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Flavivirus Enzyme-Substrate Interactions Studied with Chimeric Proteinases: Identification of an Intragenic Locus Important for Substrate Recognition

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The proteins of flaviviruses are translated as a single long polyprotein which is co- and posttranslationally processed by both cellular and viral proteinases. We have studied the processing of flavivirus polyproteins in vitro by a viral proteinase located within protein NS3 that cleaves at least three sites within the nonstructural region of the polyprotein, acting primarily autocatalytically. Recombinant polyproteins in which part of the polyprotein is derived from yellow fever virus and part from dengue virus were used. We found that polyproteins containing the yellow fever virus cleavage sites were processed efficiently by the yellow fever virus enzyme, by the dengue virus enzyme, and by various chimeric enzymes. In contrast, dengue virus cleavage sites were cleaved inefficiently by the dengue virus enzyme and not at all by the yellow fever virus enzyme. Studies with chimeric proteinases and with site-directed mutants provided evidence for a direct interaction between the cleavage sites and the proposed substrate-binding pocket of the enzyme. We also found that the efficiency and order of processing could be altered by site-directed mutagenesis of the proposed substrate-binding pocket.

The *Flaviviridae* are a family of positive-stranded RNA viruses whose genomes contain a single long open reading frame that is translated into a polyprotein. Co- and posttranslational processing of the polyprotein by viral and host cell proteinases produces 10 individual proteins, in the order C, prM(M), E, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (5, 37). The structural proteins C, prM(M), and E are cotranslationally processed in the lumen of the endoplasmic reticulum by host cell signalases (25, 32, 39). The nonstructural proteins NS1 to NS5 are processed by a combination of enzymes, including a virus-encoded trypsinlike serine proteinase (NS3) that functions in the cytosol, a host cell signal peptidase acting in the lumen of the endoplasmic reticulum, and possibly a viral enzyme of specificity similar to that of the host cell signal peptidase (6, 12, 35). NS3 cleaves between a Lys or Arg and a Gly, Ser, or Ala to generate the N termini of NS2B, NS3, NS4A, and presumably NS5.

The nonstructural proteinase of dengue virus type 2 (DEN2) is functionally contained within the first 184 amino acids of NS3, but the N-terminal boundary of the protease domain and the role of NS2B in proteolysis have not been accurately defined (2, 13, 35, 38). In vitro, the proteinase has been shown to process both the 2A/2B and 2B/3 cleavage sites in *cis*, with the initial cleavage occurring between NS2A and NS2B, followed by cleavage of the 2B/3 site. In contrast, similar experiments in vitro with the yellow fever virus (YF) proteinase (which also acts primarily in *cis*) have shown that for this virus the initial cleavage occurs between NS2B and NS3 (6).

The linear amino acid sequences surrounding the cleavage sites processed by NS3 are not well conserved, either within individual viruses such as DEN2 or among different flaviviruses, and the determinants that lead to the precise processing observed are not understood. Similarly, although some regions within the NS3 proteinase are well conserved, other regions are quite divergent. Either the rapidly evolving regions of the proteinase are not required to maintain structure or catalytic function or, alternatively, some of these changes may be important for recognition of an evolving substrate.

A number of picornavirus and retrovirus proteinases have been purified, and their biochemical and physical properties have been extensively studied (28, 30, 31, 40, 45). Biochemical and genetic experiments to examine enzyme-substrate interactions have concentrated on defining the sequence that constitutes a cleavage site, rather than identifying residues within the enzyme responsible for the specificity of recognition of these cleavage sites (18, 19, 34). We were interested in developing a biochemical assay that could identify residues important for substrate recognition. We constructed chimeric DEN2-YF polyproteins as a model system for studying the differences in enzyme-substrate interaction between these two related proteinases. Chimeras made within the protease domains themselves and site-directed mutagenesis allowed mapping of regions involved in substrate recognition. We also investigated the order of processing of DEN2, YF, and chimeric polyproteins.

MATERIALS AND METHODS

Nomenclature. All pD series constructs contain the 2A/2B cleavage site of DEN2, and all pY series constructs contain the 2A/2B (and 2B/3) cleavage sites of YF. The parental DEN2 and YF constructs are designated pD0 and pY0, respectively, a number greater than zero identifies each particular chimera, and decimals are used to designate mutant constructs. Proteins produced by in vitro translation and processing that are truncated at the N or C terminus have been designated by the prefix P. For example, pD0-programmed translations yield the precursor P2A2B3, the processing intermediate P2B3, and the products NS2B and P3 (see reference 35; in that reference, pD0 was called pT11 and the truncated NS3 product was called P3′).

Plasmid constructions. All plasmids were constructed by using cDNA clones of the PR159 strain of DEN2 (15), of the

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TABLE 1. Translation constructs

Construct	Cleavage site		NS3 amino acids		Restriction site(s)
	2A/2B	2B/3	DEN2	YF	
Parental					
pD0	DEN2	DEN2	1–303	0	*Pst*I-*Ase*I
pY0	YF	YF	0	1–299	*Pst*I-*Ava*I
Chimeric					
pD1	DEN2	DEN2	1–49	52–301	*Dra*III
pD2	DEN2	DEN2	1–49, 141–303	52–143	*Dra*III, *Hin*cII-*Hpa*I
pD3	DEN2	DEN2	1–140	144–301	*Hin*cII-*Hpa*I
pD4[a]	DEN2	YF	0	1–299	*Eco*RV
pY1	YF	YF	50–303	1–51	*Dra*III
pY2	YF	YF	50–141	1–51, 144–301	*Dra*III, *Hin*cII-*Hpa*I
pY3	YF	YF	141–303	1–143	*Hin*cII-*Hpa*I

[a] Contains NS2B residues 1 to 67 from DEN2 and residues 67 to 130 from YF.

Asibi strain of YF (YF cDNA clone 29) (14), or of the 1951 isolate of Murray Valley encephalitis virus (MVE; cDNA clone 2/1/22) (8), using standard recombinant DNA techniques (24). Plasmids pD0 (formerly pT11) and p5′L213 have been previously described (35). Recombinant proteinases were created by taking advantage of a conserved *Dra*III site located at residue 49 of DEN2 NS3 or residue 51 of YF NS3 or by using a *Hpa*I site in YF (residue 144 of NS3) that corresponds to a *Hin*cII site in DEN2 (residue 141 of NS3). All constructions are summarized in Table 1, and selected constructions are detailed below.

