Inhibition of hepatitis C virus NS3 protease by peptides derived from complementarity-determining regions (CDRs) of the monoclonal antibody 8D4: tolerance of a CDR peptide to conformational changes of a target

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Abstract **We have synthesized and characterized peptides derived from complementarity-determining regions (CDRs) of 8D4, a mouse monoclonal antibody against NS3 protease domain of hepatitis C virus. 8D4 inhibits enzymatic activity without its cofactor, NS4A peptide. One of the synthetic peptides derived from CDRs, CDR1 of the heavy-chain (CDR-H1) peptide strongly inhibited NS3 protease activity competitively in the absence of NS4A and non-competitively in the presence of NS4A. Moreover, cyclic CDR-H1 peptides bridged by disulfide inhibited NS3 protease more potently. The chain length of the CDR-H1 peptide is critical for strong inhibition, even when the peptide is circularized. This finding suggests the importance of peptide conformation. In contrast to a cognate antibody molecule, CDR-derived peptides may provide good ligands for target molecules by having a tolerance to conformational changes of the targets caused by cofactor binding or mutation.**

Key words: CDR peptide; Antibody; Conformational change; Circularization; NS3 protease

1. Introduction

Antibodies use six complementarity-determining regions (CDRs) in their variable domains to bind with high specificity and affinity to antigens [1–4]. The well-studied antigen-binding sites of antibodies hold considerable promise as model systems for the design of peptides with antibody-like recognition mechanisms [5]. The small repertoire of main-chain conformations of CDRs, referred to as 'canonical structures' [6–8], suggests that sequences of CDRs provide structural templates for designing low-molecular-weight lead compounds. Several recent reports have shown that synthetic peptides derived from CDR sequences have binding properties similar to those of the intact antibody [9–14]. This finding indicates that CDR-derived peptides can overcome several disadvantages of full antibodies, including time-consuming purification steps, instability of antibodies and antibody-producing hybridomas, and immunoreactivity.

Non-structural protein (NS) 3 of hepatitis C virus (HCV) is a multifunctional, virus-specific protein that contains serine protease activity in its N-terminal region and accounts for processing of the viral polyprotein at four cleavage sites, NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B. NS3 also contains helicase and nucleic-acid-stimulated nucleoside triphosphatase activities in its C-terminal region (see references [15–17] for review). NS3 protease requires the NS4A peptide as a cofactor for efficient cleavage of the viral polyprotein [18–19]. X-ray crystallographic analysis and nuclear magnetic resonance spectroscopic analysis of the NS3 protease domain with and without the NS4A cofactor have provided a refined picture of the NS3 structure, demonstrating that the overall topology is similar and forms N-terminal (approximately residues 1–93) and C-terminal (residues 94–180), six-stranded, anti-parallel β-barrels that are packed like those of chymotrypsin-like serine proteases [20–22]. However, several loops found in other chymotrypsin family proteases, which play a critical role in defining the shapes of the non-prime-side substrate-binding pockets, are missing from NS3, rendering the substrate-binding groove relatively featureless and therefore making the design of low-molecular-weight inhibitors challenging [20]. The interaction of NS4A with NS3 was shown to induce conformational changes in NS3 that involve both a structural reorganization of the N-terminal domain and a rearrangement of the protease catalytic site [20,22].

Recently, we developed the monoclonal antibody 8D4, which recognizes the active site of HCV NS3 protease [23]. This immunoglobulin inhibits NS3 protease activity relatively

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Abbreviations: Fv, antibody variable domain fragment; HCV, hepatitis C virus; NS, non-structural protein; K_i, inhibition constant; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CDR, complementarity-determining region; Abz, fluorophore 2-aminobenzoyl; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography

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strongly, with an inhibition constant (K_i) of 39 nM. We have also reported the DNA sequence analysis, bacterial expression, and functional characterization of the antibody variable domain fragment (Fv) and single-chain antibody (scFv) of 8D4, demonstrating that the Fv had an inhibition profile almost identical to that of the entire IgG [24]. However, an observed reduction of more than 1000-fold in the inhibitory activity of the antibody against NS3–4A complex is due to conformational changes to the NS3 caused by binding to NS4A [23]. To date, no antibody molecule except for 8D4 has been prepared against NS3–4A protease, perhaps because of its featureless antigenic structure. Thus, engineering of 8D4 or CDR peptides would be promising for construction of inhibitors.

