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Hepatitis C Virus-Encoded NS2-3 Protease: Cleavage-Site Mutagenesis and Requirements for Bimolecular Cleavage

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Cleavage at the 2/3 site of hepatitis C virus (HCV) is thought to be mediated by a virus-encoded protease composed of the region of the polyprotein encoding NS2 and the N-terminal one-third of NS3. This protease is distinct from the NS3 serine protease, which is responsible for downstream cleavages in the nonstructural region. Site-directed mutagenesis of residues surrounding the 2/3 cleavage site showed that cleavage is remarkably resistant to single-amino-acid substitutions from P5 to P3′ (GWRLL ↓ API). The only mutations which dramatically inhibited cleavage were the ones most likely to alter the conformation of the region, such as Pro substitutions at the P1 or P1′ position, deletion of both amino acids at P1 and P1′, or simultaneous substitution of multiple Ala residues. Cotransfection experiments were done to provide additional information on the polypeptide requirements for bimolecular cleavage. Polypeptides used in these experiments contained amino acid substitutions and/or deletions in NS2 and/or the N-terminal one-third of NS3. Polypeptides with defects in either NS2 or the N-terminal portion of NS3 but not both were cleaved when cotransfected with constructs expressing intact versions of the defective region. Cotransfection experiments also showed that certain defective NS2-3 constructs partially inhibited cleavage of wild-type polypeptides. Although these results show that inefficient cleavage can occur in a bimolecular reaction, they suggest that both molecules must contribute a functional subunit to allow formation of a protease which is capable of cleavage at the 2/3 site. This reaction may resemble the *cis* cleavage thought to occur at the 2/3 site during processing of the wild-type HCV polyprotein.

Hepatitis C virus (HCV) has been classified as a separate genus in the flavivirus family on the basis of its similar hydrophobicity profile and limited sequence homology to the other two genera of the family, the flaviviruses and the pestiviruses (15). All members of the flavivirus family have enveloped virions that contain a positive-strand RNA genome with a single, long open reading frame. In HCV, this open reading frame encodes a polyprotein of 3010 to 3033 amino acids with the following gene order: NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. C is a basic protein believed to be the viral capsid protein; E1 and E2 are thought to be the virion envelope glycoproteins; p7 is a protein of unknown function that is inefficiently cleaved from the E2 glycoprotein (28, 34, 47); and NS2 through NS5B are putative nonstructural proteins. The HCV nonstructural proteins were named according to their possible correspondence to the nonstructural proteins of flaviviruses and pestiviruses; however, the extent of functional similarities remains to be determined.

HCV polyprotein processing is accomplished by a combination of host and viral proteases. Host signal peptidase seems to be responsible for cleavages in the structural-NS2 region (21, 28, 34, 47). The nonstructural proteins are produced by two HCV-encoded proteases. A chymotrypsin-like serine protease located in the N-terminal one-third of NS3 cleaves at the 3/4A, 4A/4B, 4B/5A, and 5A/5B sites (2, 9, 18, 22, 33, 53). The HCV serine protease seems to require the central region of NS4A for cleavage at the 3/4A, 4A/4B, and 4B/5A sites (3, 12, 29–31). The NS3 serine protease of flaviviruses also requires an adjacent nonstructural protein for cleavage of the polyprotein, but in flaviviruses this role is fulfilled by NS2B rather than NS4A (1, 4, 6, 7, 13, 14). HCV also differs from flaviviruses in possessing a second virus-encoded protease that is responsible for cleavage at the 2/3 site (19, 22). The NS2-3 protease encompasses most of the NS2 region and the NS3 serine protease domain; however, catalytic activity of the serine protease is not required for 2/3 cleavage (19, 22). Although no homology has been found between the HCV NS2-3 protease and known proteases, it has been proposed to be a metalloprotease on the basis of the observation that its proteolytic activity is inhibited by EDTA and stimulated by $ZnCl_2$ (22). Site-directed mutagenesis of amino acids His-952 and Cys-993 (numbered according to their position in the HCV-H sequence) has revealed that they are essential for processing at the 2/3 site. The NS2-3 protease is believed to be primarily autocatalytic, but bimolecular cleavage has been observed in cell culture expression studies (19). Bimolecular cleavage is defined here as cleavage that requires two distinct polypeptides. This definition is not meant to indicate which polypeptide contains the enzymatic activity.

Although the 2/3 cleavage site has been identified by N-terminal amino acid sequencing of NS3 (19, 24), the importance of residues immediately surrounding the cleavage site for processing of NS2 and NS3 is currently unknown. Furthermore, although previous work has demonstrated bimolecular cleavage at the 2/3 site (19), the polypeptide requirements for this reaction have not been defined. Although bimolecular cleavage is inefficient and likely to be rare or nonexistent in natural HCV infections, characterization of this reaction may increase our understanding of the cleavage mechanism of the NS2-3 protease. In this study, we have analyzed the importance of residues flanking the 2/3 cleavage site for processing and

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examined the polypeptide requirements for bimolecular cleavage at this site. NS2-3 derivatives with substitutions blocking cleavage at the 2/3 site were tested for *trans* cleavage activity in comparison with the unmutated NS2-3 polyprotein and the individual NS2 and NS3 proteins. We also investigated the possibility that NS2 and NS3 remain associated as a complex after intramolecular cleavage at the 2/3 site.

