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Adaptive Mutations in Sindbis Virus E2 and Ross River Virus E1 That Allow Efficient Budding of Chimeric Viruses

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Alphavirus glycoproteins E2 and E1 form a heterodimer that is required for virus assembly. We have studied adaptive mutations in E2 of Sindbis virus (SIN) and E1 of Ross River virus (RR) that allow these two glycoproteins to interact more efficiently in a chimeric virus that has SIN E2 but RR E1. These mutations include K129E, K131E, and V237F in SIN E2 and S310F and C433R in RR E1. Although RR E1 and SIN E2 will form a chimeric heterodimer, the chimeric virus is almost nonviable, producing about 10^{-7} as much virus as SIN at 24 h and 10^{-5} as much after 48 h. Chimeras containing one adaptive change produced 3 to 20 times more virus than did the parental chimera, whereas chimeras with two changes produced 10 to 100 times more virus and chimeras containing three mutations produced yields that were 180 to 250 times better. None of the mutations had significant effects upon the parental wild-type viruses, however. Passage of the triple variants eight or nine times resulted in variants that produced virus rapidly and were capable of producing $>10^8$ PFU/ml of culture fluid within 24 h. These further-adapted variants possessed one or two additional mutations, including E2-V116K, E2-S110N, or E1-T65S. The RR E1-C433R mutation was studied in more detail. This Cys is located in the putative transmembrane domain of E1 and was shown to be palmitoylated. Mutation to Arg-433 resulted in loss of palmitoylation of E1. The positively charged arginine residue within the putative transmembrane domain of E1 would be expected to alter the conformation of this domain. These results suggest that interactions within the transmembrane region are important for the assembly of the E1/E2 heterodimer, as are regions of the ectodomains possibly identified by the locations of adaptive mutations in these regions. Further, the finding that four or five changes in the chimera allow virus production that approaches the levels seen with the parental SIN and exceeds that of the parental RR illustrates that the structure and function of SIN and RR E1s have been conserved during the 50% divergence in sequence that has occurred.

Alphaviruses are enveloped, positive-strand RNA viruses (36). The 26 members of this genus that are currently recognized are all arboviruses that alternate between infection of arthropod vectors and higher vertebrates. Virions are formed by budding of nucleocapsids formed in the cytoplasm through the cell plasma membrane to acquire a lipid envelope containing two virus-encoded glycoproteins, E2 and E1. The virion has T=4 icosahedral symmetry. There are 240 copies of capsid protein encapsidating the 11.7-kb viral genomic RNA in the nucleocapsid, and 240 copies of E2 and E1 in the envelope.

The viral glycoproteins are synthesized as a polyprotein that is cleaved by cellular proteases to form E1, a small peptide called 6K, and PE2, a precursor to E2. E1 and PE2 quickly associate to form a heterodimer (3) which is exported through the cellular secretory pathway to the plasma membrane (36). During transport, PE2 is cleaved to E2 within the trans-Golgi network by furin or a related protease (2, 10) and E1 in the heterodimer acquires its mature conformation accompanied by changes in disulfide bonding (7, 26). E2/E1 heterodimers trimerize to form a mature spike during transport or during budding, and trimerization, as well as longer range interactions between trimeric spikes, contributes to the free energy of alphavirus budding (11, 40). One-to-one interactions between the cytoplasmic tail of E2 and the nucleocapsid (8, 25, 28, 35, 43) also contribute to the free energy of budding (36, 37).

Previously, we showed that a chimeric alphavirus, referred as SIN(RRE1), in which the 6K, E1, and 3′-nontranslated regions were derived from Ross River virus (RR) and the rest of the genome was derived from Sindbis virus (SIN), was almost nonviable because of a defect in budding (41). Chimeric heterodimers between SIN PE2 and RR E1 formed and were cleaved to E2/E1 heterodimers during transport to the cell plasma membrane but had an altered conformation that did not support budding (41). Nucleocapsids in the cytoplasm did not interact with chimeric E2/E1 heterodimers in the plasma membrane as determined by electron microscopy (41). When this chimera was passaged in culture, adapted variants that grew 100 times better than the original chimera arose (42). In these variants, interactions between nucleocapsids and heterodimers were readily observed by electron microscopy. Adaptive mutations were identified in both SIN E2 and RR E1, and some of these mutations have been partially characterized (42; E. G. Strauss, E. M. Lenches, and J. H. Strauss, unpublished data). In this paper, we report a study of three of these adaptive mutations, the change from Lys-131 to Glu in the ectodomain of SIN E2, the change from Ser-310 to Phe in the ectodomain of RR E1, and the change from Cys-433 to Arg in the transmembrane domain of RR E1. The last change is of particular interest because it represents the introduction of a charged residue within what is believed to be a transmembrane anchor and because palmitoylation occurs on cysteine residues in the transmembrane domains of the membrane proteins of a number of enveloped viruses (1, 6, 17, 27, 30, 31, 44). Fatty acylation has been suggested to have roles in virus formation (12, 14, 16, 44), tissue invasiveness (17), or fusion activity (6, 27). Both E1 and E2 of SIN and of Semliki Forest virus, as well

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as the 6K protein of SIN, are known to be palmitoylated on Cys residues in transmembrane domains (12–14, 16, 30–33). The one palmitic acid in Semliki Forest virus E1 was shown by direct biochemical analysis to be covalently attached to Cys-433 in the transmembrane domain (31), suggesting that RR E1 Cys-433, the only Cys residue in the transmembrane region, also carries a palmitic acid. We show here that RR E1 is indeed palmitoylated on Cys-433 and that RR E1 containing Arg-433 is not acylated. RR containing E1 Arg-433 grows essentially indistinguishably from wild-type RR, demonstrating that under the conditions used here acylation is not required for a full yield of virus. This mutation is adaptive for the interaction of SIN E2 with RR E1 in chimeric heterodimers, however, suggesting that the transmembrane domains are important sites for the interaction of E1 and E2 in heterodimers.

MATERIALS AND METHODS

Virus and cells. BHK21 cells were used throughout, whether for transfection of RNA, infection with virus, or plaque assay. The incubation temperature was 37°C for all experiments reported here. Parental viruses were recovered from the full-length cDNA clones pToto54 (18) and pRR64 (19). Chimeric viruses were recovered from the full-length chimeric cDNA clones pSIN(RRE1) and pSIN(RR6K) as described previously (41).

