JOURNAL OF VIROLOGY, Jan. 1996, p. 127–132 Vol. 70, No. 1
0022-538X/96/$04.00+0

Identification of the Sequence on NS4A Required for Enhanced Cleavage of the NS5A/5B Site by Hepatitis C Virus NS3 Protease

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Received 19 July 1995/Accepted 15 September 1995

In addition to NS3 protease, the NS4A protein is required for efficient cleavage of the nonstructural protein region of the hepatitis C virus polyprotein. To investigate the function and the sequence of NS4A required for the enhancement of NS3 protease activity, we developed an in vitro NS3 protease assay system consisting of three purified viral elements: (i) a recombinant NS3 protease which was expressed in *Escherichia coli* as a maltose-binding protein–NS3 fusion protein (MBP-NS3), (ii) synthetic NS4A fragments, and (iii) a synthetic peptide substrate which mimics the NS5A/5B junction. We showed that the NS3 protease activity of MBP-NS3 was enhanced in a dose-dependent manner by 4A18-40, which is a peptide composed of amino acid residues 18 to 40 of NS4A. The optimal activity was observed at a 10-fold molar excess of 4A18-40 over MBP-NS3. The coefficient for proteolytic efficiency, k_{cat}/K_m, of NS3 protease was increased by about 40 times by the addition of a 10-fold molar excess of 4A18-40. Using a series of truncations of 4A18-40, we estimated that amino acid residues 22 to 31 in NS4A (SVVIVGRIIL) constituted the core sequence for the effector activity. Single-substitution experiments with 4A21-34, a peptide composed of amino acid residues 21 to 34 of NS4A, suggested the importance of several residues (Val-23, Ile-25, Gly-27, Arg-28, Ile-29, and Leu-31) for its activity. In addition, we found that some single-amino-acid substitutions in 4A21-34 were able to inhibit the enhancement of NS3 protease activity by 4A18-40. This approach has potential as a novel strategy for inhibiting the NS3 protease activity important for hepatitis C virus proliferation.

Hepatitis C viruses (HCV) are considered to be the major cause of posttransfusion and sporadic non-A, non-B hepatitis (reviewed in references 19 and 27). On the basis of their genome structures and virion properties, HCV have been proposed to be classified as a separate genus in the family *Flaviviridae*, together with pestiviruses and flaviviruses (5, 8, 12). HCV contains a positive-sense linear RNA genome of about 9.5 kb in length, which encodes a single polyprotein of about 3,000 amino acid residues (7, 23, 33). This is a precursor polyprotein from which the following 10 viral proteins are proteolytically processed: NH_2-core (C), envelope 1 (E1), envelope 2 type A (E2-A)/envelope 2 type B (E2-B), p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B-COOH (15, 18, 24, 28, 32, 37).

By using transient-coexpression systems in animal cells (2, 9, 25, 26, 35) and an in vitro transcription and translation system (16), it has been shown that proteolytic cleavages in the nonstructural protein region (NS3 to NS5B) of the HCV polyprotein are effected by two viral proteins, NS3 and NS4A. The N-terminal 180-amino-acid region of NS3, which is called the NS3 protease domain, includes sequences showing homology with the active sites of serine proteases (22, 29, 30). The histidine 1083, aspartate 1107, and serine 1165 residues (numbered according to their locations in the polyprotein of HCV subtype J [HCV-1b] [23, 34]) found in this domain have been proposed to constitute the catalytic triad of the HCV protease, like for other serine proteases belonging to the chymotrypsin family (30). Consistent with this prediction, when the presumptive catalytic serine 1165 of the polyprotein was mutated to alanine, cleavage at the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B sites was abolished (13, 17, 37). NS4A is an amphipathic protein of 54 amino acids, and it has a very hydrophobic N-terminal domain followed by a hydrophilic C-terminal domain (9). NS4A acts as an effector or cofactor of the NS3 protease activity for efficient cleavage of the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B sites, and it interacts with the NS3 protease in *trans* (2, 9, 16, 25, 26, 35). Although very recently our group and another suggested that the central region of NS4A plays a key role in NS4A-dependent processing of the NS4B/5A site (26, 35), little is known about the mechanism by which NS4A regulates NS3 protease activity, because of the unavailability of purified NS3 protease and the effector molecule.

