

Editor-Communicated Paper

Modulation of Procarboxypeptidase R (ProCPR) Activation by Complementary Peptides to Thrombomodulin

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Abstract: We designed complementary peptides (C-peptides) using a novel computer program (MIMETIC), which generates a series of peptides designed to interact with a target peptide sequence. Carboxypeptidase R (CPR) is an unstable basic carboxypeptidase found in fresh serum in addition to carboxypeptidase N (CPN) which is stable. CPR is generated from its precursor form (proCPR) by trypsin-like enzymes, and its activation is mediated by thrombin generated in the coagulation cascade. The efficiency of activation is enhanced approximately 1,200-fold when thrombin (T) is bound to thrombomodulin (TM). We attempted to generate C-peptides which recognize the T-binding site within TM assuming that some of these might interfere with the generation of T and TM complexes (T-TM). Among three peptides designed, two inhibited the enhancement in activation of proCPR by T in the presence of TM. One of the peptides at 16 μM reduced the activation of proCPR to the level obtained by T alone.

Key words: Carboxypeptidase R, Thrombin-thrombomodulin complex, Fibrinolysis, Complementary peptide

We designed complementary peptides (C-peptides) using a novel computer program (MIMETIC), which generates a series of peptides for interaction with a target peptide sequence (3). The genetic algorithm employed ranks the sequence obtained from one generation to the next by “goodness of fit” to the target.

Carboxypeptidase R (CPR) (5, 7, 11) plays two important roles, one of which appears to be control of the inflammatory response by inactivation of anaphylatoxins such as complement-derived C3a and C5a (4), and the other which inhibits fibrinolysis by cleaving C-terminal lysine residues from partially degraded fibrin, thereby attenuating tissue-type plasminogen activator (tPA)-mediated fibrinolysis (5, 6).

Carboxypeptidase N (CPN) is present in the active form in plasma, whereas CPR is generated from its precursor form (proCPR) by trypsin-like enzymes (7, 17)

such as thrombin (T). ProCPR can be also activated by elastase from leukocytes (12).

Recently, it was found that the efficiency of proCPR activation by T is enhanced approximately 1,200-fold following binding of T with thrombomodulin (TM) (1, 8).

Therefore, we attempted to generate C-peptides which recognize the T-binding site within TM expecting that some of these might interfere with the enhancement of T mediated proCPR activation by TM.

TM consists of a lectin-like amino-terminal domain, followed by a hydrophobic segment, six contiguous epidermal growth factor (EGF)-like domains (TME1-6), an *O*-glycosylated serin/threonine-rich domain, a transmembrane segment and a short cytoplasmic tail (9).

Abbreviations: C-peptide, complementary peptide; CPN, carboxypeptidase N; CPR, carboxypeptidase R; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; PPACK, Phe-Pro-Arg-chloromethylketone; T, thrombin; TAFI, thrombin-activatable fibrinolysis inhibitor; TM, thrombomodulin; TME, thrombomodulin epidermal growth factor-like domains; tPA, tissue-type plasminogen activator; T-TM, thrombin and thrombomodulin complex.

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Table 1. Target peptides in TM and generated complementary peptides

Target peptide: TME5 with sequence of Glu408-Glu426	
408ECPEGYILDDGFICTDIDE426	
Complementary peptide sequence	Molecular weight
TMT-1: NH ₂ -FAHISMHQFVSNPSISLS-OH	2,130.43
TMT-2: NH ₂ -LAKISFDKFTVKQTTVEIS-OH	2,155.54
TMT-3: NH ₂ -FMWESIYKHVSIYQSVDEL-OH	2,374.72

TME5 and part of TME6 bind to a cluster of lysine and arginine residues distant from the T active site (termed anion-binding exosite-I) (2, 10, 15, 16). Macromolecular substrates and inhibitors require an intact anion-binding exosite-I for T recognition and complex formation (2). Occupation of this region by TME56 prevents binding of procoagulant substrates (for example, fibrinogen, factor V, or protease-activated receptors), impairing their cleavage by TM bound T (9).

The minimal T-binding region of TM as the most acidic loop of TME5 with sequence of Glu408-Glu426 has been reported (18). Therefore we used Glu408-Glu426 as the target sequence.

Materials and Methods

Design of complementary peptides. We used the evolutionary software program MIMETIC (3) to design C-peptides sequences for interaction with Glu408-Glu426 from TME5. The genetic algorithm employed ranks the sequence obtained from one generation to the next by "goodness of fit" to the target.