MATERIALS AND METHODS

Plasmid constructs. All plasmids used in the transfection experiments were derivatives of pTM3 (37) or pBRTM (18, 20). The HCV cDNA sequence in these plasmids is under the control of the T7 promoter. Plasmids pBRTM/HCV1-3011, pBRTM/HCV827-3011, pBRTM/HCV827-3011 C_{993}A, pBRTM/HCV827-1137, pTM3/HCV827-1207, pTM3/HCV827-1207 H_{952}A, pTM3/HCV 1027-1207, pTM3/HCV1027-1657, pTM3/YF NS2B, and pTM3/YF NS2B-3_{181} have been described previously (6, 18, 20, 29). Numbers following the HCV designation in the names of the plasmid constructs indicate the amino acids of the HCV-H strain expressed by each construct. All plasmids containing DNA fragments derived from PCR amplification or treated with DNA-modifying enzymes such as T4 DNA polymerase or mung bean exonuclease were verified by sequencing; all other plasmids were verified by digestion with restriction endonucleases. pTM3/HCV827-1137 H_{952}A was constructed by replacing the *Spl*I-*Bst*XI fragment of pTM3/HCV827-1207 H_{952}A with the *Spl*I-*Bst*XI fragment of pBRTM/HCV827-1137. pTM3/HCV936-1039 H_{952}A was constructed by PCR amplification of pTM3/HCV827-1207 H_{952}A with primers SFN2 (5′-CCGCCA TGGTCATCATTAAGTTAGGGGCG-3′) and SF123 (5′-CCACAAGGTCTT GGTC-3′), followed by replacement of the *Nco*I-*Stu*I fragment of pTM3/HCV827-1207 with the *Nco*I- and *Stu*I-digested PCR product. The SFN2 primer consists of an HCV-1 sequence homologous to nucleotides (nt) 3147 to 3170 of the HCV-H strain plus an *Nco*I site that contains an ATG codon corresponding to amino acids 936 of the HCV-H strain. The amino acid sequence encoded by the SFN2 primer matches the HCV-H strain except for an Ala-to-Val substitution at amino acid 937. pTM3/HCV827-1026 was constructed by PCR amplification of pTM3/HCV827-1207 with primers SFN3 (5′-CCGCCATGGCGCTGA CTCTGTCACCATAT-3′) and IDT/13 (5′-TTTGGGATCCTACAGCAACCT CCACCC-3′), followed by replacement of the *Nco*I-*Bam*HI fragment of pTM3/HCV827-1207 with the *Nco*I- and *Bam*HI-digested PCR product. The SFN3 primer consists of nt 2820 to 2843 of the HCV-H strain plus an *Nco*I site that contains an ATG codon corresponding to amino acid 827 of the HCV-H strain. The IDT/13 primer consists of nt 3405 to 3419 of the HCV-H strain, which encode the C-terminal amino acids of NS2, followed by an engineered termination codon and a *Bam*HI site. pTM3/HCV936-1026 H_{952}A was constructed by replacing the *Bgl*II fragment of pTM3/936-1039 H_{952}A with the *Bgl*II fragment of pTM3/HCV827-1026. pTM3/HCV810-1026 was constructed by replacing the *Nco*I-*Bss*HII fragment of pTM3/HCV827-1026 with the *Xba*I-*Bss*HII fragment of pSINrep5/13.1 (32). pTM3/HCV810-1657 was constructed by replacing the *Bst*1107I-*Bpu*1102I fragment of pTM3/HCV810-1207-myc with the *Bst*1107I-*Bpu*1102I fragment of pTM3/HCV1027-1657. pTM3/HCV810-1207-myc was derived from pTM3/HCV810-1026 by replacing its *Bss*HII-*Pst*I fragment with the *Bss*HII-*Nsi*I fragment of pSINrep5/13.1 (32). Note that plasmids encoding polypeptides beginning with amino acid 810 also express the upstream signal sequence (residues 785 to 809) to generate the authentic N terminus of NS2, which is Leu-810 of the HCV polyprotein.

Single-amino-acid substitutions were made in the 2/3 cleavage site of the expression construct pTM3/HCV827-1207 by oligonucleotide-directed mutagenesis (27). In many cases, silent mutations were included in the oligonucleotide sequence to create novel restriction sites to facilitate the identification of correct clones. Other mutants were identified by sequencing or by the destruction of a unique *Kas*I site at nt 3419 in the wild-type HCV-H strain cDNA sequence. Two independent clones were obtained of mutants identified by creation or destruction of a restriction site and compared for their effects on cleavage at the 2/3 site to ensure that they exhibited identical phenotypes. Oligonucleotide sequences surrounding the mutations are shown below for the complementary strand to facilitate comparison with the HCV-H strain sequence. Nucleotides which differ from the HCV-H strain are shown in lowercase letters, and the codons corresponding to mutated amino acids are underlined: K1021A (CTCCgcGGGGT), K1021E (CTCtgAaGGGT), G1022A (CAAaGctTGGA), G1022V (CAAGGtc TGGA), G1022W (CAAGtGGTGGA), G1022R (CAAGcGcTGGA), G1022P (CAAGccaTGGA), W1023A (GGGGgcccGGT), R1024A (GTGGgcccTGC), R1024E (GTGGgaGcTcC), L1025A (GAGGgccCTGG), L1025E (GAGGgaG CTcG), L1026A GcTagcGGCGC), L1026E (GcTcgaGGCGC), L1026R (GTT GCgcGCGC), L1026F (GcTctTcGCGC), L1026P (GTTGCcGGCGC), A1027V (GCTGGtaCCCA), A1027R (GCTGagaCCCA), A1027D (GCTGGatCCCA), A1027P (GCTGgCGCCCA), P1028A (GGCGgCCATCA), I1029A (GCCggc CACGG), I1029E (tCCggaaACGG), I1029P (GCCCccgACcG), and ΔL1026–A1027 (AGGTTG ^ CCCATC).

pTM3/HCV827-1207 (G_{1022}-I_{1029})A, a plasmid encoding Ala at every position from P5 through P3′ of the 2/3 site, was constructed by PCR amplification of pTM3/HCV827-1207 with primers IDT/19 (5′-TAATACGACTCACTATA-3′) and IDT/108 (5′-TGGGCGTACGCCGTTGCTGCCGCTGCTGCTGCTGC CGCCTTGGAGACCATTC-3′), followed by replacement of the *Nco*I-*Spl*I fragment of pTM3/HCV827-1207 with the *Nco*I- and *Spl*I-digested PCR product. The sequence of IDT/19 corresponds to the T7 promoter region of pTM3, and primer IDT/108 consists of nt 3391 to 3442 of the HCV-H strain, altered to encode Ala from amino acids 1022 through 1029.

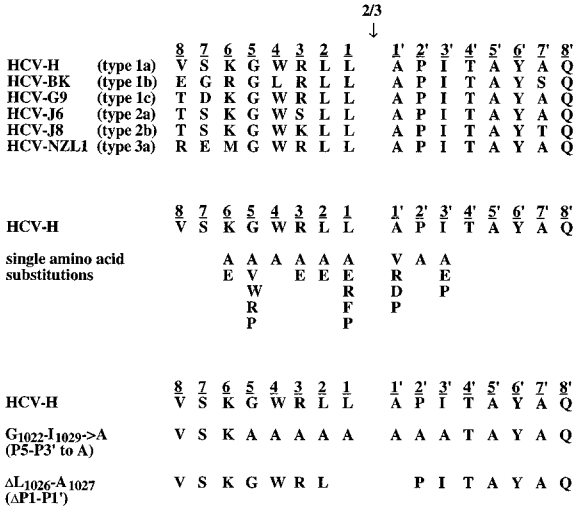

FIG. 1. Sequences flanking the 2/3 cleavage site of representative HCV strains and cleavage-site mutations. The HCV sequences shown represent three genotypes and six subtypes (25, 38–40, 44, 51) for which sequence data are available at the 2/3 cleavage site. Of six major HCV genotypes currently recognized, type 2 sequences have the greatest evolutionary distance from type 1 sequences, such as the HCV-H strain used in this study; type 3 sequences are more closely related to type 1 sequences (48, 49). The mutations analyzed for their effect on NS2-3 cleavage are shown below the wild-type amino acid sequence. The position of each amino acid relative to the cleavage site is shown above the HCV-H strain, using the nomenclature of Schechter and Berger (46).