Placement of adaptive mutations into a uniform background. Three adaptive mutations previously identified (42; Strauss et al., unpublished data), SIN E2-K131E, RR E1-S310F, and RR E1-C433R, were placed into the parental chimeric clone pSIN(RRE1) in order to generate an adapted variant that had a uniform genetic background except for the desired change or changes. The E2 changes were also placed into the SIN clone pToto54 and the chimeric cDNA clone pSIN(RR6K) (which has only the 6K gene from RR in a SIN background), and the E1 changes were also put into the RR clone pRR64. A second E2 mutation, K129E, was also constructed and put into the various cDNA clones. Full-length cDNA clones containing multiple mutations were also constructed. For the K131E mutation, a cDNA plasmid derived from the adapted variant containing this change (Strauss et al., unpublished data) was digested with *Bsu*36I and *Sph*I and the 1.1-kb fragment was cloned into a *Bsu*36I- and *Sph*I-digested intermediate vector, pRREdBM, constructed by treating pSIN(RRE1) with *Bst*XI and *Mlu*I and ligating the ends after blunt-ending. The resulting clone was called pRRE1dBM-K131E. The 1.9-kb *Pml*I- and *Sph*I-digested DNA fragment from pRRE1dBM-K131E was cloned into *Pml*I- and *Sph*I-digested pSIN(RRE1), resulting in pSIN(RRE1)-K131E. Other mutations were introduced by site-directed mutagenesis, using a recombinant PCR procedure (41). The entire PCR-derived regions were sequenced to confirm the presence of the specific mutation and the absence of other mutations. To change Lys(AAG)-129 to Glu(GAG) in SIN E2, the full-length cDNA chimeric clone pSIN(RRE1) was incubated with JSY 42 (5′-CCGCCCACATGCTATAC-3′), which binds nucleotides (nt) 8502 to 8518 of negative-sense RNA, and KM002 (5′-CGAATTTT GGTTTTATCTCGCGGGCCAGTGTAC-3′), which binds nt 9005 to 9036 of positive-sense RNA, in PCR buffer at 94°C for 30 s, 50°C for 1 min, and 72°C for 45 s for 25 cycles. Also, pSIN(RRE1) was incubated with KM001 (5′-CACTG GCCCGCGAGATAAAACCAAAATTCGTG-3′), which binds nt 9002 to 9034, and JSY44 (5′-AGGCGACGCACTGCTTG-3′), which binds nt 9299 to 9315 of positive-sense RNA. The resulting 0.5- and 0.3-kb PCR fragments were purified and combined for recombinant PCR (41) using 94°C for 30 s, 45°C for 1 min, and 72°C for 1 min 30 s for 25 cycles, with oligonucleotides JSY 42 and JSY 44. The 0.8-kb PCR product was digested with *Bsu*36I and *Sna*BI and the 0.33-kb *Bsu*36I-*Sna*BI fragment was cloned into *Bsu*36I- and *Sna*BI-digested pRREdBM. The 1.7-kb *Pml*I and *Bss*HII fragment from pRREdBM-K129E was cloned into *Pml*I- and *Bss*HII-digested pSIN(RRE1), resulting in pSIN(RRE1)-K129E. To change Ser(TCC)-310 to Phe(TTC) in E1 of RR (Strauss et al., unpublished data), pSIN(RRE1) was incubated with JSY34 (5′-TTCAGAGCAGGACAGTG-3′), which binds nt 10688 to 10704 of SIN(RRE1) negative-strand RNA and KM004 (5′-CTCCTCCGAAATCGAAGGAGTGTGTACAGACC-3′), which binds nt 10992 to 11023 of SIN(RRE1) positive-strand RNA in one reaction, and with KM003 (5′-CTGTACACACTCCTTCGATTTCGGAGGAGTTG-3′), which binds nt 10995 to 11026, and dTSacIRR40 (5′-ATTCCCGAGCTCGAATTCCGTT$_{14}$-3′), which binds the 3′ end of the SIN(RRE1) genome including part of the poly(A) tail in a second reaction, using the same PCR conditions described above. The resulting 0.35- and 1-kb PCR fragments were purified and combined for recombinant PCR using oligonucleotides JSY34 and dTSacIRR40. The resulting 1.35-kb PCR fragment was purified and digested with *Bsp*EI, and the 0.48-kb *Bsp*EI fragment was cloned into the *Bsp*EI-digested pRREdBM intermediate vector. The clone with the inserted fragment in the correct orientation was identified and called pRREdBM-S310F. The 0.75-kb *Bst*EII fragment from pRREdBM-S310F was cloned into *Bst*EII-digested pSIN(RRE1), and a clone with the insert in the correct orientation was identified and called pSIN(RRE1)-S310F. To change Cys(TGC)-433 to Arg(CGC) in E1 of RR (Strauss et al., unpublished data), pSIN(RRE1) was incubated with JSY34 and KM006 (5′-CG CATTGTTATGCGGGTTACCAAGACCAGC-3′), which binds nt 11361 to 11390, in the first reaction, and with KM005 (5′-GTCTTGGTAACCCGCATA ACAATGCGTCGG-3′), which binds nt 11365 to 11394, and dTSacIRR40 in the second reaction. The resulting 0.75- and 0.6-kb PCR fragments were purified, combined, and subjected to recombinant PCR with JSY34 and dTSacIRR40. The 1.35-kb PCR fragment was purified and digested with *Ssp*I, and the 1.1-kb *Ssp*I fragment was cloned into *Ssp*I-digested pRREdBM. A clone with the insert in the right orientation was identified and called pRREdBM-C433R. The 2-kb *Sph*I-*Xho*I fragment from pRREdBM C433R was cloned into *Sph*I- and *Xho*I-digested pSIN(RRE1), resulting in pSIN(RRE1)-C433R.

The 0.75-kb *Bst*EII fragment from pSIN(RRE1)-C433R was cloned into *Bst*EII-digested pSINRRE1-S310F, resulting in pSIN(RRE1)-S310F/C433R, abbreviated as pSIN(RRE1)-FR. The 1.9-kb *Pml*I-*Sph*I fragment from pSIN(RRE1)-K131E and from pSIN(RRE1)-K129E was cloned into *Pml*I- and *Sph*I-digested pSIN(RRE1)-S310F, pSIN(RRE1)-C433R, and pSIN(RRE1)-FR, resulting in pSIN(RRE1)-K131E/S310F, pSIN(RRE1)-K129E/S310F, pSIN(RRE1)-K131E/C433R, pSIN(RRE1)-K129E/C433R, pSIN(RRE1)-K131E-FR, and pSIN(RRE1)-K129E-FR. The 1.9-kb *Pml*I-*Sph*I fragment from pSIN(RRE1)-V237F (42) was placed into *Pml*I- and *Sph*I-digested pSIN(RRE1)-FR, resulting in pSINRRE1-V237F-FR (also called FFR).

