Synthesis of RGDFPASS-containing Cystine Peptides and Isolation of RGD-recognizing Receptor in Sand Dollar Embryo by the Peptide-Affinity Chromatography

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RGDFPASS-containing cystine decapeptides were synthesized by the solution method. These peptides strongly inhibited platelet aggregation. N-Acetyl and C-methoxy peptide ([Phe^5]-FR-1-AM) having amphiphilic structure exhibited especially high activity as a platelet aggregation inhibitor. A 240 kDa RGD-recognizing receptor was obtained from a sand dollar embryo by RGDFPASS-containing peptide-affinity chromatography.

The Arg-Gly-Asp (RGD)-containing peptides inhibit tumor progression,[1] platelet aggregation,[2] and sand dollar embryo genesis.[3] Tripeptide RGD is essential for recognizing cell surface receptor.[4] We attempted to specify the secondary structure of RGDSPASS sequence of fibronectin (FN), a cell adhesion protein, and synthesized RGDSPASS-containing cystine peptides, FR-1 and FR-1 analogs.[5a-c]

H-Cys^1-Arg^2-Gly^3-Asp^4-Ser^5-Pro^6-Ala^7-Ser^8-Ser^9-Cys^{10}-OH

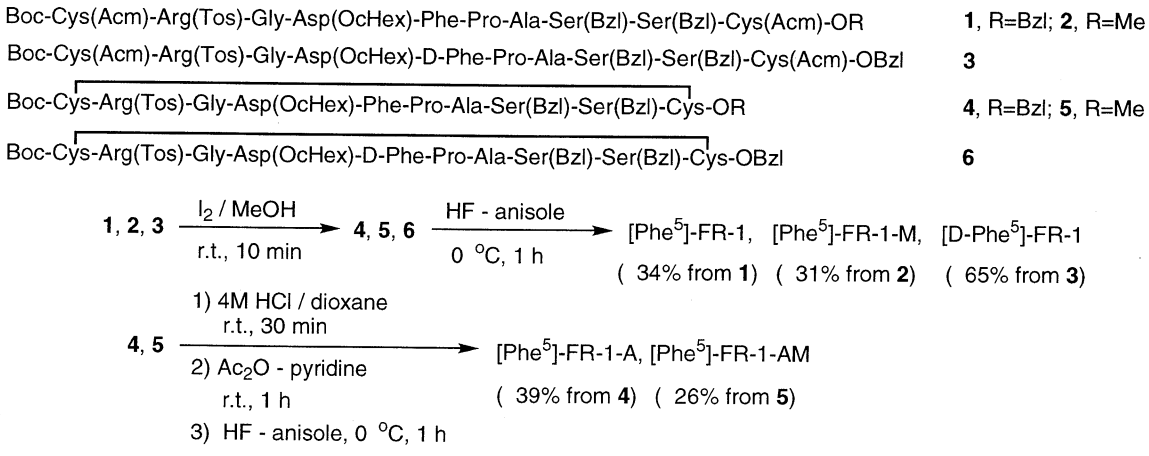
FR-1

From our previous studies,[5c] we expect that the inhibitory activity of [Phe^5]-FR-1 for platelet aggregation would be high, while that of [D-Phe^5]-FR-1 would be low. In order to investigate the relationship between the biological activity and the hydrophobicity of [Phe^5]-FR-1, we placed Ac and/or MeO at the N- and/or C-termini in the [Phe^5]-FR-1. We describe the synthesis and biological activity of [Phe^5]-FR-1 and its derivatives. We also discuss the relationship between amphiphilic structure and biological activity of RGDFPASS-containing cystine peptide. In addition, we successfully isolated RGD-recognizing receptor in the sand dollar embryo.

