Venezuelan equine encephalitis, and eastern equine encephalitis viruses have an Asp, a Leu, and a Phe, respectively, at this position. Interestingly, one or two positions downstream of position 251, depending on the virus, there is a highly conserved sequence, K/R/Q.G.K.L/V/I.H/V/L/I.PF. The function of this sequence is not known.

A late defect in virus assembly is also observed when SVSTD-infected CEF or BHK cells are maintained after infection in low-ionic-strength medium (Waite and Pfefferkorn, 1970). We recently isolated a mutant, SVLS29, which is able to replicate normally under such conditions; the amino acid changes responsible for the SVLS29 phenotype were also in the ectodomain of E2, but in the N-terminal region of the protein (Li and Stollar, 1995).

Although, as noted above, the interaction between the C protein of the nucleocapsid and the endodomain of E2 is crucial to the budding of alphaviruses, the properties of the viral mutants described in Table 5 suggest that the ectodomain of E2 must also play a vital role in the final stages of viral assembly. Just how the ectodomain influences the assembly process or budding of E2 we can only speculate. It has been suggested by Yao et al. (1996) that for the tail of E2 to be correctly positioned for interaction with the NC, the E2–E1 heterodimer may have to attain a certain conformation. Possibly an amino acid change in the ectodomain of E2 may prevent the heterodimer from attaining that conformation. But as Yao et al. also point out, there is so far no direct evidence that E2–E1 interactions can affect the orientation of the tail. Alternatively, given that the binding between the envelope proteins and the NC is a multivalent interaction, trimerization of the E2–E1 heterodimers may be crucial for driving the budding reaction. The results of Ekström et al. (1994) are in accord with this idea. If the efficient trimerization of the E2–E1 heterodimers requires a proper and specific configuration of E2, then it is reasonable to expect that this configuration could be disturbed by certain critical amino acid changes. It is also possible that an amino acid change in E2, such as that in SVLM17, might affect virus assembly at a higher level of organization, e.g., the lateral aggregation of trimers within the plane of the plasma membrane, a process necessary for formation of the icosahedral lattice on the viral surface.

A clear understanding of how alphaviruses bud through the plasma membrane and are assembled must await more information about the conformation of the viral envelope proteins.

### MATERIALS AND METHODS

#### Cells, media, and viruses

The *Ae. albopictus* mosquito cells (clone C7-10) and the preparation of primary chick embryo fibroblasts (CEF) have been described previously (Stollar et al., 1976; Durbin and Stollar, 1984).

SVSTD is a cloned derivative (Shenk and Stollar, 1973) of the HR strain of Burge and Pfefferkorn (1966). SVLM17 and SVLM21 were described above. SV Toto is virus derived from Toto 1101 (Rice et al., 1987; and see below).

Following infection, cell cultures were maintained in EA medium (Eagle’s MEM supplemented with nonessential amino acids and glutamine plus 0.1% BSA) (Durbin and Stollar, 1984).

To infect primary CEF or *Ae. albopictus* cells, confluent cultures in 60-mm plates were inoculated with 0.5 ml of virus at the indicated m.o.i. After 60 min at 34°C for adsorption, the virus was removed and the cultures were maintained in EA medium at 34°C with 5% CO₂.

Plaque assays of SV on primary CEF and on mosquito cells were carried out as described by Shenk et al. (1974) and Durbin and Stollar (1984), respectively.
Synthesis of viral cDNA and site-directed mutagenesis

The purification of viral RNA from virions and synthesis of double-stranded cDNA have been described previously (Durbin and Stollar, 1986; Li and Stollar, 1995). Site-directed mutagenesis was performed by the PCR-based method described by Landt et al. (1990). The presence of the desired mutations was confirmed by nucleic acid sequencing; in addition, in each case the entire sequence amplified by PCR was sequenced to rule out the presence of extraneous mutations. DNA was sequenced by the chain-termination method (Sanger et al., 1977) using Sequenase version 2.0 and the protocol supplied with the kit (United States Biochemicals).

Generation of infectious RNA transcripts from pToto and transfection of Ae. albopictus cells

Toto 1101 (13638 bp) is a plasmid which contains a full-length cDNA clone of SV RNA immediately downstream from an SP6 promoter (Rice et al., 1987). The preparation of SV RNA transcripts from Toto 1101 and the transfection of Ae. albopictus cells were carried out as described previously (Li and Stollar, 1995).

Labeling and gel electrophoresis of viral proteins

Labeling of viral proteins in infected cultures and preparation of cell lysates were performed as described by Li and Stollar (1995). Viral proteins were examined by means of 10% nonreducing SDS–PAGE (Durbin and Stollar, 1984).

Immunofluorescent microscopy

Viral antigen in permeabilized and nonpermeabilized virus-infected CEF cultures was visualized by the indirect fluorescent antibody method. To prepare nonpermeabilized cells, cells were fixed with 4% paraformaldehyde. To prepare permeabilized cells, cells were first fixed with paraformaldehyde and then treated with 0.05% Triton X-100 (Saraste et al., 1980). Anti-E2 rabbit serum which had been obtained from Dr. Joel Dalrymple was diluted 1:200 and used as the primary antibody; the second antibody was fluorescein-conjugated goat anti-rabbit IgG (1:200) purchased from Cappel. Cells were examined using a Zeiss Axioplan microscope equipped with an epifluorescer with fluorescein and rhodamine filters. Micrographs of cells were obtained by electronic imaging as described by Li and Stollar (1995).

Electron microscopy

Cells were infected with virus at an m.o.i. of 100 PFU/cell. At 6 h postinfection, the cell monolayers were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1.5 h, rinsed in cacodylate buffer, and postfixed with 1% osmium tetroxide for 1 h. Following dehydration in a graded ethanol series (50, 75, 95, and 100%), the cell monolayers were infiltrated with Spurr's epoxy resin (Spurr, 1969), after which pure Spurr's epoxy resin was added to the monolayers in the petri dishes. The resin was then polymerized at 60°C, after which 2- to 3-mm strips were cut through the polymerized resin, the cell monolayer, and the petri dish. Thin sections cut from these strips were stained with uranyl acetate and lead citrate and examined by electron microscopy.

ACKNOWLEDGMENTS

The studies described here were supported by the US-Japan Medical Science Program through Public Health Service Grant AI-05920 from the National Institutes of Health. We are also grateful to Lillian M. MacMath for her help in the preparation of the manuscript.

REFERENCES