We report the inhibitory activities of synthetic peptides derived from six CDRs of the antibody 8D4. One of the peptides, CDR-H1 peptide shows inhibitory activity toward NS3 and NS3–4A complex, with K_i values of 3.5 and 6.5 μM, respectively. Circularization of the CDR-H1 peptides by introducing Cys residues into their N- and C-terminus increased the inhibitory activity by more than four times. The peptides could provide structural templates for designing low-molecular-weight lead compounds of NS3–4A-specific inhibitors as anti-HCV agents.

2. Materials and methods

2.1. Peptides

All CDR peptides, listed in Tables 1 and 2, were provided by Sawady Technology (Tokyo, Japan), in which these peptides have been synthesized with an automated multiple peptide synthesizer (SYRO II), circularized with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and purified by high-performance liquid chromatography (HPLC). Design of the peptide sequence is based on a previous report of antibody sequences [24]. Substrate peptide of NS3 protease and NS4A peptide, used for enzymatic assays were synthesized and purified by HPLC using the Peptide Institute Inc. (Osaka, Japan).

2.2. Production and purification of active NS3 protease

DNA fragments encoding the N-terminal protease domain (1–190 residues) of NS3 of HCV II_J [25] were cloned into the expression vector pMT1. The vector had been constructed by replacing the tac promoter of pMK2 with a tryptophan promoter to overproduce an N-terminal domain with a hexahistidine tag (His_6-$NS3_{1-190}$) [23]. *Escherichia coli* (strain JM109) cells transformed with the resulting plasmid were cultured overnight at 25°C in M9 medium (60 g Na_2HPO_4, 30 g KH_2PO_4, 10 g NH_4Cl, and 5 g NaCl per liter of distilled water, pH 7.4) containing ampicillin (100 μg/ml) and supplemented with 0.2% casamino acids, 0.2% glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 1 mM thiamine. The cells were further propagated in 3.6 l of the same medium for 15 h at 25°C. They were then exposed to 25 μg/ml *3*-indoleacrylic acid and cultured for an additional 8 h at 20°C. Cells were then harvested, suspended in buffer [50 mM Tris·HCl (pH 7.5), 0.3 M NaCl, 20 mM imidazole, 10% glycerol], and disrupted by sonication on ice. His_6-$NS3_{1-190}$ was then purified by metal chelate affinity (Ni–NTA Superflow, Qiagen) and ion exchange chromatography (S-Sepharose Fastflow, Pharmacia). Eluted fractions were collected in CHAPS buffer [50 mM Tris·HCl (pH 7.5), 10% glycerol, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate] and pooled. NS3 protease thus prepared showed >90% purity as assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; data not shown), and was stored at −70°C after the addition of an equal volume of glycerol.

Table 1
Amino acid sequences of linear peptides derived from CDRs of antibody 8D4

Peptide[a]	Sequence[b]
CDR-H1 (15)	H-GYSFTDYVLIWVKQS-OH
CDR-H2 (17)	H-NSNPYYGRTSYNLKFKG-OH
CDR-H3 (15)	H-CARGGFYAMDYWQGQG-OH
CDR-L1 (16)	H-GSSKSLLHSDGNTYLY-OH
CDR-L2 (15)	H-LLIYRMSNLASGVPD-OH
CDR-L3 (14)	H-YYCMQHLEYPYTFG-OH
CDR-H1-7 (7)	H-TDYVLIW-OH
CDR-H1-5 (5)	H-DYVLI-OH

[a]Values in parentheses are the numbers of residues in each peptide.
[b]CDR sequences are underlined, and other sequences are derived from the sequence of parental antibody 8D4.

Table 2
Amino acid sequences of circularized peptides derived from CDR-H1 of antibody 8D4

Peptide[a]	Sequence[b]
CDR-H1-C17 (17)	H- CGYSFTDYVLIWVKQSC –OH
CDR-H1-C13 (13)	H- CSFTDYVLIWVKC –OH
CDR-H1-C9 (9)	H- CTDYVLIWC –OH
CDR-H1-C7 (7)	H- CDYVLIC –OH
CDR-H1-15 (15)[c]	H- GYSFTDYVLIWVKQS –OH

[a]Values in parentheses are the number of residues in each peptide.
[b]CDR sequences are underlined, and other sequences are derived from the sequence of parental antibody 8D4. Two cysteines for circularization by disulfide bond formation are combined with a line.
[c]From Table 1.

