Sequence analysis of three Sindbis virus mutants
temperature-sensitive in the capsid protein autoprotease
(alphavirus/serine protease/RNA virus evolution)

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ABSTRACT We have cloned and sequenced the cDNA made to the region of RNA encoding the structural proteins of three complementation group C mutants of Sindbis virus, ts2, ts5, and ts13, and of their revertants. These mutants possess defects in the posttranslational processing of their structural proteins at the nonpermissive temperature. Comparison of the deduced amino acid sequences of the mutants with those of the revertants and with the parental HR strain of virus showed all three mutants to have single amino acid substitutions in the highly conserved COOH-terminal half of the capsid protein that give rise to temperature sensitivity. ts2 and ts5 were found to have the same lesion and thus represent independent isolations of the same mutant, whereas ts13 possessed a different change. Reversion to temperature insensitivity in all three mutants occurred by reversion of the mutated nucleotide to the parental nucleotide, restoring the original amino acid. It has been previously postulated that the capsid protein possesses an autoproteolytic activity that cleaves the capsid protein from the nascent polyprotein during translation. Comparison of the amino acid sequence of the capsid protein with that of serine proteases leads us to hypothesize that histidine-141, aspartate-147, and serine-215 of the Sindbis capsid protein form the catalytic triad of a serine protease. This hypothesis is supported by the finding that all three temperature-sensitive lesions mapped near these residues: ts2 and ts5 change proline-218 to serine and in ts13 lysine-138 has been replaced by isoleucine.

Sindbis virus is a small enveloped RNA virus that belongs to the genus Alphavirus of the family Togaviridae. It consists of a nucleocapsid containing the single-stranded virus RNA complexed with a basic capsid protein, surrounded by a lipid bilayer containing the virus-encoded integral membrane glycoproteins E1 and E2 (1).

The viral structural proteins are translated as a single large precursor polyprotein from a subgenomic messenger, 26S RNA, that is the 3' terminal one-third of the genomic 49S RNA (2-6). In this precursor the NH2-terminal capsid protein is followed by the envelope protein precursor PE2 and the second envelope protein, E1. Proteolytic cleavages occur several times during processing of this polyprotein precursor. The first cleavage releases the capsid protein from the polyprotein, and the remaining portion of the nascent polypeptide is then inserted into the rough endoplasmic reticulum of the cell. During their insertion, or shortly after, the glycoproteins are glycosylated with mannose-rich oligosaccharides and cleaved into the proteins PE2 and E1 (7, 8). A final proteolytic cleavage converts the PE2 glycoprotein to proteins E2 and E3. A failure in any of these cleavage and/or insertional steps inhibits virion formation and abnormal polypeptide precursors accumulate in the infected cell.

There are three complementation groups of temperature-sensitive (ts) mutants of Sindbis virus that affect the structural proteins of the virus (9). Mutants in groups D and E are located in glycoproteins E1 (10) and E2 (unpublished data), respectively. Mutants in group C are thought to be in the capsid protein since at the nonpermissive temperature the polypeptide precursor is not cleaved and accumulates in the cytoplasm (11). No capsid protein is formed and little envelope protein is produced. It is thought that failure to remove the capsid protein causes the signal sequences for insertion of the glycoproteins to fail to function; the result is that the glycoprotein portions are not inserted or glycosylated or further processed, and the complete polyprotein results.

Several investigators have suggested that the cleavage of the capsid protein from the polyprotein precursor could occur by autoproteolytic activity of the capsid protein itself (3, 12, 13). In this model autoproteolysis ordinarily results from the nascent polypeptide acting upon itself, rather than from a diffusible protease, because proteolysis is rapid and complete even during in vitro translation where very small amounts of products are made (3).

The complete nucleotide sequences of Sindbis virus genomic RNA and of 26S messenger RNA have been published (14, 15). This has made possible the analysis of ts mutants of Sindbis virus (10). Here we report the cloning of cDNA from three group C mutants, ts2, ts5, and ts13, and of their revertants and the determination of the nucleotide sequences of the regions of 26S RNA that encode the capsid proteins and parts of the glycoproteins. Mapping of these ts mutations together with comparison of the amino acid sequences of alphavirus capsid proteins with those of serine proteases leads us to postulate that the alphavirus capsid protein is a serine protease whose catalytic triad is formed by histidine-141, aspartate-147, and serine-215 in Sindbis virus.