In an in vitro translation system and in mammalian expression systems previously used (2, 9, 16, 25, 26, 35), it was found to be difficult to adjust the amounts of enzyme, substrate, and NS4A molecules for enzymological studies because of variations in the levels of expression of products and because of their different stabilities. To investigate the enzymological characterization of NS3 protease and the sequences on NS4A important for its effector function, we developed an in vitro reconstituted assay system for NS3 protease consisting of three purified viral elements: (i) a purified maltose-binding protein–NS3 fusion protein (MBP-NS3), (ii) synthetic NS4A fragments, and (iii) a peptide substrate which mimics the NS5A/5B junction.

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Using this assay system, we demonstrated that (i) synthetic NS4A fragments change the enzymatic properties of NS3 protease in vitro, and (ii) residues 22 to 31 of NS4A constitute the putative core sequence for NS4A's effector activity. Furthermore, we found that a peptide, mcR28Q, composed of amino acid residues 21 to 34 of NS4A and having a mutation of arginine 28 to glutamine, is a potent inhibitor which disturbs the interaction of NS4A with NS3 protease.

MATERIALS AND METHODS

Preparation of MBP-NS3 fusion protein. We used an MBP-NS3 fusion protein as the source of NS3 protease activity because it is expressed as a soluble form, shows a considerably higher expression level than NS3 itself, and is able to be conveniently purified on an amylose resin (20). The expression and purification of MBP-NS3 have been described previously (20). In brief, MBP-NS3 is a protein containing an HCV polypeptide spanning amino acids 985 to 1647 fused with *Escherichia coli* MBP at the N terminus. MBP-NS3 has full NS3 protease activity but not metalloproteinase activity (cpro1) (14, 17). The purity of MBP-NS3 (about 110 kDa) was more than 95% as determined by reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme was stored at −70°C in 20% glycerol before use for biochemical characterization. The concentration of the purified MBP-NS3 was determined by amino acid composition analysis by the Pico-Tag method (4) after acid hydrolysis (6 M HCl, 110°C, 21 h).

Peptide synthesis. All peptides employed in this study were synthesized by the procedure of Fmoc chemistry (1) on an automated multiple peptide synthesizer (model PSSM-8; Shimadzu, Kyoto, Japan). The peptides were purified by preparative reverse-phase high-performance liquid chromatography (HPLC) (ODS80Tm; 2.15 by 30 cm; Tosoh, Tokyo, Japan) with 0.1% aqueous trifluoroacetic acid and acetonitrile-based mobile phases. All synthetic peptides were confirmed to be the desired ones by analyses of amino acid composition and electron spray mass spectrometry. Their purities exceeded 95%, and they were stored frozen at −20°C as 10 mM stock solutions in 100% dimethyl sulfoxide.

HCV NS3 protease assays. A synthetic peptide, NH_2-Glu-Ala-Gly-Asp-Asp-Ile-Val-Pro-Cys-Ser-Met-Ser-Tyr-Thr-Trp-Thr-Gly-Ala-COOH (S5A/5B), which mimics the NS5A/5B junction, served as the substrate for the study of HCV NS3 protease activity. By mass spectrometry of the S5A/5B digests, we confirmed that the cleavage of the synthetic peptide takes place at the peptide bond between Cys and Ser, as expected from previous observations (3, 20).

A standard assay was performed in a total volume of 50 μl of assay buffer (50 mM Tris-HCl, 30 mM NaCl, 1 mM $MgCl_2$, and 2 mM dithiothreitol, pH 7.5) containing 12 μg of MBP-NS3 (2.16 μM). MBP-NS3 in the buffer mixture was preincubated with or without NS4A fragments for 30 min at 25°C. The reaction was initiated by adding 1 μl of a 5 mM S5A/5B solution (final concentration, 100 μM) containing 5 mM internal standard peptide IS5 (NH_2-Ser-Met-Ser-Tyr-Lys-Asp-Lys-COOH) to 50 μl of assay cocktail, and the reaction mixture was incubated at 25 or 37°C. The optimal temperatures for substrate cleavage differed for reaction mixtures with and without 4A18-40 (a peptide composed of amino acid residues 18 to 40 of NS4A), since prolonged incubation of MBP-NS3 at 37°C is detrimental to the stability of NS3 protease. Therefore, comparisons of kinetic reactions with and without 4A fragments were carried out at 25°C. Other experiments relating to the characterization of NS4A fragments were performed at 37°C. After an adequate incubation period, the reaction was stopped by the addition of 2 μl of 2.5 M acetic acid, and the reaction mixture was transferred to a vial for HPLC analysis. Analytical HPLC was carried out with a Shimadzu LC-10A liquid chromatography system. Twenty microliters of each sample was analyzed by reverse-phase HPLC (ODS80Ts; 0.46 by 15 cm; Tosoh), eluting with a linear gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in 60% acetonitrile over a period of 30 min at a flow rate of 1.0 ml/min. Peptides were detected by UV A_{210}, and the cleavage products were quantitated by calculating the peak areas.