YF cDNA clone 29 was digested with *Ava*I, 3′ recessed ends were filled in with *Escherichia coli* DNA polymerase I Klenow fragment, and the plasmid was then digested with *Bam*HI to generate a fragment containing nucleotides 3801 to 5463 of YF. Plasmid p5′L213 was digested with *Pst*I, the 3′ overhang was made blunt by treatment with T4 DNA polymerase, and the plasmid was digested with *Bam*HI and ligated to the fragment described above to yield pY0. In vitro transcription and translation of RNA from pY0 produces a polyprotein that contains the first 37 amino acids of the DEN2 capsid protein, the last 127 amino acids of YF NS2A, all of YF NS2B, and the first 299 amino acids of YF NS3.

pD4 was constructed from pD1 by using a fragment derived from cDNA clone pY0. Briefly, a fragment containing nucleotides 4379 to 5463 of YF was amplified by the polymerase chain reaction (PCR), using a plus-strand adaptive primer (which created an *Eco*RV restriction site within YF NS2B) and a minus-strand primer complementary to the SP6 promoter. The PCR product was digested with *Eco*RV and *Bam*HI and was cloned into *Eco*RV- and *Bam*HI-digested pD1.

PCR mutagenesis. PCR was used to amplify cDNA containing nucleotides 4940 to 5071 of the DEN2 genome. Substrate-binding pocket mutants were created by using individual mutagenic oligonucleotide primers and an oligonucleotide primer of constant sequence to synthesize the plus and minus strands, respectively. pD0, at 100 ng/ml, was used as a template for PCR amplification. Mutagenic and constant primers were used at a concentration of 1 μM. A standard PCR cycle consisted of a 1-min denaturation step at 94°C, hybridization at 50°C for 1 min, and a 2-min 72°C polymerization step. After 15 cycles, PCR reaction mixes were phenol extracted, ethanol precipitated, and resuspended in restriction endonuclease digestion buffer.

All PCR products and vectors used for cloning were isolated from low-melting-temperature agarose gels (SeaKem) prior to ligation. Mutagenized DEN2 PCR products were digested with *Sal*I and *Asu*II and ligated to *Sal*I- and *Asu*II-digested pY1 and pD0. 2A/2B cleavage site mutants were created by using a mutagenic minus-strand primer (which introduced a *Sca*I site, to facilitate cloning), a plus-strand primer of constant sequence, and pD0 as a template. The PCR product was digested with *Bgl*II and *Sca*I and was ligated to a *Dra*I- and *Nhe*I-digested adaptor fragment derived from either pD0 or pD4. The inserts were cloned into *Bgl*II- and *Nhe*I-digested pD0 and pD4. 2B/3 cleavage site mutants were created by using a mutagenic plus-strand primer and a minus-strand primer of constant sequence. pD0 was used as a template, and the *Kpn*I- and *Nsi*I-digested PCR product was cloned into *Kpn*I- and *Nsi*I-digested pD0 and pD3. All recombinant plasmids were analyzed by DNA sequencing and by in vitro transcription and translation of encoded RNAs (see below).

In vitro transcriptions and translations. CsCl gradient-purified plasmid DNAs were linearized, digested with proteinase K, phenol extracted, and ethanol precipitated. Linearized templates were purified from low-melting-point agarose gels (SeaKem) and were resuspended in diethylpyrocarbonate-treated, RNase-free water at a concentration of 0.5 μg/ml. In vitro transcriptions and translations were conducted essentially as described previously (35). For pulse-chase experiments, translations were allowed to proceed for 15 min, and then cycloheximide and unlabeled Met were added to 0.6 mg/ml and 1 mM, respectively. All translations were carried out at 30°C for 90 min unless otherwise noted.

Immunoprecipitations. Reticulocyte lysates were diluted 10-fold in denaturing lysis buffer or were diluted 25-fold directly into RIPA buffer (35), and all immunoprecipitated proteins were resuspended by boiling in sodium dodecyl sulfate (SDS)-containing loading buffer (20). Samples were analyzed by electrophoresis on 15% polyacrylamide gels (PAGE) containing SDS, and gels were fluorographed at −80°C (4).

RESULTS

In vitro processing of chimeric polyproteins. Bazan and Fletterick (2) and Gorbalenya et al. (13) recently proposed a model for the flavivirus proteinase that is based on structural and functional analogies with the trypsinlike serine proteinases. This model has received considerable support from studies of the processing of DEN2 and YF polyproteins in

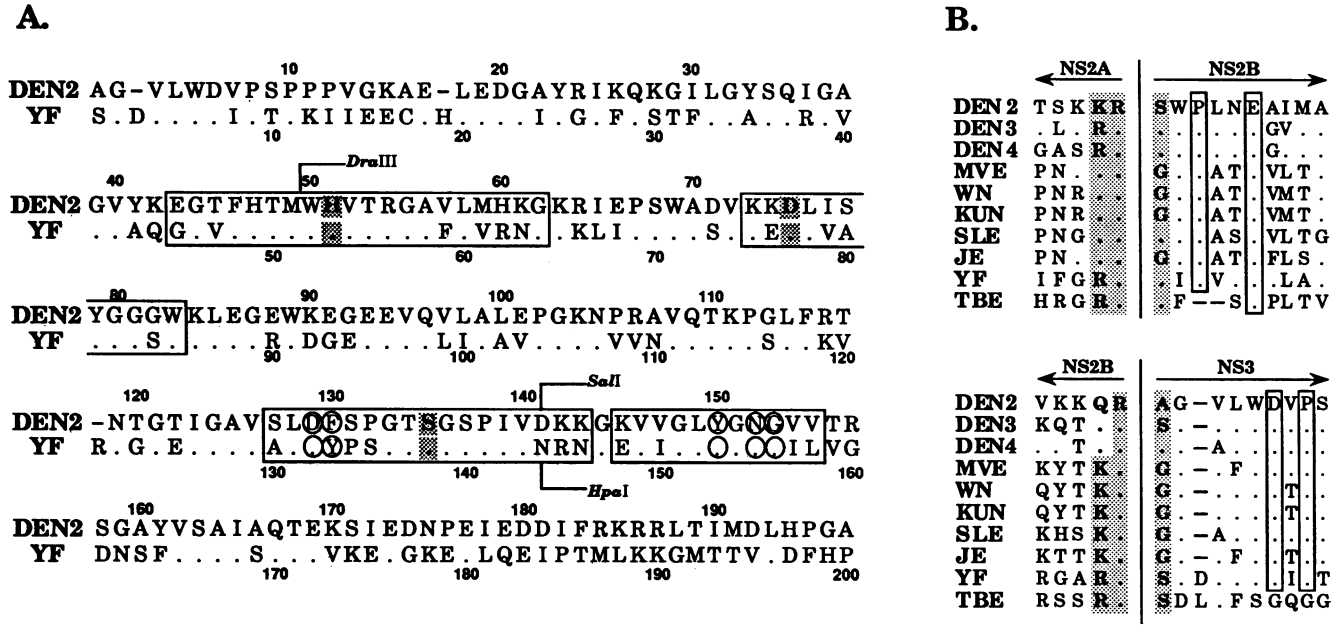

FIG. 1. Sequence comparisons of enzymes and substrates. (A) Alignment of the amino acid sequences of the N-terminal domains of the NS3 proteins of DEN2 PR159 S1 and YF Asibi. Residues are numbered from the N terminus of the protein. The three enzymatic domains and the substrate-binding pocket as postulated by Bazan and Fletterick (2) are boxed, and the amino acids which form the putative catalytic triad (His, Asp, and Ser) are shaded. Proposed contact residues are circled. The *Dra*III and *Sal*I (*Hin*cII)-*Hpa*I restriction sites which were used to make chimeric proteinases are indicated. The single-letter amino acid code is used throughout. (B) Alignment of the sequences surrounding the 2A/2B and 2B/3 cleavage sites for several flaviviruses. Cleavage usually occurs between two basic residues (shaded) and a Gly, Ser, or Ala (shaded). Conserved Pro and acidic residues downstream of the cleavage site are boxed. Viruses and references for sequences: DEN2 (15); DEN3 (33); DEN4 (22); MVE (8); Kunjin virus (KUN) (7); St. Louis encephalitis virus (SLE) (41); West Nile virus (WN) (3); YF, Asibi strain (14); Japanese encephalitis virus, Beijing 1 strain (JE) (16); tick-borne encephalitis virus (TBE) (23).