MATERIALS AND METHODS

Virus Strains. The mutants ts2, ts5, and ts13 isolated from the heat-resistant HR strain were kindly provided by B. W. Burge. We used the oldest stocks in our possession, which had not been passed since 1971, as seed stocks in this work. All strains were plaque purified at 30°C immediately before use.

Isolation of Revertants. Mutant stocks were assayed for revertants by plaque titration at 40°C and 30°C, and the reversion frequency is expressed as the ratio of plaques at 40°C to plaques at 30°C. Single virus clones of ts+ revertants were picked from the 40°C plate and the virus was eluted into 1 ml of Eagle's medium/5% fetal calf serum (16). These revertant plaque picks were used to infect a Petri plate of primary chicken embryo fibroblast cells at 40°C and the resulting stocks were plaque assayed at 30°C and 40°C and used as the infecting stocks for RNA preparation.

Virus Purification and RNA Isolation. Viruses were grown in primary or secondary chicken embryo fibroblast cells and harvested 10-20 hr after infection, depending on the mutant.
All mutants and revertants were incubated at 30°C or 40°C, respectively. Viruses were purified and RNA was isolated as described (17).

Cloning and Sequencing of DNA. The methods used for obtaining clones of cDNA made to the entire structural protein region will be described elsewhere (unpublished data). Briefly, a plasmid vector originally derived from pBR322 was cut with Sma I, thymidine-tailed with terminal deoxynucleotidyltransferase, and then cut with SAI to remove one of the tails. The resulting tailed vector was used as a primer with 49S RNA purified virions to synthesize double-stranded cDNA. Conditions for first-strand synthesis were essentially as described by Strauss et al. (14) and those for second-strand synthesis were as described by Okayama and Berg (18). The resulting product was cut with HindIII, ligated with *Escherichia coli* ligase, and cloned. Most of the plasmids that contain inserts have a 5.5-kilobase insert containing cDNA from the 3' terminal poly(A) tract of 49S RNA to the *HindIII* site at nucleotides 6266–6271 in the genomic RNA and thus contain the entire structural protein region. Plasmid DNA was isolated from these clones and sequenced by the chemical sequencing methods of Maxam and Gilbert (19), as modified by Smith and Calvo (20), as described (14).

**RESULTS**

Sequencing of Complementation Group C Mutants. Clones containing the entire structural protein region of three group C mutants, ts2, ts5, and ts13, and of revertants of these mutants were obtained by using a poly(dT)-tailed vector as a primer for cDNA synthesis with purified virion RNA. The cloned cDNA extended from the 3' terminal poly(A) tract to the *HindIII* site at position 6267 in the genomic RNA sequence. DNA from these clones was sequenced, using the methods of chemical sequencing of Maxam and Gilbert (19), from the first nucleotide of 26S RNA (nucleotide 7598 of the genomic RNA) to nucleotide 1237 of 26S RNA (nucleotide 8835 of the genome). This region encompasses the 5' untranslated region of 26S RNA, the entire capsid protein region, all of the E3 protein, and the first 68 amino acids of the E2 protein. In each case at least three independent clones were sequenced to ensure that any changes found were characteristic of the RNA population and did not represent minor variants in the population or reverse transcriptase errors. This proved to be necessary in some cases because revertants in the population or other minor variants were sequenced. The results presented in Fig. 1 in all cases represent at least three clones that agree as to sequence.

Sequence Analysis of ts2 and Its Revertant. *ts2* was produced by nitrous acid mutagenesis of the HR strain of Sindbis virus (21, 22). The sequences obtained for this mutant and its revertant are illustrated in Fig. 1. Comparing the *ts2* sequence with that of the parental HR strain (14), we found three nucleotide substitutions in *ts2* within the region sequenced. Two of these changes do not revert in the revertant and presumably have nothing to do with temperature sensitivity, probably arising during the original mutagenesis even though the changes observed are not those expected for nitrous acid. The first of these nonreverting changes is a U→C substitution at nucleotide 67, which is a silent change in the codon for phenylalanine-6 (UUU→UUC). The second nonreverting change observed is a G→U change at nucleotide 1043, which leads to a change of aspartate-4 to tyrosine (GAC→UAC) in glycoprotein E2.