Determination of kinetic constants of NS3 protease for hydrolysis of S5A/5B. Enzyme assays were carried out as described above at S5A/5B substrate concentrations ranging from 70 to 400 μM. Incubations were carried out at 25°C at times during the linear range of the enzyme reaction. The K_m and k_{cat} values were then determined by Lineweaver-Burk plots.

Inhibition of enhanced NS3 protease activity. The ability of 4A21-34 derivatives to inhibit NS3 protease activity enhanced by 4A18-40 was examined with the following experiments. MBP-NS3 was pretreated with 44 μM concentrations of various mutated forms of 4A21-34 for 10 min at 25°C and then incubated with 4.4 μM 4A18-40 for 30 min at 25°C. Proteolytic reactions were initiated by the addition of S5A/5B as a substrate as described above and were allowed to proceed for 3 h at 37°C. The percent inhibition of enhanced NS3 protease activity was calculated as follows: percent inhibition = 100 × [100 − (percent cleavage with 4A18-40 and 4A21-34 analog)]/[(percent cleavage with 4A18-40) − (percent cleavage without 4A18-40)].

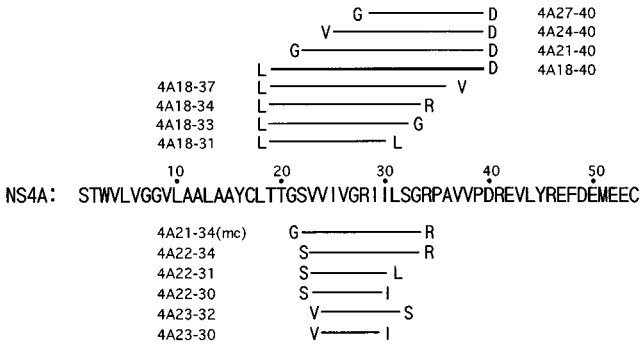

FIG. 1. Amino acid sequences of NS4A and the NS4A fragments used in this study. The names of the NS4A fragments are shown on the left. The lines depict the regions of the NS4A fragments. The numbers refer to the first and last amino acids of the NS4A fragments. Various NS4A fragments were synthesized on the basis of the amino acid sequence of NS4A of HCV strain JT (32).

RESULTS

An NS4A fragment enhances NS3 protease cleavage of the NS5A/5B junction in vitro. Previously, Failla et al. (9) suggested that the C-terminal 33 amino acids of NS4A, which consists of a total of 54 residues, are required for cleavage at the NS3/4A and NS4B/5A sites and accelerate the rate of cleavage at the NS5A/5B junction. In contrast, Lin et al. (25) showed that only the N-terminal 35 amino acids of NS4A, coexpressed with the NS3 protease domain, are required for trans cleavage at the NS4B/5A site. Tanji et al. (35) showed that the central region of NS4A is important for the activity. To elucidate whether a synthetic NS4A fragment including the central region of NS4A (Fig. 1) can accelerate proteolytic cleavage of the NS5A/5B site by NS3 protease, we synthesized a peptide from amino acid 18 to 40 of NS4A, designated 4A18-40, and developed a simple in vitro assay system consisting of three purified viral elements: a bacterially produced MBP-NS3 (20), a 4A18-40 peptide, and a synthetic peptide substrate, S5A/5B, which mimics the NS5A/5B junction.