2.3. Inhibition assay of NS3-dependent cleavage of a peptide substrate by CDR-derived peptides

A synthetic peptide carrying the NS5A/5B cleavage site and having a fluorescent moiety at its N-terminus (fluorophore 2-aminobenzoyl[Abz]-EDVVECSMSY-NH_2) was used as substrate. The reaction mixture contained 50 mM Tris·HCl (pH 8.5), 30 mM NaCl, 2% CHAPS, and 5% Glycerol. The NS3 protease (38 nM) was preincubated in the presence or absence of 100 μM of CDR-derived peptides and the 5 μM of NS4A peptide (H-LTTGSVVIVGRIILSGRPAVVPD-OH; $Pep4A_{18-40}$, designated as NS4A) [26] for 10 min at room temperature in a total volume of 80 μl. The reaction was initiated by the addition of 20 μl of the substrate peptide dissolved in 50 mM Tris·HCl (pH 8.5), 30 mM NaCl, 5 mM $CaCl_2$, and 25 mM dithiothreitol (DTT). The final concentration of the peptide substrate in the reaction mixture was 0.025 mM in the presence of NS4A or 0.2 mM in its absence, unless otherwise indicated. The reaction was continued at 37°C for 15 min with NS4A or 60 min without, and quenched by the addition of 100 μl of 0.5% trifluoroacetic acid (TFA). The fluorescent reaction product (Abz-EDVVEC-OH) was then separated on a reverse-phase HPLC apparatus equipped with a C_4 column (4.6×50 mm, Vydac) by elution at a flow rate of 2 ml/min with an aqueous solution containing 14% acetonitrile and 0.1% TFA. The fluorescence signal was detected by a spectrofluorometric detector (Shimadzu, Kyoto, Japan) with excitation and emission wavelengths at 320 and 425 nm, respectively. The amount of the product was quantified by integration of the chromatogram in relation to a chemically synthesized peptide standard with a structure identical to that of the cleavage product. Precise analyses of the inhibitory activity of CDR peptides were performed under the same conditions described above, with varying concentrations of CDR peptides before addition of the synthetic substrate peptide. Steady-state kinetic parameters and the K_i value were determined by Lineweaver–Burk plot and Dixon plot analyses, respectively [23].

2.4. Inhibition assay of NS3-dependent cleavage of a peptide substrate by circularized CDR peptides

The experimental procedures of the inhibition assay using circularized CDR peptides were in principle followed by the same conditions described above. To determine the effect of circularization of the peptides on the inhibitory activity, we reduced the circularized CDR peptides with 40 mM DTT for 30 min, then assayed the inhibitory activity as described above.

