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Nucleocapsid-Glycoprotein Interactions Required for Assembly of Alphaviruses

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We have studied interactions between nucleocapsids and glycoproteins required for budding of alphaviruses, using Ross River virus-Sindbis virus chimeras in which the nucleocapsid protein is derived from one virus and the envelope glycoproteins are derived from the second virus. A virus containing the Ross River virus genome in which the capsid protein had been replaced with that from Sindbis virus was almost nonviable. Nucleocapsids formed in normal numbers in the infected cell, but very little virus was released from the cell. There are 11 amino acid differences between Ross River virus and Sindbis virus in their 33-residue E2 cytoplasmic domains. Site-specific mutagenesis was used to change 9 of these 11 amino acids in the chimera from the Ross River virus to the Sindbis virus sequence in an attempt to adapt the E2 of the chimera to the nucleocapsid. The resulting mutant chimera grew 4 orders of magnitude better than the parental chimeric virus. This finding provides direct evidence for a sequence-specific interaction between the nucleocapsid and the E2 cytoplasmic domain during virus budding. The mutated chimeric virus readily gave rise to large-plaque variants that grew almost as well as Ross River virus, suggesting that additional single amino acid substitutions in the structural proteins can further enhance the interactions between the disparate capsid and the glycoproteins. Unexpectedly, change of E2 residue 394 from lysine (Ross River virus) to glutamic acid (Sindbis virus) was deleterious for the chimera, suggesting that in addition to its role in nucleocapsid-E2 interactions, the N-terminal part of the E2 cytoplasmic domain may be involved in glycoprotein-glycoprotein interactions required to assemble the glycoprotein spikes. The reciprocal chimera, Sindbis virus containing the Ross River virus capsid, also grew poorly. Suppressor mutations arose readily in this chimera, producing a virus that grew moderately well and that formed larger plaques.

The alphaviruses form a family of 27 plus-strand RNA viruses with a wide geographic distribution, many of which cause important human or veterinary diseases (2, 11, 25). Alphaviruses contain an icosahedral nucleocapsid with T=4 symmetry that consists of the viral RNA of 11.7 kb encapsidated with 240 copies of a capsid protein (C) of 30 kDa (24, 32). Virion formation occurs when nucleocapsids, previously assembled in the cytoplasm, bud through the cell plasma membrane, acquiring a lipoprotein envelope. The envelope contains 240 copies each of two virus-encoded glycoproteins (E1 and E2), each of about 50 kDa, arranged in a T=4 icosahedral lattice consisting of 80 trimers of E1-E2 heterodimers (6, 14, 24). The equimolar ratio of virion proteins is thought to arise because of specific interactions between the C-terminal cytoplasmic domain of glycoprotein E2 and a capsid protein subunit that drive viral assembly (10). Lateral interactions between the glycoproteins are also known to be important for the assembly of the virion (13, 36). The specificity of the interactions is attested to by the fact that host protein is excluded from the alphavirus envelope (30) and the fact that whereas alphaviruses will at times form pseudotypes with other alphaviruses, they will not form pseudotypes with other enveloped viruses (37).

Hahn et al. (12) reported that western equine encephalitis virus (WEE) arose by recombination between eastern equine encephalitis virus (EEE) and a New World virus related to Sindbis virus (SIN). The recombinant virus contained the glycoproteins of the SIN-like parent but the nucleocapsid protein of the EEE parent. They found that there were changes in both C and E2 of WEE that appeared to adapt these two proteins obtained from the two different parents to one another. In the 33-residue cytoplasmic domain of E2, there were eight differences between WEE and SIN, of which four were changes to the EEE sequence. These four amino acids, Ile-408, Val-418, Ser-420, and Ala-421 in SIN, were postulated to be directly involved in the interaction between WEE E2 (derived from SIN) and WEE C (derived from EEE). Conversely, there were only 12 changes between WEE C and EEE C in the conserved C-terminal 160 residues. Of these changes, seven were to the SIN sequence, and it was postulated that these residues, Met-137, Asn-172, Gly-201, Leu-231, Ala-234, Thr-238, and Ile-254 (SIN numbering; WEE has five fewer amino acids in C), interacted directly with WEE E2 in the envelope. The structure of the SIN capsid protein has recently been solved to 2.8 Å (0.28 nm) (3, 34a), and these seven amino acids form a linear trace across the folded molecule, consistent with this hypothesis.

We have constructed full-length cDNA clones of two alphaviruses, SIN (27) and Ross River virus (RR) (19), from which infectious RNA can be transcribed in vitro. These clones make possible the construction of chimeric viruses in which part of the genome is derived from one virus and part is derived from the second virus (17, 19). We have used these clones to explore

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the interactions between the glycoproteins and the capsid protein in alphaviruses by making reciprocal SIN-RR chimeras in which the capsid protein is derived from one virus and the envelope proteins are derived from the second virus. In these chimeras, the glycoproteins of one virus must therefore interact with the nucleocapsid of the second virus. To construct these chimeras, new restriction sites were engineered into the viral genomes so that the capsid protein could be precisely replaced with the capsid protein of the other virus. We report here results with such chimeras, which include site-specific mutagenesis experiments and passage experiments that attempt to adapt the disparate E2 and C to one another.

