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DIFFERENTIAL ROLES OF TWO CONSECUTIVE PHENYLALANINE RESIDUES IN THROMBIN RECEPTOR-TETHERED LIGAND PEPTIDES (SFFLRNP) IN THROMBIN RECEPTOR ACTIVATION

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SUMMARY: A synthetic heptapeptide H-Ser-Phe-Phe-Leu-Arg-Asn-Pro-NH_2, which corresponds to the ligand peptide latent in rodent thrombin receptors, was able to activate the thrombin receptor with no thrombin. In order to evaluate the structural requisites of two consecutive phenylalanines, three sets of analogs with substitutions at position either 2 or 3 were synthesized and examined for their stimulatory activity in phosphoinositide turnover in SH-EP epithelial-like cells. The replacement of Phe-2 by Ala completely eliminated the activity, while that of Phe-3 retained about 50% activity with a full stimulation. The Phe/Leu substitution resulted in a large increase (37-fold) in EC_{50} value for Phe-2, but in insignificant change for Phe-3. Substitution of *para*-fluorophenylalanine ((*p*-F)Phe) for Phe-2 enhanced strongly (4-fold) the activity, in contrast to a reduction by the Phe-3/(*p*-F)Phe substitution. Elimination of either Phe-2 or Phe-3 resulted in a complete loss of activity. These results indicated that Phe-2 and Phe-3 play different roles in the receptor activation. A highly specific aromatic π-π interaction was suggested between Phe-2-phenyl and thrombin receptor binding site, while Phe-3 appeared to be important for retaining a bioactive conformation.

A serine proteinase thrombin is able to evoke various biological responses from a variety of cells (1, 2). These responses of thrombin are mediated by G-protein-coupling receptors on the cell surface (3). In recent years, functional thrombin receptors from cells such as rat vascular smooth muscle cell, hamster lung fibroblasts, and human endothelial cell have been cloned, and their amino acid sequences were deduced from cDNAs (4-6). These

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Abbreviations: The abbreviations according to biochemical nomenclature by IUPAC-IUB Joint Commission, *Eur. J. Biochem.*, **138**, 9-37 (1984), are used throughout. Additional abbreviations are as follows: Boc, *t*-butoxycarbonyl; EC_{50}, the half-maximal effective concentration; HPLC, high-performance liquid chromatography; (*p*-F)Phe, *para*-fluorophenylalanine; PI, phosphoinositide and; SFFLRNP, amino acid sequence denoted by the one-letter amino acid code for Ser-Phe-Phe-Leu-Arg-Asn-Pro (all other peptides were also shown in a similar way).

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thrombin receptors have a very high sequence similarity and are a member of the seven-transmembrane domain receptors.

It has been clarified that thrombin receptors have a novel mechanism for their activation (4-6). Thrombin binds to its receptors at the long N-terminal extracellular extension moiety and cleaves the peptide bond between Arg-41 and Ser-42. The newly exposed N-terminal moiety starting from Ser-42 acts as a ligand tethered to the receptor to activate the receptor by itself. It was found that exogenously added synthetic peptides corresponding to this ligand moiety (number of residues: n = 5 ~ 17) *per se* can activate the thrombin receptor without thrombin (4, 7–13). The minimal size of peptide length to induce various cellular responses has been found to be n = 5 ~ 7, depending upon the types of cells used for assays.

The structural essentials important for receptor activation can be defined by evaluating the abilities of synthetic peptides with various chemical modifications and amino acid substitutions. For instance, the crucial importance of the N-terminal amino group was found by acetylation of ligand peptides (10-12, 14). H-Ser-Phe-Phe-Leu-Arg-Asn-Pro-NH_2 (**1**, SFFLRNP by one-letter amino acid code) derived from the rodent thrombin receptors (Fig. 1) possesses two phenylalanines at two consecutive positions of 2 and 3. Such a series of Phe residues are seen in some biologically active peptides such as neurokinin B (15), somatostatin (16) and substance P (17), and appear to play critical roles in the receptor interactions. In the present study, in order to assess the structural importance of each of the Phe-Phe residues, Phe-2 and Phe-3 of SFFLRNP were substituted by Ala (**2**), Leu (**3**) and *para*-fluorophenylalanine ((*p*-F)Phe) (**4**) (Fig. 1). In the same rationale, SFLRNP (**5**) lacking Phe-2 or Phe-3 was also synthesized. We have tested these synthetic peptides for their ability to stimulate phosphoinositide (PI)-turnover using epithelial-like SH-EP cells (18) and found that these two Phe residues have different roles in the receptor activation.

