

Site-Directed Mutagenesis of the Proposed Catalytic Amino Acids of the Sindbis Virus Capsid Protein Autoprotease

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The structural proteins of Sindbis virus are translated as a polyprotein precursor that is cleaved upon translation. The capsid protein is postulated to be a serine protease that releases itself from the N terminus of the nascent polyprotein by autoproteolysis. We have tested the importance in autoproteolysis of His-141, Asp-147, and Ser-215, previously postulated to form the catalytic triad of the protease, and of Asp-163. Several site-specific mutations were constructed at each of these positions, and the release of the capsid protein during translation in a cell-free system was examined. Because proteolysis occurs in *cis* during translation, the kinetics of release cannot be determined in this system, but the extent of proteolysis can be ascertained. Ser-215 appears to be the catalytic serine of the proteinase. Cys or Thr could substitute inefficiently for Ser-215, but substitution with Ala or Ile led to complete loss of activity. His-141 was also important for proteolysis. Substitution with Ala or Pro led to total loss of activity. Surprisingly, substitution with Arg resulted in complete proteolysis in vitro. Changes at the two Asp residues resulted in complete proteolysis of the substrate in vitro. All mutations that resulted in at least partial cleavage in vitro were incorporated into a full-length clone of Sindbis virus and an attempt was made to recover mutant virus. All changes tested were lethal for the virus except Asp-163 to Asn. Thus, production of infectious virus is either a more sensitive measure of the catalytic rate than the extent of in vitro cleavage, or these residues have necessary functions in addition to their possible role in proteolysis.

Sindbis virus is a small enveloped RNA virus belonging to the genus *Alphavirus* of the family *Togaviridae*. The virion structural proteins are translated as a single, large precursor polyprotein from a subgenomic mRNA, 26S RNA, which forms the 3'-terminal one-third of the genomic 49S RNA (27). In this polyprotein precursor, the capsid protein is at the N terminus and is followed by glycoprotein precursor PE2, a small hydrophobic peptide referred to as 6K, and the second glycoprotein, E1. Proteolysis occurs several times during the processing of this polyprotein precursor. The first cleavage releases the capsid protein from the nascent polyprotein (1, 23). The remaining cleavages occur during insertion of the protein into the endoplasmic reticulum, subsequent processing, and transport to the cell membrane (3, 9) and are postulated to be effected by cellular proteases active in subcellular organelles (8, 19). The cleavage of the capsid protein from the precursor has long been believed to be an autoproteolytic event (1, 2, 12, 15, 21, 23). We previously postulated that the capsid autoprotease is a serine protease whose catalytic triad is formed by His-141, Asp-147, and Ser-215 (12), based in part on the location of mutations that cause the capsid protein autoprotease to be nonfunctional at the nonpermissive temperature and in part upon amino acid sequence similarities between the sequences around these three residues and those surrounding the amino acids of the catalytic triads of mammalian and insect serine proteases. It was suggested recently, based upon protein modeling studies, that the three-dimensional structure of the alphavirus capsid is related to that of the capsid proteins of picornaviruses and thus that the capsid proteins of these disparate viruses may be derived from a common ancestor (7). The catalytic triad hypothesis was used in partial support of the protein-modeling results. More recently, the alphavirus cap-

sid protein has been used as a model to predict the existence of other viral serine proteases, such as NS3 of the flaviviruses (11). However, these results suggested that Asp-163 might be a component of the catalytic triad rather than Asp-147. For these and other reasons, it seemed important to examine the role of the proposed catalytic triad amino acids in proteolysis.

