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NS3-4A of Hepatitis C Virus Is a Chymotrypsin-Like Protease

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The polyprotein encoded by a single open reading frame of hepatitis C virus (HCV) is processed by host- and virus-encoded proteases. The viral protease NS3 is responsible for the cleavage of at least four sites (NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B junctions) in the nonstructural protein region. To characterize the protease function of NS3 and NS4 on various target sites, efficient *cis*- and *trans*-cleavage assay systems were developed by using in vitro transcription and translation. Deletion of the C-terminal two-thirds from NS3 in an NS3-NS4A-4B polypeptide (NS3ΔC-4A-4B) hampered cleavage of the NS3/4A junction but not that of the NS4A/4B junction. As a consequence, expression of NS3ΔC-4A-4B containing an internal deletion of NS3 results in an NS3ΔC-4A fusion protein. NS3ΔC-4A shows very efficient and specific *trans*-cleavage activity at NS4A/4B, NS4B/5A, and NS5A/5B junctions. In addition, the biochemical properties of HCV NS3ΔC-4A were further elucidated by adding known protease inhibitors in *trans*-cleavage reactions. The HCV protease NS3-4A is inhibited by chymotrypsin-specific inhibitors *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), chymostatin, and Pefabloc SC but not by trypsin-like protease inhibitors antipain, leupeptin, and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) or by the protease inhibitors E-64, bestatin, pepstatin, and phosphoramidon. This finding strongly suggests that HCV protease NS3-4A is a chymotrypsin-like serine protease.

Hepatitis C virus (HCV) is the major etiological agent of posttransfusion non-A, non-B hepatitis (1, 9, 26). The hepatitis often progresses into liver cirrhosis and hepatocellular carcinoma (6, 35). Moreover, it was proposed that HCV could escape from the host defense system by changing its major antigenic determinant sites (13, 31, 32). Development of therapeutic agents against this virus, therefore, is critical for treatment of the prevalent disease.

HCV, along with flaviviruses and pestiviruses, is a member of the family *Flaviviridae* (14, 28). Sequence data for HCV have been accumulated by cDNA sequencing of genomic RNAs derived from the sera of HCV-infected patients (9, 24 26, 32, 40, 42). HCV contains a single-stranded positive-sense RNA genome which encodes a single long polyprotein with the gene order of 5′-C-E1-E2-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3′. The structural proteins consist of a possible capsid protein (C) and envelope glycoproteins (E1 and E2). The remaining portion is composed of nonstructural proteins (NS2 to NS5B). The NS5 protein is suggested to be an RNA-dependent RNA polymerase which is an enzymatic component of the viral RNA replicase, but no biochemical activity of this protein has been shown yet. The NS3 protein contains a catalytic triad characteristic of serine proteases in the N-terminal one-third (5, 28) and amino acid sequence motifs of helicases in the C-terminal portion (15, 28, 39).

Processing of polyprotein is mediated by host and viral proteases. The signal peptidase on the endoplasmic reticulum is responsible for generation of the N termini of E1, E2, and possibly NS2 (20, 21, 23, 27, 30, 38). The NS2/3 junction was suggested to be cleaved by an HCV-encoded metalloprotease, which resides in the C-terminal portion of NS2 and the N-terminal region of NS3 (17, 22). The cleavages of the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B sites depend on the activity of serine protease NS3, as shown by expression of different parts of NS proteins in eukaryotic cell expression systems and in vitro translation systems (3, 10, 16, 17, 21, 41). A cysteine residue at the P1 position was suggested to be important for the cleavage of NS4A/4B, NS4B/5A, and NS5A/5B junctions (16, 33). Recently, it has been shown that NS4A assists NS3 in the processing of NS3/4A, NS4B/5A, and NS5A/5B junctions, analogous to the activity of NS2B on NS3 of yellow fever virus and dengue virus (2, 4, 7, 8, 11, 12, 34).

To investigate the molecular mechanism of HCV protease NS3 in detail, we developed a *trans*-cleavage assay of protease NS3 that uses in vitro transcription and translation systems. The N-terminal one-third of NS3 showed efficient and substrate-specific proteolytic activity at the NS4A/4B, NS4B/5A, and NS5A/5B junctions. NS4A, supplied either in *cis* or in *trans*, was necessary for the *trans*-cleavage activity of NS3 in this system. Kinetic studies on the protease action of NS3 and NS4A with various protease inhibitors led us to classify NS3 as a chymotrypsin-like protease.