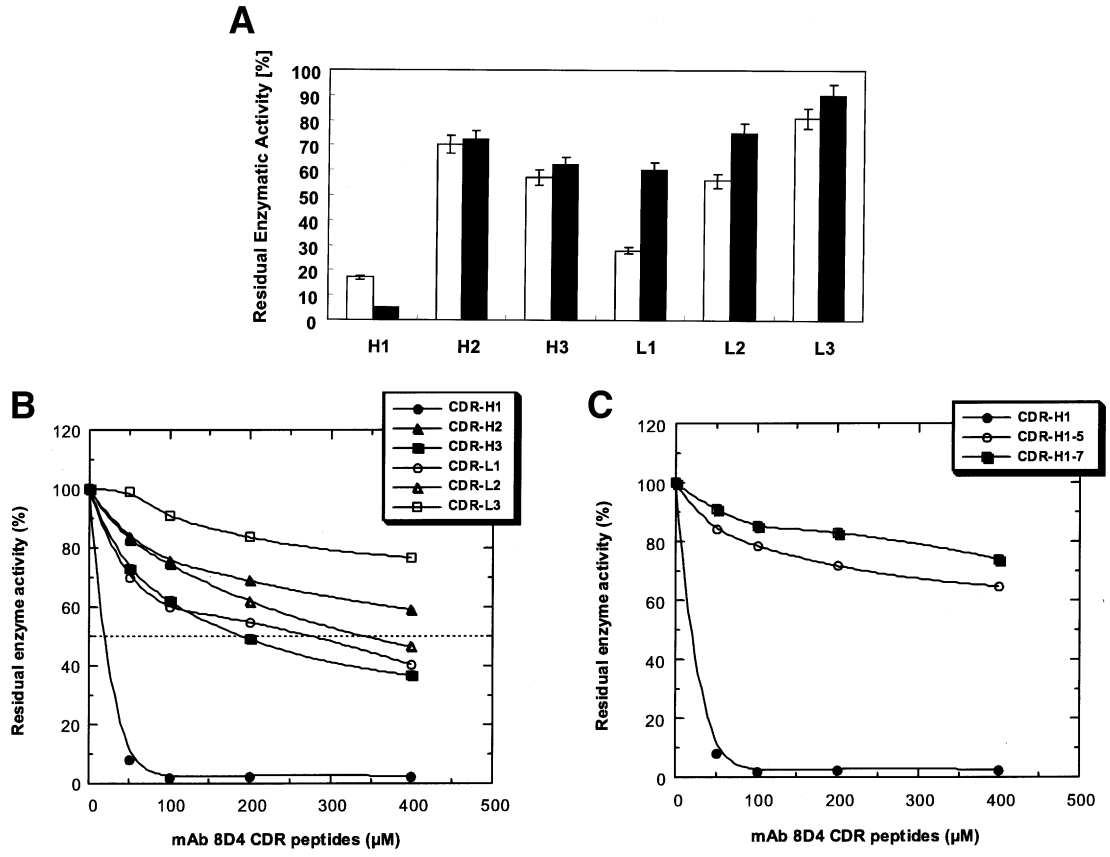

Fig. 1. A: Profile of inhibition by linear CDR peptides of hexahistidine-tagged $NS3_{1-190}$ protease with (solid bar) or without NS4A (open bar). A 38 nM solution of NS3 protease in 50 mM Tris·HCl (pH 8.5) containing 30 mM NaCl and 2% CHAPS was preincubated for 10 min with 100 μM of each CDR peptide. The reactions were started by addition of 0.2 mM oligopeptide substrate S3 (Abz-EDVVECSMSY-NH_2, where Abz is fluorophore 2-aminobenzoyl). The mixture was incubated for 1 h, and the enzymatic reaction was terminated by addition of an equal volume of 0.1% TFA. The N-terminal cleavage product of S3 was detected by the fluorescence of Abz (λ_{ex} = 320 nm, λ_{em} = 425 nm) in HPLC analysis and quantified as the ratio of the integrated area denoting the cleavage product to that of a known quantity of standard. B: Dose dependency of the inhibitory activity of CDR-H1 and CDR-L1 peptides with NS4A. Assays were performed under the same conditions as described above, with varying concentrations of CDR peptides before addition of the enzyme. C: Dose dependency of the inhibitory activity of CDR-H1, -H1-5, and -H1-7 peptides with NS4A. Assays were performed under the same conditions described above, with varying concentrations of CDR peptides before addition of the enzyme. Symbols used: solid circle, CDR-H1; open circle, CDR-H1-5; solid square, CDR-H1-7.

3. Results

3.1. Effect of CDR-derived peptides on NS3-dependent cleavage of a peptide substrate

We assessed the abilities of the CDR-derived peptides listed in Table 1 to inhibit the NS3 protease activity toward a synthetic peptide (Abz-EDVVECSMSY-NH_2) containing the NS5A/5B cleavage site as a substrate in the absence or presence of 5 μM NS4A. As a result, CDR-H1 peptide inhibited the enzymatic activity of NS3 protease in both the absence and presence of NS4A. We further examined the extent of inhibition by CDR-derived peptides in the absence and presence of 5 μM NS4A (Fig. 1B). CDR-H1 peptide had inhibitory activity toward NS3 protease in a dose-dependent man-

