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# Molecular Genetic Study of the Interaction of Sindbis Virus E2 with Ross River Virus E1 for Virus Budding

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**Glycoprotein PE2 of Sindbis virus will form a heterodimer with glycoprotein E1 of Ross River virus that is cleaved to an E2/E1 heterodimer and transported to the cell plasma membrane, but this chimeric heterodimer fails to interact with Sindbis virus nucleocapsids, and very little budding to produce mature virus occurs upon infection with chimeric viruses. We have isolated in both Sindbis virus E2 and in Ross River virus E1 a series of suppressing mutations that adapt these two proteins to one another and allow increased levels of chimeric virus production. Two adaptive E1 changes in an ectodomain immediately adjacent to the membrane anchor and five adaptive E2 changes in a 12-residue ectodomain centered on Asp-242 have been identified. One change in Ross River virus E1 (Gln-411→Leu) and one change in Sindbis virus E2 (Asp-248→Tyr) were investigated in detail. Each change individually leads to about a 10-fold increase in virus production, and combined the two changes lead to a 100-fold increase in virus. During passage of a chimeric virus containing Ross River virus E1 and Sindbis virus E2, the E2 change was first selected, followed by the E1 change. Heterodimers containing these two adaptive mutations have a demonstrably increased degree of interaction with Sindbis virus nucleocapsids. In the parental chimera, no interaction between heterodimers and capsids was visible at the plasma membrane in electron microscopic studies, whereas alignment of nucleocapsids along the plasma membrane, indicating interaction of heterodimers with nucleocapsids, was readily seen in the adapted chimera. The significance of these findings in light of our current understanding of alphavirus budding is discussed.**

Alphaviruses comprise a group of 26 animal viruses that mature by budding of a preformed nucleocapsid through the cell plasma membrane, such that the mature virus particle contains a lipid envelope with two virus-encoded glycoproteins, called E2 and E1, anchored in it. The alphavirus virion is a regular icosahedral structure with T=4 symmetry, in contrast to the less well defined structures possessed by many enveloped viruses. This symmetry arises in part because the regular geometry of the nucleocapsid, formed when 240 copies of the capsid protein encapsidate the 11.7-kb viral RNA genome, is imposed upon the glycoproteins during budding through a one-to-one interaction between individual nucleocapsid subunits and the cytoplasmic domains of glycoprotein E2; these E2 tail-capsid interactions provide much of the free energy of budding (14, 15).

The viral glycoproteins E1 and PE2, a precursor to E2, associate to form a heterodimer within minutes following their synthesis and insertion into the endoplasmic reticulum (1). As the PE2/E1 heterodimers are transported to the cell surface, PE2 is cleaved to E2 by furin or a furin-like enzyme, resulting in an E2/E1 heterodimer (reviewed in reference 14). Sometime before budding or during budding, three E2/E1 heterodimers associate to form a trimeric spike, and the 240 heterodimers on the surface of the virion thus form 80 spikes whose structure has been resolved to about 25Å (2, 11, 16). The interactions to form trimers, as well as longer-range interactions between the glycoproteins in the virus, also contribute to the free energy of budding (3, 17).

We previously found that a chimeric alphavirus that had PE2 from Sindbis virus (SIN) but E1 from Ross River virus (RR), referred to as SIN(RRE1), was almost nonviable because of a failure to bud (19). The PE2 glycoproteins of SIN and RR share only 43% amino acid sequence identity, and the E1 glycoproteins share 51% identity. Despite this extensive sequence divergence, the SIN PE2 and RR E1 encoded in the genome of SIN(RRE1) formed heterodimers that were cleaved to E2/E1 heterodimers and transported to the cell plasma membrane. However, these heterodimers differed in conformation from SIN E2/SIN E1 heterodimers (as well as from RR E2/RR E1 heterodimers), as shown by the facts that (i) the glycoproteins in the chimeric heterodimers differed from those in the parental heterodimers in their availability for biotinylation at the cell surface and (ii) the chimeric heterodimers were unable to interact with SIN nucleocapsids to drive budding. Although copious quantities of nucleocapsids were present in the cytoplasm of cells transfected with SIN (RRE1) RNA and chimeric E2/E1 heterodimers were clearly present in the plasma membrane, no evidence for interaction of nucleocapsids with the glycoproteins in the plasma membrane could be seen at the level of electron microscopy. In the present study, we have isolated variants of this chimeric virus in which the SIN E2 and RR E1 have become better adapted to each other such that the interaction between the heterodimers and the nucleocapsid is now readily observable in the electron microscope and virus budding is 100-fold more efficient.

## MATERIALS AND METHODS

**Virus and cells.** Construction and characterization of the full-length chimeric cDNA clone pSIN(RRE1) and transfection of cells with RNA transcribed in vitro from this clone have been described, as have the methods used for characterization of infected cells by electron microscopy and for assay of intracellular nucleocapsids or released virus by sucrose gradient sedimentation (19).