MATERIALS AND METHODS

Peptide synthesis: Peptides were synthesized by the manual solid phase synthesis using *t*-Boc-amino acids. To obtain C-terminal amide peptides Boc-Pro-MBHA resin was utilized. Peptides synthesized are SFFLRNP (**1**), S/Ala/FLRNP (**2**(Ala-2)), SF/Ala/LRNP (**2** '(Ala-3)), S/Leu/FLRNP (**3**(Leu-2)), SF/Leu/LRNP (**3** '(Leu-3)), S/(*p*-F)Phe/FLRNP (**4**((*p*-F)Phe-2)), SF/(*p*-F)Phe/LRNP (**4** '((*p*-F)Phe-3)) and SFLRNP (**5**). Coupling reactions were carried out with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (19) in the presence of 1-hydroxybenzotriazole (HOBt) in a mixture of *N*-methylpyrrolidone and *N*,*N*-dimethylformamide (1:2, v/v) for 30 min. Peptides were liberated from the resin by treatment with anhydrous liquid hydrogen fluoride containing 10% *p*-cresol at 0°C for 1 h, and purified by Sephadex G-15 followed by preparative RP-HPLC (Cica-Merck, LiChrospher RP-18 (5 μ): 25 x 250 mm). The purity was verified by analytical RP-HPLC (LiChrospher RP-18 (5 μ): 4.0 x 250 mm) and amino acid analysis. Boc-(*p*-F)Phe-OH was prepared from (*p*-F)Phe which was a generous gift from Asahi Glass Co. (Tokyo).

Stimulatory activity of peptides in PI turnover: Biological activity of synthetic peptides were evaluated in SH-EP cells essentially as reported previously by Ogino and Costa (18). The extent of PI hydrolysis was determined by measuring the accumulation of radio-labeled inositol following incorporation of *myo*-[^{3}H]inositol into cellular phosphoinositides. Briefly, SH-EP cells were first seeded into 24-well culture plates (1-3 x

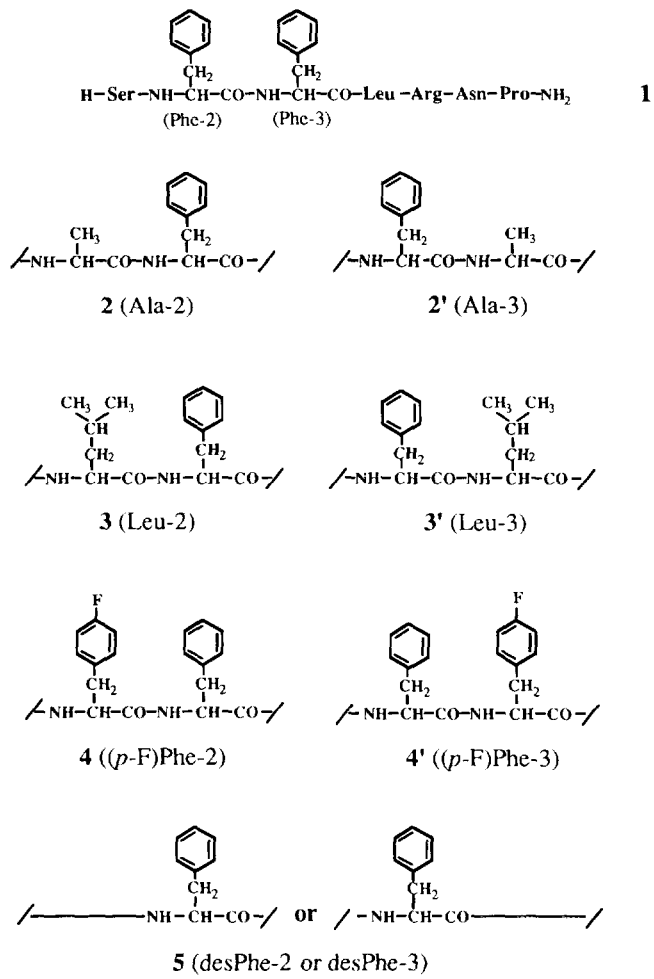

Fig. 1. Structures of the tethered peptide ligand of thrombin receptor, SFFLRNP, and its analogs with amino acid substitutions at position either 2 or 3.