MATERIALS AND METHODS

Construction of in vitro expression plasmids. Subcloning of plasmids was carried out by using standard methods (36). The HCV cDNA clones encoding amino acids 760 to 1846, which were obtained by PCR from the plasma of a patient infected with group I (type 1b) HCV, were kindly supplied by A. Nomoto and M. Kohara (The Tokyo Metropolitan Institute of Medical Science). The construction of plasmids pTHEΔ1116-1634, pTHEΔ1219-1634 (=NS3ΔC-4ΔC), and pTHE1016-1846 (=NS3-4ΔC) are described elsewhere (19). Plasmid pTHE760-1846 was completely digested with *Eco*RI and *Bam*HI, and the larger fragment was self-ligated to generate pTHE1467-1846 (=NS3ΔN-4ΔC). The HCV cDNA clones encoding amino acids 1844 to 3011 (pTZ-19R NS4-NS5) were obtained by PCR from the plasma of a patient infected with group I (type 1b) HCV. To yield pTHE1964-3011 (=NS4ΔN-5), restriction enzymes *Ase*I and *Xba*I were used for digestion of pTZ-19R NS4-NS5, and then the larger fragment

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was subcloned into the *Sma*I site of a pTH vector. Plasmid pTH NS3-4-5 encoding the C-terminal nine amino acids of NS2, NS3, NS4, and NS5 was constructed from pTHE1016-1846 and pTZ-19R NS4-NS5. The C-terminal nine amino acids of NS3, NS4, and NS5 were derived from pTZ-19R NS4-NS5 by using restriction enzymes *Sph*I and *Kpn*I. The remaining region of pTH NS3-4-5 was derived from pTHE1016-1846 by using *Sph*I and *Xho*I.

For construction of NS3ΔC-4A, HCV cDNA from nucleotides (nt) 3073 to 3654 and from nt 4903 to 5133 was amplified by PCR using DNA template NS3ΔC-4ΔC, which contains an internal deletion from nt 3655 to 4902 in NS3-4ΔC (nucleotide numbering is as described by Ogata et al. [31]). Oligonucleotides 5′ CCCAGAATTCCTGCTCCCATCACGGCC 3′ and 5′ CCCACTGCA GCGCACTCTTCCATCTCATC 3′ were used as plus- and minus-strand primers, respectively. PCR products were digested with *Eco*RI and *Pst*I, and then the largest fragment was ligated into pTM1.

In vitro transcription and translation. Plasmid DNAs were purified by the polyethylene glycol precipitation method (36) and then were linearized with appropriate restriction enzymes downstream of the translational termination codon. Linear DNAs were extracted with phenol-chloroform and ethanol precipitated. From the linearized DNAs, RNAs were transcribed with T7 RNA polymerase (New England Biolabs or KOSCO) for 90 min at 37°C as described by the manufacturer. The resulting RNAs were used for in vitro translation reactions in rabbit reticulocyte lysate (RRL; Promega Biotec). In vitro translation reactions were carried out at 30°C with or without [^{35}S]methionine using 30 to 60 nM (final concentrations) RNAs. ^{35}S-labeled proteins were analyzed on 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels, using the buffer system described by Nicklin et al. (30a). The intensity of autoradiographic images of ^{35}S was enhanced by fluorography using salicylic acid. Gels were dried and exposed to Kodak XAR-5 or Agfa Curix RP1 film for 8 to 18 h.

***trans*-cleavage reactions.** [^{35}S]methionine-labeled substrates and unlabeled enzymes were produced by in vitro translation of RNA transcripts, using the RRL system. In all reaction mixtures, 0.1% Tween 20 was included to enhance *trans* cleavage of NS3. For the production of unlabeled polypeptides, [^{35}S]methionine was replaced by unlabeled methionine. After 60 min of translation, 0.6 mg of cycloheximide per ml and 1 mM unlabeled methionine (final concentrations) were added to block further expression of labeled products. Immediately after translation blockage, 10 μl of unlabeled enzyme source was mixed with 10 μl of the substrate labeled with [^{35}S]methionine. The enzyme-substrate mixture was incubated at 30°C for 120 min or as otherwise indicated.

Inhibitor assays. Unlabeled NS3ΔC-4ΔC and [^{35}S]methionine-labeled substrates (NS3ΔN-4ΔC and NS4ΔN-5) were produced by in vitro translation in RRL separately. After blocking of translation as described above, enzymes were preincubated for 10 min at 30°C with inhibitors or with their solvents. Substrates were added to the protease-inhibitor mixtures and incubated further for 120 min at 30°C. Protease inhibitors used in this study were aprotinin, leupeptin, antipain, Pefabloc SC [4-(2-aminoethyl)-benzenesulfonyl-fluoride], chymostatin, E-64, bestatin, pepstatin, EDTA, phosphoramidon (purchased from Boehringer Mannheim), cupric ion (in the form of cupric chloride dihydrate), *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (purchased from Sigma Chemical Company), and phenylmethylsulfonyl fluoride (PMSF; purchased from Amresco).