Expression and analysis of HCV proteins. Transient-expression assays with the vaccinia virus T7 hybrid system were performed as previously described, with minor modifications (18). Briefly, BHK-21 monolayers were infected with vTF7-3 (16) at a multiplicity of infection of 10 in phosphate-buffered saline–1% fetal bovine serum (FBS) for 30 min at room temperature and subsequently transfected with 5 μl of Lipofectamine (Gibco/BRL) per μg of plasmid DNA in serum-free minimal essential medium (MEM) for 2 h at 37°C. For pulse-labeling experiments, the transfected cells were labeled for 20 min with 80 μCi of ^{35}S-protein-labeling mix (NEN) per ml plus 80 μCi of [^{35}S]cysteine (Amersham) per ml in MEM lacking methionine and cysteine and supplemented with 3% FBS. For long labeling experiments, cells were labeled for 3 to 4 h with 40 μCi of ^{35}S-protein-labeling mix per ml plus 40 μCi of [^{35}S]cysteine per ml in MEM containing 1/40 the normal concentration of methionine and cysteine and supplemented with 3% FBS. Cells were harvested in lysis buffer (50 mM Tris-Cl [pH 7.5], 0.5% sodium dodecyl sulfate (SDS), 1 mM EDTA, 20 μg of phenylmethylsulfonyl fluoride per ml), and cellular DNA was sheared by repeated passage through a 27.5-gauge needle. Lysates were stored at −70°C or analyzed immediately by immunoprecipitation with region-specific antisera and SDS-polyacrylamide gel electrophoresis (PAGE) as previously described (18).

For the coprecipitation experiments, BHK-21 cells were transfected as described above, pulse-labeled for 30 min at 37°C with 100 μCi of ^{35}S-protein-labeling mix per ml in MEM lacking methionine and supplemented with 2% FBS and chased for 30 min at 37°C in MEM containing excess methionine (1.5 mg/ml) and supplemented with 2% FBS. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS) or a buffer containing Triton X-100 (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 0.5% Triton X-100). Both buffers also contained 20 μg of phenylmethylsulfonyl fluoride per ml, 1 μg of aprotinin per ml, and 1 μg of leupeptin per ml. Lysates were centrifuged at 16,000 × *g* for 20 min at 4°C, and the supernatants were immunoprecipitated by the addition of region-specific antisera. Immunoprecipitates were collected by using *Staphylococcus aureus* Cowan 1 (Calbiochem), washed three times with lysis buffer and once with TN buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl), and analyzed by SDS-PAGE.

RESULTS

Most single-amino-acid substitutions are tolerated at the 2/3 cleavage site. The HCV 2/3 cleavage site is highly con-

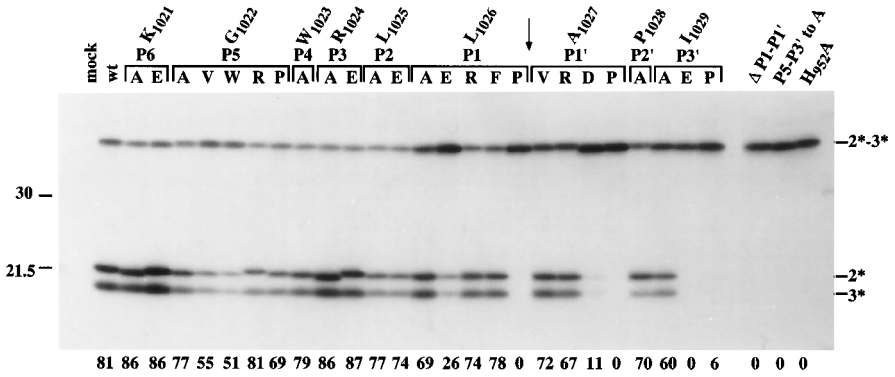

FIG. 2. Mutations of the 2/3 cleavage site. BHK-21 cells previously infected with vTF7-3 were transfected with 2 μg of pTM3/HCV827-1207 plasmid DNAs containing the indicated mutations. "mock" refers to cells transfected with pTM3 alone; wt refers to cells transfected with the unmutated plasmid as a positive control. The H-952→A mutant was included as a negative control. Transfected cells were labeled 20 min with ^{35}S-protein-labeling mix plus [^{35}S]cysteine, lysates were prepared, and the NS2-3 precursor and cleavage products were immunoprecipitated with the specific antiserum WU43 (20). Immunoprecipitated proteins were separated by SDS-PAGE (14% polyacrylamide). The protein molecular sizes standards (in kilodaltons) are indicated at the left, and HCV proteins are identified at the right. * denotes truncated proteins. The amount of total ^{35}S-protein label in the cleavage products is given below each lane, as determined by quantitation on a phosphorimager (Molecular Dynamics). 0 indicates mutations that block detectable cleavage, defined as ≤2% of the total ^{35}S-protein label in the region of the gel where the cleavage products should migrate.

served among diverse isolates of HCV, as seen in Fig. 1. Although sequence data at the 2/3 cleavage site are not available for all of the major HCV genotypes, sequences of two subtypes of type 2, the most evolutionarily distant genotype relative to the type 1 isolate used in this study, are included in Fig. 1. Amino acids at the P5 and P2 through P6′ positions are absolutely conserved, and amino acids at the P4 and P3 positions are highly conserved; the only nonconservative amino acid change in the region from P5 to P6′ is a Ser at the P3 position of the HCV-J6 strain. To investigate the importance of amino acid residues surrounding the HCV 2/3 cleavage site for efficient processing, site-directed mutagenesis was used to make various alterations in positions P5 through P3′ (Fig. 1). The processing phenotypes of these mutants were analyzed in BHK-21 cells with the vaccinia virus-T7 hybrid expression system (16).

The parental construct used in this mutagenesis study expresses a region of the HCV-H strain polyprotein corresponding to amino acids 827 to 1207. This region encodes a truncated, 42-kDa NS2-3 precursor protein (2*-3*) which lacks the first 17 amino acids of NS2 and extends through the N-terminal one-third of NS3. This region of the HCV polyprotein was previously shown to be sufficient for processing at the 2/3 site, yielding cleavage products of 22 and 19 kDa, corresponding to truncated NS2 (2*) and NS3 (3*), respectively. As shown in Fig. 2, the NS2-3 protease tolerated most single-amino-acid substitutions at the 2/3 cleavage site, including many nonconservative changes, remarkably well. For example, substitution of Ala or Glu for Lys-1021 at the P6 position seemed to have little or no effect on cleavage. Similarly, replacement of Gly-1022 at the P5 position by Ala, Arg, or even Pro had little effect on cleavage, although bulky, hydrophobic residues at this position, such as Val or Trp, seemed to be somewhat unfavorable for cleavage. Substitutions at the P4, P3, and P2 positions likewise showed little or no effect on cleavage, including R1024E, which reverses the charge of the residue at P3, and L1025E, which introduces a charged residue close to the cleavage site at the P2 position.

The P1 and P1′ positions, which contain the amino acids that form the scissile bond, were more sensitive to mutation. Replacement of Leu-1026 at the P1 position by Ala, Arg, or Phe was tolerated, but replacement by Glu or Pro was not. Similarly, substitution of Val or Arg for Ala-1027 at the P1′ position seemed to have little effect on cleavage, while substitution of Asp or Pro at this position dramatically inhibited cleavage. Also, deletion of both Leu-1026 and Ala-1027 (ΔP1-P1′) virtually eliminated cleavage, although traces of cleaved products were occasionally observed after long exposures (data not shown). Replacement of Ala for Pro-1028 at the P2′ position seemed to have little effect on the degree of cleavage; however, somewhat surprisingly in view of the extreme tolerance exhibited at other positions peripheral to the scissile-bond residues, Ile and Ala were strongly preferred at the P3′ position over Glu and Pro. Since individual Ala substitutions from P5 to P3′ seemed to have little or no effect on cleavage, a mutant containing Ala at all positions from P5 through P3′, designated G1022–I1029A, was also tested for its ability to undergo cleavage, but no cleavage was observed.