The 1.7-kb *Pml*I-*Bss*HII fragment from pSIN(RRE1)-K131E and pSIN-(RRE1)-K129E was placed into pToto54 and pSIN(RR6K) digested with the same enzymes, resulting in pToto54-K131E, pToto54-K129E, pSIN(RR6K)-K131E, and pSIN(RR6K)-K129E. For pRR64-S310F, pRR64-C433R, and pRR64-FR constructions, the 1-kb *Xma*I-*Nhe*I fragments from pSIN(RRE1)-S310F, pSIN(RRE1)-C433R, and pSIN(RRE1-FR) were each ligated with the 4.4-kb *Xma*I-*Nhe*I fragment from pRR64 and digested with *Nhe*I, and the resulting 5.4-kb fragments were each cloned into *Nhe*I-digested pRR64. All of the full-length cDNA clones with adaptive mutations were sequenced to check for the presence of the expected mutation(s).

Labeling and purification of virions, immunoprecipitation, and SDS-PAGE. BHK21 cells were infected with RR64 or RR64-C433R at 30°C. After 12 h, the medium was changed to Eagle's medium containing 5% dialyzed fetal calf serum and 0.1 mCi of [^{3}H]palmitic acid (New England Nuclear) or [^{35}S]methionine per ml and the infected cells were incubated for another 24 h. Virus was precipitated from the cell culture fluid with polyethylene glycol (29) and subjected to velocity sucrose gradient centrifugation. The virus band was recovered from the sucrose gradient and pelleted onto a 15% sucrose cushion by ultracentrifugation. Virus recovered from the cushion was disrupted with sodium dodecyl sulfate (SDS) and examined by SDS-polyacrylamide gel electrophoresis (PAGE) either directly or after immunoprecipitation with antibodies directed against E1 or E2 of RR. Virion proteins were incubated at 100°C for 5 min in nonreducing sample buffer before analysis by SDS-PAGE.

Passage of adapted viruses. Passage of reconstructed chimeric viruses containing three adaptive mutations was as previously described (42). BHK21 cells were transfected with viral RNA transcribed in vitro and incubated for 2 days at 37°C. One-fifth of the culture fluid was then used to infect to new BHK21 cells. Passage was repeated 10 or more times. Virus at each passage was titrated by plaque assay on BHK21 cells.

Sequencing of adapted viruses. Adapted variants from selected passages were used to infect BHK21 cells, and the culture fluid was harvested at 48 h. Virus was precipitated with polyethylene glycol (29), and virion RNA was extracted with phenol-chloroform. cDNA was synthesized by using the oligonucleotide primer dTSacIRR40, which is complementary to the 3′ end of RR RNA and avian myeloblastosis virus reverse transcriptase at 42°C for 1 h. PCR was performed to amplify regions of viral glycoproteins, using high-fidelity polymerase. The gel-purified PCR products were subjected to kinase treatment, blunt ended, and cloned into the *Sma*I site of pUC18. More than one cDNA clone was sequenced in each case in order to obtain consensus sequences, which eliminates most PCR artifacts from consideration.

RESULTS

Replication of cloned adapted chimeric viruses. A chimeric SIN, SIN(RRE1), in which 6K, E1, and the 3′-nontranslational region were derived from RR, was severely impaired in budding, producing only about 10^{-7} as much virus as the SIN yield (41). Upon passaging the chimera in culture, variants that had adaptive mutations in SIN E2, RR E1, or RR 6K and that yielded up to 100 times more virus than the parental chimera arose (42). We chose four adaptive mutations for further study, K131E and V237F in the ectodomain of E2, S310F in the ectodomain of RR E1, and C433R in the transmembrane domain of RR E1. These mutations were placed into the parental chimeric cDNA clone, pSIN(RRE1), and the reconstructed chimeric variants were then studied. This ensured that the mutation found in the adapted variant did indeed have a phenotypic effect and was not simply an uninteresting change or an

TABLE 1. Growth of SIN(RRE1) mutants[a] after RNA transfection of BHK cells at 37°C

Mutation(s) in:		Growth relative to parental chimera at[b]:	
SIN E2	RR E1	24 h	48 h
None	None	1	1
K131E		5	5
K129E		2	2
V237F		7	11
	S310F	16	18
	C433R	2	3
K131E	S310F	42	10
K129E	S310F	63	19
K131E	C433R	161	53
K129E	C433R	40	27
	S310F + C433R	47	94
K131E	S310F + C433R	421	200
K129E	S310F + C433R	342	250
V237F	S310F + C433R	166	181

[a] In vitro-synthesized RNAs from full-length cDNA clones with an adaptive mutation(s) in SIN(RRE1) chimeras were transfected into BHK21 cells by lipofectin. Released viruses were determined at 24 and 48 h after transfection.

[b] Fold enhancement relative to SIN(RRE1) titers of 3.8×10^2 PFU/ml at 24 h and 1.6×10^4 PFU/ml at 48 h.

TABLE 2. Plaque assay on BHK21 cells at 37°C (PFU/ml)

Virus background	Mutation(s) introduced into:		Titer of released virus at[a]:	
	SIN E2	RR E1	24 h	48 h
Toto54			2×10^8	5×10^8
	K131E		3×10^8	6×10^8
	K129E		3×10^8	8×10^8
RR6K			3×10^7	4×10^8
	K131E		4×10^7	6×10^8
	K129E		3×10^7	5×10^8
RR64			2×10^6	6×10^6
		S310F	3×10^6	2×10^7
		C433R	2×10^6	5×10^6
		S310F + C433R	4×10^6	1×10^7

[a] In vitro-synthesized RNAs from full-length cDNA clones with and without mutations were transfected into BHK21 cells by lipofectin. Titers of released virus at 24 and 48 h after transfection were determined.

artifact introduced during PCR, for example. Furthermore, this procedure eliminated any possible contribution from unmapped mutations elsewhere in the genome. All of the mutations except E2 V237F were also placed into the parental viral clones in order to determine whether they had any effect upon the parental SIN or RR viruses.

We first assayed the specific infectivity of RNA transcribed in vitro from the various clones by transfection of BHK cells using lipofectin and overlaying the transfected cells with agarose. The specific infectivity (PFU per nanogram of RNA) did not differ appreciably for any of the RNAs.