As shown in Fig. 2, we compared the time courses of the proteolytic cleavage of the S5A/5B substrate by MBP-NS3 with and without 4A18-40. MBP-NS3 alone hydrolyzed the synthetic substrate very slowly (25% hydrolysis by 6 h), and during this time course, the hydrolysis of S5A/5B was linear. On the other hand, in the presence of a twofold molar excess of 4A18-40, the rate of S5A/5B cleavage was significantly augmented. The time course of hydrolysis was linear up to 30% conversion to products, after which the reaction proceeded smoothly to complete hydrolysis (Fig. 2). S5A/5B was not cleaved when incubated with 4A18-40 alone. The initial velocity of S5A/5B cleavage with NS3 protease in the presence of 4A18-40 is about 15 times higher than that with NS3 protease alone (1.00 versus 0.069 μmol/min). In this assay system, MBP-NS3 was not processed or degraded for up to 6 h, as estimated by reduced SDS-PAGE (data not shown). Bovine serum albumin, when added instead of 4A18-40, was not able to enhance the cleavage of S5A/5B. Moreover, some of the single substitutions of 4A21-34 dramatically reduced the enhancing activity (see below). Therefore, enhancement of NS3 protease-dependent cleavage by NS4A fragments is a highly specific event based on the NS4A sequence, not a bulk effect due to an increase in the protein or peptide concentration.

Characterization of the enhanced proteolytic cleavage of S5A/5B with 4A18-40. By altering the molar ratio of 4A18-40 to MBP-NS3, we investigated the effect of the 4A18-40 concentration (0.55 to 44 μM) on the hydrolysis of S5A/5B by NS3

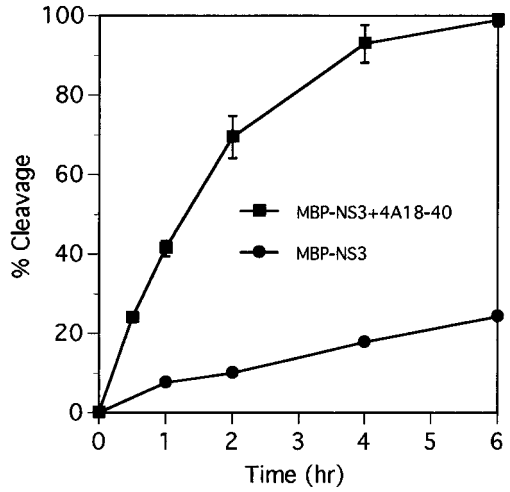

FIG. 2. Kinetic analyses of HCV NS3 protease reaction with and without 4A18-40. NS3 protease activity, which is expressed as percent cleavage of the S5A/5B substrate peptide, was measured after different incubation times at 25°C in the absence or presence of 4A18-40, as described in Materials and Methods. Twelve micrograms of MBP-NS3 (2.2 μM) in the assay buffer was preincubated at 25°C for 30 min with and without a twofold molar excess of 4A18-40 (4.4 μM). Each point is the mean ± standard deviation of results from three different experiments which gave similar results.

protease (2.2 μM). As shown in Fig. 3, the initial velocity of S5A/5B hydrolysis increased in a dose-dependent manner up to a molar ratio of 2:1 and approached a plateau at a molar ratio of around 4:1. The maximum enhancement of the proteolytic cleavage was observed at molar ratios of greater than 10:1.

We then compared the steady-state kinetic constants for the proteolytic activities of NS3 protease for S5A/5B cleavage with and without 4A18-40 by measuring the NS3 protease activity as a function of the substrate concentration. Table 1 shows that in

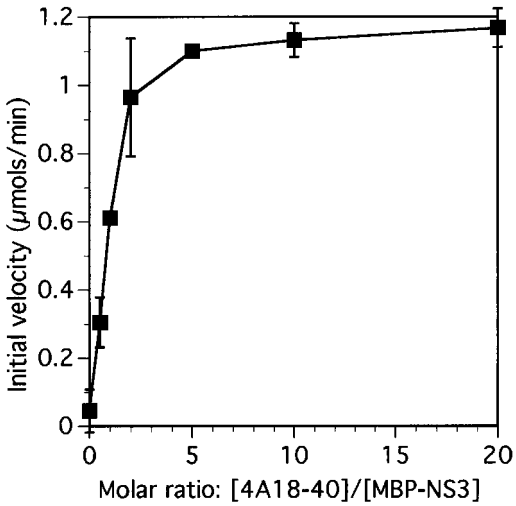

FIG. 3. Dose-dependent enhancement of NS3 protease activity by 4A18-40. Twelve micrograms of MBP-NS3 (2.2 μM) was preincubated with 4A18-40 at six different concentrations, i.e., 1.1, 2.2, 4.4, 11, 22, and 44 μM (molar ratios of MBP-NS3 to 4A18-40 are 1:0.5, 1:1, 1:2, 1:5, 1:10, and 1:20, respectively), for 30 min at 25°C. The reactions were then initiated by adding 100 μM of S5A/5B at 37°C. To determine the initial velocity, the substrate digestion was stopped before 20 to 30% conversion to products. Each point is the mean ± standard deviation of results from four different experiments which gave similar results.