Requirements for bimolecular cleavage. To examine the polypeptide requirements for bimolecular cleavage at the 2/3 site of HCV, a series of constructs were made that contained various deletions and/or mutations previously shown to abolish cleavage at the 2/3 site. Some of the smaller constructs were hoped to be suitable substrates for *trans* cleavage assays. The processing phenotypes of these constructs were examined with the vaccinia virus-T7 hybrid expression system, as for the cleavage-site mutagenesis experiments. The data shown below are representative of multiple experiments that were performed to verify the results. The 1–3011 polyprotein was processed to yield a 70-kDa NS3 protein and a 23-kDa NS2 protein as expected (Fig. 3A, lane 2). Construct 827-3011 expressed a truncated 22-kDa NS2 protein and a 70-kDa NS3 protein (lane 3), and 827-1207 expressed truncated NS2 and a truncated NS3 protein of 19 kDa (lane 5). Also, as previously demonstrated (19, 22), the substitution mutations C993A (lane 4) and H952A (Fig. 3B, lane 3) in the NS2 region abolished cleavage at the 2/3 site.

Construct 827-1207 ΔP1-P1′ also failed to undergo cleavage (Fig. 3A, lane 6), as shown above in the cleavage-site mutagenesis study. However, this construct seemed to retain proteolytic activity. In Fig. 3A, lane 9, the appearance of the truncated 22-kDa NS2 protein and full-length NS3 protein indicates that cleavage of 827-3011 C993A occurred upon coexpression with 827-1207 ΔP1-P1′. As shown in previous experiments and confirmed here, 1-3011 and 827-1207 also cleave 827-3011 C993A, as evidenced by the appearance of truncated NS2 in lane 7 and

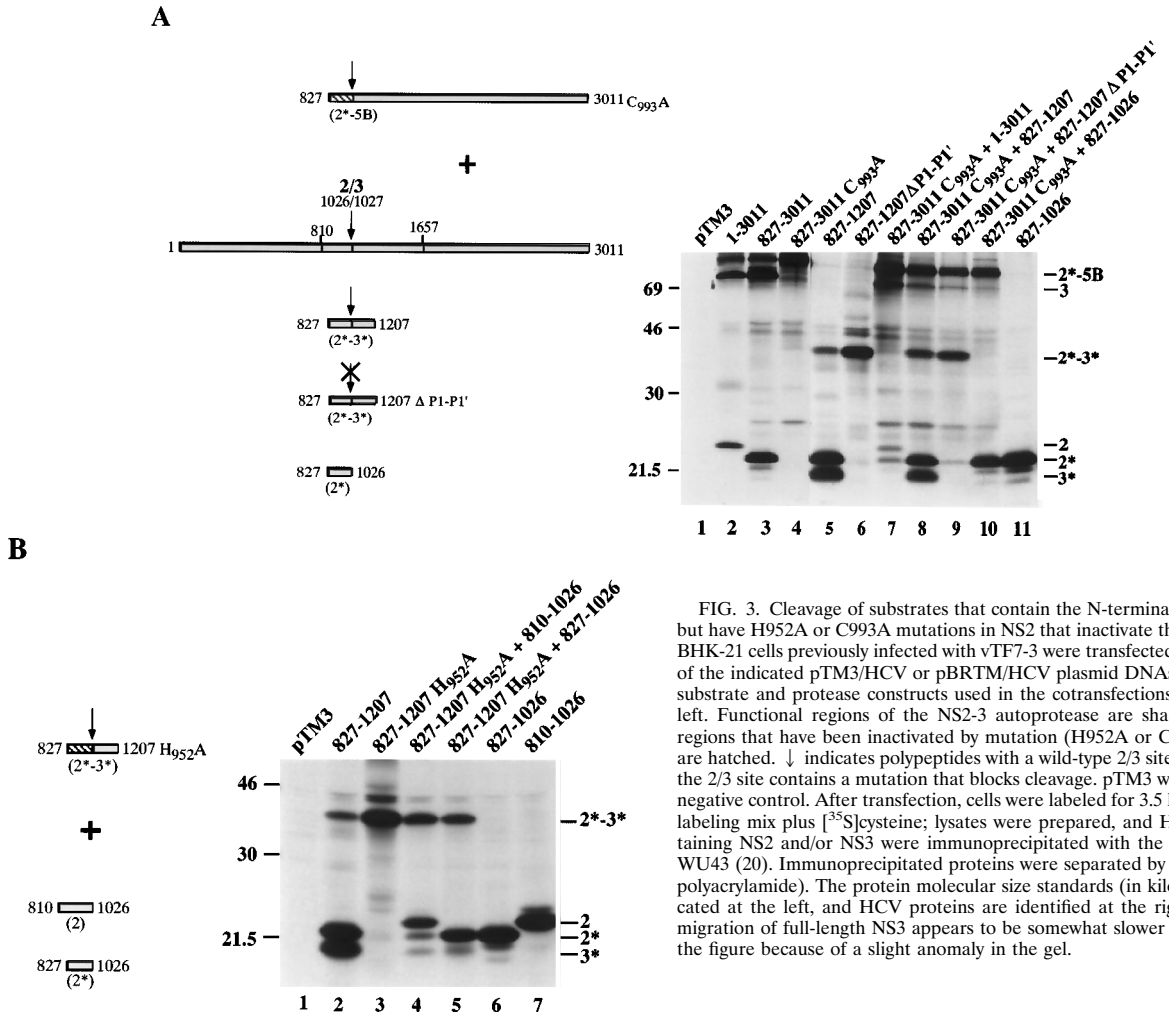

FIG. 3. Cleavage of substrates that contain the N-terminal one-third of NS3 but have H952A or C993A mutations in NS2 that inactivate the NS2-3 protease. BHK-21 cells previously infected with vTF7-3 were transfected with 2 μg of each of the indicated pTM3/HCV or pBRTM/HCV plasmid DNAs. Diagrams of the substrate and protease constructs used in the cotransfections are shown at the left. Functional regions of the NS2-3 autoprotease are shaded in solid gray; regions that have been inactivated by mutation (H952A or C993A) or deletion are hatched. ↓ indicates polypeptides with a wild-type 2/3 site; ↓̸ indicates that the 2/3 site contains a mutation that blocks cleavage. pTM3 was transfected as a negative control. After transfection, cells were labeled for 3.5 h with ^{35}S-protein-labeling mix plus [^{35}S]cysteine; lysates were prepared, and HCV proteins containing NS2 and/or NS3 were immunoprecipitated with the specific antiserum WU43 (20). Immunoprecipitated proteins were separated by SDS-PAGE (14% polyacrylamide). The protein molecular size standards (in kilodaltons) are indicated at the left, and HCV proteins are identified at the right. Note that the migration of full-length NS3 appears to be somewhat slower on the left side of the figure because of a slight anomaly in the gel.

full-length NS3 in lane 8. Although it was observed that the amount of full-length NS2 produced from cleavage of 1-3011 in lane 7 seemed to be smaller than in lane 2, such decreases seemed to be a general phenomenon resulting from reduced expression of especially large or small polypeptides upon cotransfection with plasmids encoding polypeptides of intermediate size. Since the proteolytic activity of 827-1207 ΔP1-P1′ in bimolecular cleavage reactions was similar to that of 827-1207 on the basis of comparison of production of full-length NS3 in lanes 8 and 9 and since its inability to be processed simplified interpretation of cleavage patterns, this construct was used in several subsequent cotransfections.