We conclude that the mutation responsible for temperature sensitivity in *ts2* is a change of C→U at nucleotide 701, which leads to the replacement of proline-218 by serine (CCG→UCG). In the revertant the changed nucleotide reverts to the original nucleotide, restoring the parental amino acid. It is of interest that this is the only change found in the sequenced region that has the expected characteristics of nitrous acid mutagenesis.

Sequence Analysis of ts5 and Its Revertant. *ts5* was produced by ethyl methanesulfonate mutagenesis of the HR strain (21, 22). We found that this mutant had only one change throughout the sequenced region, a C→U change at nucleotide 7598, which begins at nucleotide 7598 in the 49S RNA. Amino acids are numbered from the NH₂ terminus of each protein. All nucleotides or amino acids changed from the ancestral sequence in any of the strains shown are indicated in the representation of the ancestral sequence. In the other strains shown, any change from the ancestral sequence is indicated; if no change is shown, then the sequence is the same as the ancestral sequence. nt, Nucleotide.

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**Fig. 1.** Schematic representation of the nucleotide sequence of the first one-third of 26S RNA, which encodes the capsid protein, the E3 protein, and part of the E2 glycoprotein, for several strains of Sindbis virus. The top line represents the sequence of the ancestral HR strain (10). The remaining lines represent *ts2* (derived from HR by mutagenesis with HNO₂) and its revertant *ts2R*, *ts5* (derived from HR by mutagenesis with ethyl methanesulfonate) and its revertant *ts5R*, *ts13* (derived from HR by nitrosoguanidine mutagenesis) and its revertant *ts13R*. Nucleotides are numbered from the 5' end of 26S RNA (which begins at nucleotide 7598 in the 49S RNA). Amino acids are numbered from the NH₂ terminus of each protein. All nucleotides or amino acids changed from the ancestral sequence in any of the strains shown are indicated in the representation of the ancestral sequence. In the other strains shown, any change from the ancestral sequence is indicated; if no change is shown, then the sequence is the same as the ancestral sequence. nt, Nucleotide.
otide 701 of 26S RNA that leads to replacement of proline-218 of the capsid protein by serine (CCG→UCG) (Fig. 1). In the ts5 revertant, this change reverts to the original nucleotide (Fig. 1). Thus despite the fact that different mutagens were used to produce them, ts2 and ts5 represent independent isolations of the same mutation. Their independent history is confirmed by the fact that ts5 lacks the two extraneous changes found in ts2.

Sequence Analysis of ts13 and Its Revertant. ts13 was produced by nitrosoguanidine mutagenesis of the HR strain (21, 22). ts13 also has a single base substitution in the region sequenced. The change is A→U at nucleotide 462, resulting in the change of lysine-138 to isoleucine (AAA→AUA) in the capsid protein. In the ts13 revertant, this nucleotide reverts to the original nucleotide (Fig. 1).

**DISCUSSION**

**Nature of the Mutational Events.** The three temperature-sensitive mutants of the C complementation group isolated by Burge and Pfefferkorn (21, 22) have been widely studied. All mutants in this group studied to date accumulate the large polyprotein precursor called the ts2 protein or NVP130 at the nonpermissive temperature (9, 11, 23). cDNA sequence analysis of the structural protein region of these three mutants shows that amino acid substitutions in the COOH-terminal half of the capsid protein cause this ts2 protein accumulation. It has long been thought that group C mutants are defective in the capsid protein, but no firm evidence for this has existed.

In each case, reversion to temperature insensitivity occurred by single-base reversion, which is consistent with our work on ts mutants in proteins E1 (10) and E2 (unpublished data). This has greatly simplified the task of identifying the changes responsible for temperature sensitivity. It is not completely clear why second-site pseudorevertants have not been seen. Either they do not occur or they do not accumulate in the virus population. In most cases the ts mutant grows more slowly than the wild type even at the permissive temperature, the wild type being highly evolved for rapid growth. Once true revertants arise they are quickly amplified in the population, and it is possible that second-site pseudorevertants, even if they arose, might not possess such a selective advantage and might not be amplified in the virus population.