MATERIALS AND METHODS

Cell lines. BHK-21 (clone 15) and Vero cells were grown in Eagle's minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal bovine serum.

Construction of chimeric viruses. Plasmids were constructed by standard methods (28). Transcription of RNA in vitro and transfection into permissive cells have been described elsewhere (18, 19, 27).

Full-length clones of SIN (pToto54) and RR (pRR40b), which have an engineered *Mlu*I site at positions 7603 and 7524, respectively, will be described elsewhere (17). For the present work, a new *Bsp*EI site was introduced into full-length clones of SIN (pToto51) and RR (pRR64) (19) by site-specific mutagenesis of M13mp18 subclones as described previously (18); these clones were named pToto58 and pRR131, respectively. Full-length clones containing both the *Mlu*I and *Bsp*EI sites, for exchanging the capsid proteins, were constructed as follows. A SIN full-length clone, pToto59, containing the *Mlu*I and *Bsp*EI sites was constructed by replacing the *Pml*I-*Bss*HII fragment of pToto54 with that of pToto58. The RR construct with the *Mlu*I and *Bsp*EI sites, pRR201, was obtained by a three-piece ligation in which plasmid pRR40b digested with *Apa*I and *Pml*I was ligated to the *Apa*I-*Bst*EII fragment from pRR40b and to the *Bst*EII-*Pml*I fragment from pRR131.

The chimeric viruses were constructed as follows. SIN(RRc) was obtained by a three-piece ligation in which the *Mlu*I-*Bsp*EI fragment of pRR201 was ligated to the *Mlu*I-*Xho*I fragment and the *Bsp*EI-*Xho*I fragment from pToto59. The RR(SINc) clone was obtained by a three-piece ligation in which the *Mlu*I-*Bsp*EI fragment from pToto59 was ligated to the *Mlu*I-*Sma*I fragment and the *Bsp*EI-*Sma*I fragment of pRR201.

Site-directed mutagenesis. Oligonucleotide-directed mutagenesis was performed with a subclone in which the *Xho*I-*Sma*I fragment (nucleotides 9376 to 10689) from the full-length clone pRR64 was inserted into M13mp18 digested with *Sma*I. Mutagenesis was essentially as described previously (20), using synthetic oligonucleotides about 30 nucleotides in length that contained the desired nucleotide substitutions. M13 plaques were screened by sequencing in the region of the mutation. Double-stranded replicative-form DNA was prepared from M13-infected cells containing the desired mutation, and the *Csp*I-*Xma*I fragment (nucleotides 9568 to 10689) was used to replace the corresponding fragment in full-length pRR64 or pRR(SINc). In each construct, the region around the mutations was sequenced directly in the full-length cDNA clones, and two independent constructs were tested for infectivity and growth properties in order to rule out extraneous changes in the constructs.

Assay of nucleocapsids and released virus. Subconfluent monolayers of BHK-21 (clone 15) cells were removed from 10-cm-diameter petri plates with trypsin treatment, washed, and resuspended in ice-cold phosphate-buffered saline (PBS) of Dulbecco and Vogt (5) at a concentration of 10^7 cells per ml. To 0.45 ml of cells was added 10 μg of RNA transcript, and the mixtures were transferred to 0.2-cm cuvettes. Electroporation was at room temperature, using two consecutive pulses of 1.5 kV and 25 μF in a Gene Pulser apparatus (Bio-Rad), as described by Liljeström and Garoff (21). The electroporated cells were diluted to 10 ml with Eagle's medium containing 10% fetal calf serum and nonessential amino acids, seeded into 10-cm-diameter petri plates, and incubated at 37°C for 8 h. The medium was then removed and replaced with Eagle's medium containing 5% dialyzed fetal calf serum and 1 μg of dactinomycin per ml, and the cells were incubated for an additional 4 h. The medium was again removed and replaced with Eagle's medium containing 5% dialyzed fetal calf serum and 20 μCi of [^{3}H]uridine per ml (but lacking dactinomycin), and the cells were incubated for an additional 10 to 12 h. Virus released into the medium and intracellular nucleocapsids were then assayed from the same infection.

To assay virus released into the medium, the virus was first precipitated from the culture fluid with polyethylene glycol (26). The precipitated virus was resuspended in 1 ml of 200 mM NaCl–50 mM Tris-Cl (pH 7.5)–1 mM EDTA, the solution was clarified by brief centrifugation, and the virus was sedimented in linear 15 to 30% sucrose gradients in the same buffer but containing 0.3% fetal calf serum for 1.5 h at 32,000 rpm and 5°C in an SW41 rotor. Fractions of 0.4 ml were assayed for radioactivity by liquid scintillation counting.