Cotransfection of a plasmid encoding full-length NS2 (data not shown) or the truncated 22-kDa (Fig. 3A, lane 10) form was also sufficient for cleavage of 827-3011 C993A, as evidenced by the appearance of the 70-kDa NS3 band. Similar results were obtained with other constructs containing deletions and/or mutations in NS2. For example, cotransfection of NS2-expressing constructs with 827-1207 H952A allowed cleavage to occur (Fig. 3B, lanes 4 and 5). The 19-kDa cleavage product corresponding to truncated NS3 was clearly visible in both lanes. However, some additional bands were present in the 827-1026 lanes, possibly because of degradation of the truncated NS2 protein or alternative initiation.

The results thus far have shown that constructs containing point mutations in NS2 which inactivate the NS2-3 protease were cleaved when cotransfected with constructs expressing at least amino acids 827 to 1026 of NS2. Similarly, constructs with larger N-terminal deletions in NS2, such as 936-3011 or 936-1207, were cleaved when coexpressed with forms of NS2 that included the region from amino acids 827 to 1026, although cleavage of these constructs was more inefficient than of polypeptides beginning with amino acid 827 (data not shown). These data suggest that addition of unmutated NS2 in any form that includes amino acids 827 to 1026 will result in the cleavage of polypeptides with deletions or inactivating mutations in NS2, as long as they also contain the N-terminal one-third of NS3.

The ability of polypeptides containing the N-terminal one-third of NS3 to participate in the bimolecular cleavage of polypeptides with C-terminal deletions in the NS3 region that inhibit or abolish NS2-3 protease activity was also examined. Previous work has shown that cleavage at the 2/3 site was sharply reduced by C-terminal deletions in NS3 to amino acid 1137 or 1186 (19). In agreement with this work, a faint band corresponding to the truncated 22-kDa NS2 protein in Fig. 4, lane 3, indicates low-level cleavage of the singly expressed 827-1137 polypeptide. Cleavage of 827-1137 would also be expected to produce a truncated NS3 protein of approximately 12 kDa, but this product was never detected, presumably be-

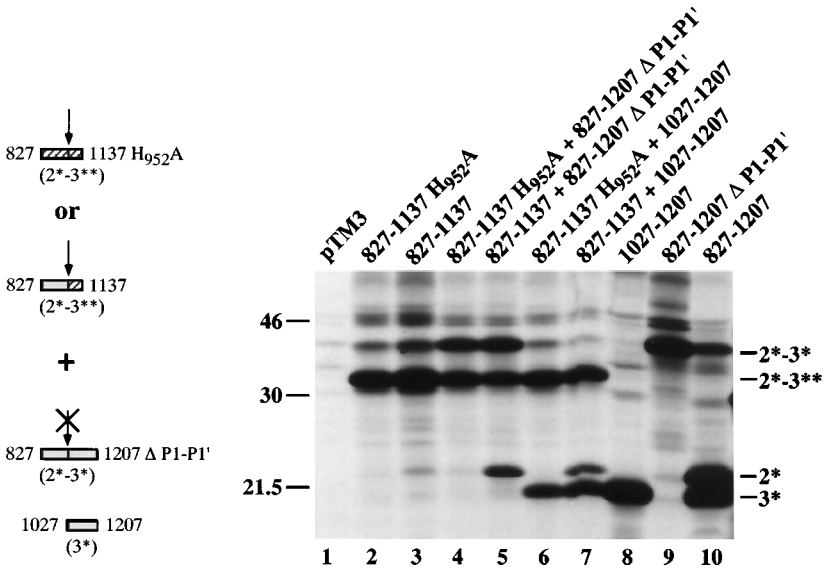

FIG. 4. Cleavage of substrates that contain a functional NS2 region but lack part of the N-terminal one-third of NS3. The experiment was performed as described in the legend to Fig. 3.

cause it was not recognized by any of the antisera used in these experiments. The band corresponding to truncated NS2 disappeared when the H952A mutation was introduced (Fig. 4, lane 2), although a background band with a slightly faster migration was present. This background band and others may reflect susceptibility of 827-1137 and 827-1137 H952A to degradation by cellular proteases. Cleavage of 827-1137 increased significantly when it was coexpressed with the N-terminal region of NS3 encoded by construct 1027-1207 or 827-1207 ΔP1-P1′ (lanes 7 and 5, respectively). No cleavage occurred when 827-1137 H952A was coexpressed with 1027-1207 (lane 6), presumably because of the absence of a functional NS2 domain.

Interestingly, very little if any cleavage of 827-1137 H952A was seen in cotransfections with 827-1207 ΔP1-P1′ (Fig. 4, lane 4), even though the necessary regions of NS2 and NS3 were present and seemed to be functional in 827-1207 ΔP1-P1′. As shown above, 827-1207 ΔP1-P1′ is capable of cleaving polypeptides which lack a functional NS2 region, such as 827-3011 C993A, and those which lack part of the NS3 region necessary for optimal NS2-3 protease activity, such as 827-1137. However, 827-3011 C993A contains a complete, unaltered NS3 region and 827-1137 contains the functional region of NS2. Both of these regions are disrupted in 827-1137 H952A—the NS2 region by point mutation and the NS3 region by C-terminal deletion. The extremely low level of truncated NS2 observed may be due to complementation of 827-1137 H952A by the NS2 region of 827-1207 ΔP1-P1′ to form a protease analogous to 827-1137 alone or to trace cleavage of 827-1207 ΔP1-P1′ itself, although such cleavage of 827-1207 ΔP1-P1′ usually was observed only after long exposures (data not shown).

The results with 827-1137 H952A suggest that polypeptides must contain a functional NS2 region or the N-terminal one-third of NS3 to be cleaved in a bimolecular reaction. Other constructs with deletions and/or mutations in both the NS2 and NS3 regions were investigated either to find a construct with the proper conformation for cleavage by a *trans*-acting protease or to support the results obtained with 827-1137 H952A. 936-1039 H952A, which contains disruptions in both of the NS2 and NS3 regions necessary for NS2-3 protease activity was one of the additional constructs tested for its ability to undergo bimolecular cleavage. Cleavage of this construct would be expected to produce a 10-kDa product. However, no cleavage of this construct was observed in multiple cotransfections with constructs that express a functional NS2-3 protease. The results of two typical experiments are shown in Fig. 5. Successful expression of construct 936-1026 H952A, a marker for the potential 10-kDa cleavage product, suggests that failure to observe this product is not due to inherent instability of the protein (lanes 3 and 4). Similar results were obtained with the substrate 936-1137 H952A, which contains a smaller deletion in the NS3 region (data not shown). Together, these data suggest that bimolecular cleavage is possible if the polypeptide to be cleaved contains either a functional NS2 region from amino acids 827 to 1026 or the N-terminal one-third of NS3 and is complemented by a polypeptide which supplies the missing region. This result implies that potential substrates must contribute a functional domain to form an active NS2-3 protease.

