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Improvement of Biological Activity and Proteolytic Stability of Peptides by Coupling with a Cyclic Peptide

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Abstract—The cyclic moiety of an endothelin antagonist peptide RES-701-1, composed of 10 amino acids with an amide bond between α-NH_2 of Gly1 and β-COOH of Asp9, was coupled to some biologically active peptides aiming to improve their activities and stabilities against proteolytic degradation. Coupling of the cyclic peptide to the N-terminal of RGD-peptides, maximally 4-fold improvement of in vitro activity compared to the original peptide has been achieved. Coupling of it to protein farnesyltransferase inhibiting peptides resulted to improve in vitro activity maximally 3-fold. These peptides coupled with the cyclic peptide also showed enhanced stability against some typical proteases. These results indicate that this cyclic peptide can stabilize the conformations of the peptides coupled to its C-terminus. Coupling of our cyclic peptide is anticipated to be a novel conformational stabilizing method for biologically active peptides, results to improve their activity and stability.

An endothelin (ET) antagonist RES-701-1 (**1**, Fig. 1) has a unique cyclic structure in its N-terminus.[1] The cyclic structure of RES-701-1 is essential for its biological activity and also is important for its high stability against proteolytic degradation.[1,2] Although the cyclic moiety of RES-701-1, RES-701-1 (1-10) (**2**, Fig. 1) has no detectable binding activity to ET receptors, we found that the chimeric peptide produced by coupling of **2** to the N-terminus of a weak ET antagonist [Ala15] ET-1 (12-21) (**3**, Fig. 1) exhibited remarkably improved receptor binding activity.[2] The linear peptide **4**, which has the same sequence as **3**, had similar binding activity for ET_B receptor,[3] however, the stability against proteolysis was proved to be much inferior to **3**. We previously demonstrated that degradation of **4** by subtilysin, one of the typical protease, was much faster than **3** (unpublished). RES-701-1, as well as endothelins, has been reported to have a β-turn structure in its C-terminal 'tail' part, residues 9–12. The hydrophobic core containing β-turn structures are considered to be important for their receptor binding activity.[4,5] We had predicted from these results that the cyclic peptide **2** can stabilize the solution conformation, particularly the turn structures, of the peptides coupled to its C-terminus, which results to improve their biological activity and stability against proteolysis.[6,7]

To prove this prediction, we have coupled the cyclic peptide **2** to known cell adhesion inhibiting RGD-peptides **5a** and **6a**[8] and protein farnesyltransferase (FTase) inhibiting peptides **7a** and **8a**[9,10] as shown in Figure 1, which have been reported to have γ- or β-turn structures in their sequences respectively,[11,12] and evaluated their

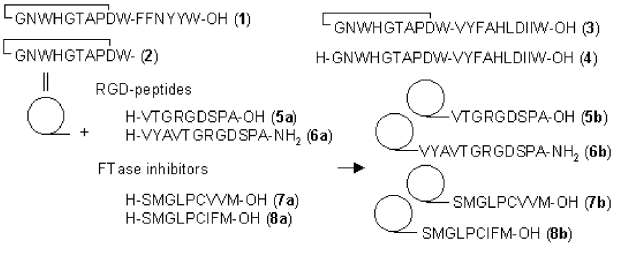

Figure 1. Structures of RES-701-1 (**1**) and biologically active peptides coupled with the cyclic moiety of **1** (**2**).

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biological activities and stabilities against proteolytic degradation.

Materials and Methods

(a) *Peptide synthesis*. The cyclic peptide of RES-701-1, RES-701-1(1-10), was obtained by combination of standard solid-phase and liquid-phase peptide synthetic strategy[13] briefly as below. N-terminally Fmoc-protected fragment of RES-701-1(1–8) and the dipeptide, H-Asp(O*t*Bu)-Trp-OBzl, was coupled to obtain the fully protected fragment peptide. Then N-terminal and side chain protecting groups were removed followed by cyclization between N-terminal and side-chain of Asp. Finally, the cyclic peptide was obtained after deprotection of C-terminal benzyl ester followed by purification by semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC). The cyclic peptide was coupled to side chain-protected peptides synthesized on appropriate solid support resin followed by cleavage and deprotection to obtain the peptides coupled with **2**. Peptides coupled with **2** were purified by RP-HPLC and their structures were confirmed by mass spectrometry and amino acid analyses.