To assay intracellular nucleocapsids, the cells were washed twice with ice-cold PBS after removal of the medium, scraped from the plate with a rubber policeman into 5 ml of PBS, and pelleted at 2,000 rpm for 10 min at 4°C. The cells were resuspended in 1 ml of 10 mM NaCl–10 mM Tris-Cl (pH 7.5)–20 mM EDTA and kept on ice for 15 to 20 min; 200 μl of 20% Triton X-100 was added, the cells were vortexed briefly, and the nuclei were removed by sedimentation at 4,000 rpm for 10 min at 4°C (31). The nucleocapsids in the supernatant were sedimented in linear 10 to 40% sucrose gradients in 50 mM Tris-Cl (pH 7.5)–100 mM NaCl–1 mM EDTA–0.1% Triton X-100 for 2.5 h at 5°C at 32,000 rpm in an SW41 rotor. Fractions of 0.4 ml were assayed for radioactivity by liquid scintillation counting.

RESULTS

Production of chimeric viruses. We report elsewhere the introduction of a unique *Mlu*I site into the nontranslated junction region between the nonstructural and structural domains of full-length clones of SIN and RR (17). These sites can be used to exchange the structural and nonstructural regions of these two viruses. For the current study, we introduced a new *Bsp*EI near the end of the capsid protein to allow exchange of capsid proteins. This new site is illustrated in Fig. 1. The last eight amino acids of the RR and SIN capsid proteins are identical. Introducing one silent change into SIN (C-8417→T) and two silent changes in RR (G-8354→T and A-7835→G) resulted in the introduction of *Bsp*EI sites at the corresponding positions of the two genomes. This new site is unique within the structural region, and because the amino acid sequences of the two virus capsid proteins are identical at this new site and downstream of this site, these *Bsp*EI sites allow the precise exchange of the capsid proteins of the two viruses.

Two reciprocal chimeric viruses were produced for this study and are illustrated schematically in Fig. 2. The *Mlu*I site in the junction region and the *Bsp*EI site at the end of the capsid protein were used to exchange the capsid proteins so that the chimeras contained all of their sequence from one of the

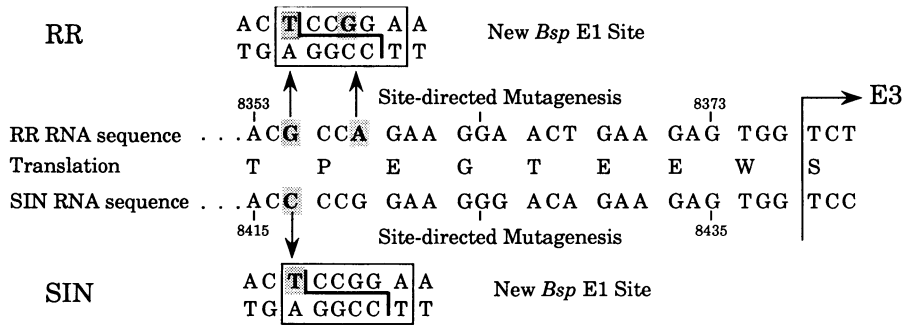

FIG. 1. Introduction of a new *Bsp*EI site in RR and SIN cDNAs. The translated cDNA sequences in the C-terminal regions of the capsid protein genes of RR and SIN are shown. The start of the E3 gene is indicated. Nucleotides are numbered from the 5′ end of the genomic RNA. Note that the eight C-terminal residues of RR and SIN capsid proteins are identical, although there are third-codon substitutions. The nucleotides changed by mutagenesis are shaded, and the new sequences are shown above or below the parental sequences. The changes introduced are silent and result in the introduction of a new *Bsp*EI site at the same corresponding position in the two genomes.

viruses except for the capsid protein, which was derived from the other virus. SIN containing the RR capsid protein is referred to as SIN(RRc), and RR containing the SIN capsid protein is referred to as RR(SINc).

RR containing the SIN capsid protein. RR(SINc) produced extremely low virus yields. In a primary transfection plaque assay in which the transfected cells were overlaid with agarose and plaques were allowed to develop, BHK cells transfected with RR or with RR(SINc) RNA transcribed in vitro gave rise to the same number of plaques per unit of RNA within the reliability of the assay. Thus, RR(SINc) RNA is as infectious as RR RNA, and the results obtained do not appear to arise from the presence of mutant RNAs present in low amounts. The plaques produced by RR(SINc) were tiny in comparison with those produced by RR, however, and took about 2 days longer to develop to the point where they were readily visible. When RR or RR(SINc) plaques were picked from the transfection plate and used to infect BHK cells, the resulting RR stocks had titers on the order of 10^7 PFU/ml, whereas stocks of RR(SINc) had titers less than 10^2. Similar titers were obtained when cells transfected with RNA were incubated in liquid medium and the titers of the resulting virus stocks were determined. Attempts to passage the chimeric virus in either Vero or BHK cells resulted in loss of the virus; growth of the virus was too feeble to maintain a passage series, and mutants capable of better growth did not arise.