We next incubated transfected cells under liquid medium for 24 or 48 h and assayed for the production of progeny virus by plaque assay on BHK cells. By using transfection of RNA for these experiments, complications due to the selection of adapted variants that grow better were largely avoided. The results are shown in Tables 1 and 2. For all of the results in these two tables, the experiments were performed three or more times with consistent results, and the data represent the average from the different experiments. Furthermore, within each experiment, the different chimeras were always compared to the parental chimera and to each other on the same day so that the cells and other conditions were the same during transfection and during plaque assay.

The different individual mutations led to increases in the yield of chimeric virus of between 2-fold (C433R) and 18-fold (S310F), and results obtained after 24 or 48 h of growth were comparable (Table 1). Thus, some of these changes had little effect upon the growth of the chimera when present singly, whereas others led to an appreciable increase in virus yield.

The effects of the mutations on the growth of parental SIN or RR were also tested. The SIN E2-K131E mutation had little effect on the growth of the parental SIN (Table 2). Similarly, the RR E1-S310F and E1-C433R mutations had only marginal effects upon the growth of the parental RR virus (Table 2). Note that as previously described our strains of SIN grow to higher titers in mammalian cells than do our strains of RR (although, interestingly, RR grows to higher titers in mosquito cells than does SIN) (19).

In order to further probe the requirements for adaptation of the disparate SIN and RR proteins in the chimeric heterodimer, we also constructed the site-specific mutant SIN E2-K129E. This mutation did not arise in any of the passage series examined, but it occurs at a position only two residues removed from the K131E mutation and leads to an identical change in charge. This mutation led to a marginal increase in chimeric virus production (Table 1) and had little effect upon the growth of parental SIN (Table 2).

We have also previously studied a SIN-RR chimera in which only the 6K protein is derived from RR. Thus, in this chimera, called SIN(RR6K), both E2 and E1 are derived from SIN and any differences in virus assembly arise from the effects of the 6K protein in promoting assembly. In this study, the yield of SIN(RR6K) was only 15% that of SIN after 24 h, whereas after 48 h the yield was 80% that of SIN (Table 2). Thus, there is a pronounced delay in the assembly of virions in SIN(RR6K). The two E2 mutations K131E and K129E had little effect upon maturation of SIN(RR6K) ($\leq$1.5-fold enhancement). Thus, the major effect of the modest but significant adaptation (fivefold enhancement) demonstrated by the K131E mutation in SIN(RRE1) appears to lie in the interaction of SIN E2 with RR E1 rather than with RR 6K.

Replication of virus with multiple adaptive mutations. During multiple independent passage series of the parental chimeric SIN(RRE1), all of the adapted variants that were present after 10 passages had more than one change in the envelope glycoproteins (42; Strauss et al., unpublished data). To examine the effect of multiple adaptive mutations in the same virus, we constructed SIN(RRE1) variants that had two or three of the individual changes combined in the same virus. Combining two mutations in the same chimeric virus led to a marked increase in the yield of progeny. At 24 h, the doubly mutant chimeras released 40- to 160-fold more virus than did the parental chimera (Table 1). After 48 h, the fractional increase in yield was less marked but the yield of progeny now approached 10^6/ml for most of the constructs. Several features of the results deserve comment. The E2-K131E/E1-C433R double mutant occurred naturally during one of the passage series (Strauss et al., unpublished data), and this combination gave the greatest increase in virus yield after 24 h, illustrating its selective advantage (Table 1). It is remarkable that E2-

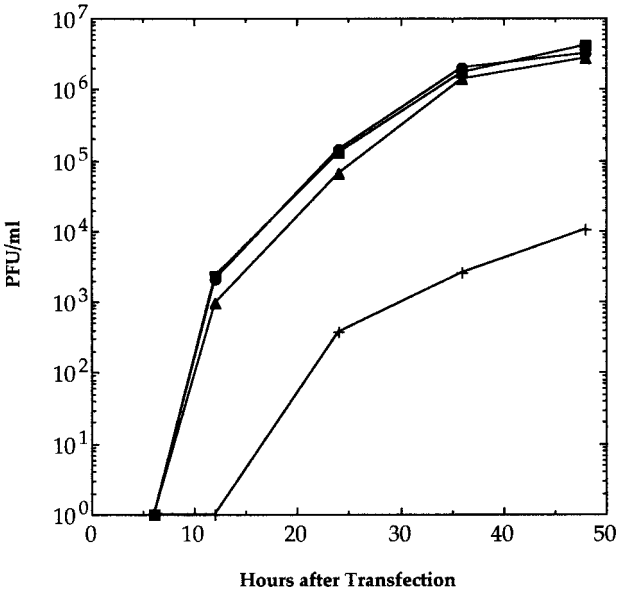

FIG. 1. Growth curve of cloned variants with three adaptive mutations in the background of SIN(RRE1). The E2 changes, Lys-131→Glu, Lys-129→Glu, or Val-237→Phe, were introduced into the SIN(RRE1) chimera containing Ser-310→Phe and Cys-433→Arg changes in RR E1, resulting in three variants, each having three adaptive mutations. The in vitro-transcribed RNA from full-length cDNA clones was transfected into BHK21 cells using lipofectin. At the indicated times, 0.2 ml of culture fluid (of 2 ml total) was removed and replaced with fresh medium, and released virus was titrated. The results are the average of two independent experiments. Symbols: +, parental chimera SIN(RRE1); ●, chimera with SIN E2 Lys-131→Glu and RR E1 Ser-310→Phe and Cys-433→Arg mutations; ▲, chimera with E2 Lys-129→Glu and E1 Ser-310→Phe and Cys-433→Arg mutations; ■, chimera with E2 Val-237→Phe and E1 Ser-310→Phe and Cys-433→Arg mutations.