**Passage of chimeric virus to produce adapted variants.** RNA was transcribed from the chimeric cDNA clone pSIN(RRE1) and transfected into BHK-21 cells, using Lipofectin, as previously described (19). The culture fluid was harvested after 3 days, and released virus was quantitated by plaque assay on BHK cells. Five well-separated plaques were picked and used to initiate five independent passage series by infection of confluent monolayers of BHK cells. After incuba-

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tion for 3 days at 37°C, the culture fluid in each series was diluted fivefold and used to infect a new plate of BHK cells. Subsequent passages used the same procedure but with harvest after 2 days rather than 3 days, and a total of 10 passages were carried out for each of the five series. The culture fluid from the 10th passage (P10) was used to infect cells for preparation of RNA or virus.

An additional six passage series were initiated by transfecting BHK cells with RNA as described above and harvesting the culture fluid after only 2 days. The culture fluid was divided into six parts, and each was used to initiate an independent passage series. Subsequent passages in each series were carried out as before. The culture fluid from P10 was analyzed by plaque assay, and a single plaque was picked and used to prepare a stock of virus.

**Sequencing of variants.** For P10 of passage series 1, the culture fluid was diluted and used to infect a 150-mm-diameter petri plate of confluent BHK cells. After incubation for 36 h at 37°C, the cells were harvested and total cytoplasmic RNA was isolated by using RNAzol B as described by the manufacturer (TelTest Inc.), followed by isolation of poly(A)-containing RNA by using an Oligotex kit. First-strand cDNA was made by using a $dT_{14}$ primer and avian myeloblastosis virus reverse transcriptase at 42°C for 1 h. Second-strand cDNA was synthesized by using RNase H and DNA polymerase I as described by Gubler and Hoffmann (4). The double-stranded cDNA was blunt ended, fractionated on low-melting-temperature agarose, ligated to phosphorylated *Eco*RI linkers, and cloned into the *Eco*RI site of pGEM3Z as previously described (13). Clones containing the E1 and E2 regions were identified by colony lift hybridization. The sequence of the entire structural region was obtained with Sequenase, using appropriately chosen synthetic primers.

For sequencing of P5 of series 1 as well as of P10 of series 2 to 11, 150-mm-diameter petri plates of BHK cells were infected, released virus was harvested after 48 h, precipitated with polyethylene glycol (12), and resuspended in Tris-EDTA buffer, and RNA was extracted with phenol-chloroform. Reverse transcription-PCR was used to amplify regions of the viral glycoprotein, followed by cloning of the cDNA into the *Sma*I site of pGEM3Z. In the case of P5 of series 1, the entire E2-E1 region was sequenced, whereas in the case of passage series 2 to 11, only selected regions were sequenced, as described in the text. In either case, first-strand cDNA was primed with 5′ ATTCCCCTCGAGGAATTCCCT$_{15}$ 3′, and PCR amplification used one of two sets of primers. Primer set 1, for cloning of E2 sequences, consisted of sense primer 5′ CCTGGAATAGTAAA GGGA 3′ and antisense primer 5′ GCTCGTAAGCTTTTGCGG 3′; primer set 2, for cloning of E1 sequences, consisted of sense primer 5′ CGGAACCAACC AGTGAAT 3′ and antisense primer 5′ ATTCCCCTCGAGGAATTCCCT 3′. The PCR product was purified on low-melting-temperature agarose gels, blunt ended, phosphorylated, and cloned into *Sma*I-cut, dephosphorylated pGEM3Z; in some cases the PCR DNA itself was sequenced so as to obtain a consensus sequence that is not affected by PCR errors or clonal variation; in other cases the cloned cDNA was sequenced, in which case more than one clone was normally sequenced so as to obtain a consensus sequence.

**cDNA clones of adapted variants.** Mutations identified in variant clones were moved into the full-length pSIN(RRE1) through intermediate shuttle vectors. The 319-nucleotide (nt) E2 fragment *Sna*BI-*Pfl*MI (SIN positions 9221 to 9550) containing the E2 change at position 248 was cloned into an intermediate vector consisting of the *Ssp*I-*Ssp*I fragment from pSIN(RR6K) inserted into the *Eco*RI site of pGEM3Z. The *Mlu*I-*Bss*HII fragment from this shuttle vector was then inserted into SIN(RRE1) by two-piece ligation. For the E1 mutation, the 1,044-nt *Sma*I-*Nhe*I fragment (RR coordinates 10689 to 11733) was cloned into an intermediate clone consisting of the full-length RR clone pRR40 from which nt 2745 to 6468 had been deleted. The *Eco*R47III-*Nhe*I fragment from this shuttle clone was then cloned into pSIN(RRE1). To combine the two mutations, the *Bss*HII-*Xho*I fragment from the full-length clone containing the E1 mutation was used to replace the corresponding fragment in the full-length clone containing the E2 mutation.