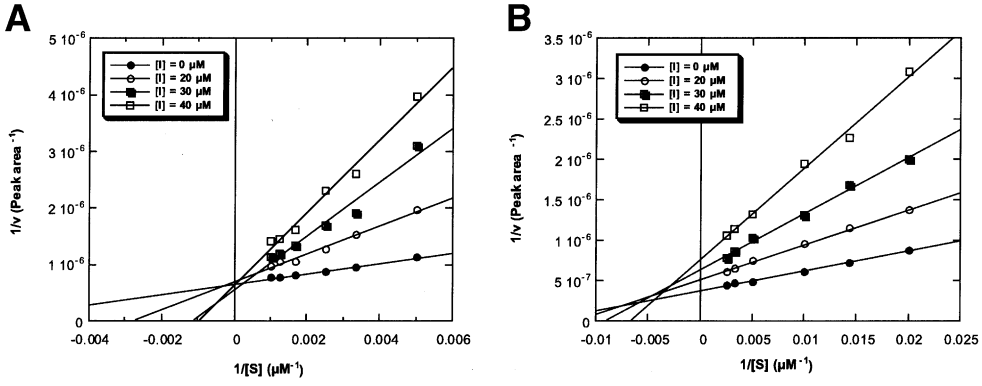

Fig. 2. Lineweaver–Burk plot analyses of the inhibition of linear CDR-H1 peptides for NS3 protease without (A) or with NS4A (B). [S] is concentration of oligopeptide substrate (Abz-EDVVECSMSY-NH_2, where Abz is fluorophore 2-aminobenzoyl). See reference [23] for details.

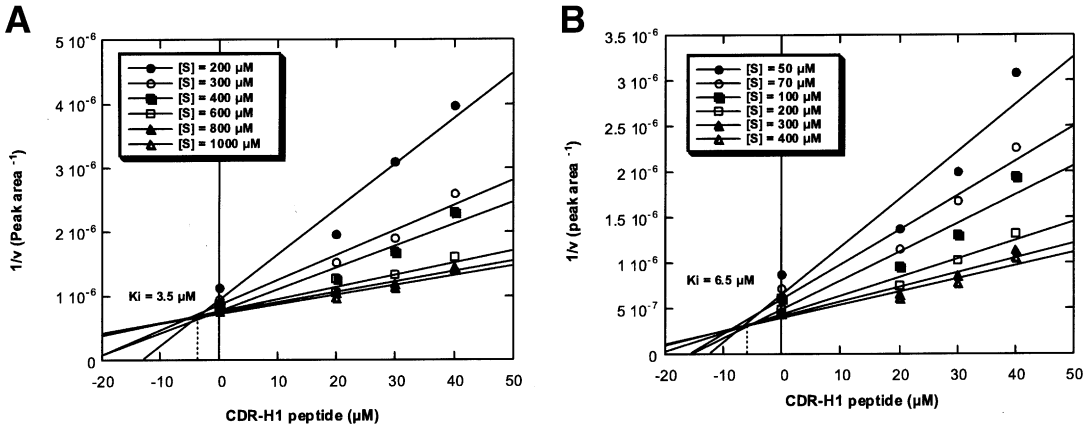

Fig. 3. Estimation of K_i of linear CDR-H1 peptide without (A) or with NS4A (B). See reference [23] for details.

ner in both the absence and presence of the NS4A peptide (Fig. 1B). This result suggests that CDR-H1 is tolerant to structural changes. CDR-H1-derived peptides with 5 and 7 residues showed reduced inhibition (Fig. 1C), suggesting the importance of the remaining residues within CDR-H1 for strong inhibition.

We then conducted steady-state kinetic experiments to analyze the mechanism of inhibition by CDR-H1 in the absence and presence of NS4A. As shown in Fig. 2A, CDR-H1 showed linear competitive inhibition of the proteolytic cleavage of the peptide substrate by the protease without NS4A cofactor, indicating that CDR-H1 directly blocked the binding of the substrate peptide in the absence of NS4A, in a competitive inhibitory manner. On the other hand, as shown in Fig. 2B, CDR-H1 inhibited the NS3 protease activity in the presence of NS4A non-competitively. The K_i values of CDR-H1 inhibition of the protease, as determined by Dixon plot analysis, were 3.5 μM in the absence of the cofactor and 6.5 μM in its presence (Fig. 3). These results indicate that CDR-H1 has relatively strong inhibitory activity toward NS3 protease in both the absence and the presence of NS4A cofactor, using different mechanisms of inhibition.