RESULTS

Deletion of the helicase domain did not hamper the *trans*-cleavage activity of NS3. To characterize protease NS3, the processing patterns of polyprotein NS3-4-5 and its derivatives were investigated by an in vitro transcription-translation system. The mRNAs were generated by T7 RNA polymerase, and then the resulting RNAs were used in translation reactions using RRL. All of the mRNAs contained the 5′ nontranslated region of encephalomyocarditis virus for efficient translation (25).

The *trans*-cleavage activity of the virus-encoded protease in HCV polyprotein was tested by incubating [^{35}S]methionine-labeled NS4ΔN-5 (Fig. 1, lane 1) with unlabeled polypeptides containing NS3-4-5 or its derivatives. Translation of the mRNA corresponding to NS3-4-5 revealed polypeptides with an apparent molecular mass of 70 kDa (Fig. 1, lane 8) and minor bands of 65, 49, and 27 kDa which could be detected well when the gel was overexposed. Considering the sizes of the polypeptides, these are most likely to be NS3, NS5B, NS5A, and NS4B, respectively. This finding indicates that an active protease resides in NS3-4-5 and that the polyprotein is cleavable as expected. When a substrate of HCV protease (NS4ΔN-5 labeled with [^{35}S]methionine) was incubated with unlabeled NS3-4-5, the substrate was processed into 5A,

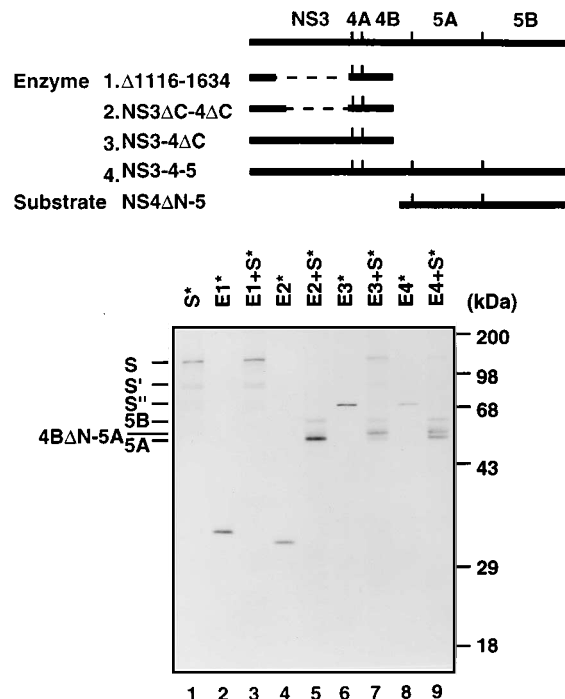

FIG. 1. Deletion of the helicase domain of NS3 does not alter the *trans*-cleavage pattern of protease NS3. A schematic diagram of polypeptides used as enzyme sources and substrates is shown at the top. Bold lines represent polypeptides, and dashed lines indicate deleted regions. [^{35}S]methionine-labeled NS4ΔN-5 (lane 1) and unlabeled pTHEΔ1116-1634, NS3ΔC-4ΔC, NS3-4ΔC, and NS3-4-5 were produced by in vitro translation in RRL. Tween 20 to a final concentration of 0.1% was added in all reaction mixtures for efficient *trans* cleavage. To show the status of proteases in protease sources, pTHEΔ1116-1634 (lane 2), NS3ΔC-4ΔC (lane 4), NS3-4ΔC (lane 6), and NS3-4-5 (lane 8) were translated in the presence of [^{35}S]methionine. After 60 min of incubation, all samples were treated with 0.6 mg of cycloheximide per ml and 1 mM of unlabeled methionine on ice to block translation. Labeled substrate (NS4ΔN-5) was mixed with unlabeled protease sources pTHEΔ1116-1634 (lane 3), NS3ΔC-4ΔC (lane 5), NS3-4ΔC (lane 7), and NS3-4-5 (lane 9). The samples were incubated further for 180 min at 30°C and then analyzed on an SDS–12% polyacrylamide gel. Substrates and processed products (S, full-length substrate; S′ and S″, by-products of substrate generated in the in vitro translation reaction) are indicated on the left; protein molecular size markers are indicated on the right. Asterisks indicate labeled polypeptides used as enzymes (E) and substrates (S).