To probe the reasons for the very limited production of infectious virus, cells were transfected with RR RNA derived from cDNA clone pRR64 or with RNA from the chimera RR(SINc), using electroporation so that a large percentage of the cells were infected, and the transfected cells were labeled with [^{3}H]uridine. Virions released into the supernatant were examined by sucrose gradient sedimentation (Fig. 3A). Whereas RR infection led to the release of large amounts of labeled 280S virions, RR(SINc) infection led to no detectable release of labeled virus. Thus, RR(SINc) does not assemble detectable levels of virus particles, whether infectious or noninfectious, and the results from plaque titer and from sucrose gradient analysis of labeled virus are concordant.

Nucleocapsids present in the transfected cells were also examined in the same experiment. For this, nucleocapsids were extracted from the cells with neutral detergent and sedimented in sucrose gradients; the results are shown in Fig. 3B. Comparable amounts of labeled 140S nucleocapsids were found in cells infected with either RR or RR(SINc). Thus, SIN capsid protein can support the replication and encapsidation of RR RNA with high efficiency, and the dramatic reduction in virus yield is not due to a failure to synthesize RNA or structural proteins or to encapsidate the RNA. It has been shown previously that transport of alphavirus glycoproteins to the cell surface occurs when the glycoproteins alone are expressed, and thus transport is independent of capsid protein expression and formation of nucleocapsids or of other steps in virus replication (38). Therefore, the RR envelope glycoproteins, which are wild-type RR proteins in this chimera, are expected to be transported normally to the cell surface. We conclude that the

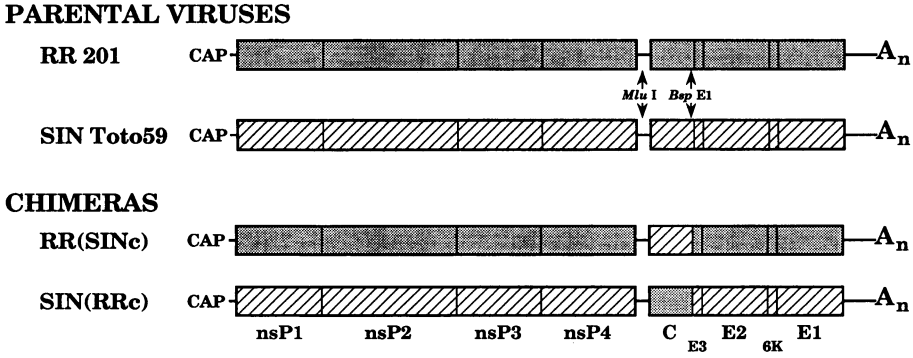

FIG. 2. Chimeric viruses. The genomes of RR and SIN are diagrammed schematically. The positions of new *Mlu*I and *Bsp*EI sites are shown. These sites were used to construct the two chimeric genomes illustrated below.

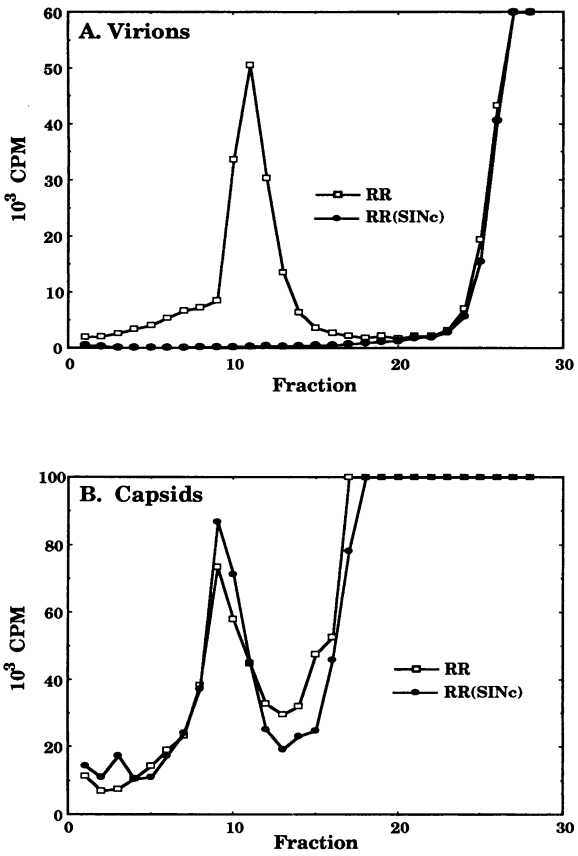

FIG. 3. Sucrose gradient analysis of virions and nucleocapsids assembled by RR and by RR(SINc). BHK cells were transfected with in vitro-transcribed RNA from pRR64 or pRR(SINc) by electroporation and labeled with [^{3}H]uridine from 12 to 20 h after electroporation. (A) Virus in the cell culture fluid was precipitated with polyethylene glycol, and the resuspended virus was layered onto linear 10 to 40% sucrose gradients and sedimented for 1.5 h at 32,000 rpm and 5°C in an SW41 rotor. The gradients were fractionated and assayed for radioactivity, and the gradients with RR and RR(SINc) are plotted on the same graph. (B) Nucleocapsids in the cell cytoplasm were extracted by lysing the cells with Triton X-100 and sedimented in linear 10 to 40% sucrose gradients for 2.5 h at 32,000 rpm and 5°C in an SW41 rotor. The gradients were fractionated and assayed for radioactivity, and the gradients with RR and RR(SINc) are plotted on the same graph.

dramatic reduction in formation of virus must arise from an incompatibility between RR glycoproteins and SIN capsid proteins during virus budding.