Some constructs which do not undergo autocatalytic cleavage may inhibit cleavage of wild-type constructs. During the bimolecular cleavage experiments, it was observed that cleavage of the unmutated 827-1207 construct seemed to be inhibited in cotransfections with certain constructs that lacked NS2-3 protease activity. Closer examination of this phenomenon suggested that with one exception, cleavage of the 827-1207 construct was inhibited only by those polypeptides capable of participating in bimolecular cleavage reactions, such as 827-3011 C993A and 827-1137 (Fig. 6).

Cotransfection with any translated plasmid decreases expression of the 827-1207 (truncated NS2-3) construct (Fig. 6, lanes 11 and 12), perhaps as a result of competition for ribosomes or some factor(s) involved in translation since cotransfection of pTM3 had little effect on the level of protein expression (lanes 9 and 10). An alternative but less likely explanation is that the reduced expression of 827-1207 results from an increase in proteolytic degradation upon coexpression with other polypeptides. Lanes 13 and 14 verify that the 1972-2420 (NS5A) construct was expressed in lanes 11 and 12. Although the amount of NS5A expressed does not seem to increase in proportion to the amount of DNA added (compare lanes

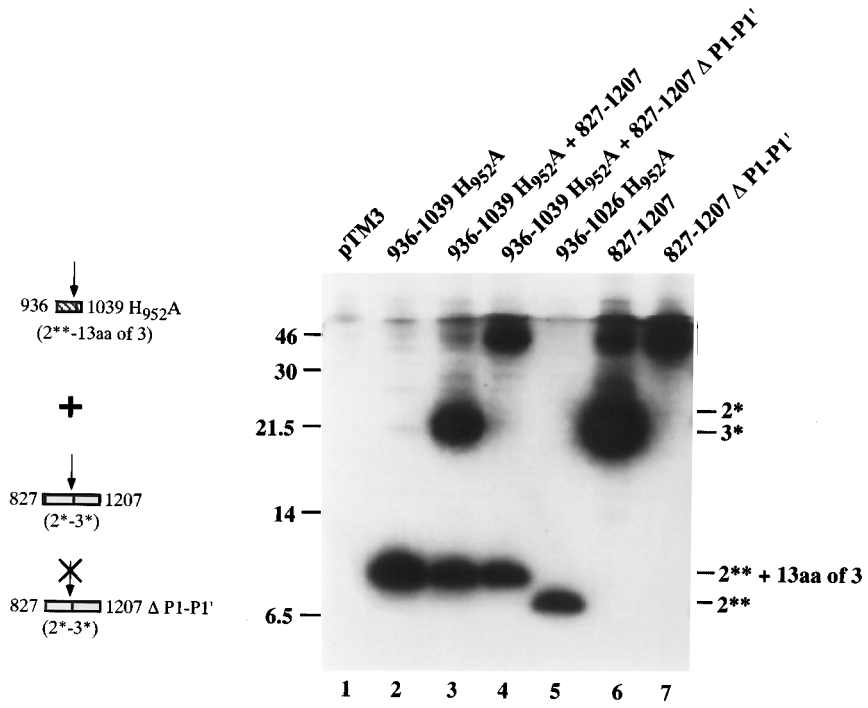

FIG. 5. No cleavage of substrates that have deletions or mutations that inactivate both of the NS2 and NS3 regions. The experiment was performed as described in the legend to Fig. 3, except that the immunoprecipitated proteins were separated by SDS-Tricine-PAGE (16% polyacrylamide) (45). aa, amino acids.

13 and 14), the decreased expression level of the NS2-3 polypeptide and its cleavage products (lane 12) suggests that a larger proportion of the translated protein in lanes 14 and 12 consists of NS5A compared with lanes 13 and 11. Although cotransfection of larger amounts of DNA seems to decrease the overall expression level of the 827-1207 polypeptide and its cleavage products, coexpression of the 827-3011 C993A or 827-1137 constructs increases the precursor/product ratio of proteins translated from the 827-1207 construct. In contrast, the 936-1039 H952A construct and 1972-2420 (NS5A), which do not participate in bimolecular cleavage reactions, decrease the overall expression of the 827-1207 construct but do not seem to change the precursor/product ratio (lanes 7 and 8). The exception to the general trend was 827-1137 H952A, which seemed to inhibit cleavage of the 827-1207 construct even though it is not a viable substrate for bimolecular cleavage (lanes 5 and 6). Perhaps the 827-1137 H952A construct is still able to interact with the 827-1207 precursor and interfere with cleavage, although it seems to lack some feature necessary for bimolecular cleavage.

The NS2 and NS3 proteins do not form demonstrable heterodimers. Members of the HCV-related flavivirus genus form stable, NS2B-3 heterodimers that are detectable by immunoprecipitation under nondenaturing conditions. Formation of this heterodimer in yellow fever virus and dengue 2 virus, for example, is thought to be required for cleavage activity of the serine protease located in the N-terminal region of the NS3 protein (1, 6). HCV differs from these two viruses in that NS2 sequences are not necessary for cleavage by the serine protease. However, the observed requirement for extended regions of both NS2 and NS3 in cleavage of the HCV 2/3 site raises the possibility that a similar, stable heterodimer exists between HCV NS2 and NS3 after cleavage at the 2/3 site. Such an interaction might have a role in replication, as proposed for flaviviruses (5). However, when nondenaturing immunoprecipitation with RIPA buffer or a buffer containing 0.5% Triton X-100 was performed on lysates from cells expressing the HCV NS2 and NS3 proteins, using multiple antisera, no association of these proteins was detected after cleavage (Fig. 7; data not shown). Small amounts of NS2 or NS3 may have precipitated with antiserum to the other protein, but the level of interaction was not readily distinguishable from the background levels (compare lanes 6 and 7 and lanes 10 and 11). In contrast, under the same conditions, the YF NS2B protein clearly coprecipitated with the serine protease domain of NS3 when antiserum directed against NS3 was used (Fig. 7, lane 3). HCV antisera tested include WU107, directed against NS2; WU110, directed against the N-terminal one-third of NS3; and WU117, which was raised against the NS3 NTPase/helicase domain (20). Since the NS3 NTPase/helicase domain is not required for cleavage at the 2/3 site, WU117 would not be expected to disrupt an interaction between NS2 and NS3. HCV NS2-3 constructs with a C-terminal c-*myc* tag were also tested with a monoclonal antibody to the c-*myc* peptide (Myc1-9E10) (11), but the results were similarly negative (data not shown).

To address the possibility that an interaction occurs between NS2 and NS3 that is disrupted by detergents, cells were treated with the bifunctional cross-linking agent DSP [dithio-*bis*(succinimidylpropionate)] before lysis with SDS and immunoprecipitation under nonreducing conditions. No NS2-NS3 complexes were detected, although positive controls showed that the Sindbis virus glycoproteins E1 and E2 were effectively cross-linked under the same conditions (reference 43 and data not shown). Although we cannot exclude the possibility that HCV NS2 and NS3 form a complex after cleavage, such complexes were not detected under any of the conditions tried for the coprecipitation or cross-linking experiments.