vitro (6, 35). The amino acid sequences of the NS3 protease domains of DEN2 and YF are aligned in Fig. 1A, and the amino acid sequences (for several flaviviruses) surrounding two of the cleavage sites processed by this proteinase are aligned in Fig. 1B. The DEN2 and YF protease domains in NS3 are 55% identical overall. Four conserved regions are boxed in Fig. 1A. The first three include the three proposed catalytic residues (shaded). The fourth box is referred to in the structural model of Bazan and Fletterick (2) as the substrate-binding pocket, containing residues important for interaction with the substrate. The model predicts that the three circled residues in this domain are in direct contact with bound substrate and that two other conserved residues found near the catalytic Ser, also circled, are also in direct contact with the substrate and form part of the substrate binding domain.

In contrast to the conservation in NS3, especially within the core region of the protease, the sequences around the 2A/2B and 2B/3 cleavage sites are not well conserved (overall the small hydrophobic proteins NS2A and NS2B are only 19 and 33% conserved, respectively). NS3 cleaves between a basic residue (usually Arg) and a Gly, Ser, or Ala (Fig. 1B), but helix-breaking and acidic residues (boxed in Fig. 1B) may also be important for substrate recognition.

The rapid evolution of viral proteinases and their cleavage sites suggests that viral proteinases should process homologous cleavage sites with greater efficiency than heterologous cleavage sites, and recent studies using chimeric picornavirus 3C proteinases have provided support for this view (10, 11, 21). To investigate whether these two divergent flavivirus NS3 proteinases could process heterologous cleavage sites which have diverged significantly, we constructed plasmids that would allow expression of chimeric polyproteins of YF and DEN2 (Fig. 2A). The cleavage sites present in each construct and the origins of the NS3 amino acids in these constructs are listed in Table 1.

We have previously described the construction of an expression plasmid, herein called pD0 (35). In vitro transcription of this plasmid with T7 RNA polymerase produces an mRNA that contains the authentic 5′ leader of DEN2 followed by the sequences encoding the N-terminal 37 amino acids of the C protein fused in frame to the sequences encoding the C-terminal 118 amino acids of NS2A, all of NS2B (130 residues), and the N-terminal 303 amino acids of NS3. Translation of this RNA in rabbit reticulocyte lysates produces protein products that are correctly cleaved at the 2A/2B and 2B/3 sites. The processing intermediate P2B3 and the product P3 are always observed in pD0-programmed translations (Fig. 2B), as is the full-length unprocessed translate (P2A2B3). The last migrates as a diffuse band in SDS-gels, presumably because of hydrophobic sequences present in the polyprotein. P2A is also expected to be present but is very hydrophobic and difficult to detect by direct SDS-PAGE analysis. For this project we also constructed plasmid pY0, in which the leader sequence and the domain encoding the first 37 amino acids of the capsid protein of DEN2 were fused in frame to YF sequences encoding the C-terminal region of NS2A, all of NS2B, and the first 299 residues of NS3 (Fig. 2A). In vitro transcription and translation of RNA from pY0 resulted in efficient processing of the YF proteins at the 2A/2B and 2B/3 sites (Fig. 2B). The processing is so efficient that no unprocessed