Processing of the structural proteins in vitro. The Sindbis virus structural proteins are translated in vivo from a 26S subgenomic mRNA which is 4,106 nucleotides in length exclusive of the 3'-terminal poly(A) tract. Translation of this mRNA in vitro has been shown to result in production of capsid protein and of other structural proteins (3, 9, 23). We constructed a plasmid expression vector, pT7SVSP, which contains the 26S RNA region of Sindbis virus next to a T7 RNA polymerase promoter. For this, a 4,157-nucleotide fragment of Sindbis virus cDNA which begins at an *Ava*II site 7 nucleotides upstream of the 26S RNA start point and continues through the poly(A) tract was inserted into the *Sma*I-*Eco*RI site of pGEM4. Linearization of pT7SVSP with *Eco*RI and transcription in vitro with T7 RNA polymerase resulted in an RNA transcript which had 55 extra nucleotides at the 5' end and 7 extra nucleotides at the 3' end following the poly(A) tract, but which was otherwise identical to the 26S subgenomic mRNA. Translation of this RNA, which was capped with a cap analog during transcription, in a rabbit reticulocyte lysate gave protein products which were indistinguishable from those produced by cell-free translation of 26S mRNA isolated from infected cells (Fig. 1). Proteins synthesized included the capsid protein (29,300 daltons) and polypeptide p107, which contains the sequences of the envelope protein region (PE2, 6K, and E1) and which accumulated because no membranes were present that would allow insertion and processing of the envelope proteins.

Construction of site-specific mutations. An *Nco*I fragment of Sindbis virus (strain HRSP) cDNA (genomic coordinates

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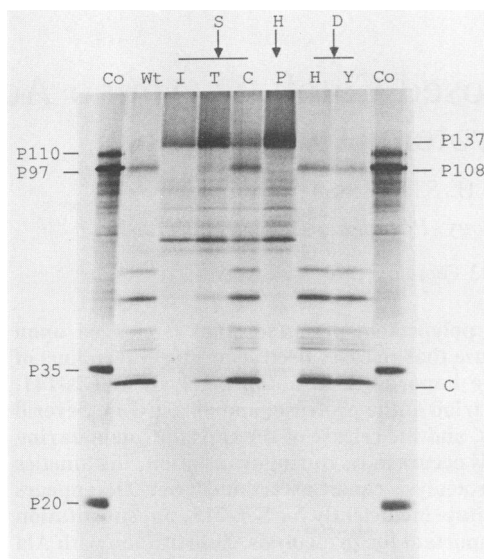


FIG. 1. Sodium dodecyl sulfate (SDS)-polyacrylamide gel analysis of in vitro translation products from in vitro transcripts of pT7SVSP. Large-scale preparations of plasmid pT7SVSP and its mutagenized derivatives were purified by isopycnic centrifugation twice in CsCl. The purified plasmids were linearized by digestion with *Eco*RI, which cuts just past the poly(A) tail of the Sindbis virus cDNA insert, and the linearized plasmids were used as templates for run-off transcription with T7 RNA polymerase. The reaction mixture contained 40 mM Tris chloride (pH 8.0), 8 mM MgCl₂, 25 mM NaCl, 2 mM spermidine hydrochloride, 5 mM dithiothreitol, 1 mM each of the four NTPs, and 10 to 50 μg of linearized DNA template per ml. To increase the efficiency of translation of the RNA transcript, a cap analog (New England BioLabs) was present at 1 mM during transcription. The RNA was purified from the transcription mixture by phenol-chloroform extraction, followed by ethanol precipitation, and translated in vitro in a rabbit reticulocyte translation system (Promega Biotec) for 1 h at 30°C as described by the manufacturer, in the presence of [³⁵S]methionine. The resulting translated polypeptides were analyzed by electrophoresis in 15% SDS-containing polyacrylamide gels. Outside lanes (Co) contain the products of in vitro translation of total brome mosaic virus RNA, and their molecular weights are indicated at the left. Lane 1 contains transcripts from pT7SVSP (parental HRSP). In lanes 2, 3, and 4, Ser-215 (S) has been changed to Ile (I), Thr (T), or Cys (C), respectively, in the plasmids used for transcription and translation. Similarly, in lane 5, His-141 (H) has been replaced with Pro (P), and in lanes 6 and 7, Asp-147 (D) was replaced with His (H) and Tyr (Y). The amount of RNA used was normalized before translation so that the same amount was present in each translation mix. The positions of capsid protein (C), p107, and p137 are indicated at the right. The extra bands located between capsid protein and p107 probably arise from premature termination in the stretch of hydrophobic amino acids located at the C-terminus of glycoprotein E2 and within the 6K protein.