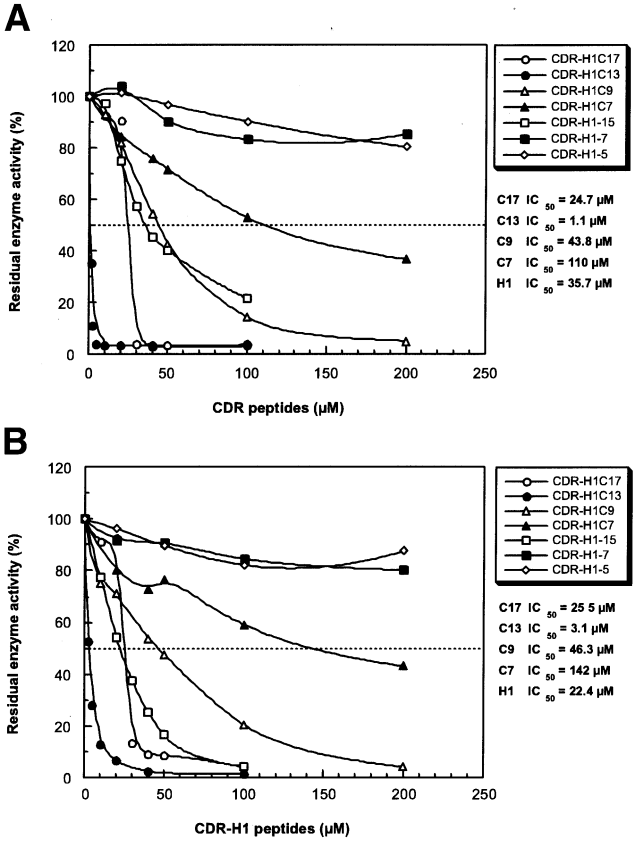

Fig. 4. Profile of inhibition by cyclic CDR-H1 peptides of NS3 protease without (A) or with NS4A (B). A 38 nM solution of NS3 protease in 50 mM Tris·HCl (pH 8.5) containing 30 mM NaCl and 2% CHAPS was preincubated for 10 min with various concentrations of cyclic CDR peptides, and the reactions were started by addition of 0.2 mM oligopeptide substrate S3 (Abz-EDVVECSMSY-NH_2, where Abz is fluorophore 2-aminobenzoyl). The mixture was incubated for 1 h, and the enzymatic reaction was terminated by addition of an equal volume of 0.1% TFA. The N-terminal cleavage product of S3 was detected by the fluorescence of Abz (λ_{ex} = 320 nm, λ_{em} = 425 nm) in HPLC analysis and quantified as the ratio of the integrated area denoting the cleavage product to that of a known quantity of standard.

3.2. *Effect of circularization of CDR-H1-derived peptides on the inhibitory activity*

We next synthesized 4 CDR-H1-derived peptides circularized with a disulfide bond by introducing Cys residues into the N- and C-terminus of the peptides (Table 2). We examined the inhibitory activity of the circularized peptides toward NS3 protease in the absence or presence of NS4A cofactor. As shown in Fig. 4, CDR-H1C13, with 13 amino acids, had en-

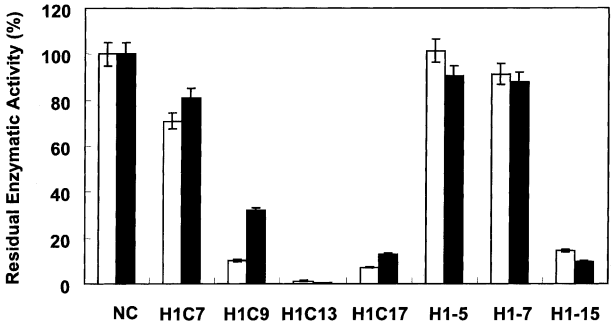

Fig. 5. Effect of DTT preincubation on the inhibitory activity of cyclic CDR-H1 peptides toward NS3 protease in the presence of NS4A. Open bar, without DTT incubation; solid bar, with DTT incubation. Each peptide was preincubated with 40 mM DTT for 30 min, and enzymatic activity of NS3 was assayed as described in Section 2.