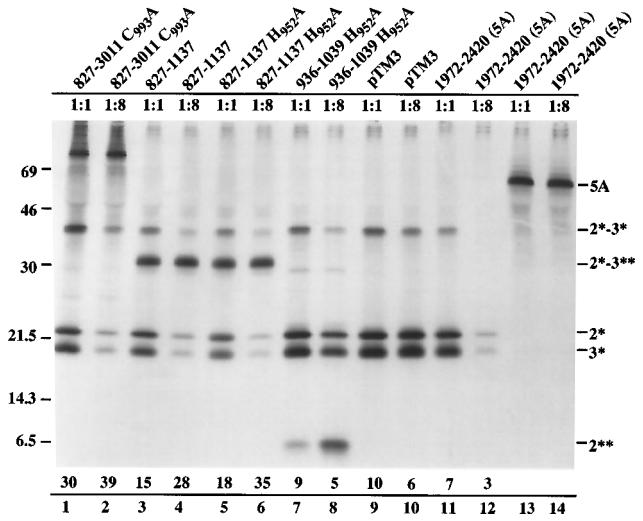

FIG. 6. Inhibition of wild-type cleavage by some protease-defective constructs. BHK-21 cells previously infected with vTF7-3 were cotransfected with 250 ng of pTM3/HCV827-1207 DNA and 250 ng (1:1) or 2 μg (1:8) of the indicated pTM3/HCV or pBRTM/HCV plasmid DNAs. After transfection, cells were labeled for 20 min at 37°C with ^{35}S-protein-labeling mix plus [^{35}S]cysteine, lysates were prepared, and in lanes 1 through 12, HCV proteins containing NS2 and/or NS3 were immunoprecipitated with the specific antiserum WU43 (20). Lanes 13 and 14 are identical to lanes 11 and 12, respectively, except that the lysates were immunoprecipitated with the NS5A-specific antiserum WU123 (20). Immunoprecipitated proteins were separated by SDS-PAGE (14% polyacrylamide). The amount of ^{35}S-protein label in the 827-1207 (2*-3*) precursor and its cleavage products was determined by quantitation on a phosphorimager (Molecular Dynamics). The percentage of radioactivity in the precursor is given below each lane.

DISCUSSION

Previous studies have shown that the NS2-3 junction of HCV-H is cleaved by a protease which resides between polyprotein residues 827 and 1207 (19). This region includes most of NS2 and the N-terminal one-third of NS3, which overlaps with the NS3 serine protease domain; however, catalytic activity of the NS3 serine protease is not necessary for cleavage at the 2/3 site, indicating that a distinct proteolytic activity is responsible.

The HCV NS2-3 protease cleaves between Leu-1026 and Ala-1027 in the wild-type polyprotein and, as we have shown, readily accepts hydrophobic or basic amino acid substitutions such as Ala, Phe, or Arg at the P1 position and Val or Arg at the P1′ position but is inhibited by substitution of the acidic amino acids Glu and Asp at P1 and P1′, respectively. Hirowatari et al. (24) also analyzed five mutations at the P1 and P1′ positions of the 2/3 site for their effects on cleavage of an in vitro translated precursor consisting of amino acids 722 through 1908 of the HCV-J strain (26), a member of subtype 1b. Two of these mutations, L1026R and A1027V, were also analyzed in this report. Hirowatari et al. (24) observed that the L1026R mutation had little or no effect on cleavage at the 2/3 site and that the A1027V mutation caused a slight decrease in the intensity of the cleavage products, similar to the results shown in Fig. 2. The other three mutations examined, L1026I, L1026W, and A1027I, partially inhibited cleavage at the 2/3 site (24). In neither analysis did substitutions of one hydrophobic amino acid for another abolish NS2-3 processing.

Observation that the NS2-3 protease activity is stimulated by $ZnCl_2$ and inhibited by EDTA, a chelator of divalent metal cations, has prompted the suggestion that the HCV NS2-3 protease is a Zn^{2+} metalloprotease (22). The apparent preferences observed for the HCV NS2-3 protease at the P1 and P1′ positions of the 2/3 site are similar to the preferences of neutral metalloproteases, which tend to cleave next to hydrophobic amino acids. Thermolysin, the prototype of this group, shows specificity for hydrophobic amino acids at the P1′ position, unlike the serine and cysteine proteases, which show greater specificity for residues in the P1 position (reviewed in reference 36). The amino acid preferences at the 2/3 site that were observed for the NS2-3 protease particularly resemble the cleavage-site specificity of endopeptidase 24.15, a mammalian metalloprotease thought to be involved in the proteolytic processing of peptide hormones (reviewed in reference 10). Like the NS2-3 protease, this enzyme prefers to cleave between hydrophobic residues but tolerates basic amino acids on either side of the cleavage site (41). Furthermore, endopeptidase 24.15 seems to have a hydrophobic pocket in the S3′ subsite (46) of the substrate-binding region. Although a large number of substitutions were not tested at the P3′ position of the HCV 2/3 site, Ile and Ala were strongly preferred over Glu and Pro. It should be emphasized, however, that no solid evidence demonstrates that the NS2-3 protease is a metalloprotease and that the observed stimulation of protease activity by $ZnCl_2$ and inhibition by EDTA could be indicative of a structural role for zinc rather than a catalytic role.

Since the NS2-3 protease is believed to be autocatalytic, amino acids important for cleavage-site recognition are not readily distinguishable from those with a role in proteolysis. The correct conformation of the NS2-3 autoprotease and the 2/3 cleavage site may be more important for cleavage than particular amino acid side chains at the cleavage site, similar to many other *cis*-cleaving proteases. The few mutations that severely inhibited cleavage at the 2/3 site, such as the Pro substitutions at the P1, P1′, and P3′ positions, deletion of the P1 and P1′ residues, or conversion of amino acids in the P5-P3′ region to Ala might act by disrupting proper folding of the autoprotease, the cleavage site, or both. Another issue to be considered is the effect of mutations on the stability of the precursor or products. For example, the P1028A mutation

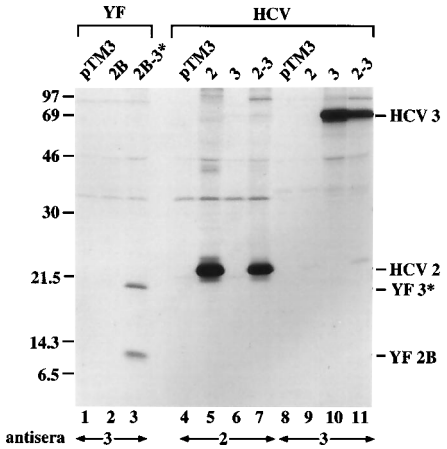

FIG. 7. Complexes between NS2 and NS3 of yellow fever virus (YF) but not HCV are readily detected by coimmunoprecipitation. BHK-21 cells previously infected with vTF7-3 were transfected with 1 μg of pTM3/YF or pTM3/HCV plasmid DNAs. After transfection, cells were labeled for 30 min at 37°C with ^{35}S-protein-labeling mix and chased for 30 min at 37°C in medium containing excess cold methionine. Lysates were prepared, and yellow fever virus or HCV proteins were immunoprecipitated under nondenaturing conditions with specific antisera. Antiserum R258 specific for yellow fever virus NS3 (provided by Marc Collett [6]) was used in lanes 1 through 3, WU107 to HCV NS2 was used in lanes 4 through 7, and WU117 to the NTPase/helicase domain of NS3 was used in lanes 8 through 11 (20).

seemed to destabilize the truncated NS3 cleavage product, which could cause the degree of cleavage to be underestimated in our assay. However, none of the other mutations seemed to have a significant effect on protein stability.