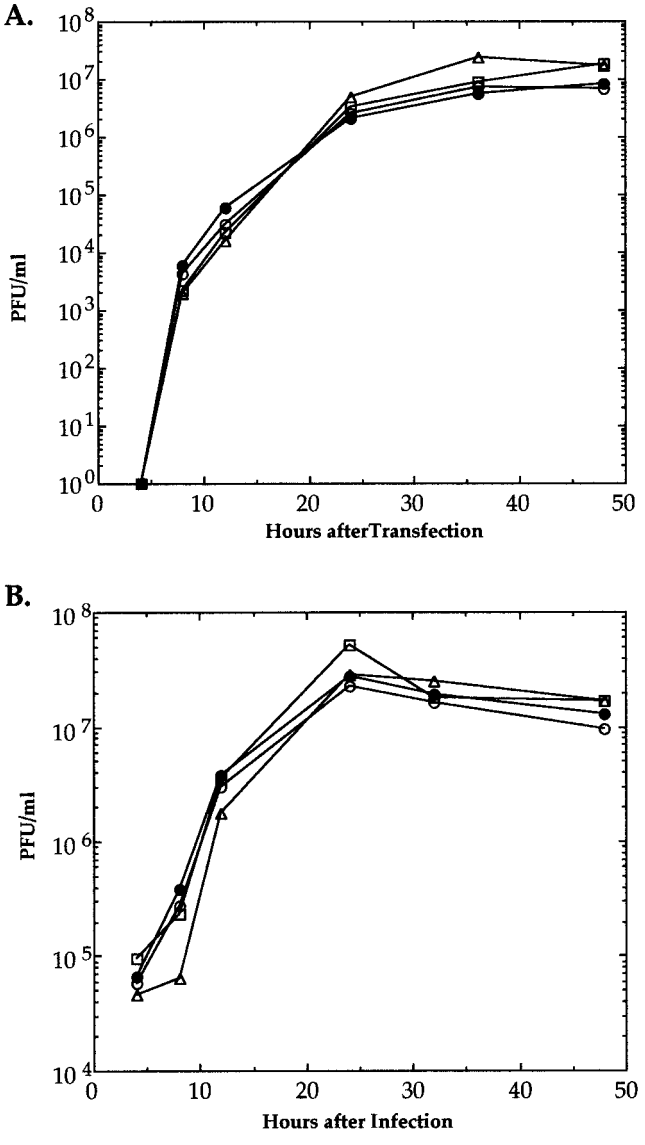

FIG. 2. Growth of RR having the adaptive mutations. The E1 changes Ser-310→Phe and Cys-433→Arg were introduced individually or in combination into the full-length RR clone pRR64. RNAs transcribed in vitro from pRR64 (as a wild-type control) or from pRR64 containing the mutation(s) were transfected into BHK21 cells by using lipofectin (A), or viruses rescued from RNA transfections were used to infect BHK21 cells at a multiplicity of infection of 2 (B). At the indicated times, 1/10 of the culture fluid was removed and replaced with fresh medium, and the released virus was titrated. The results are the average of two independent experiments. Symbols: ●, RR64; □, RR64 S310F; ○, RR64 C433R; △, RR64 S310F/C433R.

K131E alone gave a 5-fold increase and E1-C433R alone produced only a 2-fold increase, yet the combination led to a 160-fold increase when examined early after infection. It is also noteworthy that the E2 K129E change, which did not occur naturally, nevertheless led to a large increase in yield when combined with other adaptive changes (Table 1). This suggests that a key component of the adaptation in this case is the change in charge. Finally, combining the two E1 mutations into the same chimera also led to a large increase in yield (47- to 94-fold), and thus combining adaptive mutations within the same protein also leads to multiplicative increases in yield. Combining the two E1 mutations in the parental RR virus led to only a twofold increase in yield, however (also see below).

We also produced three triple mutants in which the two RR E1 mutations (S310F and C433R) were combined with the three different SIN E2 mutations (Table 1). The triple mutants grew 160- to 420-fold better than the parental chimera, demonstrating that combining up to at least three mutations continues to lead to multiplicative effects on virus production.

Growth curves of adapted variants. In order to assay more accurately the relative growth rates of variants, growth curves of the three triple mutant variants were determined using multiple time points for harvesting (Fig. 1). Progeny virus was detectable 12 h after transfection in the case of the triple mutants, whereas 24 h was required with the parental chimera. Progeny continued to accumulate rapidly in the case of the triple variants so that at any time point the mutants had produced several hundredfold more virus than the parental chimera. The yield of mutant virus after 48 h was about 3×10^6, a level about 1% that of SIN at this time point but equivalent to the yield of RR after 48 h (Table 2). These results make clear that assembly of progeny occurs much more efficiently in the triple variants than in the parental chimera.

Growth curves of RR containing E1 changes. Because of our interest in the RR E1-C433R mutation, we also determined more exact growth curves of RR containing this mutation. For comparison, we included RR containing the S310F mutation and RR containing both mutations. Figure 2A shows the results from a transfection experiment, and Fig. 2B shows the results of an experiment using virus infection of BHK21 cells. The kinetics of production of progeny are very similar in all cases. There is a tendency for the two S310F mutants to show

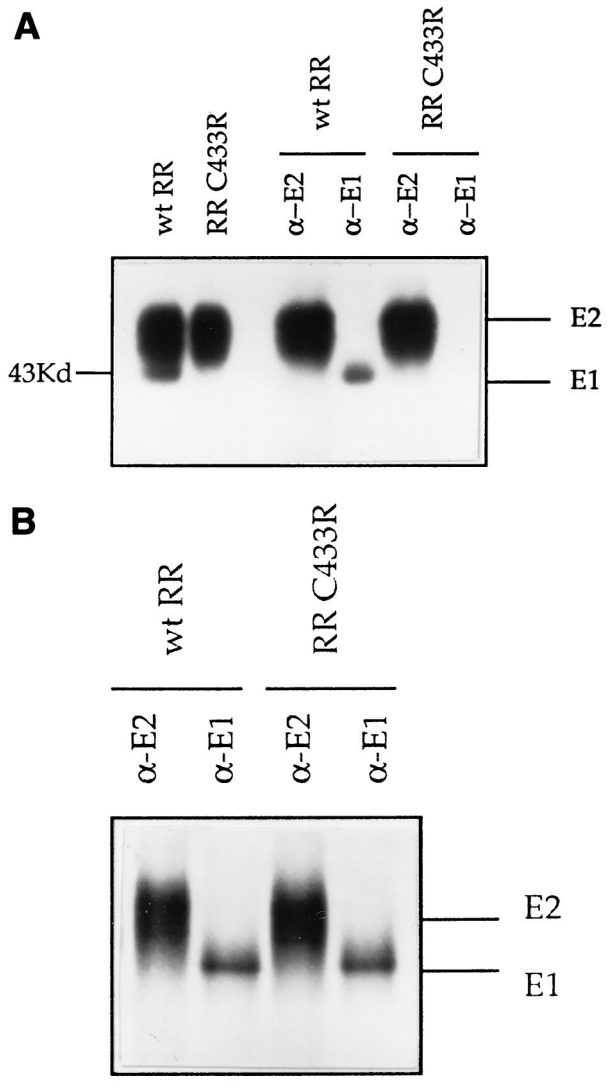

FIG. 3. Palmitoylated virion envelope proteins of wild-type RR and the RR E1-C433R mutant. RR64 or RR64 C433R virions were metabolically labeled and partially purified by precipitation and sucrose gradient sedimentation. Virions were disrupted with SDS, and the virion proteins were analyzed by SDS–8.5% PAGE either directly or after immunoprecipitation with anti-RR E2 or anti-RR E1 antibodies. (A) Proteins labeled with [^{3}H]palmitic acid; (B) proteins labeled with [^{35}S]methionine.

a slight delay in virus production, but they show a twofold increase in final yield (see also Table 2), and it is clear that the C433R mutation has little or no effect upon virus production. The results with transfection or infection are essentially indistinguishable.