## RESULTS

**Adaptation of RR E1 to SIN E2.** SIN(RRE1) is a chimeric virus whose genome is entirely derived from SIN except for the 6K gene, the E1 gene, and the 3′ nontranslated region, which have been replaced with the corresponding regions of RR. Infection of cells with this chimera leads to production of virus at a rate only about $10^{-7}$ that of SIN, due to the failure of the SIN E2/RR E1 heterodimers to interact with nucleocapsids (19). When this chimeric virus was passaged 10 times in BHK cells, variants arose that formed larger plaques and that produced about 100-fold more virus than did the parental chimera during growth in BHK cells. Variants present in one passage series were characterized in detail, and an overview of this passage series is shown in Table 1. After five passages, variants that resulted in the production of 30-fold more virus than did the parental chimera were present, whereas after 10 passages, variants that produced 200-fold more virus were present.

TABLE 1. Passage of chimeric virus[a]

| Passage no. | Yield (PFU/ml) | Amino acid changes in[b]: | |
|---|---|---|---|
| | | SIN E2 | RR E1 |
| 0 | $1 \times 10^4$ | | |
| 5 | $3 \times 10^5$ | Asp-248→Tyr | None |
| 10 | $2 \times 10^6$ | Asp-248→Tyr | Gln-411→Leu |

[a] Passage series 1. P0 was initiated by infection of BHK cells with a single plaque, and the yield is that after 72 h. For P1 to P10, the culture fluid from the preceding passage was diluted fivefold and used to infect a new plate of cells, and the yield is that after 48 h of infection.

[b] The entire region encoding structural proteins in virus present in P5 and P10 virus was cloned and sequenced, and the sequence was compared with that in the chimeric cDNA clone pSIN(RRE1).

The structural regions from the P5 and P10 viruses were cloned and sequenced. More than one clone was sequenced, and only changes present in all clones are reported in order to eliminate changes that may have been introduced during PCR. Only one change, Asp-248→Tyr in SIN E2, was found in the P5 virus, whereas two changes, Asp-248→Tyr in SIN E2 and Gln-411→Leu in RR E1, were found in the P10 virus (Table 1). Thus our preliminary conclusion was that the change in SIN E2 arose first and allowed the virus to grow 30-fold better and was followed by a second change in RR E1 that led to a further sevenfold increase in virus production.

To further define the individual contributions of these two changes to the phenotype of better growth and to rule out the possibility that other changes in the chimeric virus genome were responsible for the ability of the viruses to grow to higher titers, the changes in E2 and E1 were placed individually or together into the parental chimeric cDNA clone. The growth rates of viruses rescued from the reconstructed clones are shown in Fig. 1; in this growth curve, transfection of RNA was used to initiate infection so as to reduce the possibility that further changes might occur in the variants during the experiment. Each mutation separately allowed the virus to grow better than the parental chimera; after 48 h, the E2 mutant had produced 15-fold more virus than the parent and the E1 mu-

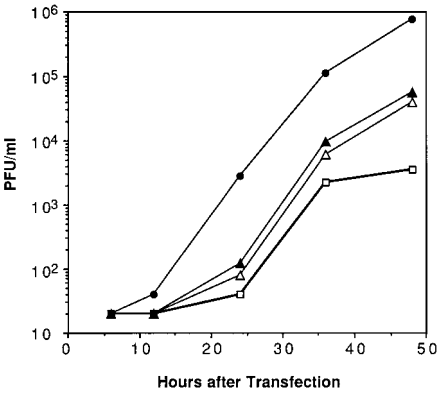


FIG. 1. Growth curves of cloned variants of the chimeric SIN(RRE1). The E2 change Asp-248→Tyr and the E1 change Gln-411→Leu were inserted individually or together into the full-length chimeric clone pSIN(RRE1). RNA was transcribed from the resulting clones and used to transfect BHK cells, using Lipofectin, as previously described (19). At the indicated times, 0.5 ml of culture fluid (of 3 ml in total) was removed and replaced with fresh medium, and the plaque titer of released virus in the sample portion was determined. □, parental chimera SIN(RRE1); △, chimera containing the RR E1 mutation Gln-411→Leu; ▲, chimera containing the SIN E2 mutation Asp-248→Tyr; ●, chimera containing both the E1 and the E2 mutations.