TABLE 1. Effect of 4A18-40 on steady-state kinetic constants[a] for proteolytic cleavage of S5A/5B by NS3 protease

Enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}/\mu M$)
MBP-NS3 alone	411	0.14	3.66×10^{-4}
MBP-NS3 + 4A18-40	108	1.64	1.53×10^{-2}

[a] Data are the mean values from three independent experiments.

the absence of 4A18-40, the K_m for S5A/5B was 411 μM and the k_{cat} was 0.14 min^{-1}. In contrast, in the presence of a 10-fold molar excess of 4A18-40, the K_m (108 μM) was about four times lower than that in the case of enzyme alone, and the k_{cat} (1.64 min^{-1}) was about 12 times higher than that for MBP-NS3 alone. As a result, the coefficient for proteolytic efficiency, k_{cat}/K_m, was increased about 42 times in the presence of 4A18-40.

To investigate whether the effect of 4A18-40 on NS3 protease activity was substrate dependent, we measured the proteolytic cleavages of different synthetic substrates corresponding to NS4A/4B and NS4B/5A junctions in the presence and absence of 4A18-40. To compare the substrate specificities, we synthesized peptide substrates having the same length (14 amino acids). Whereas S5A/5B14, a truncated substrate of S5A/5B (NH_2-GDDIVPC-SMSYTWT-COOH), was cleaved inefficiently with MBP-NS3 alone, the rate of cleavage was accelerated in the presence of 4A18-40. However, MBP-NS3 did not show any proteolytic activity on the substrates S4A/4B (NH_2-EFDEMEAC-APHLPY-COOH) and S4B/5A (NH_2-NEDCSTPC-SGSWLK-COOH), which are composed of 14 amino acid residues surrounding the NS4A/4B and NS4B/5A junctions, respectively, even in the presence of 4A18-40 (data not shown).

Identification of the minimal NS4A sequence required for NS4A effector activity. Since a peptide containing the central region of NS4A had accelerated the cleavage of S5A/5B by MBP-NS3 in vitro, we tried to determine the borders of the minimal sequence required for NS4A effector activity by using a series of truncated derivatives of 4A18-40 (Fig. 1). As shown in Fig. 4, 4A21-40, with the N-terminal truncation to residue 21, enhanced the cleavage of S5A/5B, whereas 4A24-40 and 4A27-40, with N-terminal truncations to residues 24 and 27, respectively, showed no effector activity. While the C-terminal truncations to residues 37 and 34 (4A18-37 and 4A18-34, respectively) retained the effector activity at the same level as that of 4A18-40, 4A18-33 and 4A18-31 lowered the level. These results suggest that 4A21-34 is sufficient to enhance NS3 protease activity. Actually, 4A21-34, and even the further N-terminally truncated form, 4A22-34, showed enhancing activity similar to that of 4A18-40. Although 4A22-31 still showed detectable activity at a high concentration (22 μM), we could not narrow down the active domain further; that is, 4A22-30, 4A23-30, and 4A23-32 did not exhibit the effector activity (Fig. 4). From these truncation experiments, we postulated that although residues 22 to 34 are necessary for full activity, the 10 amino acids spanning residues 22 to 31 (SVVIVGRIIL) of NS4A are the core sequence (or active domain) for the effector activity.

Analysis of the importance of each amino acid residue in and around the core sequence for effector activity. As expected from these truncation analyses, single Ala substitutions at Ser-22 (S22A) and Ser-32 (S32A) and single Ser substitutions at Gly-33 (G33S) and Arg-34 (R34S) of 4A21-34, which are all located outside or on the border of 4A22-31, did not impair the