10^4 cells/well) and allowed to grow until about 90% confluent. Cells were then labeled in growth medium containing 1% FCS and 2-4 μCi/ml of *myo*-[^{3}H]inositol (90 *Ci*/mmole; Amersham, Buckinghamshir, England) for 48-72 h. After washing, the cells were exposed to the reaction buffer, which included 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 6 mM Na_2HPO_4, 20 mM Na/HEPES (pH 7.45), 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM EGTA, 11.1 mM glucose, 0.5 mg/ml bovine serum albumin, 10 mM LiCl and test peptides or thrombin (human, 3200 U/mg from Dr. J. W. Fenton, II). Reactions were conducted at 37°C for 30 min and terminated by the addition of ice-cold methanol (1 ml) containing 60 mM HCl. After centrifugation, the reaction mixture was applied onto anion-exchange columns (AG 1 x 8, formate form) to elute mono- and bisphosphates in a single fraction.

Each peptide was assayed 3-5 times and the concentration-response curves were analyzed by the ALLFIT computer program (20). In one assay, for instance, the maximal stimulation was 7,200±150 dpm for thrombin and 7,180±180 dpm for SFFLRNP (**1**) with 290 dpm background. Other active peptides exhibited similar maximal stimulation. As the level of maximal stimulation differed from assay to assay (6,500-11,500 dpm), Fig. 2 was depicted using normalized % activity from repeated assays.

RESULTS AND DISCUSSION

Epithelial-like SH-EP cells are known to respond to thrombin with strong stimulation of PI-turnover. The inositol phosphate species that accumulate are mainly inositol mono- and bisphosphates. The activity of thrombin can be therefore assessed by measuring the total increase of both phosphates when incubated with thrombin at several different concentrations (18). When synthetic heptapeptide SFFLRNP (**1**) was tested for activation of SH-EP cells, it was found that it can elicit the full stimulation of PI-turnover in a dose-dependent manner (the half-maximal effective concentration EC_{50} = 1.32 μM) (Fig. 2A, Table 1). The dose-response curve constructed for peptide **1** was almost parallel to that of thrombin, although their effective concentration ranges were considerably apart from each other. The EC_{50} value

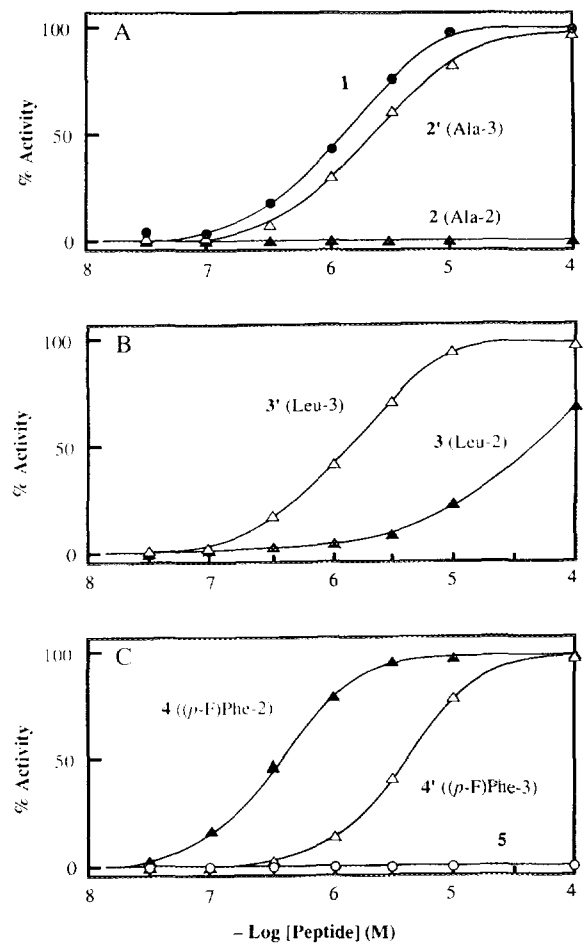

Fig. 2. Dose-response curves of SFFLRNP and its analogs in phosphoinositide turnover assay using epithelial-like SH-EP cells. Effects of substitutions of Phe-2 and Phe-3 by Ala (A), Leu (B), and (*p*-F)Phe (C). SFFLRNP (**1**, ●—●), analogs with substitution at position 2 (▲—▲), analogs with substitution at position 3 (△—△), and SFLRNP (**5**, ○—○).