Although the NS2-3 protease is believed to be autocatalytic, bimolecular cleavage has been observed (19). Constructs containing amino acids 827 to 1207 or more of the HCV polyprotein with a H952A or C993A mutation that abolished proteolytic activity were found to be cleaved in cotransfections with various sources of wild-type protease, although cleavage was inefficient in comparison to the unmutated construct. The results of those experiments have been extended here to identify the regions of the substrate necessary for it to be cleaved in a bimolecular reaction. Polypeptides which contain the mutations H952A or C993A and/or which lack the N-terminal 126 amino acids of NS2 do not undergo intramolecular cleavage but are cleaved bimolecularly when coexpressed with functional NS2; however, these polypeptides must also contain the N-terminal one-third (181 amino acids) of NS3. Similarly, polypeptides which contain a functional NS2 region but have a C-terminal deletion in NS3 to amino acid 1137 do not undergo intramolecular cleavage, but are cleaved bimolecularly when coexpressed with the NS3 region from amino acids 1027 to at least 1207. However, polypeptides which contained a C-terminal deletion in NS3 to amino acid 1137 and a mutation or N-terminal deletion in NS2 that inactivated the NS2-3 protease were not cleaved in *trans*. These results imply that polypeptides containing a 2/3 cleavage site must contribute a functional domain to the formation of an active NS2-3 protease in order to undergo bimolecular cleavage. Polypeptides containing a 2/3 site that is cleaved bimolecularly seem to be capable of supplying either the N-terminal one-third of NS3 or a functional NS2 region to form the NS2-3 protease. However, the inefficient cleavage observed for constructs which contain the N-terminal one-third of NS3 but lack the N-terminal 126 amino acids of NS2 suggests that certain regions of NS2 may be necessary but not sufficient for the polypeptide to be permissive for bimolecular cleavage. Deletion of a significant portion of NS2 might also disrupt the folding and conformation necessary for cleavage in a bimolecular reaction.

Polypeptides with a defective NS2-3 protease were also shown to interfere with wild-type cleavage. Polypeptides 827-3011 C993A and 827-1137 inhibited cleavage of the active polypeptide 827-1207, whereas 936-1039 H952A did not. These results were reminiscent of the bimolecular cleavage experiments, in which polypeptides 827-3011 C993A and 827-1137, but not 936-1039 H952A, were cleaved bimolecularly. However, 827-1137 H952A, which did not undergo bimolecular cleavage, was still able to interfere with cleavage of the active polypeptide. One interpretation of these data is that proteolytically inactive domains of 827-1137 H952A were sufficient for interaction with wild-type domains leading to *trans*-dominant inhibition of cleavage but not for participation in self-cleavage, while the shorter 936-1039 H952A polypeptide lacked some region necessary for this interaction. Alternatively, the 827-3011 C993A, 827-1137, and 827-1137 H952A but not the 936-1039 H952A polypeptides might have interacted with some host factor essential for cleavage, in competition with the active polypeptide.

The requirement for portions of both NS2 and NS3 for intramolecular and bimolecular cleavage at the 2/3 site suggests that these two regions interact. While such an interaction could involve direct association of the NS2 and NS3 regions, host factors or metal ions may also be involved. Besides forming an active autoprotease leading to cleavage at the 2/3 site, a stable interaction between NS2, with a hydrophobic N terminus produced by signalase cleavage (19, 28, 35, 47), and NS3, whose C-terminal portion probably functions as an RNA helicase (17, 50), might be important for membrane association of the HCV RNA replication complex. Such a role has been proposed for the NS2B protein of flaviviruses, where stable NS2B-NS3 complexes persist after autocatalytic cleavage at the 2B/3 site (1, 6). For HCV, however, we were unable to obtain evidence for stable NS2-NS3 complexes either by coimmunoprecipitation with multiple antisera or by cross-linking with DSP. These results are consistent with the observation that expression of a self-cleaving NS2-3 polyprotein in vitro, in the presence of microsomal membranes, does not lead to membrane association of NS3 (23). Rather, the NS4A protein of HCV may fulfill a role analogous to the NS2B protein of flaviviruses. HCV NS4A is required for NS3 serine protease-dependent processing at several cleavage sites (3, 12, 29), forms a detergent-stable complex with the serine protease domain (31), and contains a hydrophobic N-terminal region which presumably leads to membrane association of NS3 after *cis* cleavage at the 3/4A site (30). Besides being membrane associated (54) and forming a stable complex with NS3, the NS2B protein of flaviviruses is also required for processing at all serine protease-dependent cleavage sites (1, 4, 6, 13, 14, 42, 56).

The p54 protein of the pestivirus bovine viral diarrhea virus, which lies N-terminal to p80 (the NS3 homolog), may have functions distinct from HCV NS2 and flavivirus NS2B. Bovine viral diarrhea virus exists in two biotypes which can be distinguished by their cytopathogenicity in cell culture (reviewed in reference 52). In noncytopathic strains, p80 is not cleaved from its precursor, p125, which includes the p80 region preceded by p54 (which contains both a hydrophobic region and zinc finger motif [8]). This suggests that the pestivirus RNA helicase domain can function in the context of uncleaved p125. By a variety of mechanisms, cytopathic strains, which are associated with development of fatal mucosal disease in cattle, all produce both p125 and p80 (52). Like the NS2 region of HCV, the majority of p54 is dispensable for downstream polyprotein cleavages catalyzed by the p80 serine protease. Rather, the bovine viral diarrhea virus serine protease appears to require sequences C-terminal to p80, perhaps analogous to HCV NS4A, for processing at some sites (55).

Taken together, these observations suggest that considerable divergence has occurred in the sequences upstream of the serine protease domain in the flavivirus family. No sequence homology has been found among the HCV NS2, pestivirus p54, and flavivirus NS2B regions. Although both flavivirus NS2B and HCV NS2 regions are required for the autocatalytic cleavages producing the N terminus of NS3, they do so by distinct mechanisms. NS2B and the NS3 serine protease domain are required for cleavage at the 2B/3 site and form the active flavivirus serine protease complex. For HCV, NS2 and the N-terminal domain of NS3 are also required for cleavage at the 2/3 site, but this proteolytic activity is distinct from the serine protease responsible for cleavage at downstream sites. Further studies on the HCV NS2-3 autoprotease are clearly needed to define the catalytic mechanism and elucidate the role of zinc or other metal ions. Structural analysis of mutant forms of the protease, incapable of autocatalytic cleavage at the 2/3 site but still active as assayed in bimolecular cleavage reactions, may prove useful for these studies. In addition, our observation that inactivated forms of the protease can inhibit autocatalytic processing at the 2/3 site suggests that it may be possible to design inhibitors of this intramolecular cleavage.

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