Palmitic acid attachment in E1 and virus assembly. By comparison of RR with Semliki Forest virus, we would expect that Cys-433 of RR to be palmitoylated (31). To test this directly, BHK cells infected with RR64 or RR64-C433R were labeled with [^{3}H]palmitic acid. Released virions were purified and examined on SDS-PAGE gels, either directly or after immunoprecipitation of dissociated proteins with anti-E2 or anti-E1 antibodies. Both E2 and E1 from RR64 virions were labeled with [^{3}H]palmitic acid (Fig. 3A). In the case of RR64-C433R, E2 was labeled with [^{3}H]palmitic acid to the same extent as RR64 but E1 was not labeled. Thus RR E1 is indeed palmitoylated on Cys-433, which is believed to lie within the transmembrane domain, and palmitoylation is abolished by the change from Cys to Arg. As a control, virions of RR64 and RR64-C433R were also labeled with [^{35}S]methionine. There was no detectable difference in the intensities of [^{35}S]methionine labeling of E2 and E1 between RR64 and RR64-C433R virions (Fig. 3B).

The intensity of [^{3}H]palmitate labeling of E2 and E1 in RR64 is different in part because E2 has five possible palmitic acid attachment sites, two cysteine residues in the transmembrane domain and three in the cytoplasmic domain of E2 (reviewed in reference 36), whereas E1 has only the single palmitoylation site. Labeling of SIN (16, 33) and Semliki Forest virus (32) with palmitic acid has shown that E2 does carry about five times the number of palmitic acid residues as does E1. It also appears from the intensity of methionine labeling that immunoprecipitation of E1 is not as quantitative as that of E2, but this does not affect the conclusion that RR E1 with Cys-433 is palmitoylated whereas E1 with Arg-433 is not.

We conclude that Cys-433 of RR is palmitoylated, but this palmitic acid residue, or even the presence of an uncharged residue at this position, is not important for the assembly of virions, at least under the conditions tested here.

Further passage of adapted chimeras. We wanted to identify additional mutations that would further adapt SIN E2 and RR E1 to one another. For this purpose, the three triple mutant chimeras described above were used. All three triple mutants possess the same two changes in RR E1 (S310F and C433R) but differ in their SIN E2 mutations (K131E, K129E, or V237F). These three triple variants were passed in BHK cells more than 10 times. Increasing yields of virus were obtained with passage up to passage level 8 from one passage series and passage level 9 from a second experiment. Following this, virus titers started to fluctuate, perhaps because defective interfering particles had arisen. Growth curves of passage level 9 virus from the second passage series are shown in Fig. 4 and compared with growth curves for the parental triple variants. The growth of the parental variants lags behind that of the passaged variants by about 8 h, suggesting that the passaged variants package virus more efficiently, presumably as a result of additional mutations that were selected during passage. However, the 48-h yields of the passaged mutant viruses were only twofold greater than those of their unpassaged parents. The rate of virus release by these passaged variants approaches that for the parental SIN. The final yield of virus is within about 10% of the yield of SIN and exceeds that of RR in similar experiments.

To identify the new adaptive mutations in these passaged chimeras, virus from each of the three passaged triple chimeras was precipitated with polyethylene glycol, RNA was extracted, and cDNA from the structural region of the genome was prepared by reverse transcription-PCR and cloned into a plasmid vector for sequencing. At least two clones of each passaged variant were sequenced in order to obtain a consensus sequence and reduce the probability that the changes might have been introduced during PCR. The results are shown in Table 3. Two mutations were found in the passaged K131E triple mutant, Val-116→Lys in SIN E2 and Thr-65→Ser in RR E1. Thr-65→Ser in RR E1 was also found in the passaged K129E triple mutant. The passaged V237F triple mutant also had only a single change, E2 Ser-110→Asn.

Although the importance of these additional changes has not been proven by insertion of these changes, alone or in combination, back into various chimeric constructs, the results strongly suggest that these changes are in fact adaptive, at least in the context of the mutations studied. First, the finding that the E1 mutation T65S arose in two independent passage series

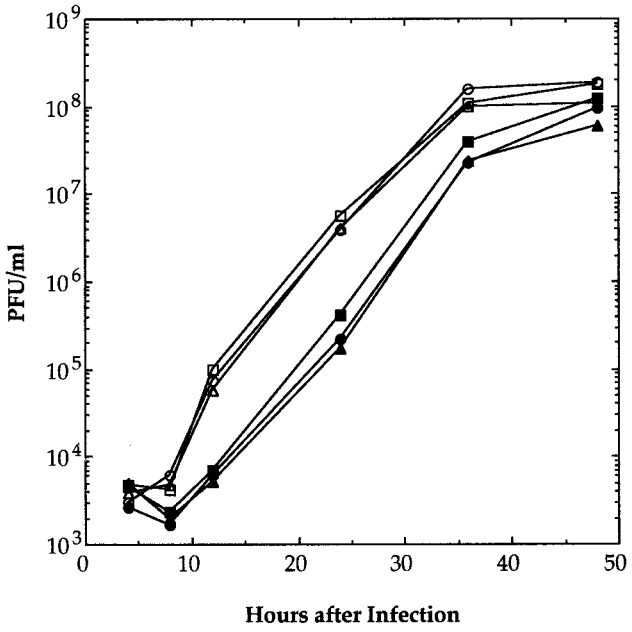

FIG. 4. Growth curves of adapted variants after passage. After nine passages of the three SIN(RRE1) chimeric viruses containing three adaptive mutations (K131E/S310F/C433R, K129E/S310F/C433R, or V237F/S310F/C433R), these passaged variants and the parental triple variants were used to infect BHK cells at a multiplicity of infection of 1. At the indicated times, 1/10 of the culture fluid was removed and replaced, and the released viruses were titrated. The results are the average of two independent experiments. Closed symbols represent the parental triple mutants, and open symbols represent passage 9-adapted virus. Symbols: ■ and □, K131E/S310F/C433R; ● and ○, K129E/S310F/C433R; ▲ and △: V237F/S310F/C433R.