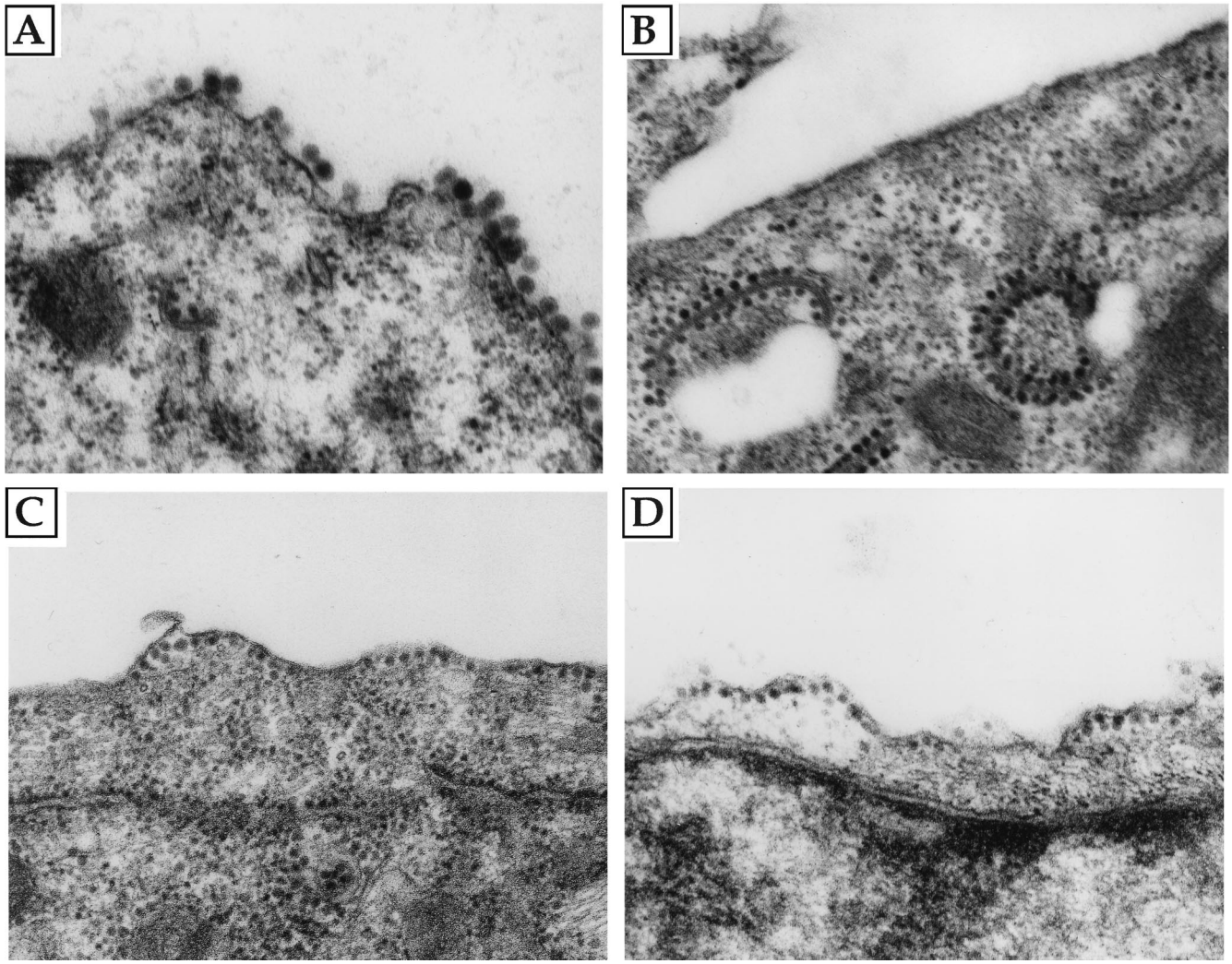


FIG. 2. Electron microscopy of infected or transfected cells. BHK cells were transfected with RNA (A, B, and D) or infected with virus (C), and the cells were prepared for electron microscopy after 12 h (A, C, and D) or 24 h (B). (A) Transfection with RNA from pToto54 as a wild-type SIN control; (B) transfection with RNA from the parental chimera pSIN(RRE1); (C) infection with P10 virus from passage series 1; (D) transfection with RNA from the reconstructed adapted chimera pSIN(RRE1)(E2:D248Y/E1:Q411L). Note the abundance of budding virus in panel A and the alignment of nucleocapsids along the plasma membrane in panels C and D, which are not present in panel B.

tant had produced 11-fold more virus. Combined, the two mutations had a multiplicative effect on virus growth; after 48 h, the double mutant had produced 200-fold more virus than the parental chimera. The growth rate of the double mutant is still considerably less than that of SIN, but it is clear that each of the changes adapts SIN E2 and RR E1 to one another so as to allow an increase in the efficiency of budding, leading to the production of much more virus than from the parental chimera.