8039 to 8866, covering the C-terminal 133 amino acids of the capsid protein and the N-terminal 144 amino acids of PE2) was cloned into phage M13mp19, and oligonucleotide-directed site-specific mutagenesis was performed by using M13 single-stranded DNA as a template and synthetic oligonucleotides as primers essentially as described before (6, 22, 29). Following mutagenesis, the resulting virus plaques were screened by plaque hybridization with sequentially higher-stringency washes, using the same primers as were used in the mutagenesis reactions as radiolabeled probes. Mutant plaques were sequenced by the dideoxy method with synthetic oligonucleotides as primers (20, 25). The 827-nucleo-

tide *Nco*I fragment from M13 RF was then used to replace the corresponding fragment in pT7SVSP in order to analyze the effect of the mutation on proteolysis during translation in vitro.

The mutations made in the capsid protein are diagrammed in Fig. 2. Also indicated are the locations of four temperature-sensitive mutations that rendered the proteinase inactive at 40°C. Mutant RNAs were translated in rabbit reticulocyte lysates, and the amount of capsid protein cleaved from the precursor was determined. Representative translation results are shown in Fig. 1, and a summary of all the results is given in Table 1. Translation of wild-type RNA under these conditions led to complete cleavage of the polyprotein precursor to capsid protein and p107. Similar results were found with the two mutations introduced at Asp-147, the two mutations at Asp-163, and the Arg substitution for His-141. On the other hand, the change of His-141 to Ala or Pro abolished all protease activity in this system; the full-length translated p137 accumulated, and no capsid protein was detectable. The changes introduced at Ser-215 resulted in 0 to 60% cleavage, depending upon the change.

Effect of the mutations on virus viability. The seven single-amino-acid substitutions for which at least some proteolytic activity was detectable in vitro were tested for their effect on virus viability. For this, the 827-nucleotide *Nco*I fragment from M13 RF was used to replace the corresponding fragment in cDNA clone pSIN3', and the *Hpa*I-*Bss*HII fragment from the resulting clone was used to replace the corresponding fragment in Sindbis virus cDNA clone Toto1101 (18) (these enzymes cleave unique sites in the Sindbis virus genome at nucleotides 6917 and 9804, respectively). The resulting clones were checked by dideoxy sequencing for the presence of the mutation. RNA was transcribed in vitro with SP6 RNA polymerase and tested for its infectivity in secondary chicken embryo fibroblast cells at both 30 and 40°C (14, 18). RNA transcribed from the parental clone Toto1101 served as a control. No infectious virus could be recovered after transfection with RNA from any of these mutants except for RNA containing the Asp-163 to Asn change (Table 1). The plaques produced by this mutant were clearly different from those produced by the wild type, being small in comparison, especially so at 40°C, and 10-fold fewer plaques were produced at 40°C (i.e., the mutant was somewhat temperature sensitive in comparison to the parental virus). Because the specific infectivity of the RNA at 30°C was approximately the same as that of RNA from Toto1101, this difference in phenotype appears to be due to the mutation introduced rather than to a second-site reversion. In any event, all but one of these mutations, although retaining all or some proteolytic activity as assayed in vitro, were lethal for the virus.

Attempts to demonstrate cleavage in trans. We have been successful in demonstrating cleavage in *trans* by the Sindbis virus nonstructural protease by translating constructs in which the protease activity has been abolished and using these as substrates for the wild-type protease (13). Similar attempts with the capsid protease have been negative. When either the Ser-215 to Ile or the His-141 to Pro mutant was translated in the presence of [³⁵S]methionine and the uncleaved products were mixed with unlabeled wild-type translation products, no cleavage of the labeled substrate was detected. Either the polyprotein cannot be cleaved after completion of translation or the capsid protease cannot cleave in *trans*, at least within the sensitivity of our assay.

Serine protease inhibitor studies. We performed translations of RNA in the presence of either TPCK (L-1-tosyl-

—OH of Ser or Thr or the —SH of Cys is required for proteolysis. Carter and Wells (4) found that changing the active-site Ser-221 of subtilisin to Ala resulted in a decrease in the catalytic rate to 6×10^{-7} of the wild-type rate. Even so, however, the catalytic rate was some three orders of magnitude faster than proteolysis in buffer alone, suggesting that the mutant protein had activity analogous to that of catalytic antibodies (17, 28), in which the binding of the protein to the substrate enhances proteolysis in aqueous solution by stabilizing transition state complexes.