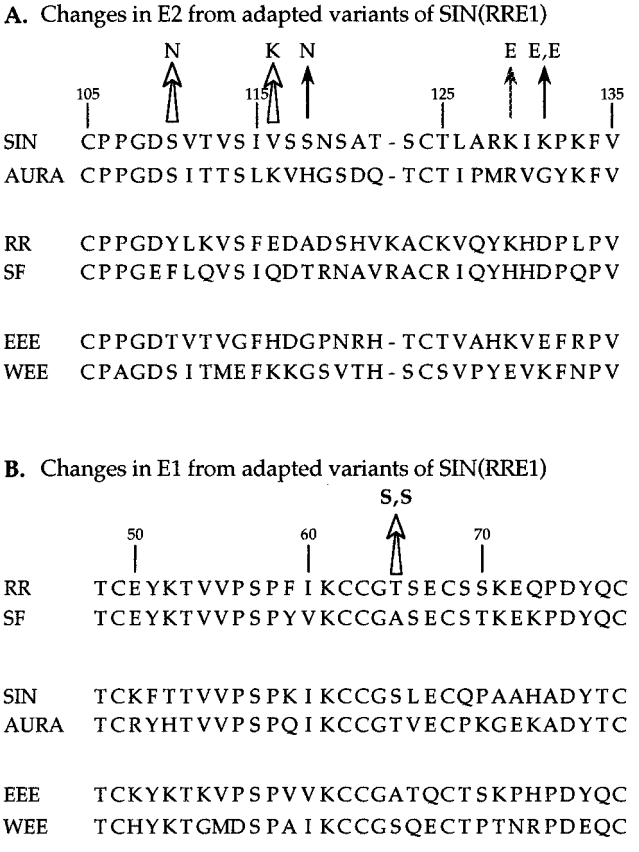

FIG. 5. Comparison of amino acid sequences of six alphaviruses around the newly found adaptive mutations. Aligned protein sequences in a short region of E2 (A) and a short region of E1 (B) are shown. The residue numbering is for SIN E2 or RR E1. Open arrows indicate the newly found adaptive mutations, solid arrows indicate changes found in the original passage series of Yao et al. (42; Strauss et al., unpublished data), and the hatched arrow indicates the mutation introduced at residue 129 of SIN E2.

starting from different mutants is indicative of its importance in adapting SIN E2 and RR E1, and it is interesting that the starting mutants differed only in having a Lys→Glu mutation in E2 at position 129 rather than at position 131. Second, the two mutations observed in E2, V116K and S110N, lie close to one another, suggesting that this domain of E2 is important for the interaction of SIN E2 with RR E1. The importance of this E2 domain for interaction with E1 is also suggested by the fact that Ser-118→Asn arose in SIN E2 during one passage series of the original SIN(RRE1) chimera (Strauss et al., unpublished data). Figure 5 illustrates short regions of SIN E2 and RR E1 surrounding these new mutations, which emphasizes the clustering of a number of mutations in SIN E2 that are important for adaptation.

DISCUSSION

Alphavirus glycoprotein-glycoprotein interactions. It has been shown by a number of different approaches that direct interaction of the alphavirus nucleocapsid with the C-terminal domain of the E2 protein is essential for virus budding (8, 25, 28, 35, 43). This E2 domain lies within the membrane when first synthesized but is retracted into the cytoplasm during transport of the heterodimers (24, 37). Phosphorylation has been implicated in the retraction event, possibly at Thr-398 or Tyr-400 (SIN numbering) (23), followed by dephosphorylation, before a productive interaction with the nucleocapsid required for virus budding can occur (24). The interaction of the cytoplasmic tail of the E2 protein with a hydrophobic pocket in the capsid protein has been modeled recently (9, 20, 21, 28, 34). These models predict that Tyr-400 and Leu-402 residues are key residues for the interaction of E2 with this hydrophobic pocket.

It is also clear that lateral interactions between the glycoproteins, which include heterodimerization of E1 and E2, trimerization of these heterodimers, and longer-range trimer-trimer associations, are also important for alphavirus budding (reviewed in references 15 and 37). Studies that have explored these interactions have included electron microscopic observations that the glycoproteins will form a regular lattice even in the absence of nucleocapsids (38, 39), complementation studies where it was found that the budding of wild-type virus was inhibited by coexpression of a budding-incompetent variant that lacked the nucleocapsid binding site in the cytoplasmic tail

TABLE 3. E2 and E1 changes after undiluted passages of reconstructed chimeras

Reconstructed chimeras after passages[a]	E2 changes	E1 changes
SIN(RRE1) K131E/S310F/C433R (P9)	Val 116→Lys	Thr 65→Ser
SIN(RRE1) K129F/S310F/C433R (P8)	None	Thr 65→Ser
SIN(RRE1) V237F/S310F/C433R (P8)	Ser 110→Asn	None

[a] Three different SIN(RRE1) chimeras reconstructed to contain the adaptive mutations shown were passaged independently either eight (P8) or nine (P9) times as described in Materials and Methods. Virus from the last passage was cloned and sequenced throughout the region encoding SIN E2 and RRE1.

(11), the demonstration that expression of E2 in the absence of E1 expression does not result in budding even though E2 is transported to plasma membrane (4), and our studies of chimeric SIN-RR viruses (25, 41, 42). The chimeric SIN-RR viruses studied in this paper contain SIN E2 protein and SIN capsids so that the E2-tail-nucleocapsid interactions should be normal, but these interactions do not occur even though SIN E2/RR E1 heterodimers are formed and are transported to the plasma membrane. The failure of budding must therefore be due to disruption of the normal lateral interactions between the glycoproteins.