These conclusions are supported by studies of another RR-SIN chimera in which the nonstructural proteins are derived from RR and all three structural proteins are from SIN. This virus grows moderately well (17), which also indicates that the poor growth of RR(SINc) results from an incompatibility between the envelope glycoproteins and the capsid protein.

Adaptation of RR E2 to the SIN capsid. There are 11 amino acid differences between RR and SIN in the C-terminal cytoplasmic domain of E2 (Fig. 4). We reasoned that some or all of these changes could be responsible, in whole or in part, for the failure of glycoproteins and capsids to interact properly during virus budding. Because the phenotype of the chimera RR(SINc) was stable, and the chimera was virtually nonviable, this chimera is ideal for site-specific mutagenesis experiments that attempt to adapt the RR E2 to the SIN capsid. The effects of changes in 10 of the amino acids that are different were tested by changing these amino acids from the RR sequence to the SIN sequence in nine different combinations, as illustrated in Fig. 4. These changes were introduced into the chimera and, as a control, into the parental RR. In primary transfection plaque assays, the specific infectivity of the different RNAs was the same in all chimeric and RR viruses except for RR(SINc) mutants 7 and 8, in which about 1/10 and 1/3, respectively, as many plaques were observed. Thus, the phenotypes observed for the different RR and RR(SINc) mutants, with the possible exception of RR(SINc) mutants 7 and 8, appear to result from the mutations introduced and not from the appearance of suppressor mutations. The plaques observed with the chimeric viruses were all very small and developed more slowly than RR plaques, suggesting poor growth, except for RR(SINc) mutants 4, 5, and 6, which produced medium-size plaques that were larger than those of the other chimeras but still smaller than RR plaques.

To quantitate the growth of the various mutant chimeric and RR viruses, plaques were picked from the transfection plate and used to infect BHK cells. The average titers of two stocks prepared in this way for chimeric virus and for each mutant are shown in Fig. 4. In the same experiment, titers of RR stocks containing the various mutations were all between 0.4×10^7 and 2×10^7 (data not shown), and thus the mutations had only modest effects on RR growth.

The results clearly show that certain of the changes allowed the chimeric virus to grow to much higher titers. The most important changes were Thr-402→Ala and Gly-404→Asn. Mutant 4, containing only these two changes, produced 400-fold more virus (and larger plaques) than did the parental chimera. However, other changes are also clearly important because mutant 6, in which nine changes have been introduced, produced 17-fold more virus than did mutant 4; this mutant produced almost 10^4 more virus than did the parental chimera and about 1/40 the amount produced of RR. In this mutant, the entire C-terminal 28 residues of the E2 cytoplasmic domain have been converted to the SIN sequence. Overall, the results suggest that the most important differences between RR and SIN are Thr-402–Pro-403–Gly-404→Ala-Pro-Asn followed by the block Ala-417–Pro-418–Arg-419→Val-Arg-Ser. The results provide convincing evidence that the E2 cytoplasmic domain and the nucleocapsid interact in a sequence-specific fashion during virus budding.

Although the mutations introduced into chimera mutant 6 resulted in a dramatic increase in virus titer, by changing most of the RR E2 tail to that of SIN, these changes together or in groups had only modest effects on the growth of RR, as described above. Thus, the block to interaction in the chimera between RR E2 and SIN C is not reciprocal, and the cytoplasmic domain of SIN E2 appears to be able to interact with RR C fairly efficiently in the context of RR (but also see below).

Surprisingly, the change Lys-394→Glu was deleterious for the chimera. In mutant combinations 7 and 8, less virus was produced than in the parental chimera, and in combination 9, the virus grew only slightly better than the parental chimera. Thus, the RR(SINc) chimera with 10 changes to the SIN sequence grows very poorly, whereas a mutant with 9 of these 10 changes grows well. The results suggest that the Lys→Glu change might disrupt glycoprotein-glycoprotein interactions, and thus that the N-terminal region of the cytoplasmic domain of E2 may be important for interactions involved in dimerization of E1 and E2 or in the lateral interactions involved in virus assembly. The Lys-394→Glu change also had a definite but modest effect on RR. In the experiment reported in Fig. 4, RR