**Electron microscopy.** BHK cells were infected with virus or transfected with RNA from the different virus strains studied here, and the cells were examined by thin-section electron microscopy at 12 or 24 h after infection. In Fig. 2 are shown micrographs of cells 12 h after transfection with RNA from pToto54 (as a wild-type SIN control) or RNA from pSIN (RRE1)(E2:D248Y/E1:Q411L) (the reconstructed chimeric clone containing the E1 and E2 mutations), of cells 12 h after infection by uncloned virus from P10 of series 1 (for comparison with the reconstructed clone), and of cells 24 h after transfection with RNA from the parental chimera pSIN (RRE1). Abundant virus budding from the plasma membrane is seen in cells transfected with SIN RNA (Fig. 2A). In contrast, no budding is seen in cells transfected with SIN(RRE1) RNA, nor are there nucleocapsids observed aligned along the plasma membrane (Fig. 2B). In cells infected for 24 h as in Fig. 2B, nucleocapsids are frequently seen aligned along internal membranes for both wild-type infection and infection by the chimera, whereas 12 h after transfection these internal membranous structures are uncommon and nucleocapsids are found scattered throughout the cytoplasm (19). The binding of nucleocapsids to these internal membranes may indicate that the interaction of the nucleocapsid with the chimeric heterodimers is not totally defective, although interaction with chimeric heterodimers in the plasma membrane appears to be nonexistent.

Cells transfected with RNA from the doubly variant clone pSIN(RRE1)(E2:D248Y/E1:Q411L) or infected with the uncloned P10 virus exhibit few budding figures, but there is now clear association of nucleocapsids with the plasma membrane (Fig. 2C and D). The lack of budding figures is consistent with

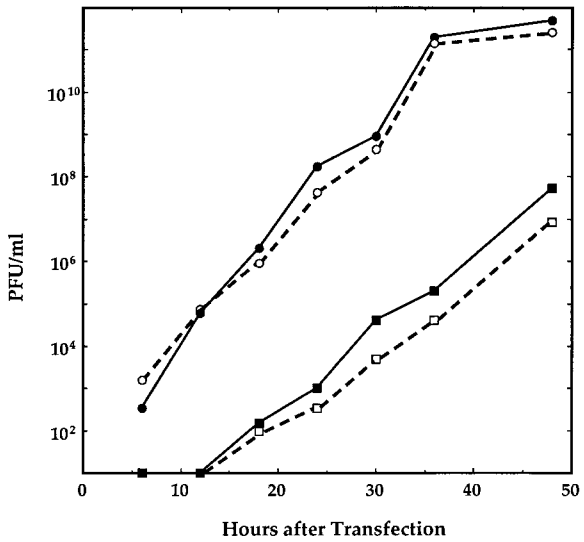


FIG. 3. Growth of SIN or RR containing the suppressing mutations. The E2 change Asp-248→Tyr was inserted into the full-length SIN clone pToto54, and the E1 change Gln-411→Leu was inserted into the full-length RR clone pRR40. RNA transcribed in vitro from pSIN Toto54 (as a wild-type control) or from pSIN Toto54(E2:D248Y) containing the E2 mutation, or RNA transcribed from pRR40 (as a wild-type control) or from pRR40(E1:Q411L), was used to transfect BHK cells, using Lipofectin. After 1 h at 37°C, the transfecting mix was removed and the cells were overlaid with 3 ml of medium. At the times indicated, 0.5 ml of cell culture fluid was removed and replaced with new medium, and the released virus was titrated by plaque assay. ●, SIN Toto54; ○, SIN (E2:D248Y); ■, RR64; □, RR(E1:Q411L).

the observation that the yield of virus from the variant chimeras is $<10^{-4}$ that of wild-type virus. The alignment of nucleocapsids along the plasma membrane, however, makes it clear that the mutations in E2 and E1 in the variant chimera result in an increase in interactions between nucleocapsids and heterodimers in the plasma membrane, allowing an increase in virus budding and the observed production of 200-fold more virus than for the parental chimera.

**Effect of E2 Tyr-248 on SIN and E1 Leu-411 on RR.** These results show that the change Asp-248→Tyr in SIN E2 adapts SIN E2 to RR E1 and the change Gln-411→Leu in RR E1 adapts RR E1 to SIN E2. We wished to examine whether these changes would interfere with the interaction of E2 and E1 in the parental viruses. For this, the Asp-248→Tyr mutation was placed into SIN clone pToto54 and the Gln-411→Leu mutation was placed into RR clone pRR64, and the growth of the resulting viruses was compared with that of SIN derived from pToto54 and with that of RR derived from pRR64. Figure 3 shows growth curves in which infection was initiated by transfection of BHK cells with RNA transcribed from the different clones (to minimize the possibility that the results could be affected by changes selected during the experiment). The E2 mutation had only a modest effect on growth of SIN virus in this experiment, and yields of the mutant were within about a factor of 2 of the parental virus over the entire time span of the experiment. The E1 mutation had a somewhat greater effect on the growth of RR, with yields depressed by about a factor of 5.