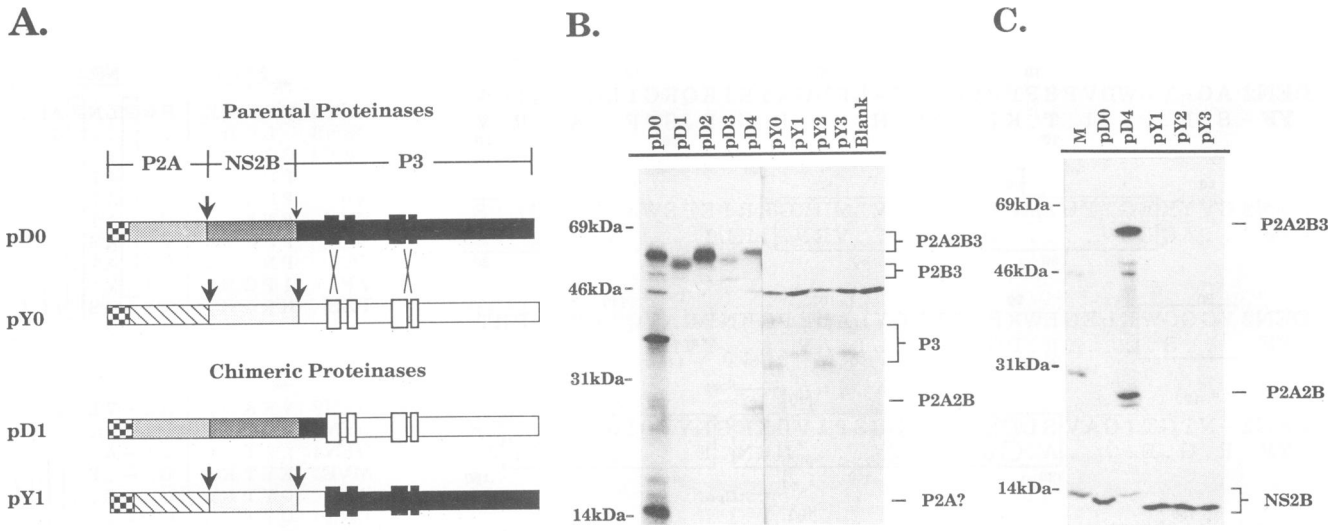

FIG. 2. Protein products produced by translation of chimeric polyproteins. (A) Schematics of parental and chimeric constructs. Processing at the 2A/2B and 2B/3 cleavage sites is indicated by arrows; efficient or inefficient cleavage of a site is indicated by a large or a small arrow, respectively. The DEN2 and YF putative catalytic and substrate binding-pocket residues are indicated by solid and open boxes, respectively. DEN2 capsid protein, P2A, NS2B, and P3 sequences are designated by checkered, lightly shaded, darkly shaded, and solid boxes, respectively. YF P2A, NS2B, and P3 sequences are designated by lightly diagonally shaded, lightly shaded, and open boxes, respectively. (B) Autoradiograph of an SDS-polyacrylamide gel displaying protein patterns from in vitro translation mixes programmed with RNA transcribed from the constructs in panel A. All in vitro translations were programmed with equivalent amounts of input RNA and were incubated for 90 min. Lanes pY0, pY1, pY2, pY3 and Blank display fivefold more reticulocyte lysate than do lanes pD0, pD1, pD2, pD3, and pD4. The molecular sizes of ^{14}C-labeled protein standards (Amersham) are indicated. Precursor P2A2B3, intermediates P2B3 and P2A2B, products P3, and putative P2A are indicated. (C) Immunoprecipitation of the translation products. Aliquots of pD0-, pD4-, pY1-, pY2-, and pY3-programmed reticulocyte lysates were denatured and immunoprecipitated with DEN2 (lane pD0) or YF (lanes pD4, pY1, pY2, and pY3) anti-NS2B antisera (35); immune complexes were dissolved in SDS-containing loading buffer and analyzed by SDS-PAGE. The construct from which RNA was transcribed is indicated above each lane. The molecular sizes of ^{14}C-labeled protein standards (Amersham) are also indicated (lane M).

precursor remains, in contrast to the case with the DEN2 construct.

Two reciprocal chimeras, pD1 and pY1, were constructed by using a conserved *Dra*III restriction site, indicated in Fig. 1A, that lies just upstream of the putative catalytic His. In essence, the 2A/2B and 2B/3 sites in each chimera are derived from one virus and the enzymatic domains are derived from the other, so that we can examine the activity of each proteinase on a heterologous substrate. Translation of RNA transcribed from pD1 produced an unprocessed full-length translation product (Fig. 2B), and we conclude that the YF protease domain in this construct was unable to cleave DEN2 cleavage sites, either because of an inability of the YF proteinase to recognize DEN2 sites or because of misfolding of the chimeric polyprotein. In contrast, translation of RNA from pY1 produced fully processed P3 and NS2B, suggesting that the DEN2 protease domain is correctly folded and can recognize YF cleavage sites (Fig. 2B and C). Small amounts of higher-molecular-weight P3 polypeptides were sometimes observed in constructs containing YF P2A2B (see below), suggesting that cryptic cleavage sites within P2A or NS2B of YF were recognized with a low frequency. The efficiency of processing of the pY1 polyprotein was similar to that observed with the parental pY0 polyprotein. Thus, the YF sites are processed efficiently by both the YF and the DEN2 NS3 proteinase, whereas the DEN2 sites are processed inefficiently by the DEN2 protease and not at all by the YF protease. It is of considerable interest that the DEN2 protease domain processes heterologous YF cleavage sites more efficiently than homologous DEN2 cleavage sites (compare lane pY1 with lane pD0).

Effect of the substrate-binding pocket. The catalytic triad of the DEN2 proteinase was replaced with the YF catalytic triad in plasmid pD2. This chimera contains the catalytic triad of YF combined with the substrate-binding pocket and cleavage sites of DEN2 (Fig. 2A). Translation of RNA from pD2 produced a pattern that differed in experiments using independent lots of reticulocyte lysate. For consistency, all translation patterns presented in this report were obtained from the same lot of reticulocyte lysate under identical conditions, and the pattern in Fig. 2B shows an unprocessed polyprotein. In some experiments the translation patterns from pD2 were consistent with processing at the 2A/2B and 2B/3 cleavage sites (data not shown), suggesting that in the experiment in Fig. 2B processing of the pD2 polyprotein was

blocked because proper protein folding did not occur. It appears that under ideal conditions the chimeric pD2 proteinase can process DEN2 cleavage sites, but the processing efficiency varies with the lot of reticulocyte lysate used.

The reciprocal construct pY2 contains the catalytic triad of DEN2 combined with the substrate-binding pocket and cleavage sites of YF. pY2-programmed translations produced a highly active proteinase which efficiently processed the YF 2A/2B and 2B/3 cleavage sites (Fig. 2B and C).

In plasmid pD3, the substrate-binding pocket of DEN2 was replaced with the YF substrate-binding pocket. Translation of pD3 RNA produced partially processed P2B3 and a small amount of fully processed P3 that can be observed upon overexposure of the autoradiogram (Fig. 2B). The amount of P2B3 that was generated varied from experiment to experiment, and Fig. 2B shows an example of the most efficient processing of the pD3 polyprotein. P2B3 was inefficiently processed to yield mature NS2B (data not shown). The partial activity of the pD3 proteinase suggested that although this chimeric proteinase could cleave the DEN2 sites, it could interact with DEN2 cleavage sites only inefficiently, although misfolding of the proteinase may be responsible for the low activity. In the reciprocal construct pY3, the DEN2 substrate-binding pocket is placed into the YF polyprotein. pY3-programmed translations produced a highly active proteinase, which efficiently processed the YF 2A/2B and 2B/3 cleavage sites (Fig. 2B and C).

Thus, the YF sites are processed efficiently by all chimeras tested. The DEN2 sites are never processed efficiently. The DEN2 proteinase will process these sites, whereas the YF proteinase will not. Chimeric proteinases with domains derived in part from DEN2 and in part from YF will process the DEN2 sites with an efficiency that differs with the construct tested. Although the chimeric proteinases did not allow direct mapping of regions involved in substrate recognition (21), the results with them do suggest that residues 144 to 180 are important for substrate interaction, although not the only determinant of substrate specificity, and set the stage for experiments to assay the effects of cleavage site and substrate-binding pocket mutations on the order and efficiency of processing (see below).

It is noteworthy that in in vitro translations in which extensive processing occurred (pY0, pY1, pY2, and pY3), less [^{35}S]Met was incorporated than in translations in which less processing occurred (pD0, pD1, pD2, pD3, and pD4). The reduction in incorporation of [^{35}S]Met was approximately fivefold (per unit of input RNA). It is possible that more active proteinases inhibit translation by cleaving essential components in the reticulocyte lysate.

The importance of NS2B in the recognition of cleavage sites. NS2B has been found to be important for proteolysis of flavivirus polyproteins, as deletions in NS2B can cause inactivation of NS3 (35). In addition, NS2B and NS3 form a complex in vitro and in vivo, suggesting that NS2B may modulate the activity of the nonstructural proteinase (6, 34a). We wished to explore the possibility that YF NS3 does not interact properly with DEN2 NS2B, resulting in the failure of the YF proteinase to cleave DEN2 sites. A chimera, pD4, was constructed that combined the DEN2 NS2A/2B cleavage site, the YF NS2B/NS3 cleavage site, and YF NS3 (Fig. 2A). Translation of pD4 RNA produced fully processed P3 and NS2B and partially processed P2A2B (Fig. 2B and C). (Note that the electrophoretic mobility of NS2B is altered, presumably because of the chimeric nature of this protein.) Several points emerge from this analysis. First, the homologous YF 2B/3 site in the D4 chimera is processed efficiently, as has been found for all YF sites. Second, the heterologous DEN2 2A/2B site is also processed, but inefficiently as we have found for all DEN2 sites. Thus the YF proteinase in construct pD4 is able to process this DEN2 site, in contrast to the result with pD1. It appears that either the YF NS3 requires YF NS2B (or at a minimum the C-terminal 63 residues) for function or the YF sequences in the N terminus of NS3 allow proper folding of NS3, or both. Third, the processing order appears to be first cleavage of the 2B/3 site followed by cleavage of the 2A/2B site, as has been found for YF processing (6; see also below).