4BΔN-5A (5A with a part of 4B), and 5B (Fig. 1, lane 9), in order of size. This finding indicates that a protease in NS3-4-5 cleaves the substrate at the NS4B/5A and NS5A/5B junctions in *trans*. The processed 4BΔN was too small to be detected in this system. However, we confirmed the specific cleavage of the NS4B/5A and NS5A/5B junctions by using different substrates containing systemic deletions in NS4-5 (data not shown). NS3-4ΔC was self-cleaved at the NS3/4 junction, and p70 (NS3) was detected as a major band (Fig. 1, lane 6). NS3-4ΔC showed qualitatively the same *trans*-cleavage activity as NS3-4-5, as shown in lane 7 of Fig. 1 (compare lane 7 with lane 9 in Fig. 1). The effect of the putative RNA helicase domain on the protease activity of NS3 was tested by deleting the C-terminal part of HCV NS3. A part of NS4 (N-terminal 20 kDa of NS4) was retained in all of the C-terminal deletion mutants of NS3, since NS4 was essential for the *trans*-cleavage activity of NS3 (4, 11). This will be discussed further in relation to Fig. 2. The NS3-4 polypeptide with a deletion of two-thirds of NS3 (amino acids 1016 to 1846 with an internal deletion of amino acids 1219 to 1634; this polypeptide is designated NS3ΔC-4ΔC) was self-cleaved into two polypeptides (Fig. 1, lane 4; also see Han et al.

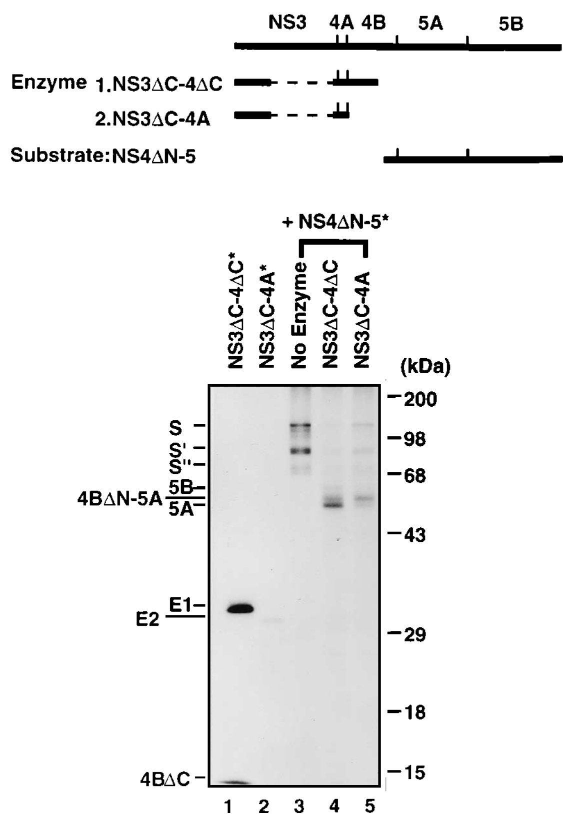

FIG. 2. NS4A is sufficient for the proteolytic activity of NS3. A schematic diagram of polypeptides is depicted at the top. [^{35}S]methionine-labeled NS4ΔN-5 (lane 3), NS3ΔC-4ΔC (lane 1), and NS3ΔC-4A (lane 2) were produced in RRL to show labeled polypeptides used in protease sources. After blocking of translation, labeled substrate (NS4ΔN-5) was mixed with unlabeled protease sources NS3ΔC-4ΔC (lane 4) and NS3ΔC-4A (lane 5). The samples were incubated further for 120 min at 30°C and then analyzed on an SDS–12% polyacrylamide gel. Lanes, polypeptides, and molecular size markers are as indicated in the legend to Fig. 1.