(b) *Proteolysis*. Examined peptides were dissolved (25 μg/mL) in phosphate-buffered saline (pH 7.2) containing 0.05% sodium aside and 0.1 mom $CaCl_2$, followed by adding trypsin (Sigma) and incubated at 37 °C. Residual ratio of examined peptides were determined by comparing the peak height in RP-HPLC analyses before and after treatment with trypsin. Treatment with chymotrypsin and prolylendopeptidase was carried out in a similar manner to that with trypsin.

(c) *Cell adhesion inhibitory activity*. Mouse melanoma cell B16-F10 was labeled with biotin by incubating cells with NHS-LC-Biotin (Pierce). These labeled cells were added to the 96-well plates (Sumitomo Bakelite) coated with mouse fibronectin (Telios) in the presence or absence of peptides. After incubation, adherent cells were fixed with glutalaldehyde followed by adding peroxidase-labeled avidin D (Vector). Absorbances at 415 nm developed by the addition of ABTS were measured.

(d) *Platelet aggregation inhibitory activity*. Platelet-rich plasma (PRP) was prepared by centrifugation (900 rpm) of citrated rabbit heart blood. Residual fraction was centrifuged additionally at 2500rpm to prepare platelet-poor plasma (PPP). Aggregation ratio was measured by a platelet aggregometer TE-500 (Erma Optical Works, Ltd., Tokyo, Japan), using as a standard value of PRP for negative control (no aggregation) and that of PPP for positive control (100% aggregation). PRP was aggregated by 10 mg/mL of collagen in the presence or absence of peptides.

(e) *Farnesylation of recombinant Ras protein*. Assays were performed as reported.[14] Radioactivity was measured by liquid scintillation counter on recombinant Ras protein (v-Ki-Ras p21) farnesylated by FTase isolated from bovine brain and [^{3}H]-farnesylpyrophosphate (Amersham) in the presence or absence of peptides.

(f) *Farnesylation of peptide substrate (SPA enzyme kit assay)*. Assays were performed according to the manufacture's manual. Radioactivity was measured using Farnesyltransferase [^{3}H]-Scintillation Proximity Assay kit (Amersham) on biotinylated lamin B peptide (biotinyl-YRASNRSCAIM) farnesylated as above.

Results and Discussion

Selected RGD peptides and FTase-inhibiting peptides in this study are shown in Figure 1. Two typical RGD-peptides, **5a** and **6a**, were derived from the sequence of human fibronectin.[8] FTase inhibiting peptides, **7a** and **8a**, were derived from the C-terminal sequence of human N-Ras.[9]

We have evaluated the biological activity of these RGD-peptides by assaying the inhibitory activity against adhesion of mouse melanoma cell B16-F10 to mouse fibronectin. Peptide **5a** inhibited the cell adhesion in IC_{50} of 1.1 mM while **5b**, which coupled with the cyclic peptide **2**, in IC_{50} of 0.29 mM, that is, the inhibitory activity of **5a** has been improved about 4-fold by coupling with the cyclic peptide **2**. Peptide **5b** also showed about four times higher inhibitory activity against the collagen-stimulated aggregation of isolated rabbit platelet than **5a** (Table 1). Coupling of **2** to **5a** has also resulted to improve its stability against tryptic degradation as shown in Figure 2a. A half of **5a** was degraded within 2 h while about 60% of **5b** was remained as intact form even after 9 h in a condition that enzyme/substrate ratio was 1/20 (w/w) at 37 °C. By coupling of **2** to **6a**, cell adhesion–inhibitory activity has improved only slightly (IC_{50} of 1.0 mM for **6a** and 0.8 mM for **6b**), while the stability against proteolysis by chymotrypsin has improved remarkably (Fig. 2b). A half of **6a** was degraded within an hour while about 95% of **6b** was remained as intact form even after 6 h in a condition that enzyme/substrate ratio was 1/10 (w/w) at 37 °C. On the other hand, stability of **6b** against trypsin was not improved compared with **6a** (data not shown). The cleavage site of these peptides by trypsin should be the C-terminal of Arg residue in the RGD part, which is the active center for the binding to integrins. Stabilization of the three dimensional structure around RGD sequence is considered to improve not only the biological activity but also the resistance to trypsin. In the case of **5b**, three-dimensional structure around RGD sequence might be stabilized as a desirable form by the cyclic peptide **2**, while **6b** could not be. In the case of **6b**, stabilized part might be the part around Tyr residue, the

Table 1. Inhibitory activity of peptides **5a** and **5b** against platelet aggregation (inhibitory ratio in %)

Peptide/Conc.	0.1 mM	0.3 mM	1.0 mM
5a	—	20.3	61.2
5b	28.3	78.4	92.1

second residue of **6a**, which is supposed to be the cleavage site by chymotrypsin. That might resulted to improve stabilization against chymotrypsin degradation.