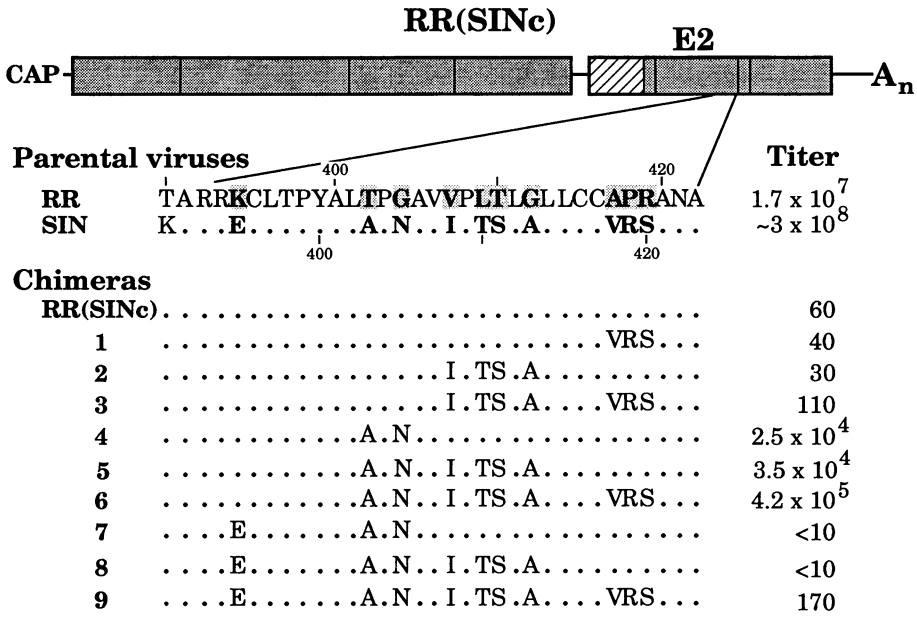

FIG. 4. Mutational analysis of E2-nucleocapsid interactions in RR(SINc). A schematic of the genome of RR(SINc) is shown, with the E2 cytoplasmic domain expanded below. Amino acid numbers refer to positions within E2. The sequences of the cytoplasmic domains of the two parental viruses are shown, as are the sequences of the different mutant chimeras. The mutant chimeras are numbered from 1 to 9. Shown are plaque titers from an experiment in which plaques formed upon transfection of cells with RNA were picked and used to infect one well of a six-well plate of cells; the averages of duplicate stocks produced in this way are shown. This experiment has been repeated in different ways with very similar results, although the absolute numbers vary somewhat; in the experiment shown, all infections and titrations were performed at the same time so as to obtain comparable numbers.

containing Glu-394 produced an average of 50% of the titer of RR containing Lys-394. Thus, the severity of the effect of the change is context dependent.

SIN containing Lys-395. The change Lys-394→Glu in RR E2 was severely deleterious for the maturation of chimera RR(SINc) and slightly deleterious for the maturation of RR. We wanted to test whether the reciprocal change, Glu-395→Lys, would be deleterious for SIN. Lys-395 was introduced in SIN E2, and its effect on virus growth was assayed. In addition, the change Glu-395→Asp was inserted into SIN to test the effect of this mutation. The results are shown in Table 1. At 12 h after infection, there was very little difference in the rates of virus release for the three viruses; the release rates at 6 and 24 h suggest that there may be slight differences in the kinetics of virus growth, however. Thus, the effects of substitutions at residue 395 in SIN are minimal.

Passage of RR(SINc) mutant 6. Mutant 6 of RR(SINc) was passaged three times in BHK cells, as were mutants 1, 2, 3, and 9. From mutant 6, large-plaque variants that grew ~10-fold better than RR(SINc) mutant 6 and almost as well as RR were obtained. We assume that these variants have one or more additional changes, probably in the structural proteins, that further adapted E2 and C to one another and allowed more efficient budding. No large-plaque variants were obtained from the other mutants or from the parental chimera, and we assume that multiple changes are required in these cases to obtain a virus that matures efficiently.

SIN with the RR capsid. SIN(RRc) also grew very poorly, apparently producing titers of about 10^3 PFU/ml, or about 10^{-5} that of the parental SIN. Work with this chimera was made difficult by the rapid appearance of large-plaque-forming virus which produced titers in excess of 10^5 PFU/ml. Both large and small plaques were present upon transfection of BHK cells with RNA, and it was difficult to obtain a pure stock of the small-plaque-forming virus for further study. We assume that

TABLE 1. Growth of SIN containing changes at E2 position 395

Virus	Virus yield (PFU/ml/h)		
	6 h postinfection	12 h postinfection	24 h postinfection
SIN	2.4×10^5	3.6×10^7	1.1×10^8
SIN(E2: E-395→K)	7.5×10^5	8.2×10^7	7.0×10^7
SIN(E2: E-395→D)	5.2×10^6	6.5×10^7	3.0×10^7

TABLE 2. Passage of SIN(RRc) in Vero cells[a]