[19]). Interestingly, the NS4A/4B junction was the major cleavage site in this polypeptide even though the NS3/4A junction and the surrounding amino acids remained intact in the polypeptide. Only the larger fragment is shown in this experiment. Both larger and smaller fragments are shown in Fig. 2, lane 1. Polypeptide NS3ΔC-4ΔC processed the substrate NS4ΔN-5 at the NS4B/5A and NS5A/5B junctions (Fig. 1, lane 5) and showed qualitatively the same cleavage patterns as the polypeptides NS3-4-5 and NS3-4ΔC (compare lane 5 with lanes 7 and 9 in Fig. 1). This finding indicates that the RNA helicase domain of NS3 is not necessary for the *trans*-cleavage activity of protease NS3. Moreover, NS3ΔC-4ΔC showed a *trans*-cleavage activity higher than that of any of the other protease sources tested in our system, judging from the NS4B/5A junction cleavage (compare the 5A band in lane 5 with the band in lanes 7 and 9 in Fig. 1). A further deletion (amino acids 1116 to 1218) of NS3 abolished the *cis*- and *trans*-cleavage activities of the polypeptide (Fig. 1, lanes 2 and 3). This result is consistent with previous results indicating that serine 1165 is the essential component of protease NS3 (16, 22, 41). This finding indicates that the N-terminal one-third of NS3 possesses an essential element for protease activity and that the C-terminal two-thirds of NS3 is dispensable for protease function.

NS4A is essential for the in vitro *trans*-cleavage activity of NS3. To investigate the role of NS4 in proteolytic processing of HCV polyprotein, we used an in vitro *trans*-cleavage assay with various polyproteins. Without NS4, protease NS3 could not cleave substrates containing the NS4B/5A and NS5A/5B junctions. Addition of NS4 restored the proteolytic cleavage activity of NS3 (data not shown). This result is consistent with the published data (4, 11) except that NS4 is also necessary for efficient *trans*-cleavage activity of NS3 at the site of the NS5A/5B junction in vitro. This discrepancy may be due to differences in the assay systems used.

To test whether NS4A is sufficient for assisting the *trans*-cleavage activity of protease NS3 in vitro, we constructed NS3ΔC-4A, which contains a part of NS3 and NS4A (amino acids 1025 to 1218 and 1635 to 1712) as described in Materials and Methods. With this polypeptide, therefore, we could rule out the possible effects of the remaining NS2, NS4B, and other junction sequences in NS3ΔC-4ΔC on viral protease activity. Polypeptide NS3ΔC-4ΔC yielded two polypeptides, E1 and 4BΔC, as described above (Fig. 2, lane 1). NS3ΔC-4A was translated into one polyprotein (E2; Fig. 2, lane 2). Note that E1 is slightly larger than E2 because it contains the C-terminal 9 amino acids of NS2 and an additional junction sequence (19 amino acids) at the N terminus. The NS3/4 junction of NS3ΔC-4A, like that of NS3ΔC-4ΔC, was not cleaved. When [^{35}S]methionine-labeled substrate NS4ΔN-5 was treated with unlabeled NS3ΔC-4A, it was processed into NS5B, NS4ΔN-5A, and NS5A (Fig. 2, lane 5). Qualitatively, NS3ΔC-4A revealed the same processed products as did NS3ΔC-4ΔC. These results indicate that NS4A alone is sufficient for assisting NS3 in *trans* cleavage at the NS4B/5A and NS5A/5B junctions.

Protease NS3 is a chymotrypsin-like protease. Many protease inhibitors block translation in vitro. Therefore, it was impossible to test the effects of protease inhibitors on protease activity shown in *cis*-cleavage reactions of polyprotein translated in vitro. Development of the *trans*-cleavage assay system described above enabled us to test the actions of protease inhibitors on NS3-4A activity. Unlabeled NS3ΔC-4ΔC produced by in vitro translation was preincubated with various protease inhibitors or with their organic solvents and then was mixed with [^{35}S]methionine-labeled substrate NS3ΔN-4ΔC produced separately by in vitro translation (Fig. 3A and B, lanes 2). Polypeptide NS3ΔC-4ΔC was chosen as a protease source in protease inhibitor assays because of its high *trans*-cleavage activity. NS3ΔN-4ΔC was processed by NS3ΔC-4ΔC only at the NS4A/4B site and revealed 32- and 14-kDa polypeptides (Fig. 3A and B, lanes 3). This finding is consistent with in vivo and in vitro data showing that the NS3/4A junction is not cleaved in *trans* (3, 19). Among tested inhibitors, four protease inhibitors showed strong effects on NS3-4A activity. These were Pefabloc SC (Fig. 3A, lane 10), TPCK (Fig. 3A, lane 12), chymostatin (Fig. 3B, lane 5), and copper (Fig. 3B, lane 13). The inhibitory effect of cupric ion on HCV protease activity was shown by Han et al. (19). Protease inhibitors blocking processing by NS3 other than cupric ion were chymotrypsin inhibitors. Chymostatin is a specific inhibitor of α-, β-, γ-, and δ-chymotrypsin, and TPCK inhibits chymotrypsin strongly. Pefabloc SC is an inhibitor of serine proteases including chymotrypsin, trypsin, plasmin, and thrombin. On the other hand, TLCK, which specifically inhibits trypsin, did not inhibit the protease NS3 (Fig. 3A, lane 9). Similarly, leupeptin and antipain, which inhibit trypsin-like and many cysteine proteases, had no effect on NS3 protease (Fig. 3A, lanes 5 and 8, respectively). Two other serine protease inhibitors, PMSF and aprotinin, showed little effect on the *trans*-cleavage activity of NS3 in our reaction conditions (Fig. 3A, lanes 7 and 4, respectively). The cysteine protease inhibitor E-64, the aspartate protease