The amino acid sequences of three peptides with the highest score were TMT-1, TMT-2 and TMT-3 (Table 1). These were synthesized by BIO-SYNTHESIS, Inc. (Lewisville, Tex., U.S.A.) for evaluation *in vitro*.

Blood sampling. Blood samples were obtained from healthy volunteers following informed consent by venipuncture from the antecubital vein into 3.8% sodium citrate. To obtain plasma, the samples were centrifuged at 3,000 rpm for 15 min at 4 C. Plasma samples were stored at -80 C until use.

Materials. Thrombin (T) was from Nihon Pharmaceutical Co., Ltd. (Tokyo). Recombinant human soluble thrombomodulin (TM) was a generous gift from Asahi Kasei Corporation (Tokyo). Phe-Pro-Arg-chloromethylketone (PPACK) was from Calbiochem (San Diego, Calif., U.S.A.). Hippuryl-L-arginine was from the Peptide Institute Inc. (Osaka, Japan), cyanuric chloride was from Tokyo Kasei Kogyo Co., Ltd. (Tokyo), dimethyl sulfoxide (DMSO) and 1,4 dioxane were from Wako (Tokyo).

Activation of proCPR by thrombin-thrombomodulin complex (T-TM). Generation of CPR activity in plasma

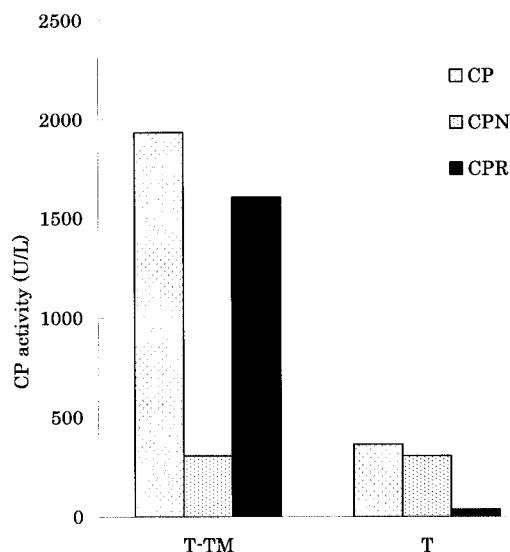


Fig. 1. The effect of TM on activation of proCPR by T in our experimental system. Comparison of the enzyme activity of CPR generated by T with and without TM. CP activity was determined with a colorimetric assay. The values obtained for plasma samples were regarded as CPN activity. The values obtained for plasma samples activated by T and TM were regarded as total CP activity. Each value was compared with those on a hippuric acid standard curve. CPR activity was calculated as the difference between total CP and CPN in plasma.

by T and TM was performed by the method of Komura, et al. (14), which showed that a 1:4 ratio of T and TM achieved maximum activation of proCPR in human, guinea pig, rat and rabbit plasma *in vitro*. Therefore, we used T and TM at a molar ratio of 1:4 throughout these experiments.

A recombinant human soluble TM (2.5 μ l of 0.92 μ M), human T (2.5 μ l of 0.23 μ M) and CaCl₂ (5 μ l of 100 mM) were added to 3.5 μ l of plasma.

To determine the effect of C-peptides on proCPR activation by T with TM, different concentrations of C-peptides were added to the system as follows: Plasma, C-peptide and TM were mixed and preincubated at room temperature for 10 min. T and CaCl₂ were then added. The volume was adjusted to 45 μ l with the peptide solvent buffer (50 mM Tris, 4.0% DMSO, pH 8.0). After incubation at room temperature for 10 min, PPACK (5 μ l of 160 μ M) was added as a specific inhibitor of T to