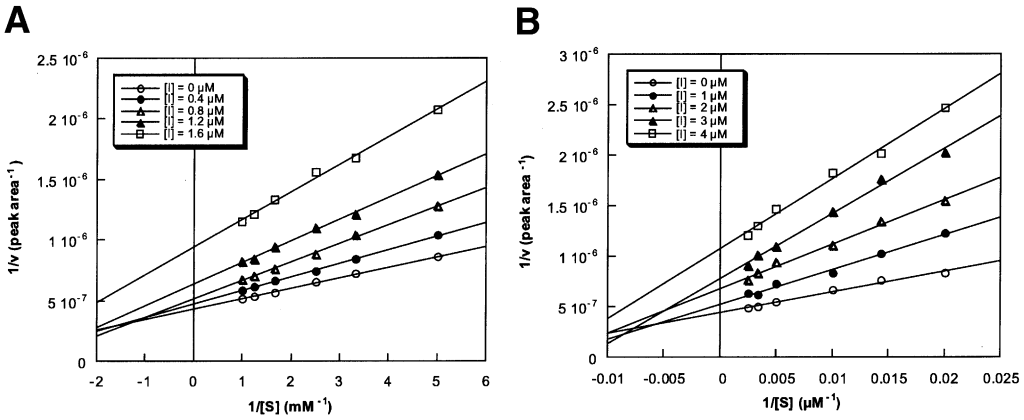

Fig. 6. Lineweaver–Burk plot analyses of the inhibition by CDR-H1C13 of NS3 protease without (A) or with NS4A (B).

hanced affinity for NS3 protease in both the absence (Fig. 4A) and presence (Fig. 4B) of NS4A, with a IC_{50} value of 1–3 μM. The other peptides had less inhibitory activity. This result suggests that CDR-H1C13 has an appropriate conformation for strong inhibition of the protease activity.

To determine the effect of circularization of the peptides on the inhibitory activity, we reduced the circularized CDR peptides, and the inhibitory activity has been assayed (Fig. 5). Reduction of the peptides did not decrease their inhibitory activity. This result strongly suggests that the conformation of the CDR peptides created by their amino acid sequences is critical for binding activity toward the target.

We then conducted kinetic experiments to analyze the mechanism of inhibition by CDR-H1C13 in the absence and presence of NS4A. As shown in Fig. 6, CDR-H1C13 inhibited the enzymatic activity of NS3 non-competitively in both the absence and presence of NS4A. Dixon plot analysis showed that CDR-H1C13 has a K_i for NS3 protease of 0.9 μM in the presence of NS4A peptide and 1.5 μM in its absence (Fig. 7).

4. Discussion

We have shown that a peptide derived from the antibody CDR, CDR-H1 has strong inhibitory activity toward HCV NS3 protease. The peptide has fewer than 20 amino acids (Table 1), which makes it feasible for development of protease inhibitors. The parental antibody 8D4 has strong inhibitory activity toward NS3 in the absence of NS4A cofactor. In the presence of the cofactor, conformational changes to NS3 via NS4A binding reduce the inhibition [23,24]. However, our results clearly indicate that CDR-H1 and the circularized CDR-H1C13 can bind to the antigen in both the absence and the presence of the cofactor. This finding indicates that CDR-derived peptides derived from the parental antibody molecules can recognize cognate binding sites even if conformational changes are induced by cofactor binding.

The candidate recognition site of the parental antibody 8D4 has been proposed as the sequential linear peptide chain DQDLV (from site 79 to 83 of NS3), which includes one of the catalytic residues of NS3 protease, Asp81 [23]. 8D4 inhibited NS3 protease competitively. Our results indicate that CDR-H1 inhibits the protease activity competitively, suggesting that CDR-H1 recognizes the region around Asp81. On the other hand, restriction of the conformation of CDR-H1 by circularization enhanced the inhibitory activity, even though the inhibition was non-competitive, suggesting that cyclic CDR-H1 recognizes a distinct region on the substrate-binding site. Recent reports of the solution structures of NS3 and single-chain NS3–4A proteases have suggested that residues Val51-Asp81 of the NS3–4A protease are in a slow exchange with an NS4A-free conformation of NS3, but only a few structural rearrangements around Asp81 have been observed [27–30]. Therefore, it might be proper to conclude that the cyclic CDR-H1 peptide recognizes a certain conformation of the region around Asp81 in a different manner from the way the linear peptide does so.