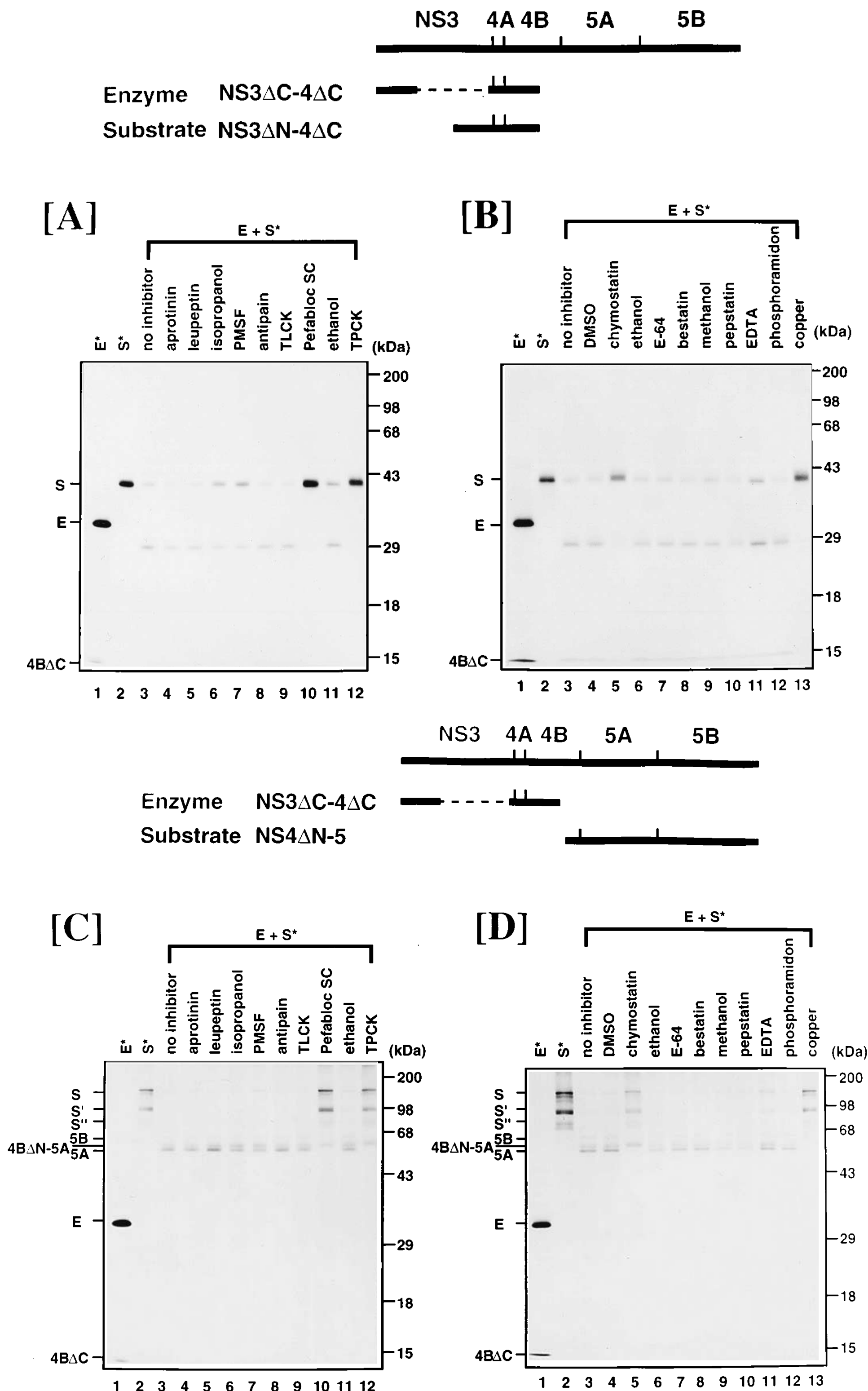

FIG. 3. Effects of protease inhibitors on NS3ΔC-4A protease activity. Schematic diagrams of polypeptides NS3ΔC-4ΔC, NS3ΔN-4ΔC, and NS4ΔN-5 are shown. The [35S]methionine-labeled enzyme source (NS3ΔC-4ΔC; E*) is shown in lane 1 of each panel. (A and B) [35S]methionine-labeled substrate NS3ΔN-4ΔC (5*) is shown in lanes 2 in panels A and B. After blocking of translation, unlabeled NS3ΔC-4ΔC was preincubated with inhibitors or solvents for 10 min at 30°C and then incubated with NS3ΔN-4ΔC for 120 min at 30°C. Lanes 3 in panels A and B contain no inhibitor. Protease inhibitors or their organic solvents tested are as follows: (A) 100 μM aprotinin (lane 4), 400 μM leupeptin (lane 5), 2.5% isopropanol (lane 6), 1 mM PMSF in isopropanol (lane 7), 500 μM antipain (lane 8), 300 μM TLCK (lane 9), 8 mM Pefabloc SC (lane 10), 1.7% ethanol (lane 11), and 300 μM TPCK in ethanol (lane 12); (B) 3.0% dimethyl sulfoxide (DMSO; lane 4), 1 mM chymostatin in DMSO (lane 5), 2.7% ethanol (lane 6), 300 μM E-64 in ethanol and H_2O (1:1) (lane 7), 300 μM bestatin (lane 8), 3.5% methanol (lane 9), 50 μM pepstatin in methanol (lane 10), 2 mM EDTA (lane 11), 1 mM phosphoramidon (lane 12), and 500 μM copper (lane 13). (C and D) Unlabeled NS3ΔC-4ΔC and [35S]methionine-labeled NS4ΔN-5 (lane 2) were used as the enzyme source and substrate. The inhibitors and method used for panel A were used in inhibitor assays with substrate NS4ΔN-5.