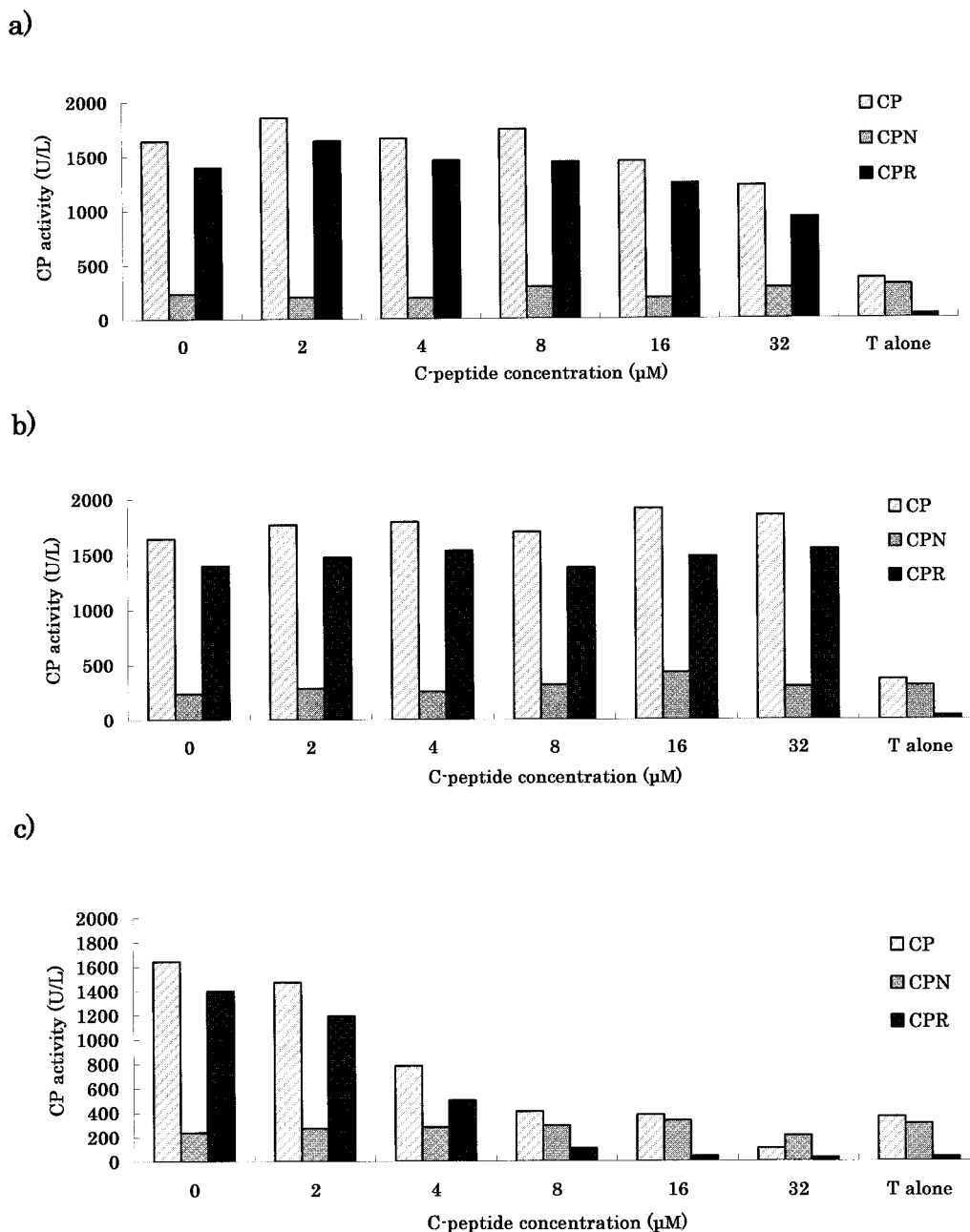


Fig. 2. The effect of C-peptides on activation of proCPR by T-TM. (a): TMT-1, (b): TMT-2, (c): TMT-3. Different concentrations of C-peptides were added to the system as follows: Plasma, C-peptide and TM were mixed and preincubated. T and CaCl_2 were then added.

stop further generation and degradation of CPR. The final 50 μl mixture contained 10% plasma, 46 nM TM, 11.5 nM T, 10 mM CaCl_2 , and 16 μM PPACK, respectively.

Measurement of CP activity. CP activity was determined with a colorimetric assay using hippuryl-L-arginine as a synthetic substrate (13). Briefly, 10 μl of diluted samples in 50 mM Tris with 4.0% DMSO (pH 8.0) and 5 μl of 30 mM hippuryl-L-arginine in 50 mM HEPES (pH 8.2) as the substrate solution were mixed and then incu-

bated at 37 C for 45 min. After incubation, 100 μl of 0.25 M phosphate buffer (pH 8.3) was added to the mixtures and placed on ice. Then 75 μl of 3% cyanuric chloride in 1.4 dioxane were added and mixed well. The mixtures were centrifuged at 5,000 rpm for 10 min to remove denatured protein and precipitates, and 100 μl of the supernatant was transferred to a microtiter plate for determination of absorbance at 405 nm.