The in vitro activities of the peptides derived from the C-terminal sequences of human N-Ras were evaluated by assaying the inhibitory activity against farnesylation of v-Ki-Ras protein by isolated bovine FTase.[14] Peptide **7b** showed three times higher inhibitory activity than **7a**, IC_{50} of 0.56 and 1.6 μM, respectively, while **8b** showed slightly improved activity (data not shown). Peptide **7b** also showed three times higher activity than **7a** in SPA enzyme kit assay using a peptide substrate, IC_{50} of 0.42 and 1.2 μM, respectively. We chose prolylendopeptidase to evaluate the stability of these peptides against proteolysis because they contain Pro residue in their sequences but no cleavage site by trypsin or chymotrypsin. Stabilities against prolylendopeptidase cleavage were remarkably improved by coupling the cyclic peptide both to **7a** (Fig. 3a) and to **8a** (Fig. 3b). In a condition that enzyme/substrate ratio was 1/500 (w/w) at 37 °C, a half of **7a** was degraded within an hour while approximately 60% of **7b** was remained as intact form even after 7 h. In the same condition, 12% of **8a** while 63% of **8b** was remained after an hour. Because of the positions of Pro residues franking to the farnesylated Cys residues, stabilization of their conformation is considered to bring improvements not only of their inhibitory activity but also their stability against proteolysis by prolylendopeptidase.

The fact that coupling of the cyclic peptide **2** to some biologically active peptides, such as endothelins, RGD peptides and farnesyltransferase inhibitors, resulted to improve their biological activity and/or stability against proteolysis indicates the existence of some interactions between the cyclic peptide moiety and the peptide chain in its C-terminal. By the interactions, the three dimensional structure of each peptide is considered to be stabilized in a certain conformation. In some cases like

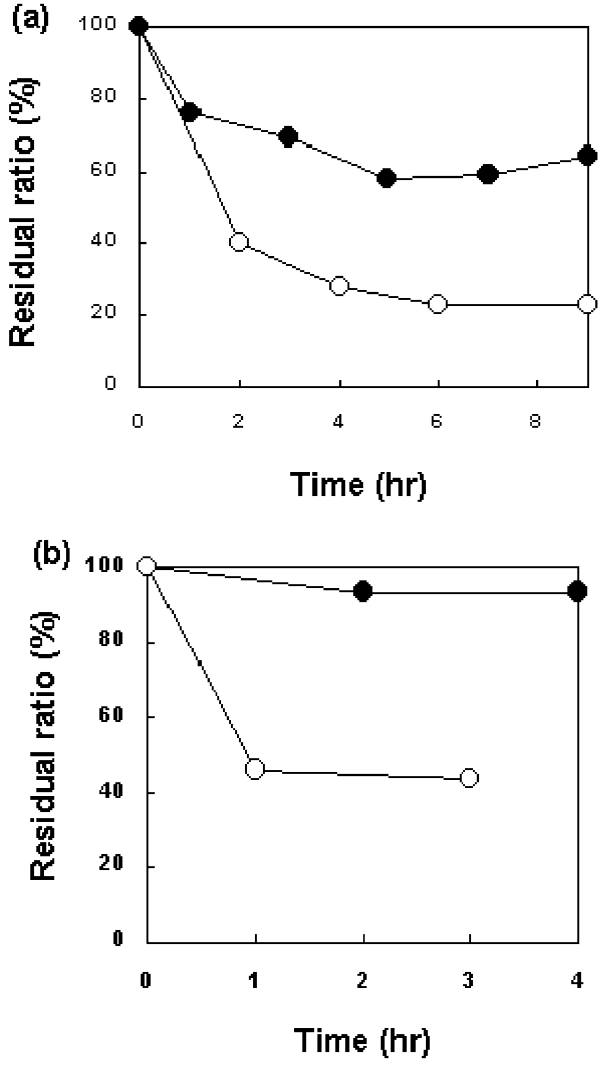

Figure 2. Time course of degradations of RGD peptides by proteases at 37 °C. (a) **5a** (open circle) and **5b** (closed circle) by trypsin in enzyme/substrate ratio of 1/20 (w/w); (b) **6a** (open circle) and **6b** (closed circle) by chymotrypsin in enzyme/substrate ratio of 1/10 (w/w). Representative data of triplicate experiments are shown.