Plaque	Passage	PFU/ml	Plaque size
1S	1	1×10^4	Small
	2	3×10^5	10% large, 90% small
	3	3×10^5	50% large, 50% small
2S	1	1×10^3	Small
	2	3×10^3	Small
	3	3×10^2	Small
3S	1	1×10^5	Small
	2	1×10^6	50% large, 50% small
	3	5×10^5	60% large, 40% small
1L	1	7×10^5	Large
2L	1	7×10^6	Large
3L	1	5×10^5	Large

[a] Three small plaques (1S, 2S, and 3S) were picked from an RNA transfection plate and passaged three times in Vero cells. At each passage, small plaques were picked and used to infect cells for the following passage. Three large plaques (1L, 2L, and 3L) were also picked from the RNA transfection plate and used to infect Vero cells. Because only large plaques were obtained, these stocks were not further passaged.

the small-plaque virus represents the original chimera and the large-plaque viru s represents a virus with a suppressor mutation that allows it to grow better, consistent with the finding that large-plaque virus produced higher titers than the small-plaque virus did.

When the large plaques and small plaques produced upon primary RNA transfection were picked separately and passaged, the large-plaque formers produced stocks of uniformly large-plaque-forming virus, whereas small-plaque formers produced virus stocks that contained both large and small plaques. Small plaques were replaqued through a total of three isolations in an attempt to obtain a uniform stock. The results of these passages are shown in Table 2. Stocks grown from small plaques that contained only small plaques usually had titers on the order of 10^3 PFU/ml. Stocks grown from large plaques had titers 2 to 3 orders of magnitude higher. The variability in the titers of the stocks suggests that different suppressor mutations might be present in different stocks, and the finding that some small-plaque stocks produced relatively high titers suggests that suppressor mutations might be present in these higher-titered stocks but the plaque-phenotype remains small.

We conclude that SIN(RRc) is viable but grows poorly and that suppressor mutations that allow the virus to grow better arise readily. We assume that these mutations better adapt the capsid and E2 to one another. Because of the rapid appearance of such suppressors, it was not possible to perform site-specific mutagenesis experiments on this chimera.

DISCUSSION

Our results clearly demonstrate that the cytoplasmic domain of glycoprotein E2 of alphaviruses interacts with the nucleocapsid in a sequence-dependent fashion during virus assembly. RR containing the SIN capsid protein is able to assemble large numbers of nucleocapsids but is unable to produce progeny virions. Replacement of nine amino acids in the RR E2 cytoplasmic domain with the corresponding amino acids from SIN E2 adapted the RR E2 to the SIN nucleocapsid in the chimera, at least in part, and allowed virus to form 4 orders of magnitude more efficiently.

It has long been proposed that in alphaviruses there is a specific interaction between the cytoplasmic domain of glycoprotein E2 and the nucleocapsid that leads to budding of the nucleocapsid through the plasma membrane (10). The specificity of the interactions involved in alphavirus assembly was attested to by the facts that no host protein is incorporated into alphavirus envelopes (30) and glycoproteins from viruses belonging to other enveloped virus families are not used to assemble alphaviruses (37). Early evidence for binding of the glycoproteins by the nucleocapsid included the finding that treatment of Semliki Forest (SF) virions with the detergent octylglucoside removed the lipid bilayer but left most of the glycoproteins attached to the nucleocapsid (15). More recently, it has been shown that a synthetic peptide of 31 residues corresponding to the cytoplasmic domain of SF virus E2 would bind to SF virus nucleocapsids, either in solution or after coupling the peptide to a solid matrix (23). It has also been shown that alphavirus assembly requires expression of both glycoproteins and capsid protein (34). Only very limited data that address the sequence specificity of such interactions have been previously reported, however.

In addition to the nucleocapsid-E2 interactions, lateral interactions between the glycoproteins are also important for virus assembly. von Bonsdorff and Harrison (36) found that SIN glycoproteins would form regular hexagonal arrays that were isomorphous in local packing to the virus surface lattice, showing that the T=4 surface lattice on the virus arises not just from interactions with the T=4 nucleocapsid but also from highly specific lateral interactions between the glycoproteins. The importance of such lateral interactions for virus assembly are shown by results with SIN mutant *ts*20. This mutant has a single amino acid change in the ectodomain of E2 (His-291→Leu) that prevents virus maturation at the nonpermissive temperature (22), even though the glycoproteins are transported to the cell surface, where they bind nucleocapsids (1). Another mutation, *ts*103 (Ala-344→Val), in the ectodomain of SIN E2 results in the formation of multicored particles, presumably because the lateral interactions of the glycoproteins during virus assembly are disrupted; the effect of this mutation can be partially suppressed by a compensating mutation in glycoprotein E1 (Lys-227→Met) (13, 31). Our results with the alteration at RR E2 residue 394 in the chimera RR(SINc) suggest that the effect of this mutation may be to disrupt lateral interactions between the glycoproteins, and thus that the E2 tail is involved in glycoprotein-glycoprotein interactions as well as in glycoprotein-nucleocapsid interactions. Although the effects of a mutation may result from changes in conformation in a region removed from the position of the mutation, the simplest hypothesis from our results is that the N-terminal region of the E2 cytoplasmic domain is involved in glycoprotein interactions and the more C-terminal region is involved in interactions with the nucleocapsid.