The abnormal interactions between SIN E2 and RR E1 in the chimeric viruses lie at least in part at the level of the structure of the heterodimer, although longer-range interactions are also probably affected by changes in the heterodimer. The conformation of the chimeric heterodimer was found to differ from that of SIN E2/SIN E1 (41). The inhibition of virus assembly, at least early after infection, by RR 6K protein in the chimera that contains SIN E2 and SIN E1 but RR 6K also suggests that the conformation of the heterodimer is critical. First, the SIN E2/SIN E1 heterodimer differs in conformation when it is formed in the presence of RR 6K (41), and, second, 6K is only a minor component of the mature virion (13, 22), so that the primary effect of 6K appears to be facilitating the maturation of the heterodimer to an assembly-competent form. In light of this, it is interesting that the effect of RR 6K demonstrated in Table 2 of this paper is to delay the production of SIN containing both glycoproteins from SIN while having no effect on the final titer achieved.

Adaptive mutations in the chimeras. We have now identified several compensating mutations in both SIN E2 and RR E1 that result in the production of much more virus much more quickly. The major effect of these mutations appears to be a facilitation of the interactions of the disparate proteins in a chimeric heterodimer to allow more efficient budding. First, the individual changes have little effect upon the parental viruses and the effects of the changes are thus specific to the chimera. This indicates that the changes adapt the two glycoproteins to one another rather than simply facilitating the interaction of one or the other with, for example, cellular receptors or other cellular components. Second, the results of the growth curves show that virus budding is accelerated by 8 h or more by the different mutations, making it unlikely that changes in entry or other early events are responsible for the increase in yield. Entry is usually complete within 1 h of alphavirus infection, and virus release into the culture fluid begins by 3 to 4 h in wild-type virus (36), and it is thus probable that it is assembly that is accelerated by the mutations in the chimeric viruses, as is also indicated by the electron microscopic results of Yao et al. (41, 42).

Most of the individual mutations have only modest effects upon virus production but when combined have more dramatic effects. Consistent with this finding, all of the passaged variants examined to date have multiple changes that when combined give rise to the better-growing virus observed upon passage. The order in which these multiple changes arose has been investigated in only one instance (42), but in those cases in which a mutation yielding only marginal improvement by itself was paired with a change resulting in a more marked improvement in yield, we suggest that the latter change probably occurred first, followed by the other change.

In the case of the chimeras containing four or five mutations in the glycoproteins (Fig. 4), virus yield at 48 h was about 10% that of wild-type SIN under the conditions used here, although virus production was still delayed relative to that of wild-type virus. It is interesting that the primary effect of the last one or two mutations selected was to accelerate the production of virus, with only minor effects on final yield. It is unclear whether the adaptive mutations alter the conformation of E1 or E2 in order to allow more effective interactions between them or whether the changes alter contact residues in the E1-E2 interaction. The selected mutations are found in several different regions of both E2 and E1, and in both the ectodomains and the transmembrane domains, suggesting that interactions over large parts of the glycoproteins are important for virus assembly. The overall conformations of SIN E1 and RR E1 must be very similar, despite 50% amino acid sequence divergence, in order that such efficient assembly can be achieved with such a small number of amino acid changes in E2 or E1. It seems clear that the function of the glycoproteins, which includes their ability to interact with one another, has been conserved during evolution of the genus.

Although changes have been observed in several regions of E2 and E1, it is striking that many of the mutations cluster into a few small regions (42). Two new adaptive changes in E2 identified here, V116K and S110N, lie close to one another and to changes previously identified, such as the K129E and K131E changes also studied here (Fig. 5). The occurrence of several adaptive changes in this region suggests that it makes contact with E1 in the heterodimer. These findings are also of interest because the S114R change accelerates the entry of SIN into BHK cells (9a). Although the acceleration induced by S114R is measured in minutes rather than in hours as for the E2 changes studied here, it nonetheless suggests that this region of E2 might interact with cellular receptors (as well as with E1?) and that at least part of the effect of these E2 changes might relate to entry.

Palmitoylation of alphavirus glycoproteins. Alphavirus glycoproteins are palmitoylated on cysteine residues via a thioester linkage (12–14, 16, 30, 31). These cysteine residues lie within transmembrane domains or the cytoplasmic domains of the proteins. As far as is known, all E2 glycoproteins are multiply palmitoylated. In particular, three conserved cysteine residues are palmitoylated in the case of SIN and presumably all alphaviruses. These residues are present initially in a transmembrane domain but lie within the cytoplasmic domain of E2 once the tail is retracted, and changes in these cysteine residues are not well tolerated (12, 14, 16), indicating that palmitoylation is required for efficient budding. The transmembrane anchor of SIN E2 has also been shown to be palmitoylated on cysteine residues immediately adjacent to the cytoplasmic domain (30), and since all alphaviruses examined possess one or more cysteine residues in this region, it is to be expected that all are similarly palmitoylated. Removal of these cysteine residues in SIN had minor effects on budding, resulting in slightly slower release of progeny, and on the stability of the released virion, making it more sensitive to heat or detergents.

Glycoproteins E1 of SIN (30), Semliki Forest virus (31), and RR (this paper) are palmitoylated on a cysteine residue believed to lie within the transmembrane domain near the cytoplasmic domain. In the case of SIN, substitution of Ala for the E1 Cys that is palmitoylated had effects similar to the substitution for the E2 transmembrane Cys that is palmitoylated, namely, a slightly slower release of virus and a virion that was somewhat less stable (30). Although these changes would be expected to make the virus less fit in nature, it is noteworthy that not all alphavirus E1s possess a cysteine in the E1 transmembrane region and so cannot be palmitoylated on E1 (36). Consistent with this, we could detect no difference in the growth rate of the RR E1-C433R mutant, although a more-detailed analysis might reveal subtle differences, as was seen for the SIN mutants. The nature of the change selected for

adaptation of RR E1 to SIN E2 was surprising, however. Positively charged residues such as Arg are not often found buried within lipid bilayers. Nonetheless, this change is adaptive for the chimera and, as stated, is well tolerated by the wild-type RR. This suggests that the wild-type Cys may not in fact be present within the bilayer but may be immediately adjacent to it or that the substitution of Arg for Cys may lead to a change in the alpha helix within the bilayer so that it is slightly stretched out and the Arg is removed to the cytoplasmic domain, with the result that the cytoplasmic domain of E1 is now six rather than two residues. The cytoplasmic domain of E1 was found to have little effect upon the budding of Semliki Forest virus (5), but this (altered) E1 tail appears to contribute to the budding of the chimeric virus studied here. Alternatively, the change in the structure of the alpha helix in the transmembrane domain might be important for the interaction between SIN E2 and RR E1 in the chimeric heterodimers, which would suggest that interactions within the transmembrane region are important for the formation of the heterodimers.

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