Mutagenesis of the 2A/2B cleavage site. The inefficient processing of DEN2 cleavage sites in all constructs tested, including the DEN2 proteinase itself, suggests that DEN2 cleavage sites have evolved a suboptimal sequence or structure. To further explore this, we wished to change the amino acid sequence of the DEN2 2A/2B and 2B/3 cleavage sites to resemble that of YF and assay the effects of these changes in different genetic backgrounds.

In vitro studies using viral proteinases indicate that seven to nine residues are often required by proteinases in order to optimally recognize cleavage sites (29). Five residues surrounding the DEN2 2A/2B cleavage site were changed to their YF counterparts. These changes include both conservative and nonconservative amino acid substitutions (Fig. 3A). The resulting sequence is identical to that of YF for four residues upstream and seven residues downstream of the cleavage site. The mutations were engineered into both the wild-type DEN2 background (pD0) and the chimera pD4 (which does not efficiently process the DEN2 2A/2B cleavage site) to generate mutants pD0.8 and pD4.8. The efficiency of polyprotein processing to produce P3 in a pD0.8-programmed translation (Fig. 3B) was similar to that produced by a parental pD0-programmed translation, rather than the efficient processing seen in construct pY1. Thus, the linear sequence immediately surrounding the 2A/2B cleavage site, and in particular the pair of lysine residues upstream of the cleavage site, is not entirely responsible for the low processing efficiency of the DEN2 proteinase.

The efficiency of polyprotein processing to produce P3 and P2A2B in a pD4.8-programmed translation (Fig. 3B) was similar to that produced by a parental pD4-programmed translation. Thus, the linear sequence immediately surrounding the 2A/2B cleavage site does not limit the processing efficiency of the YF proteinase. In conclusion, the changes did not increase or decrease the apparent processing efficiency in two different genetic backgrounds, suggesting that other unknown factors limited the efficiency of the 2A/2B cleavage site processing.

Mutagenesis of the 2B/3 cleavage site. Seven residues surrounding the DEN2 2B/3 cleavage site were changed to their YF counterparts, including the insertion of an Asp residue (Fig. 4A). The resulting cleavage site is identical to that of YF for six residues upstream of the site and seven residues downstream. The mutations were engineered into both the wild-type DEN2 background (pD0) and the chimera pD3 (which has the YF substrate-binding pocket in an otherwise DEN2 background) to generate mutants pD0.7 and pD3.7. The efficiency of polyprotein processing to produce P3 in a pD0.7-programmed translation (Fig. 4B) was similar to that produced by a parental pD0-programmed translation, rather than the efficient processing seen in construct pY1. Thus, the linear sequence immediately surrounding the 2B/3 cleavage site, and in particular the sequence Gln-Arg at the cleavage site rather than the Lys-Arg found in other flaviviruses, is not completely responsible for

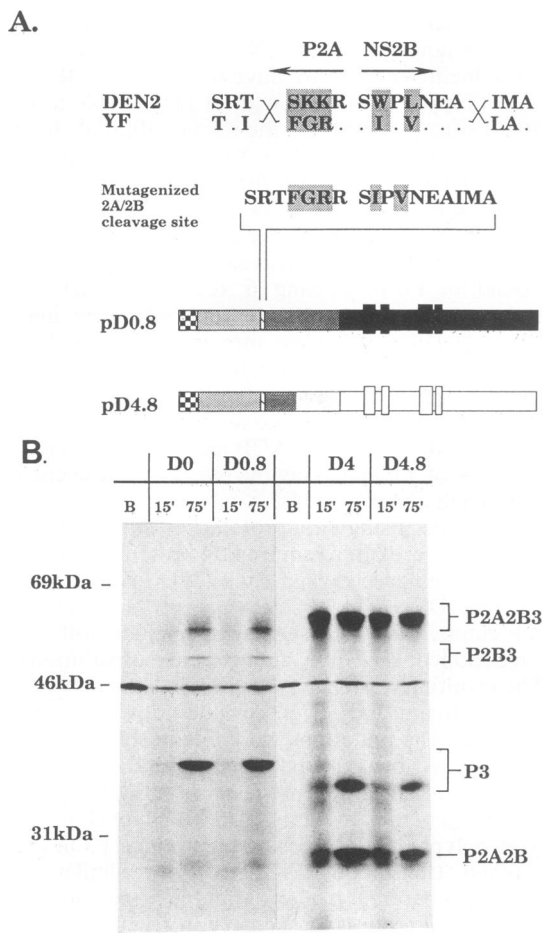

FIG. 3. Mutagenesis of the 2A/2B cleavage site. (A) Residues surrounding the DEN2, YF, and chimeric 2B/3 cleavage sites. Residues subjected to mutagenesis are shaded, and identical residues are represented as dots. The chimeric DEN2 2A/2B cleavage site present in pD0.8 and pD4.8 is functionally a double crossover, as indicated by two sets of crossed lines. The transcription templates pD0.8 and pD4.8 are indicated, using the same conventions as in Fig. 2A. The chimeric 2A/2B cleavage site is designated by a small, lightly diagonally shaded box. (B) Pulse-chase patterns of pD0.8- and pD4.8-programmed in vitro translations. Translations were labeled with [^{35}S]Met for 15 min (15′) and chased for 75 min (75′). Lanes B contain translation mixes without added RNA. Unprocessed P2A2B3, partially processed P2B3 and P2A2B, and completely processed P3 are indicated, as are the molecular sizes of ^{14}C-labeled protein standards (Amersham).