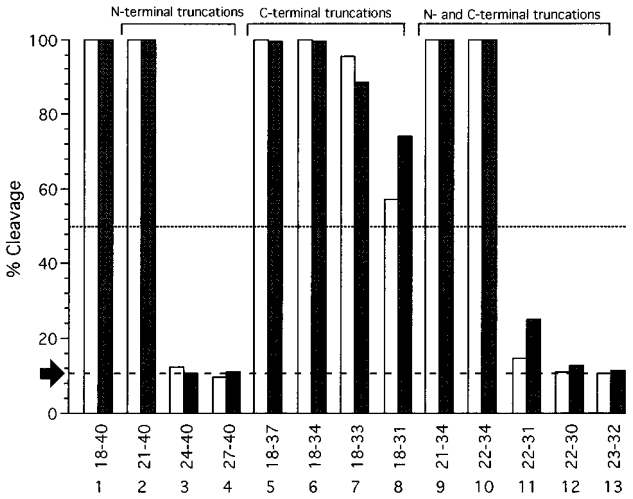

FIG. 4. Mapping of the minimal NS4A sequence required for effector activity. Twelve micrograms of MBP-NS3 was preincubated with various truncated analogs of 4A18-40 at two concentrations, 4.4 μM (a 2-fold molar excess) (white bars) and 22 μM (a 10-fold molar excess) (black bars), for 30 min at 25°C prior to addition of S5A/5B. The results are expressed as percent cleavage of the substrate after 3 h of incubation at 37°C. About 10% of the substrate was cleaved by MBP-NS3 alone (arrow).

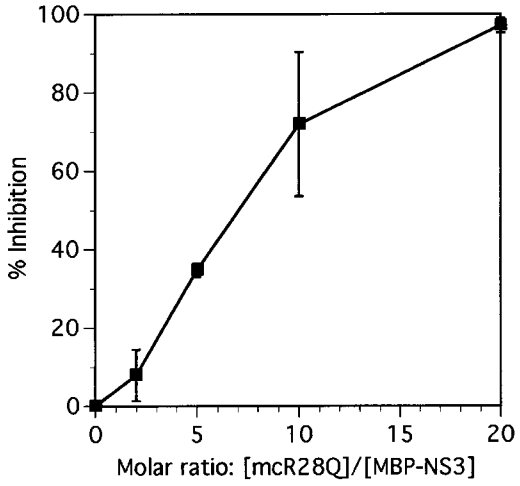

FIG. 6. Inhibition of NS3 protease activity in the presence of 4A18-40 by mcR28Q. MBP-NS3 was pretreated with mcR28Q at four different concentrations, i.e., 4.4, 11, 22, and 44 μM, for 10 min at 25°C before preincubation with 4.4 μM 4A18-40, and the enzyme reactions were performed for 3 h at 37°C. The results (means ± standard deviations) are expressed as percent inhibition of the enhanced NS3 protease activity, which was calculated as described in Materials and Methods.

effector activity (Fig. 5, bars 2 to 5). In HCV subtype 1 (HCV-1a) (7), NS4A contains three Cys residues at positions 17, 22, and 54. Cys-17 and Cys-54 are common among all known HCV subtypes, while Cys-22 is changed to Ser in subtype JT (HCV-1b) (34). Since 4A18-40, which is derived from subtype 1, possessed effector activity similar to that of strain JT, Ser-22 and Cys-22 seem to be functionally interchangeable (data not shown). Although a basic residue (Arg or Lys) at position 34 is common among all known HCV subtypes (25), the result with mcR34S suggests that Arg-34 is not essential for the effector activity. Since the core sequence does not possess the distinctive amphipathic nature of NS4A, it is concluded that the amphipathic nature is not responsible for the effector activity.

Because there is a distinctive cluster of hydrophobic amino acid residues in the putative core sequence, we evaluated the importance of each residue by using single-Ser-substituted analogs of 4A21-34 in the same assay system. Homologous or identical amino acids are at residues 25, 29, and 30 (Ile); 23, 24, and 26 (Val); and 31 (Leu). Nevertheless, single Ser substitutions at Ile-30, Val-24, and Val-26 slightly reduced the effector activity, but those at Val-23, Ile-25, Gly-27, Ile-29, and Leu-31 suppressed it strongly (Fig. 5, bars 6 to 13). The most drastic reduction was seen with a Ser substitution at Ile-29 (mcI29S). These data suggest that among the residues in the core sequence, the five hydrophobic residues (Val-23, Ile-25, Gly-27, Ile-29, and Leu-31) aligned alternately in the sequence are particularly important to the effector activity. Furthermore, we investigated the significance of Arg-28 because it is the only hydrophilic residue in the core sequence and is strongly conserved among all known HCV subtypes. Substitution of Lys for Arg-28 (R28K) had no effect on the effector activity, whereas substitution of Asp for Arg-28 (R28D) strongly impaired the activity. The replacement of Arg-28 by a neutral amino acid (Ser or Gln) also impaired the effector activity. mcR28Q and mcR28S exhibited about 10 and 50% of the activity of 4A21-34, respectively (Fig. 5, bars 14 to 17). These data indicate the necessity of a positive charge at position 28 for the strong effector activity of NS4A.