All three mutants were reported by Burge and Pfefferkorn (21) to have a reversion frequency of 0.5–1 × 10⁻³. We found the same reversion frequency for ts2 and ts5 but a considerably higher reversion frequency, 4 × 10⁻³, for ts13, which suggests that the ts13 revertant may have arisen early in this experiment. In any event the reversion frequency of all three mutants is consistent with the being single mutants as determined from nucleotide sequencing.

As we had previously observed with Sindbis ts mutants (10), the changes found in this study were often not those most commonly produced by the mutagens used (24). In the case of ts5, which was derived by ethyl methanesulfonate mutagenesis, the C→U transition found is one of the changes expected from the action of the alkylating agent. For ts2, which was derived by HNO₂ mutagenesis, the major changes expected are C→U or A→G transitions produced by deamination. The mutational single event giving rise to temperature sensitivity was a C→U transition, but a U→C transition and a G→U transversion were also found, although in the last two cases it cannot be shown that the changes occurred during HNO₂ mutagenesis. Finally, for ts13, which was derived by nitrosoguanidine mutagenesis, the A→U change found is not one predicted for this agent, but this agent has previously been found to cause many different changes (10).

**Autoproteolysis by the Capsid Protein.** Several investigators have proposed that the formation of capsid protein could be accomplished by autoproteolysis. This was first suggested by the finding that cleavage occurred normally during *in vitro* translation in lysates of rabbit reticulocytes (3). It has also been found that group C mutants can be complemented by other mutants (22) and that during complementation the ts2 polyprotein is apparently cleaved by a diffusible factor (12). Finally, during *in vitro* translation with amino acid analogues the cleavage of capsid protein from the polyprotein is inhibited (13). The ts lesions at lysine-138 and proline-218 of the capsid protein, which result in failure of the cleavage to occur at the nonpermissive temperature, provide further evidence for the significance of autoproteolysis by the capsid protein and presumably implicate these regions in proteolysis. *In vitro* translation experiments (25) have shown that cleavage occurs when fewer than 100 amino acids of the P2E2 protein have been translated, indicating that translation of the E2 protein is not required for proteolysis. The fact that aspartate-4 of E2 can be replaced by another amino acid without apparent effect on the proteolytic activity (in the ts mutant studied here) and aspartate-5 of E2 can similarly be replaced (our large-plaque strain of HR has aspartate-5 replaced by glycine (10) is consistent with this conclusion. Whether sequences within the E3 region are involved in any way in the proteolysis step is still open.

Cleavage of the capsid protein occurs after a tryptophan residue, tryptophan-264 of the polyprotein precursor, and is therefore a chymotryptic-like cleavage. Furthermore, Pfefferkorn and Boyle (26) have reported that the chymotrypsin inhibitor 1-tosylamido-2-phenylethyl chloromethyl ketone inhibits this cleavage in infected cells. Because this cleavage specificity is characteristic of serine proteases, we searched for possible homology between the highly conserved COOH-terminal half of the alphavirus capsid protein and active sites of mammalian and insect serine proteases. The results are given in Fig. 2 and show that a suggestive homology exists between animal serine proteases and alphavirus capsid proteins. The spacings between the components of serine proteases that form the active site and the corresponding homologous regions of alphavirus capsid proteins are different, but the three amino acids that constitute the catalytic triad (histidine-57, aspartate-102, and serine-195) and their surrounding amino acids show some homology with regions in the COOH-terminal half of the capsid proteins. In particular, serine-215 in Sindbis capsid protein is found in the sequence Gly-Asp-Ser-Gly, which is conserved in all the serine proteases, as has been previously noted (33). Although the sequence homology is limited, we suggest that histidine-141, aspartate-147, and serine-215 of the Sindbis capsid protein perform the same function during autoproteolysis as histidine-57, aspartate-102, and serine-195, respectively, of animal serine proteases. Furthermore, all three mutants have amino acid substitutions within the homologous regions between serine proteases and alphavirus capsid proteins around these three key residues. ts13 has a change of lysine-138 to isoleucine, which is near the possible charge transmitter, histidine-141. ts2 and ts5 have a change of proline-218 to serine, which is just next to the possible catalytic serine-215. The sites of these mutations support the hypothesis that the capsid protein possesses proteolytic activity and lend support to the hypothesis that histidine-141, aspartate-147, and serine-215 are active in this proteolysis. The hypothesis that these three residues form the catalytic triad of a serine protease should be testable in a number of ways; in particular the importance of these residues for proteolysis can be directly tested by site-specific mutagenesis.