In a second experiment, BHK cells were transfected with RNA transcribed from different cDNA clones and the transfected cells were labeled with [$^3$H]uridine from 8 to 24 h after transfection in the presence of dactinomycin. Nucleocapsids were harvested from transfected cells at 24 h and sedimented on sucrose gradients, and virus released into the medium over the transfected cells was examined on a second set of sucrose gradients. The amount of label in virions and nucleocapsids was quantitated, and the results are shown in Table 2 as a ratio relative to that produced during SIN Toto54 transfection. The amount of radiolabeled virus released from cells transfected with SIN(E2:D248Y) RNA was 60 to 100% of that released from cells transfected with SIN RNA in two different experiments, consistent with the results in Fig. 3. The amount of radiolabel in nucleocapsids within the infected cell was 20 to 30% greater in the case of SIN(E2:D248Y) than SIN; it is unclear whether this difference is significant, but it might result from a slight accumulation of nucleocapsids in the case of the mutant. In the same experiment, the parental chimera and the doubly variant chimera produced too little virus to be visible on sucrose gradients. The amount of label in nucleocapsids in the cells transfected with the parental chimera was slightly less than that in SIN-transfected cells, as was found previously (19), whereas the doubly variant chimera produced about the same amount of labeled nucleocapsids as did SIN. The low level of radioactivity in nucleocapsids and virus in RR-infected BHK cells relative to SIN-infected cells (Table 2) is consistent with other results. As is clear from Fig. 3, RR infection of BHK cells leads to the production of much less virus than does SIN infection, at least under the conditions used here. Furthermore, previous studies have shown that much more virus RNA is made following infection of Vero cells by SIN than by RR (5, 6), which would be expected to lead to the production of lesser amounts of nucleocapsids. RR-infected BHK cells produce much more virus than do the chimeras, however.

TABLE 2. Radioactivity in nucleocapsids and virions 24 h posttransfection[a]

| RNA | Radioactivity relative to that in SIN | |
|---|---|---|
| | Nucleocapsids | Virions |
| Expt 1 | | |
| SIN Toto54 | 1 | 1 |
| SIN(E2:D248Y) | 1.2 | 1.0 |
| SIN(RRE1) | 0.6 | <0.001 |
| SIN(RRE1)(E2:D248Y/E1:Q411L) | 0.9 | <0.001 |
| RR64 | 0.06 | 0.16 |
| Expt 2 | | |
| SIN Toto54 | 1 | 1 |
| SIN(E2:D248Y) | 1.3 | 0.6 |
| SIN(RRE1) | 0.9 | <0.002 |
| SIN(RRE1)(E2:D248Y/E1:Q411L) | 1.3 | <0.002 |

[a] BHK cells were transfected with RNA and labeled with [$^3$H]uridine from 8 to 24 h, and radiolabel in intracellular nucleocapsids and in released virions at 24 h was assayed by sucrose gradient sedimentation.

From the data in Table 2 and in Fig. 1 to 3, we conclude that the E2 mutation has at most modest effects on the growth and assembly of the parental SIN while enabling the chimeric virus to assemble more virus than does the parental chimera. The E1 mutation also enables the chimera to assemble virus more efficiently but has a more pronounced depressing effect on the growth of the parental RR.

**Other adaptive mutations in E2 and E1.** To determine if other changes in SIN E2 or in RR E1 would adapt the disparate glycoproteins to one another, SIN(RRE1) was blindly passed in several independent passage series. In every case, the P10 virus produced about 100-fold more virus than did the original chimera (results for series 6 to 11 are shown in Table 3). The E2 and E1 genes from the P10 virus were sequenced in the region of the changes found in passage series 1 (amino

TABLE 3. Growth of P10 variants[a]

| Virus | Plaque titer ($10^6$ PFU/ml) |
|---|---|
| Parental chimera | 0.01 |
| P10 virus from passage series: | |
| 6 | 2.8 |
| 7 | 0.5 |
| 8 | 1.8 |
| 9 | 1.5 |
| 10 | 0.5 |
| 11 | 1.5 |

[a] BHK cells were infected with P10 virus from passage series 6 to 11, and titers of released virus at 48 h after infection were determined. The unpassaged parental chimera was included as a control.