Role of His-141 in proteolysis. His-141 has been hypothesized to be a component of the catalytic triad of the alphavirus capsid protease based upon limited sequences similarity with the active-site His of known serine proteases (the similarity is not nearly as convincing as that for Ser-215), the location of a temperature-sensitive mutation three residues upstream, and protein-modeling studies (11, 12). The fact that replacing His-141 with Ala or Pro abolished activity was consistent with this hypothesis. Carter and Wells (4) found that replacement of the active-site His-64 with Ala in the catalytic triad of subtilisin had the same effect as substitution of the catalytic Ser, a reduction in rate to 6×10^{-7} of the wild-type rate. The results of the Arg-141 substitution are puzzling, however. In well-studied serine proteases the function of the His is to act as a general base to help deprotonate the Ser and increase its nucleophilicity. The high pK_a of Arg would seem to rule out its participation in proteolysis in the same way. It is possible, however, that Arg does in fact substitute for His in this system and that although the high pK_a might result in a decrease in reaction kinetics of several orders of magnitude, the intramolecular cleavage is complete. There is only one other conserved His in the alphavirus capsid protein, His-192, which is found in an almost invariant domain, Tyr-Asn-Trp-His-192-His-Gly-Ala-Val-Gln-Tyr, that could possibly play a role in catalysis. There is no conserved His downstream of Ser-215 that could be involved, as the first conserved His is 233 residues into the glycoprotein domain and cleavage occurs in the nascent polyprotein before this His is inserted.

Asp-147 and Asp-163. Asp-147 was postulated to be the third component of a serine protease catalytic triad by virtue of quite limited sequence similarities to known serine proteases and its proximity to a temperature-sensitive lesion at residue 138 (12). More recent protein-modeling studies have implicated Asp-163 as the active component (11); in addition, this residue is close to a temperature-sensitive lesion at residue 170 (26). Two different mutations introduced at each position resulted in complete proteolysis. The presence of Asp in the active site is not as critical as that of His and Ser, however, in well-studied serine proteases. In rat trypsin, it has been suggested that the function of the Asp is to maintain the active His in a tautomeric form that functions efficiently to maintain the nucleophilicity of the active Ser (24). Craik et al. (5) found that when the active-site Asp-102 of rat trypsin was replaced by Asn, the catalytic activity decreased to 1×10^{-4} to 6×10^{-2} of the wild-type rate, depending on the pH. In subtilisin, substitution of the active Asp-32 by Ala led to a decrease in the activity to 4×10^{-5} of the wild-type rate, which is still 100-fold higher than that found after substitution of the catalytic Ser or His by Ala in the same protein (4). Thus, in either case, the effect is less severe than replacement of Ser or His. It is possible that in this *in vitro* system, autocatalysis will go to completion even if the rate of catalysis is slowed by several orders of magnitude, and we cannot assess with certainty the importance of either Asp at the current time. There are three other conserved Asp

residues in alphavirus capsid proteins, Asp-113, Asp-214 (in the sequence Gly-Asp-Ser-Gly characteristic of the active-site Ser of serine proteases), and Asp-221, none of which appear to be likely candidates for the active-site Asp. It is also possible that the function supplied by Asp in serine proteases is supplied in some other way in the alphavirus protease.

Relationship of catalysis to infectivity. All of the amino acid substitutions tested were lethal to the virus except for one, the Asp-163 to Asn change. There are two possible reasons for the lethality. The capsid protein performs essential functions in virus assembly, and the changes effected could prevent nucleocapsid formation or the nucleocapsid-glycoprotein interactions required for virus budding. The second possibility is that the activity of the protease is reduced to levels that are lethal, even though partial or complete cleavage occurs *in vitro*. *In vivo*, a reduced catalytic rate could lead to abortive processing of the structural polyprotein precursor if the capsid moiety were not removed rapidly enough. Further studies on the effects of these mutations *in vivo* would be useful.

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