Decapeptides **1**, **2**, and **3** were synthesized by the solution method in the same manner as with FR-1.[5a] The FABMS data of **1**, **2**, and **3** were as follows, 1790 $(M+H)^+$, 1714 $(M+H)^+$, and 1790 $(M+H)^+$, respectively. The formation of an intramolecular disulfide bond was achieved by I_2 oxidation in high dilution ($1x10^{-3}$ M) for 10 min at room temperature (Scheme 1). The cyclization yields of **4**, **5**, and **6** were 75, 55, and 87%, respectively. Cyclic peptides **4**, **5**, and **6** were confirmed by FABMS: 1646 $(M+H)^+$, 1592 $(M+Na)^+$, and 1646 $(M+H)^+$, respectively. Protected peptides **4**, **5**, and **6** were treated with liq. HF (1 h, 0 °C) to obtain [Phe^5]-FR-1, [Phe^5]-FR-1-M, and [D-Phe^5]-FR-1, respectively (Scheme 1). On the other hand, cyclic peptides **4** and **5** were treated with 4M HCl/dioxane (30 min, room temperature) for deprotection of the Boc group. These free N-terminal peptides were treated with Ac_2O and pyridine (1 h, room temperature) and were treated with liq. HF (1 h, 0 °C) for deprotection (Scheme 1). The final products[6] were purified by HPLC. Peptide **6** was prepared in a remarkably high yield (87%). The high yield reflects that the side chains of D-Phe^5 and Pro^6 do not interact with each other and the turn structure of [D-Phe^5]-FR-1 is rigid.

RGD-containing peptides bind to fibrinogen receptor on the platelet surface and inhibit the platelet aggregation.[7] Using human platelet rich plasma, the IC_{50} of FR-1, [Phe^5]-FR-1, [Phe^5]-FR-1-A (A: N-terminal acetyl), [Phe^5]-FR-1-M (M: C-terminal methyl ester), [Phe^5]-FR-1-AM, and [D-Phe^5]-FR-1 were found to be 7.6, 2.5, 0.74, 1.8, 0.23, and 515 μM, respectively. The binding potency of [Phe^5]-FR-1 to the fibrinogen receptor was stronger than those of FR-1 and [D-Phe^5]-FR-1. These results show that the side chains' repulsion between AA^5 and Pro^6 (i+1 and i+2 positions in the turn) is important for binding to the receptor. Hydrophobic [Phe^5]-FR-1-AM exhibited the highest activity as a platelet aggregation inhibitor in our cyclic peptides.

Previously, we found that dansyl-labeled FR-1 specifically



Scheme 1.

bound to the basal surface of the ectoderm and to the cytoplasm of primary mesenchyme cell in embryo of the sand dollar, *Clypeaster japonicus*.[8] This indicates that RGD-recognizing receptor (FR-1 receptor) is present in the sand dollar embryo.

We attempted to isolate this receptor in the sand dollar embryo. An affinity matrix was prepared by coupling between activated CH-Sepharose 4B (Pharmacia Inc.) and [Phe^5]-FR-1 by the method described by Izzo and Gantt.[9] The solution of the sand dollar embryo lysate was loaded to the peptide-affinity column for isolation of FR-1 receptor. Specific elution of FR-1 receptor was performed by RGDS peptide solution.

Despite the presence of numerous protein bands separated from mesenchyme blastula lysate (Figure 1 lane 1), FR-1-affinity column has successfully separated a single band at 240 kDa region in 10% SDS-PAGE gel under non-reducing condition (Figure 1 lane 2) and 57 kDa region under reducing condition (treated with 2-mercaptoethanol) (Figure 1 lane 3). This suggests that FR-1 receptor is a tetrameric protein. Lane 4 in figure 1 shows that FR-1 receptor preserved its binding potency after affinity purification. On the other hand, FR-1 receptor was not obtained efficiently by the use of affinity chromatography with [D-Phe^5]-FR-1. This result indicates that the side chains' repulsion between AA^5 and AA^6 in cyclic decapeptide is also important for binding to FR-1 receptor.

The results of the inhibitory activity suggest that the more hydrophobicity the cyclic peptide had, the higher its affinity was to the fibrinogen receptor on the platelet surface. [Phe^5]-FR-1-AM had hydrophilic groups (the side chains of Ser, Asp, and Arg) and hydrophobic groups (the side chains of Pro and Phe, and C-terminal Me and N-terminal Ac). We predicted that this amphiphilic structure is important for binding to fibrinorgen receptor on the cell surface.