acids 225 to 270 in E2 and 375 to 416 in E1), and the results are shown in Table 4. In SIN E2, one other change at Asp-248 (Asp-248→Ala) and three changes at other amino acids (Val-237→Phe, Asp-242→Gly, and Leu-243→Ser) were found that presumably adapt SIN E2 to RR E1. In RR E1, the Gln-411→Leu change was found in a second passage series (note that in passage series 1 this change occurred at some time after P5, and this change in passage series 4 is certainly an independent event), and one change at a different amino acid (Phe-399→Ser) was also observed. Thus, five different changes in SIN E2 within the region from residues 237 to 248 and two different changes in RR E1 in the region 399 to 411 appear to have been selected because they lead to increased virus production. Because no changes were found within these regions in 4 of the 11 series, it is clear that there must be other adaptive mutations, presumably within E2 or E1, that have led to increased virus production in these four series. Furthermore, because only a single change was found in these regions in five series although two changes were required to give the full 100-fold effect on virus production in series 1, where the entire sequence was obtained, it seems probable that there are additional adaptive changes within E2 or E1 of these five passage series as well.

The sequences of SIN and RR in the two relevant regions, together with the sequences for four other alphaviruses for comparison, are shown in Fig. 4. It is of interest that none of the changes observed results in the conversion of the SIN amino acid to the corresponding RR amino acid, or vice versa, and that in general the affected residues show little conservation among alphaviruses.

TABLE 4. E2 and E1 changes during independent passage series

| Passage series[a] | E2 changes (residues 225–270) | E1 changes (residues 375–416) |
|---|---|---|
| 1 | Asp-248→Tyr | Gln-411→Leu |
| 2 | Leu-243→Ser | None |
| 3 | Val-237→Phe | None |
| 4 | None | Gln-411→Leu |
| 5 | None | Phe-399→Ser |
| 6 | Asp-248→Ala | None |
| 7 | Asp-242→Gly | None |
| 8 | None | None |
| 9 | None | None |
| 10 | None | None |
| 11 | None | None |

[a] Eleven independent passage series were initiated with the chimera SIN-(RRE1) as described in the Materials and Methods, and virus from P10 of each series was sequenced in the E1 and E2 regions. For series 1, all of E1 and E2 were sequenced, and only one change was found in each of E1 and E2; for series 2 to 11, only small regions around the changes found in series 1 were sequenced (225 to 270 in E2 and 375 to 416 in E1). The passage series numbers within series 2 to 5 and within series 6 to 11 have been changed for presentation in order to group similar results together.

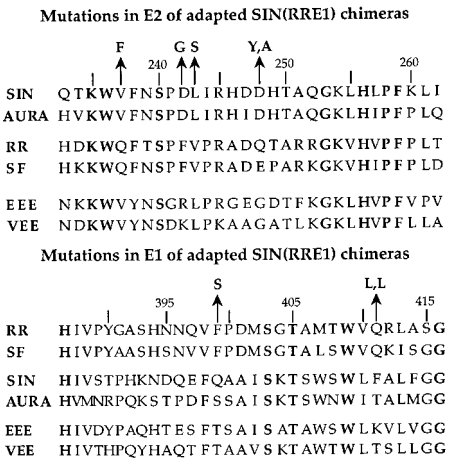


FIG. 4. Comparison of amino acids sequences of six alphaviruses around the suppressing mutations. Aligned amino acid sequences of six alphaviruses are shown for a short region of E2 and a short region of E1. The changes identified in SIN E2 or RR E1 that allow more efficient interaction in chimeric heterodimers are shown. Residues conserved in all six alphaviruses are in boldface. Residue numbering is for SIN E2 and for RR E1. These six viruses represent three distinct lineages of the alphavirus evolutionary tree (18). SIN and Aura viruses belong to a common lineage and share 59% sequence identity in their glycoproteins. RR and Semliki Forest (SF) viruses represent a second lineage and share 74% sequence identity in their glycoproteins. Eastern equine encephalitis (EEE) and Venezuelan equine encephalitis (VEE) viruses represent a third lineage and share 51% sequence identity in the glycoproteins. SIN and RR share 46% sequence identity in the glycoproteins, and SIN and EEE share 47% identity.

## DISCUSSION

Shortly after their synthesis, PE2 and E1 form a heterodimer (1) that is cleaved to an E2/E1 heterodimer during transport to the plasma membrane (reviewed in reference 15). The PE2/E1 heterodimer is more stable to treatment with low pH than is the E2/E1 heterodimer, and it is thought that synthesis as a precursor allows the heterodimer to remain intact during transit to the plasma membrane. The effect of the subsequent cleavage to an E2/E1 heterodimer is to potentiate the disassembly of the heterodimer upon exposure of the virion to the low pH of the endosomal compartment during infection of a new cell, allowing exposure of the fusion domain in E1 (9). A second function of PE2 in the heterodimer is thought to be as a chaperone to promote the proper folding of E1 in the heterodimer; E1 undergoes a number of folding steps that involve the breakage and formation of disulfide bonds (10), and association with PE2 is required for this folding. At some point the PE2/E1 or E2/E1 heterodimer trimerizes, but it is not clear whether trimerization occurs early and it is the trimer that is incorporated into the virion or whether trimerization occurs during budding. It is known that the energy of trimerization is an important component of the driving force for virus budding (3), as is the energy provided by the one-to-one interactions between the cytoplasmic tails of E2 and nucleocapsid subunits (reviewed in reference 15).