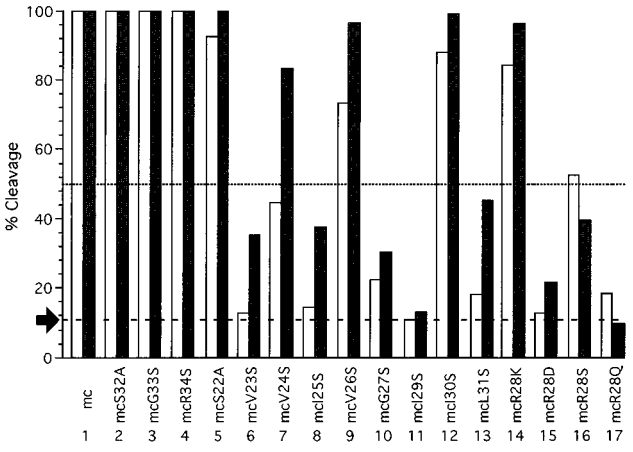

FIG. 5. Effector activities of 4A21-34 analogs with single-amino-acid substitutions. The original peptide (4A21-34) was designated mc. The effector activity of each substitution was analyzed at two concentrations, 4.4 μM (a 2-fold molar excess) (white bars) and 22 μM (a 10-fold molar excess) (black bars), as described for Fig. 4. About 10% of the substrate was cleaved by MBP-NS3 alone (arrow).

Substitution analogs of 4A21-34 inhibit enhancement of NS3 protease activity by 4A18-40. Finally, we tested whether some of the singly substituted analogs of the mc peptide can inhibit the NS3 protease activity enhanced by 4A18-40. After MBP-NS3 was pretreated with the mc analogs listed in Fig. 5, the proteolytic activity of MBP-NS3 was measured in the presence of 4A18-40, as described above. We found that a single substitution of Gln for Arg-28 (mcR28Q) inhibited the enhanced cleavage of S5A/5B strongly and in a dose-dependent manner (Fig. 6). Fifty percent inhibition was observed at around 20 μM mcR28Q. mcR28S also showed a similar inhibitory effect, but analogs with other substitutions did not (data not shown). The inhibitory effect was eliminated in the presence of a higher concentration of 4A18-40 but not in the presence of a higher concentration of substrate (Table 2).

TABLE 2. Effect of concentration of 4A18-40 or S5A/5B on inhibitory activity of mcR28Q[a]

[4A18-40]/[MBP-NS3]	[S5A/5B] (μM)	% Inhibition
2	100	97
5	100	70
10	100	38
20	100	24
2	200	100
2	400	100

[a] Inhibition of the enhanced NS3 protease activity by mcR28Q was analyzed as described in the legend to Fig. 6 in the presence of increasing concentrations of 4A18-40 or of the S5A/5B substrate. [mcR28Q]/[MBP-NS3] was 20 in each case. The percent inhibition was calculated as described in Materials and Methods.

These results suggest that mcR28Q does not directly interact with the substrate. Rather, this peptide may be bound to NS3 protease at the same site where 4A18-40 interacts, but the binding seems not to show potency in enhancing the NS3 protease activity toward S5A/5B. Thus, we were able to show the possibility that mcR28Q is a novel inhibitor for NS3 protease activation.

DISCUSSION

In this study, using an in vitro assay system for NS3 protease, we estimated that residues 22 to 31 of NS4A (SVVIVGRIIL) constitute the active domain for NS4A effector activity toward NS5A/5B cleavage by NS3 protease. Previously, Tanji et al. (35) proposed that the region is the minimum functional domain of NS4A. Recently, Lin et al. (26) also reported that residues 21 to 33 or residues 22 to 34 were essential for NS4A-dependent cleavage of NS4B/5A as determined with an in vivo transient-coexpression system and an in vitro cell-free translation system. Although the assay systems and substrates used in their study are different from those in ours, the active domains identified show good agreement. This may suggest that the core sequence of NS4A enhances NS3 protease activity irrespective of the substrate sequence.