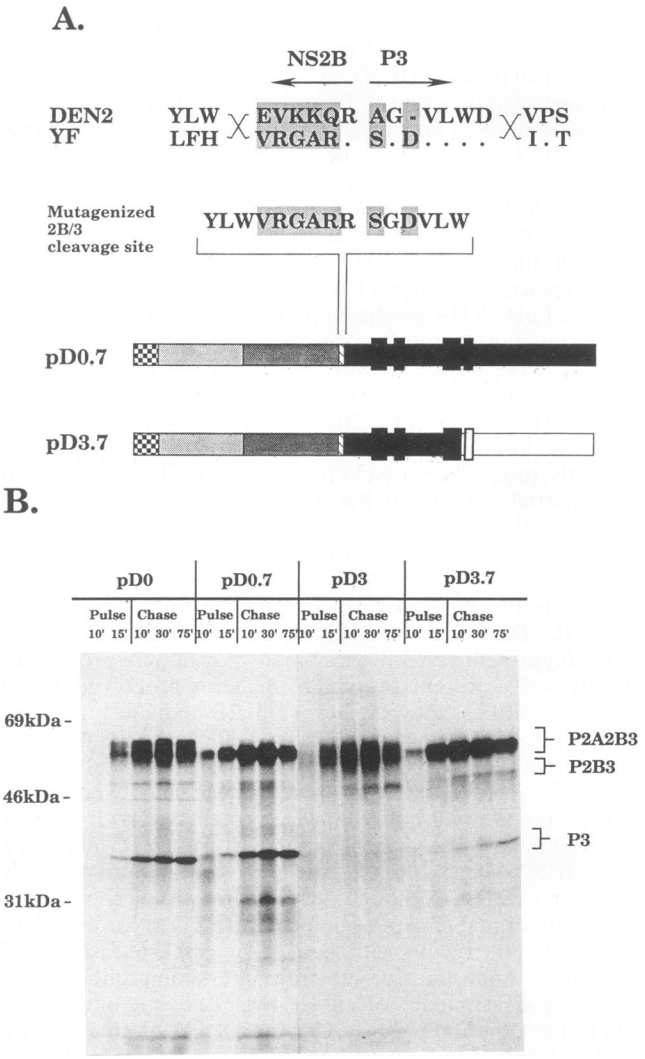

FIG. 4. Mutagenesis of the 2B/3 cleavage site. (A) Amino acid residues surrounding the DEN2, YF, and chimeric 2B/3 cleavage sites, displayed as described for Fig. 3A. The chimeric 2B/3 cleavage site is designated by a small, lightly diagonally shaded box. (B) Pulse-chase patterns of pD0-, pD0.7-, pD3- and pD3.7-programmed in vitro translations. Translations were labeled with [^{35}S]Met for 15 min (15′) and chased for various periods of time. A portion was removed after 10 min of labeling, another after 15 min of labeling, and others after 10, 30, and 75 min of chase. Unprocessed P2A2B3, partially processed P2B3, and completely processed P3 are indicated, as are the molecular sizes of ^{14}C-labeled protein standards (Amersham).

the lower processing efficiency of the DEN2 proteinase in a DEN2 background.

In pD0.7-programmed translations there were also variable amounts of a 31-kDa protein, which depended on the quality of the transcribed RNA. This protein reacted with NS3 antiserum but not with NS2B antiserum (data not shown), suggesting that this species is a processed product that results from premature termination during translation.

In contrast to the results with pD0.7, pD3.7-programmed translations produced increased amounts of P3 (Fig. 4B) and NS2B (data not shown) relative to pD3-programmed translations. In the case of pD3, little or no cleavage of the 2B/3 bond was seen and P2B3 accumulated, whereas in pD3.7, P2B3 appeared to be processed to produce P3 (Fig. 4B). This finding suggests that the failure to process the 2B/3 bond in pD3 is not due to misfolding, but that the YF substrate-binding pocket cannot interact with the wild-type DEN2 2B/3 cleavage site. This mutant provides direct evidence for an interaction of amino acids 144 to 180 of the YF NS3 protease domain with residues at the 2B/3 cleavage site. To confirm the results obtained with pD3.7, we made a new chimeric construct equivalent to pD3 in which MVE sequence was used instead of YF sequence. This chimera resulted in an active proteinase (data not shown). When the (YF) mutations in pD3.7 were introduced into the 2B/3 site of this DEN2-MVE chimera, the efficiency of processing of the 2B/3 site increased (data not shown), as in the case of

pD3.7, suggesting that the MVE substrate-binding pocket interacts with YF sites more efficiently than with DEN2 sites. The mutated 2B/3 cleavage site did not increase processing efficiency in a pD1 background, however (data not shown), and a simple change in folding efficiency induced by the mutations cannot explain the results. These results strongly suggest that the observed increase in processing efficiency is due to optimization of the 2B/3 cleavage site/substrate-binding pocket interactions.

Modulation of processing efficiency. The experiments described above pinpointed amino acids 144 to 180 of the flavivirus NS3 protease domain as being important for substrate recognition, and the model of Bazan and Fletterick (2) predicts that residues 130 to 158 are of particular import for substrate binding. The amino acid sequences of DEN2 and YF are very similar throughout the region from 130 to 158 (Fig. 1A). In particular, of the five residues predicted to contact the substrate, Asp-129, Phe-130, Tyr-150, Asn-152, and Gly-153 (DEN2 numbering) (circled in Fig. 1A), four are the same in the two viruses and cannot be responsible for the observed differences in the enzymes, and the fifth represents a conservative change, Phe in DEN2 and Tyr in YF. (It is of note that in DEN2 the mutation Tyr-150 to Phe was essentially wild type in processing, whereas Asn-152 to Lys was proteolytically inactive; data not shown). There is, however, a stretch of seven amino acids within this region in which five differences occur between DEN2 and YF, and three of these changes are nonconservative; these differences could therefore be important for the differences in substrate specificity. These residues are predicted to form part of a loop and beta-strand structure adjacent to the proposed catalytic serine, and they have a high probability of being found at the surface of the protein (2). To test the possible importance of these residues in substrate recognition, PCR mutagenesis was used to change four of the DEN2 residues to the corresponding YF amino acids (Lys-142 to Arg, Lys-143 to Asn, Lys-145 to Glu, and Val-147 to Ile; Asp-141 of DEN2 was not changed to the corresponding Asn of YF because of cloning considerations). Mutants that contained different subsets of these mutations were constructed, and nonconservative mutations were also examined individually; the effects of the mutations were examined in two different genetic backgrounds. The mutants and their designations are illustrated in Fig. 5A.

All mutations tested in a parental pD0 background, including the quadruple mutant pD0.1, failed to significantly alter the efficiency of processing (Fig. 5B), suggesting that the DEN2 proteinase can readily tolerate these substitutions at positions 142, 143, 145, and 147. Note that the electrophoretic mobility of P3 was altered in mutant pD0.2 (a shift in electrophoretic mobility was also occasionally observed with mutant pD0.1), presumably because of the amino acid changes introduced into P3.

Although multiple mutations within the DEN2 substrate-binding pocket (pD0.1) did not affect proteinase activity (Fig. 5), more complex mutations (chimera pD3) almost completely inactivated the proteinase (Fig. 2). In contrast, complex mutations within the YF substrate-binding pocket produced a highly active proteinase (chimera pY3) (Fig. 2). Taken together, these results suggest that the NS3 substrate-binding pocket functions as a minidomain (17) and that the phenotype of each substrate-binding pocket mutant depends on the genetic background in which the mutations are assayed (see below).