Fig. 3 shows the percentage homology as a moving average between the capsid proteins and the E3 proteins of Sindbis
The homology between the highly conserved COOH-terminal half of the alphavirus capsid protein and mammalian and insect serine protease active sites. Upper three lines, partial amino acid sequences of Sindbis virus (SIN), Semliki Forest virus (SF), and Ross River virus (RR) capsid proteins [sequences are from Rice and Strauss (15), Garoff et al. (27), and Dalgaro et al. (28), respectively; numbering of amino acids is for the SIN capsid protein]; middle lines, sequences of hornet chymotrypsin (HRN) (29) and silkworm coconase (SLK) (30); bottom three lines, active-site domains of three mammalian serine proteases: bovine trypsin (TRP), bovine chymotrypsin (CHY), and porcine elastase (ELA), respectively. The lower numbers are the standard numbering of amino acids for chymotrypsinogen (31, 32). Solid triangles indicate the three active amino acids that form the catalytic triad (histidine-57, aspartate-102, and serine-195) and asterisks indicate amino acids that are highly conserved between the capsid proteins and the animal serine proteases; regions of highest homology are boxed. Solid arrows indicate locations of mutations in ts2, ts5, and ts13. The single-letter amino acid code is used.

Fig. 2. Homology between the highly conserved COOH-terminal half of the alphavirus capsid protein and mammalian and insect serine protease active sites. Upper three lines, partial amino acid sequences of Sindbis virus (SIN), Semliki Forest virus (SF), and Ross River virus (RR) capsid proteins [sequences are from Rice and Strauss (15), Garoff et al. (27), and Dalgaro et al. (28), respectively; numbering of amino acids is for the SIN capsid protein]; middle lines, sequences of hornet chymotrypsin (HRN) (29) and silkworm coconase (SLK) (30); bottom three lines, active-site domains of three mammalian serine proteases: bovine trypsin (TRP), bovine chymotrypsin (CHY), and porcine elastase (ELA), respectively. The lower numbers are the standard numbering of amino acids for chymotrypsinogen (31, 32). Solid triangles indicate the three active amino acids that form the catalytic triad (histidine-57, aspartate-102, and serine-195) and asterisks indicate amino acids that are highly conserved between the capsid proteins and the animal serine proteases; regions of highest homology are boxed. Solid arrows indicate locations of mutations in ts2, ts5, and ts13. The single-letter amino acid code is used.

Fig. 3. Homology between the capsid proteins and the E3 proteins of Sindbis virus and Ross River virus plotted as a moving average with a string length of 20. The first shaded region is the possible serine protease active site between lysine-135 and lysine-155, which contains the histidine-141 and aspartate-147 residues (Fig. 2). The second shaded region is the part of the possible serine protease active site between proline-206 and asparagine-222, which contains the Gly282-Asp-Ser-Gly286 conserved sequence. Arrows indicate the locations of the mutations in ts2, ts5, and ts13.
proteases that might perform such a function has been reported, whereas several virus-encoded proteases active in the cytosol have been described, as noted above. It seems intuitively unlikely that so many viruses would have evolved their own protease activities if cellular proteases were readily available to perform such functions, although more evidence is clearly needed to resolve this issue. Finally, the question arises as to the evolutionary origin of these viral enzymatic activities. The similarities found that these proteases may share a common ancestral origin, although convergent evolution cannot be ruled out in view of the limited extent of homology found. It has previously been noted that alphaviruses and certain plant viruses appear to be evolutionarily related (40, 41). Since these plant viruses appear to lack proteases, one possibility is that a protoalphavirus acquired the protease activity after diverging from the plant virus line and that the enzyme could have been captured from the host cell.

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