The values obtained for plasma samples were regarded as CPN activity. The values obtained for plasma

samples activated by T and TM were regarded as total CP activity.

Each value was compared with those on a hippuric acid standard curve. CPR activity was calculated as the difference between total CP and CPN in plasma.

Results

The Effect of TM on Activation of proCPR by T

To confirm the effect of TM on the activation of proCPR by T in normal plasma, we compared the enzyme activity of CPR generated by T with and without TM.

As shown in Fig. 1, CPR activity generated in the presence of TM was 1,606 U/liter, whereas with T alone was 37.0 U/liter under our experimental conditions.

The Effect of C-Peptides on Activation of proCPR by T and TM

Conversion of proCPR to CPR by T and TM in the presence of C-peptides is shown in Fig. 2. Generation of CPR was strongly inhibited by TMT-3 and weakly inhibited by TMT-1, whereas TMT-2 had no effect.

Compared with CPR generation by T-TM, generation by T alone resulted in conversion of only 2.3% of the total activatable proenzyme as shown in Fig. 1. TMT-3 reduced CPR generation by T and TM dose dependently. Sixteen μM TMT-3 resulted in a reduction in CPR activity down to 2.8% which was almost the same as that obtained using T alone (Fig. 3).

Although reduction of CPR activity was observed by 32 μM and 37.6 μM TMT-1, the activity was decreased to 66.2 % and 62.4 %, respectively (data not shown).

Discussion

CPR was first described as a novel basic carboxypeptidase in fresh serum which functions as an anaphylatoxin inactivator (5, 11). Subsequently, proCPR, the zymogen of CPR was shown to be the same molecule as thrombin-activatable fibrinolysis inhibitor (TAFI) (3). The discovery of CPR as an important link between the inflammatory and fibrinolytic systems prompted the development of new concepts in regulation of the hemostatic system. Although T has the capacity to activate proCPR, the ability of T can be enhanced more than 1,000 fold following complexing with TM (1). In agreement with a previous study (14), T mediated proCPR activation was also significantly enhanced by TM in our experimental system.

Using MIMETIC, we attempted to generate C-peptides that recognize the T-binding site within TM expecting that some of these might interfere with the generation

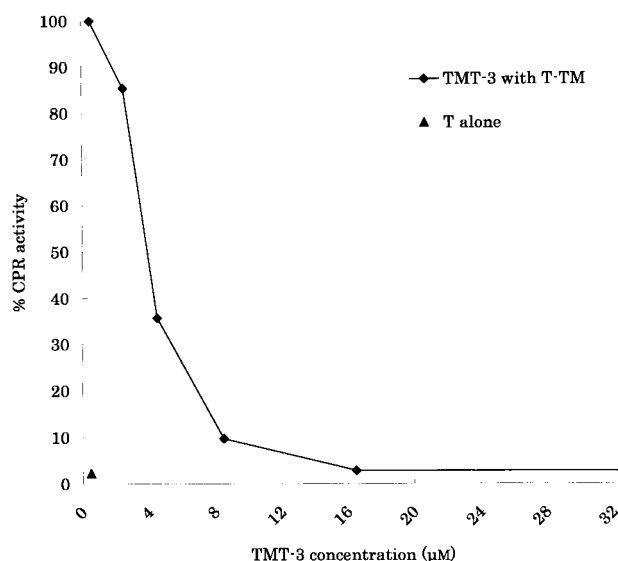


Fig. 3. Rate of CPR activity. Different concentrations of TMT-3 were added to the system. TMT-3 reduced CPR activity to almost the level obtained by T alone.

of T-TM complexes.

As the target sequences of C-peptides, the Glu408-Glu426 peptide in TME5 in TM was used, and three C-peptides with the highest score were synthesized and evaluated *in vitro*.

Two out of three C-peptides, TMT-1 and TMT-3 were able to suppress enhancement by TM in proCPR activation. Although TMT-1 reduced CPR activity to 62.4% even at 37.6 μM , 16 μM TMT-3 resulted in a reduction in CPR activity down to 2.8% which was almost the same as that obtained using T alone.

Although the mechanism responsible for the effect of TMT-3 on TM remains to be characterized, the peptide could contribute to our understanding of the mechanism by which TM enhances T activity in proCPR activation. Although, TMT-3 may be useful for regulation for proCPR activation *in vivo*, the effect of TMT-3 on protein C activation by T-TM in the regulation of coagulation remains an urgent question to be answered.

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