inhibitor pepstatin, the aminopeptidase inhibitor bestatin, and the metalloprotease inhibitors EDTA and phosphoramidon did not show any inhibitory effect on NS3 protease (Fig. 3B, lanes 7, 10, 8, 11, and 12, respectively). The inhibition mechanism of cupric ion remains to be elucidated.

The effects of protease inhibitors on NS3-4A protease were further characterized by using NS4ΔN-5 as a substrate (Fig. 3C and D, lanes 2). This polypeptide contains NS3-dependent NS4B/5A and NS5A/5B cleavage sites. As shown previously, NS3ΔC-4ΔC cleaved NS4ΔN-5 into 5A, 4BΔN-5A (5A and a part of 4B), and 5B (Fig. 3C and D, lanes 3). Similarly, with substrate NS3ΔN-4ΔC, Pefabloc SC, TPCK, chymostatin, and cupric ion strongly inhibited the *trans*-cleavage activity of protease NS3 (Fig. 3C, lanes 10 and 12, and Fig. 3D, lanes 5, and 13, respectively). The other inhibitors showed little or no effect. Full-length NS3-4ΔC and NS3ΔC-4A were also used in inhibitor assays to exclude the effect of a C-terminal deletion in NS3 and additional amino acid residues of NS3ΔC-4ΔC on inhibitor assays. Similarly to NS3ΔC-4ΔC, NS3-4ΔC and NS3ΔC-4A were inhibited by Pefabloc SC, TPCK, chymostatin, and cupric ion but not by other compounds (data not shown). We further characterized the inhibitory effect of TPCK and cupric ion on NS3-4A protease by changing the concentrations of inhibitors. TPCK showed a greater inhibitory effect (50% inhibitory concentration of ~120 μM) than did cupric ion (50% inhibitory concentration of ~170 μM) (data not shown). The inhibitor study led us to conclude that protease NS3 is a chymotrypsin-like protease.