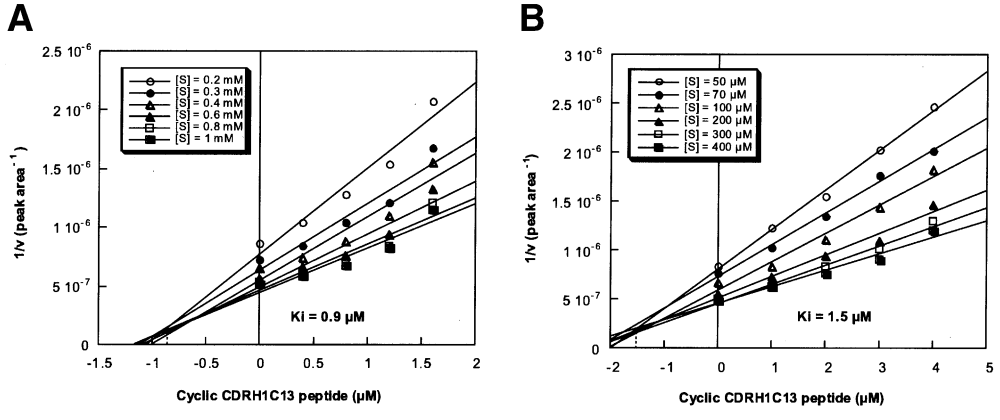

Fig. 7. Estimation of K_i of cyclic CDR-H1C13 peptide without (A) or with NS4A (B).

The sequence around CDR-H1 is SFTDYVLIWVK (CDR region is underlined; Table 2). The recent discovery of potent peptide inhibitors has shown that the inhibitor was derived from the N-terminal region of the substrate (called the P region) upon cleavage by NS3–4A, i.e. product inhibitors [31]. Interestingly, the sequence of substrate NS4A/4B is DEMEE (from P6 to P2), and the optimized natural amino acid sequences are: D(Y/E)(L/I/V)(I/L/E)(L/D/E) [31]. The results have shown that the optimized sequences shared high homology with the sequence of CDR-H1 (DYVLI) of the antibody 8D4. Shortening the length of CDR-H1 (i.e. CDR-H1-7 and CDR-H1-5, Table 1) dramatically reduced the inhibitory activity toward NS3 in the absence and presence of NS4A. This finding indicates the significant contribution of other amino acids in CDR-H1 to the inhibition of enzymatic activity, perhaps through maintaining the loop structure. CDR-H1C17 and CDR-H1C9 had less inhibitory activity than CDR-H1C13. Thus, the conformation of CDR-H1C13 might be appropriate for binding to the antigen in a similar manner to CDR-H1-15 and the cognate antibody. The canonical structure of CDR-H1 might be critical for strong inhibition of NS3 and NS3–4A.

Our results highlight the usefulness of CDR-derived peptides for the design of peptides targeted to specific molecules. Several CDR-derived peptides have been used to inhibit receptor-ligand interaction or cell adhesion [9–14]. A recent elegant peptide design achieved by combining a rational design approach based on CDR with a combinatorial technology produced matured peptides with a higher affinity at a smaller size [32]. Incorporation of non-natural amino acids or other organic compounds into CDR-derived peptides would be promising for enhancement of affinity. Crystal structures of antigen–antibody complexes have shown that only a few residues in CDRs make a critical contribution to the Gibbs energy of interactions [2,3,33]. Therefore, at a cost of some energy loss, CDR peptides might be promising for the design of target-specific peptides.

In conclusion, a CDR peptide, CDR-H1, derived from the NS3 protease antibody 8D4, strongly inhibits the protease activity competitively in the absence of the cofactor NS4A and non-competitively in the presence of NS4A. The results suggest the significance of conformation of the peptide. CDR-derived peptides may provide good ligands for target molecules by having a tolerance to conformational changes of the targets caused by cofactor binding or mutation.

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