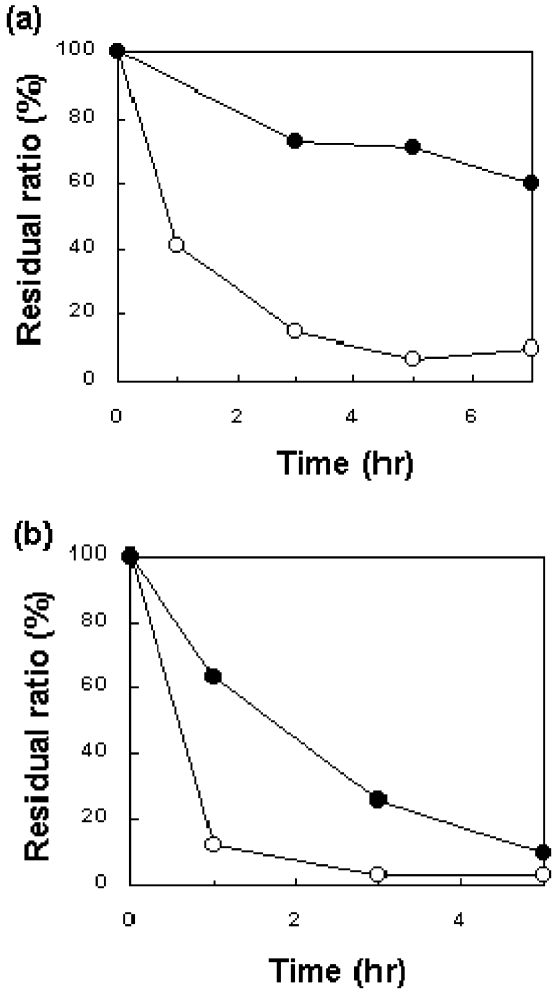

Figure 3. Time course of degradations of FTase-inhibiting peptides by prolylendopeptidase at 37 °C in enzyme/substrate ratio of 1/500 (w/w). (a) **7a** (open circle) and **7b** (closed circle); (b) **8a** (open circle) and **8b** (closed circle). Representative data of triplicate experiments are shown.

peptides **5a** and **7a**, of which biological activities have been improved 3- or 4-fold, stabilized structures by the cyclic peptide moiety were supposed to be suitable for binding to their target molecules. On the other hand, in the case like peptides **6a** and **8a**, of which biological activities have been only slightly improved, although conformational stabilizations have probably been achieved because their stabilities against proteolytic degradation have been improved clearly, their structure might not be so suitable for binding to their targets. If the precise character of interactions between the cyclic moiety and C-terminal peptide chains is revealed, we will be able to design the peptide lengths or sequences suitable for coupling with the cyclic peptide in order to improve the activity and/or stability against proteolytic degradation. In addition, it may become possible to design a small chemical unit, in spite of this cyclic peptide, which can stabilize the structures of bioactive peptides. Structural analyses of some peptides are now under investigation to observe the direct interaction between the cyclic peptide and the tail part.

Results obtained here are supposed to indicate the validity of our prediction; the cyclic peptide can stabilize the solution conformations of the peptides coupled to its C-terminus. To improve stability of a biologically active peptide is one of the key issues to convert it into a drug.[15] To stabilize the conformation of a peptide, cyclization of itself has been widely used, and shown to be able to stabilize the solution structures of certain peptide backbones.[15–17] On the other hand, because cyclization confers a structural constraint that reduces conformational flexibility of the peptide backbone, it may cause inactivation of the peptide. In our method, a peptide coupled on the C-terminus of the cyclic peptide can afford to take a certain conformation that the peptide tends to take potentially. Although it is necessary to disclose the character of interactions between the cyclic peptide and the peptides on its C-terminus by conformational analyses, coupling of our cyclic peptide is expected to give a novel and additional approach to stabilize the conformations of biologically active peptides, which results to improve their activity and stability.

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