HCV, flaviviruses, and pestiviruses, which have been classified as three genera in the family *Flaviviridae* (12), all encode a chymotrypsin-like serine protease (3, 22, 30). It is known that these proteases require a second viral protein for successful processing of the nonstructural proteins, although the requirement of a second viral protein for viral polyprotein processing differs in each virus: flavivirus NS3 protease requires NS2B (6, 11), HCV NS3 protease requires NS4A (2, 9, 25), and pestivirus p80 (possibly equivalent to HCV NS3) requires p10 (which may be a functional homolog of HCV NS4A [38]). Recently, Falgout et al. (10) identified a 40-amino-acid domain of NS2B of dengue virus type 4 that is essential for NS3 protease activity. However, this domain is relatively hydrophilic, and there is no amino acid sequence homology between the active domains of dengue virus NS2B and HCV NS4A. In addition, a comparison of NS2B, NS4A, and p10 did not reveal any obvious homology throughout their sequences.

Although the core sequence of HCV NS4A has a pivotal role for the enhanced cleavage of the NS5A/5B site by NS3 protease, we cannot yet conclude that this core sequence is sufficient for the enhanced cleavage in vivo. A requirement of the core sequence for the cleavage at the NS4A/4B and NS4B/5A sites by NS3 protease in this assay system was not proven. Because the N- and C-terminal sequences of NS4A are well conserved among all known HCV subtypes (25), these regions seem to have other important functions for the processing of viral proteins and/or other steps in viral replication. In fact, it is worth mentioning that NS5A protein is phosphorylated in an NS4A-dependent manner (21, 36). Recently it was suggested that a direct interaction between NS3 and NS4A is required for efficient cleavages at NS4B/5A and NS5A/5B (31), whereas a 10-fold molar excess of 4A18-40 over NS3 protease is required for maximum enhancement of the protease activity in this assay system. This suggests that the interaction of 4A18-40 is weak and may not be sufficient for maintaining NS3 protease activity with a low concentration. Possibly either the N- or C-terminal domain, or both, of NS4A is involved in forming the stable complex between NS4A and NS3.

The mechanism by which NS4A fragments enhance NS3 protease activity is not yet clear. Since MBP-NS3 shows weak proteolytic activity on the S5A/5B substrate even in the absence of NS4A fragments, the catalytic center of NS3 protease seems to have been formed without NS4A fragments. Although proteases which belong to the chymotrypsin family are usually activated by limited digestion of their precursor (reviewed in reference 30), MBP-NS3 does not undergo any detectable digestion during incubation with NS4A fragments. NS3 protease may not require any processing for the enhancement of its activity.

An interaction between NS3 and full-length NS4A has been reported (18, 26, 31). Therefore, it is likely that NS4A fragments also act as effector or activator molecules for NS3 protease itself through direct interaction between them. The interaction of NS4A fragments with MBP-NS3, rather than with substrates, could be proposed on the basis of the following three results. (i) Preincubation of MBP-NS3 with NS4A fragments was necessary for the maximal proteolytic activity of NS3, but preincubation of S5A/5B substrate with NS4A fragments showed little effect on the NS3 protease activity (data not shown). (ii) During the analysis of kinetic parameters (Table 1), the enhanced cleavage was not inhibited by increasing concentrations of the substrate. (iii) The inhibitory activity of a substitution analog of 4A21-34 was not affected by increasing concentrations of the substrate (Table 2). The most promising hypothesis regarding the roles of NS4A fragments is that they bind to NS3 protease and then induce alterations in the enzyme structure, leading to a more favorable conformation for catalysis and/or substrate binding. Structural analysis of HCV NS3 protease with NS4A is required in order to determine the molecular mechanism by which NS4A enhances the NS3 protease activity.

NS4A is important for proteolytic processing of the HCV polyprotein (2, 9, 16, 24, 25, 35), and the NS3 protease-NS4A interaction is an alternative to NS3 protease itself as a good target for anti-HCV drugs. Our in vitro NS3 protease assay system with NS4A fragments is expected to be useful for screening of inhibitors of NS3 protease itself and its activation. We have found that some of the singly substituted analogs of 4A21-34, for example, mcR28Q, strongly inhibited the enhancement of NS3 protease activity by 4A18-40. This may provide a potent parent compound which will interrupt the interaction between NS4A and NS3 protease. Investigation is now under way to clarify whether a point mutant of R28Q of NS4A is capable of showing dominant-negative suppression of the processing of the nonstructural region of the HCV polyprotein at the cell level.

ACKNOWLEDGMENTS

We thank Shin-ichi Ishii for his helpful suggestions and critical reading of the manuscript.

This work was supported in part by a grant-in-aid from the Ministry

of Health and Welfare for a second-term Comprehensive 10-Year Strategy for Cancer Control.

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