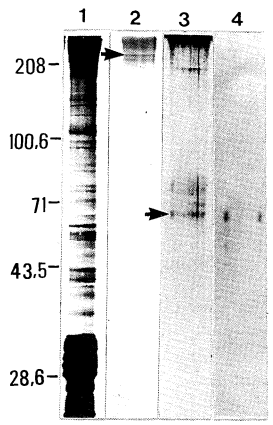


Figure 1. Affinity purification of FR-1 receptor. Silver stained 10% SDS-PAGE gel analysis of whole embryo lysate (lane 1), affinity-purified FR-1 receptor under non-reducing condition (lane 2 arrow), and reducing condition (lane 3). FR-1 receptor blotted to nitrocellulose filter bound to horseradish peroxidase-conjugated FR-1. This lane was visualized by 0.5 mg/ml DAB (lane 4).

The present receptor's relative molecular mass indicates that FR-1 receptor is not integrin β subunit, a known 90-110 kDa RGDS peptide receptor.[10] However, further thorough studies ought to be done to specify molecular properties of FR-1 receptor.

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References and Notes

Abbreviations: Acm, acetamidomethyl; Boc, *t*-butoxycarbonyl; Bzl, benzyl; cHex, cyclohexyl; DAB, 3, 3'-diaminobenzidine; Tos, *p*-toluenesulfonyl.

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6 **[Phe^5]-FR-1**:FABMS m/z 1040 $(M+H)^+$; Amino acid analysis(theoretical): Asp 1.00(1), Ser 1.78(2), Gly 0.98(1), Ala 1.00(1), $(Cys)_2$ 0.91(1), Phe 1.00(1), Arg 1.02(1), Pro 0.98(1); Found: C, 39.68; H, 4.42; N, 11.94%. Calcd for $C_{41}H_{61}N_{13}O_{15}S_2 \cdot 3TFA \cdot 3H_2O$: C, 39.28; H, 4.87; N, 12.67%. **[Phe^5]-FR-1-A**: FABMS m/z 1082 $(M+H)^+$; Found: C, 41.03; H, 4.41; N, 11.94%. Calcd for $C_{43}H_{63}N_{13}O_{15}S_2 \cdot 3TFA \cdot 1.5H_2O$: C, 40.52; H, 4.41; N, 12.54%. **[Phe^5]-FR-1-M**: FABMS m/z 1054 $(M+H)^+$; Amino acid analysis (theoretical): Asp 0.98(1), Ser 1.71(2), Gly 0.96(1), Ala 1.00(1), $(Cys)_2$ 0.70 (1), Phe 0.96(1), Arg 0.87(1), Pro 0.93(1); Found: C, 39.92; H, 4.56; N, 12.00%. Calcd for $C_{42}H_{63}N_{13}O_{15}S_2 \cdot 3TFA \cdot 3.5H_2O$: C, 39.51; H, 5.04; N, 12.00%. **[Phe^5]-FR-1-AM**: FABMS m/z 1096 $(M+H)^+$; Found: C, 40.76; H, 5.49; N, 12.87%. Calcd for $C_{44}H_{65}N_{13}O_{15}S_2 \cdot 2TFA \cdot 5H_2O$: C, 30.57; H, 4.83; N, 12.49%. **[D-Phe^5]-FR-1**: FABMS m/z 1040 $(M+H)^+$; Amino acid analysis (theoretical): Asp 1.01(1), Ser 1.85 (2), Gly 1.00(1), Ala 1.00(1), $(Cys)_2$ 0.68(1), Phe 1.01(1), Arg 0.97(1), Pro 0.99 (1); Found: C, 38.54; H, 4.96; N, 12.60%. Calcd for $C_{41}H_{61}N_{13}O_{15}S_2 \cdot 3TFA \cdot 4.5H_2O$: C, 38.58; H, 5.03; N, 12.44%.
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