DISCUSSION

HCV protease NS3 is a good therapeutic target molecule for use in developing anti-HCV drugs since its activity is considered to be essential for viral proliferation. Recently, characterizations of proteolytic activities of NS3 and NS3-4A have been accomplished by using in vitro translation and eukaryotic gene expression systems (3, 4, 10, 11, 16, 18, 22, 41). Biochemical properties of NS3 and NS3-4A proteases, on the other hand, have remained unknown because no proper in vitro *trans*-cleavage assay system for the protease has been available so far. Here we present an HCV protease assay system testing *trans* cleavage of protease NS3 combined with its auxiliary factor NS4A. Expression of the protease and substrates was performed by using in vitro transcription and translation systems. Cell-free translation systems were modified to enhance the *trans*-cleavage reaction of protease NS3. First, the C-terminal part of NS3 was deleted. Instead, NS4A-4B was fused in frame to the C-terminal end of the NS3 protease domain (NS3ΔC-4ΔC). The C-terminal two-thirds of NS3 contains a putative RNA helicase domain and is essential for *cis* cleavage of the NS3/4A junction (19). The polypeptide NS3ΔC-4ΔC was processed into NS3ΔC-4A and NS4BΔC by an autocleavage of the NS4A/4B junction. Newly generated fusion protease NS3ΔC-4A showed the same substrate specificity as NS3-4A-4B and NS3-4-5 at NS4A/4B, NS4B/5A, and NS5A/5B junctions. Moreover, it showed higher *trans*-cleavage activity than polypeptides containing full-length NS3. This may be due to easier intramolecular complex formation between NS3ΔC and NS4A, which remains as a single molecule in NS3ΔC-4A, than intermolecular complex formation between NS3 and NS4A processed into two separate molecules in NS3-4. The possibility of protein-protein interaction between NS3 and NS4A was suggested by coimmunoprecipitation of NS3 and NS4A (22). Moreover, a deletion of the N terminus of NS3 up to amino acid 1049 reduced the stability of NS4A produced by a *cis*-cleavage reaction of NS3 in RRL (19a). This finding suggests that the N-terminal part of NS3 interacts with NS4A. Very recently, the necessity of NS4A in *trans* cleavage of NS3 was shown by Failla et al. (11) and Bartenschlager et al. (4). The authors used vaccinia virus expression systems in testing the effect of NS4A on NS3 protease activity. In this report, we show that NS4A is also necessary for *trans*-cleavage reactions by NS3 in vitro. Second, the detergent Tween 20 was added in *trans*-cleavage reactions. Without the addition of Tween 20, NS3ΔC-4A cleaved the NS5A/5B junction to some extent but could not cleave the NS4A/4B and NS4B/5A junctions to detectable levels (data not shown). Tween 20 may provide a microenvironment facilitating enzyme-substrate interaction and/or enhancing proper folding of NS3ΔC-4A. The latter might be accomplished by preventing protein aggregation mediated by the hydrophobic domain in NS4A.

We characterized the HCV protease NS3-4A by investigating the effects of well-known protease inhibitors on *trans*-cleavage reactions of NS3ΔC-4A at NS4A/4B, 4B/5A, and 5A/5B junctions. The chymotrypsin-specific inhibitors TPCK, chymostatin, and Pefabloc SC inhibit the *trans*-cleavage activity of NS3-4A in vitro. Among them, TPCK, which is known to alkylate histidine residues in catalytic triads of serine proteases (37), shows the highest inhibitory effect (50% inhibitory concentration of ~120 μM) on HCV protease. In contrast, PMSF, which is known to react with serine residues of catalytic triads of serine proteases, does not show any inhibitory effect on HCV protease at 1 mM in our system. On the other hand, Pefabloc SC, a substitute for PMSF, inhibits NS3 protease. This might be due to the presence of a scavenger of PMSF in RRL. An analogous situation has been found in sera in which thrombin activity is blocked with Pefabloc SC but not with PMSF (29). Trypsin-like protease inhibitors antipain, leupeptin, and TLCK do not block the activity of NS3ΔC-4A, which strongly suggests that HCV protease NS3 is a chymotrypsin-like serine protease. Other protease inhibitors such as E-64 (a cysteine protease inhibitor), bestatin (an aminopeptidase inhibitor), pepstatin (an aspartic protease inhibitor), and EDTA and phosphoramidon (metalloprotease inhibitors) do not block HCV protease. We also showed that NS3 protease is activated by zinc ion slightly (data not shown) but inhibited by cupric ion (Fig. 3B and D, lanes 13) (19). We do not know how divalent ions influence the protease activity of NS3. One speculation is that zinc ion may play an indirect role in HCV protease function, possibly by stabilizing the correct conformation of the protease. Cupric ion, on the other hand, may disturb the conformation of the protease, resulting in inhibition of the protease. Alternatively, cupric ion may influence the active site of the protease directly. Investigations of substrate specificity determinants and mechanisms of protease inhibitors will provide the fundamental knowledge required for the development of anti-HCV drugs based on HCV protease inhibitors.

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