The experiments reported here lead to conclusions similar to those resulting from the experiment in nature in which WEE arose by recombination between EEE and a SIN-like virus. In adapting the SIN-like E2 to the EEE nucleocapsid, four changes in the E2 cytoplasmic domain appear to have been important (12). The WEE results combined with the present results indicate that at a minimum, amino acids 403 to 420 (SIN numbering) are important for the interaction between E2 and the nucleocapsid.

Vaux et al. (35) and Kail et al. (16) reported that the eight C-terminal amino acids of the E2 tail of SF virus (corresponding to SIN residues 416 to 423) contained the recognition domain in E2 for the interaction with the capsid. In these studies, internal image anti-idiotype antibodies were used to reconstruct ligand-receptor-like interactions. This conclusion has been called into question, however, by a report that the anti-idiotypic antibodies used by the authors did not possess the specificity believed, and that rather than interacting with nucleocapsids, the antibodies interacted with some component of the RNA replication machinery (33). This observation might explain why Vaux et al. (35) found that these antibodies also interacted with flavivirus-infected cells, leading them to suggest that the flavivirus and alphavirus glycoprotein-nucleocapsid binding signals were similar. Our results indicate that the interactions of alphavirus E2 with the nucleocapsid are sequence specific, and it would be surprising to find that the flaviviruses, which are only distantly related to alphaviruses (29), used the same glycoprotein-capsid interaction signal as the alphaviruses do.

Schlesinger and colleagues have used different approaches to study the interactions of capsids and glycoproteins during alphavirus maturation. In one study, synthetic peptides corresponding to various regions of the E2 tail of SIN were applied to infected cells and the inhibition of production of virus was quantitated (4). These peptides inhibited virus assembly by up to 80%, presumably by interfering either with the nucleocapsid-E2 interactions or with glycoprotein-glycoprotein interactions. The most effective inhibitor was a hexapeptide containing residues 396 to 402 of SIN E2. Peptides C terminal to this did not inhibit, possibly because of inefficient uptake by the

infected cells. A second approach used site-specific mutagenesis of the cytoplasmic tail of SIN E2 (9, 15a). Several different mutants with changes in the cytoplasmic tail of E2, Cys-396→Ser, Pro-399→Gly, Tyr-400→Phe, Ala-401→Ile or Lys, Pro-404→Gly, Cys-416–Cys-417→Ser-Ala, and Ser-420→Cys, were found to be altered in virus maturation. The double mutation at positions 415 and 416 was lethal, whereas the other changes led to virus that matured more slowly than the wild type did, particularly in mosquito cells, and that produced multicored particles, indicative of altered interactions between capsid and E2 or of altered lateral interactions between the glycoproteins during budding. It was suggested that the change at cysteine residues led to a failure of the E2 cytoplasmic domain to orient properly with respect to the cell plasma membrane, because these positions are normally acylated, and that the other changes altered the interactions of E2 and C directly. These authors also found that changes in the small protein of 55 amino acids found in the gene sequence between E2 and E1 led to defective virus assembly, including formation of multicored particles, and suggested that this protein contributes to nucleocapsid-E2 binding or to glycoprotein-glycoprotein interactions (7, 8).

It is of interest that the amino acid substitutions studied by us have nonreciprocal effects and are context dependent. Interaction of E2 with the SIN nucleocapsid in the chimera RR(SINc) required that the cytoplasmic domain be largely derived from SIN. Similarly, SIN(RRc) grew poorly, showing that in this context, the SIN E2 cytoplasmic domain interacted poorly with the RR capsid. However, changing the RR E2 cytoplasmic domain in RR to the SIN sequence resulted in a virus that grew well, showing that in this context, the SIN E2 cytoplasmic domain can interact with the RR capsid. It is possible that in wild-type RR in which all of the interactions are optimal, changes in E2 that destabilize E2-nucleocapsid interactions can be tolerated, whereas in the chimeras, a number of interactions are suboptimal and unstable E2-nucleocapsid interactions are not tolerated. Subtle differences in the structure of the chimeric nucleocapsid resulting from suboptimal interactions between the SIN capsid protein and RR RNA, for example, or differences in the structure of the E2 cytoplasmic domain and its interaction with nucleocapsids caused by differences in the interactions of the glycoproteins with one another could lead to a requirement for more effective nucleocapsid-glycoprotein interactions for efficient virus formation. Other suboptimal interactions in the chimeras that could lead to more stringent requirements for effective nucleocapsid-glycoprotein interactions can also be envisaged. Mapping of suppressor mutations that arise so readily and allow the chimeras to grow better may be of considerable value in defining other interactions important for virus assembly and help identify the reasons for the nonreciprocal effects of the mutations. Detailed structural studies of alphaviruses are also of obvious importance for our understanding the interactions that occur during virus assembly.

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