The mutations present in pD0.1 through pD0.6 were cloned into a pY1 genetic background to test the effects of these changes on the efficiency of processing of YF cleavage sites. Two single mutants, pY1.5 (Lys-143 to Asn) and pY1.6 (Lys-145 to Glu), and a double mutant that contained two conservative substitutions, pY1.3 (Lys-142 to Arg and Val-147 to Ile), had little or no effect on processing and produced patterns very similar to that from the parental construct pY1. However, mutants that contained two nonconservative mu-

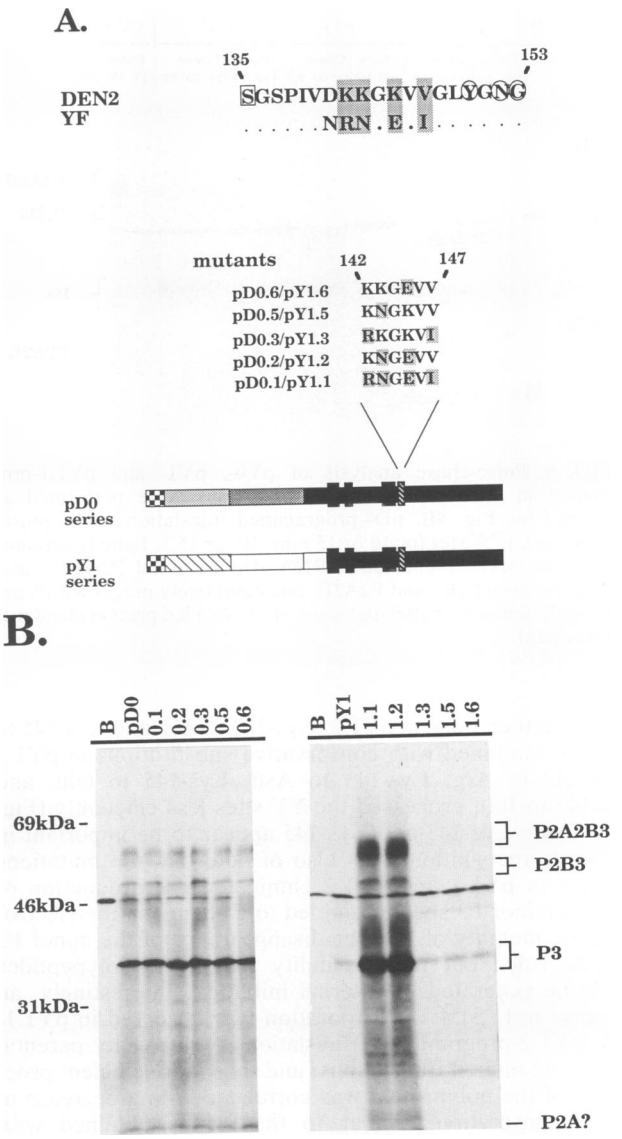

FIG. 5. In vitro translation of substrate-binding pocket mutants. (A) Alignment of partial amino acid sequences of the putative substrate-binding pockets of the DEN2 PR159 S1 and YF Asibi NS3 protease domains. The putative catalytic serine residue and proposed contact residues are outlined with an open box and circles, respectively. DEN2 substrate-binding pocket residues that were mutagenized are highlighted with shaded boxes, and the partial individual amino acid sequences of five mutants are displayed. (B) SDS-PAGE analysis. In vitro translations programmed with RNA transcribed from the indicated constructs were resolved on an SDS-PAGE gel and autoradiographed. Lanes B contain translated mixes without added RNA. Unprocessed P2A2B3, the processing intermediate P2B3, P3, and putative P2A species are indicated, as are the molecular sizes of ^{14}C-labeled protein standards (Amersham).

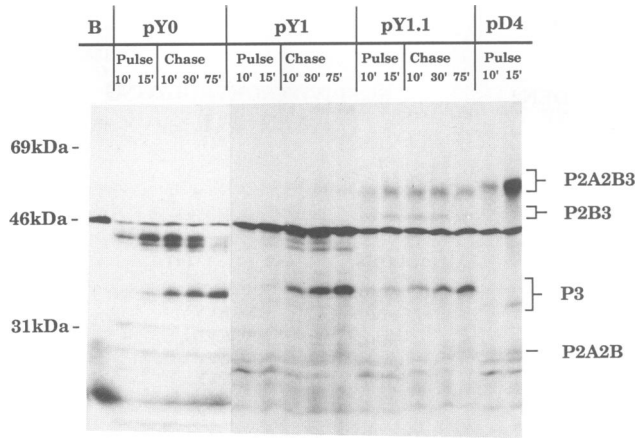

FIG. 6. Pulse-chase analysis of pY0-, pY1- and pY1.1-programmed in vitro translations. Translations were performed as described for Fig. 4B. pD4-programmed translations were pulse-labeled with [^{35}S]Met for 10 or 15 min (10′ or 15′). Lane B contains translation mix without added RNA. Unprocessed P2A2B3, partially processed P2B3 and P2A2B, and completely processed P3 are indicated, as are the molecular sizes of ^{14}C-labeled protein standards (Amersham).

tations, either alone in pY1.2 (Lys-143 to Asn and Lys-145 to Glu) or combined with conservative substitutions in pY1.1 (Lys-142 to Arg, Lys-143 to Asn, Lys-145 to Glu, and Val-147 to Ile), processed the YF sites less efficiently (Fig. 5B). Thus, Lys-143 and Lys-145 appear to be important in substrate recognition. It is also of note that the mutations present in pY1.1 and pY1.2 eliminated the production of several minor P3 species and led to a change in the electrophoretic mobility of P3. The disappearance of the minor P3 species ruled out the possibility that these polypeptides could be generated by internal initiation. Interestingly, an increase in [^{35}S]Met incorporation was observed in pY1.1- and pY1.2-programmed translations relative to parental pY1-programmed translations, and thus more efficient processing of the polyprotein was correlated with a decrease in Met incorporation, similar to the results obtained with chimeric proteinases (Fig. 2B).

Order of processing. In vitro, DEN2 NS3 processes the 2A/2B site before the 2B/3 site (35), whereas YF NS3 processes the 2B/3 site before the 2A/2B site (6). Functionally, DEN2 NS2B is produced by cleavage of a P2B3 precursor, whereas YF NS2B is produced by cleavage of a P2A2B precursor. We considered two related hypotheses for the difference in the order of processing in these viruses: it could be due to differences in NS2B (a global structural effect), to differences in the affinities of the proteinases for the cleavage sites (a local sequence-specific effect), or both. If the order and efficiency of cleavage were determined chiefly by the structure of NS2B, then a chimeric polyprotein expressing YF NS2B and the DEN2 proteinase (pY1) would be processed similarly to the parental YF polyprotein (pY0). On the other hand, if the order and efficiency of cleavage were determined chiefly by the affinity of the proteinase for cleavage sites, then it should be possible to mutagenize the substrate-binding pocket or cleavage sites to modulate the order.