We previously found that SIN PE2 will form a heterodimer with RR E1 even though these proteins share only about 50% sequence identity with their cognates (19), and thus the structures of these proteins and their interaction domains must have

been highly conserved during the evolution of the alphaviruses. However, although the chimeric heterodimer is cleaved and transported to the plasma membrane, it is not functional for budding. The block to budding cannot lie in the interaction of the glycoprotein tails and the nucleocapsid per se, because only E2 has a significant cytoplasmic tail required for budding, and in the chimera SIN E2 should be free to interact with SIN nucleocapsids. Thus, incompatibilities between the two glycoproteins must have arisen during speciation that lead to a block in virus assembly. We report here that a single change in SIN E2 and a single change in RR E1 allows these two glycoproteins to interact with one another more efficiently in a chimeric heterodimer, such that demonstrable interaction between the chimeric heterodimer and SIN nucleocapsids now occurs at the plasma membrane and assembly of progeny virus is increased about 100-fold. It is unclear whether these two amino acids are present in contact domains for the E1-E2 interaction and directly influence the interaction or whether they alter the conformation of the glycoproteins and indirectly affect virus assembly. Our finding that changes in four closely spaced amino acids within a small domain of E2 and two amino acids within a small domain of E1 all appear to increase the efficiency of virus budding suggests that these regions may in fact interact directly. The E1 domain affected lies just outside the lipid bilayer, adjacent to the membrane anchor which is predicted to begin at Leu-413 (Fig. 4). Cheng et al. (2) have interpreted their cryoelectron microscopic reconstructions of RR as showing that the three E1/E2 heterodimers that form a spike are entwined around one another in a stalk region immediately adjacent to the lipid bilayer (reviewed in reference 15). Our finding that this region of E1 is important for the interactions of the glycoproteins is consistent with this interpretation. The E2 domain affected by the suppressor mutations identified to date lies in the middle of the linear sequence forming the ectodomain, and no structural information exists to predict how this region might interact with E1.

It is unknown whether the changes selected during passage of the chimera allow folding of E1 to proceed more efficiently so as to produce a properly folded heterodimer or whether a closer interaction between PE2(E2) and E1 allows more efficient packaging into virions, perhaps because trimerization can now proceed. In any event, the results suggest that changes in conformation allow the proper positioning of the E2 tail in the heterodimer for interaction with the nucleocapsid, whereas no interaction of capsids with heterodimers in the plasma membrane are demonstrable with the parental chimera. It is of interest that the effects of the two suppressing changes studied in detail are not entirely reciprocal. The E2 change in SIN E2 allows SIN PE2(E2) to interact more efficiently with RR E1 to produce adapted chimeric virus but has at most a modest effect upon virus assembly during SIN infection. The E1 change in RR has a more reciprocal effect; it enables RR E1 to interact more efficiently with SIN PE2(E2) but results in less efficient interaction with RR PE2(E2), at least as assayed by the production of infectious virus.

There are at least two models that could explain our results with the suppressor mutations. In one model, E2 and E1 must interact with one another in a favorable way in order that the tail of E2 be properly positioned for interaction with the nucleocapsid, and in the chimera the interactions are not favorable. For example, it is believed that the tail of PE2(E2) spans the bilayer when first synthesized but is later retracted into the cytoplasm during transport, accompanied by phosphorylation, dephosphorylation, and fatty acid acylation (7, 8). We do not know if the tail of E2 in the parental chimera has been retracted, and it is possible that proper folding of E1 or close interaction between E2 and E1 must occur before the events that lead to retraction of the E2 tail can occur. Furthermore, even if the tail is retracted, it is possible that faulty E1-E2 interactions result in improper positioning of the tail in the cytoplasm for interaction with nucleocapsids. In a second model, the trimerization of the heterodimers is blocked by suboptimal interactions in the chimeric heterodimers. In this model, in order to explain the electron microscopy results, we suppose that the interaction of a single E2 tail with a nucleocapsid is not sufficient to hold the nucleocapsid at the cell surface and that trimerization, whether before or during budding, is required before a stable interaction can occur. Similarly, it is also possible that if trimerization occurs during transport of the proteins to the plasma membrane, it might be required for retraction of the E2 tail. These various models are not mutually exclusive, and a closer understanding of the details of virus budding will be required to distinguish between them.

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