A pulse-chase analysis of pY0 and pY1 polyprotein processing is presented in Fig. 6. Processing of both polyproteins is very efficient, and the translation patterns are nearly identical. The high efficiency of processing makes it difficult to detect either full-length unprocessed precursors or expected intermediates. The analysis is complicated by the transient appearance of bands of 44 and 42 kDa which appear to be intermediates in processing. The 42- and 44-kDa species react with both NS2B and NS3 antisera (data not shown) but do not appear to be identical to P2B3 (predicted molecular size of 48 kDa). It is important to note that these translations produce homogeneous NS2B (Fig. 2C), and therefore it is unlikely that aberrant processing or internal initiation of translation is responsible for these bands. Thus, we cannot determine the processing order in the parental pY0 or the chimeric pY1 polyprotein, but the processing phenotypes appear to be the same. Chambers et al. (6) have reported that for YF, cleavage of the 2B/3 site occurs before cleavage of the 2A/2B site.

Pulse-chase analysis of pY1.1-programmed translations reveal a full-length unprocessed P2A2B3 precursor and a P2B3 intermediate of 48 kDa that appeared to chase into P3 (Fig. 6). It seems clear that NS2B and P3 are generated from a P2B3 precursor in pY1.1-programmed translations. A similar result was observed with mutant pY1.2 (Fig. 5B). These two mutants have two nonconservative mutations in the substrate-binding region (Fig. 5A). Mutants with only one nonconservative change in the substrate-binding region appeared to process like the parental pY1 (Fig. 5B). Thus, changing two residues in the substrate-binding pocket of DEN2 to the corresponding YF amino acids reduces the processing efficiency and converts the processing pathway to that characteristic of DEN2. Presumably, the efficiency and order of processing were modulated by differentially decreasing the efficiency of the interaction between the substrate-binding pocket of the proteinase and the cleavage sites.

The data described above with chimeric and mutant proteinases provide evidence that both global structural and local sequence-specific effects can modulate the efficiency and order of polyprotein processing. The data also suggest that processing of flaviviral polyproteins occurs with a preferred order, but the order is not obligatory. In support of this view, in vitro expression experiments have shown that DEN2 NS3 can be generated from two different intermediates, NS34A and NS2B3 (34b).

DISCUSSION

Molecular chimeras have been used to exchange antigen-binding loops, DNA recognition helices, or whole domains involved in ligand binding and have demonstrated that minidomains of large proteins can function independently (17, 26, 43, 44). Experiments with bacterial subtilisins demonstrated that the substrate specificity of two serine proteases (which are 69% conserved, but which have virtually superimposable three-dimensional structures) could be interconverted by mutagenizing selected amino acids within the substrate-binding pocket (42). In the bacterial subtilisins, four short stretches of amino acids (which are distributed over a 126-amino-acid segment of the protease) are brought into close proximity with the substrate by tertiary folding of the polypeptide chain (42). By analogy, it seems likely that multiple determinants of substrate specificity are distributed to a number of regions within the flaviviral nonstructural proteinase. We have used chimeric polyproteins which express a portion or all of the protease domain of one virus and the cleavage sites of another, as well as site-specific mutagenesis, to map residues which are important for substrate

recognition. In particular, we found direct evidence that residues near to or within the so-called substrate-binding pocket defined by Bazan and Fletterick (2) interact directly with the cleavage site (the substrate), and that residues in this domain of the proteinase which differed between YF and DEN2 could account for some of the differences in cleavage efficiency. In general, only multiple simultaneous changes within the protease domain noticeably affected cleavage efficiency or specificity, and the phenotype of the mutant often depended on the genetic background in which the mutations were assayed. As the in vitro expression assay used in our experiments is not sensitive enough to detect small changes in efficiency, the detection of subtle effects of multiple or single mutations may require biochemical assays using purified proteinases or biological analysis using infectious clones (36).

Even though the protease domains of NS3 are only 55% conserved between YF and DEN2, the fact that chimeric polyproteins are often active suggests that enzyme and substrate must possess similar structures. The pY1, pY2, and pY3 chimeric proteinases are more efficient than the parental DEN2 proteinase, which suggests that the activity of the DEN2 proteinase is limited by an inefficient enzyme-substrate interaction. Although partially active or inactive proteinases may result from a perturbed structure, in at least one case, pD3, the activity is limited by an inefficient interaction with the 2B/3 cleavage site. This may be true for other partially active or inactive chimeras as well.

The cleavage generating the N terminus of DEN2 NS3 is unique to the dengue virus subgroup. Cleavage occurs between a single Arg and an Ala or Ser, whereas other flaviviruses generate the N terminus of NS3 by cleaving between two basic amino acids and a Ser or Gly. Our site-specific mutagenesis experiments indicated that this altered sequence is at least partially responsible for the failure of the YF proteinase to process DEN2 sites but does not seem to be responsible per se for the low cleavage efficiency of the DEN2 proteinase upon DEN2 sites. The low efficiency of the DEN2 proteinase upon DEN2 sites, contrasting with the efficient cleavage by this proteinase of YF sites, suggests that DEN2 cleavage sites may have evolved a suboptimal sequence in order to regulate the concentration of processing intermediates, which can have functions different from those of the end products (9, 46). Analysis of the results with the DEN2 proteinase and the various chimeras is complicated by difficulties in correct folding of the DEN2 proteinase during translation in rabbit reticulocytes, however. The DEN2 enzyme appears to be more dependent on cell factors for proper folding than is the YF enzyme, and the processing efficiency is reduced in vitro compared with that observed in infected cells. But it is important to note that the order (12a) and specificity (35) of cleavage in infected cells are identical to those observed in cell-free lysates, and we believe that the in vitro cleavage events accurately reflect the events that occurs in vivo.

The NS3 proteinase is a member of a large superfamily of proteases, which includes the picornaviral 3C cysteine proteinases (2). The structural and functional similarity between the picornaviral 3C and the flaviviral NS3 proteinases was further emphasized by recent experiments which localized a major determinant of picornaviral substrate recognition to the carboxy-terminal portion of the 3C proteinase domain (21). In addition, mutations within the poliovirus 5′ noncoding region can be suppressed by mutations within the 3C proteinase (1). The second-site suppressor mutations occur at residues within the substrate-binding pocket that are structurally analogous to the residues that we have identified as being important for substrate recognition. It is intriguing that mutagenesis of amino acids adjacent to the catalytic serine or cysteine residues in the flavi- and picornaviral nonstructural proteinases can modulate either protein-protein (this report) or RNA-protein (1) interactions in these two families of positive-stranded RNA viruses.

The remarkable cleavage specificity of viral proteinases makes them ideal biochemical targets for antiviral drugs, and recently developed proteinase inhibitors offer hope for the treatment of incurable disease (27). We have described a system that allows identification of residues which are directly or indirectly involved with enzyme-substrate interaction and can define the substrate preferences of individual members of a viral family. These studies can be extended to other members of the flavivirus family to expand our understanding of substrate recognition, and they are potentially useful for the design of flavivirus proteinase inhibitors.

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