Table 1. Biological activity of ligand peptide of thrombin receptor and its analogs in phosphoinositide (PI) turnover in SH-EP cells

Peptides		EC_{50} (μM)	Relative potency
SFFLRNP	**1**	1.32 ± 0.16	100
S/Ala/FLRNP	**2** (Ala-2)	inactive	0
SF/Ala/LRNP	**2'**(Ala-3)	2.77 ± 0.39	48
S/Leu/FLRNP	**3** (Leu-2)	49.2 ± 7.83	2.7
SF/Leu/LRNP	**3'**(Leu-3)	1.50 ± 0.14	88
S/(*p*-F)Phe/FLLRNP	**4** ((*p*-F)Phe-2)	0.33 ± 0.57	400
SF/(*p*-F)Phe/LLRNP	**4'**((*p*-F)Phe-3)	4.18 ± 0.36	21
SFLRNP	**5**	inactive	0

of thrombin was approximately 40 pM. The fact that a ligand peptide is tethered to the receptor molecule appears to be responsible for this extremely high potency of thrombin (4).

Through substitutions of phenylalanines in SFFLRNP (**1**) by three different types of amino acids, distinguishable roles of the Phe-2 and Phe-3 residues in receptor activation has become prominent. The Phe/Ala substitution at position 2 was found to eliminate the activity completely. No hydrolysis of phosphoinositides was observed even at a concentration of 100 μM of S/Ala/FLRNP (**2**(Ala-2)) (Fig. 2A). In contrast, the Phe/Ala substitution at position 3, namely peptide SF/Ala/LRNP (**2'**(Ala-3)), elicited a full stimulation of PI-turnover. It sustained almost a half of activity of parent peptide **1** (Table 1). These results imply that the Phe-phenyls at positions 2 and 3 function differently in the activation of thrombin receptors. The phenyl group of Phe-2 is indispensable, whereas that of Phe-3 is not essential.

Inactivity of **2**(Ala-2) is due affirmatively to the removal of the phenyl group at position 2. This prompted us to examine the effect of hydrophobicity and aromaticity of this group on the receptor activation and we have incorporated Leu and (*p*-F)Phe into position 2. As shown in Table 1, drastic differences in activity were found by these modifications. The potency of S/Leu/FLRNP (**3**(Leu-2)) relative to **1** was only 3%, while S/(*p*-F)Phe/FLRNP (**4**((*p*-F)Phe-2)) was four times more active than **1** (Table 1, Figs. 2B and 2C). We also recently found that the substitution of Phe-2 with (*p*-F)Phe enhances several times the activity of ligand derived from human thrombin receptor (21). These results indicate that the aromaticity of Phe-2 phenyl group of SFFLRNP (**1**) is a strict requisite to the ligand-receptor interaction.

When these substitutions were made for Phe-3, the reverse results were obtained (Table 1). SF/Leu/LRNP (**3'**(Leu-3)), which corresponds to the peptide derived from human thrombin receptors, was almost equipotent to **1** (Fig. 2B), while SF/(*p*-F)Phe/LRNP (**4'**((*p*-F)Phe-3)) exhibited considerably diminished activity (21%) (Fig. 2C). These results might indicate that the hydrophobicity of Phe-3 phenyl is important. However, as mentioned above, **2'**(Ala-3) retained 50% of the activity of **1** and thus the hydrophobic character of Phe-3 appeared not to be so important. Indeed, a Gly-3 derivative of **1**, SF/Gly/LRNP, was yet considerably potent (20% activity of **1**) in stimulating the PI turnover and showed a full

receptor activation. These results all together strongly suggested that only the backbone structure of Phe-3 is necessary for interaction of ligand peptide with thrombin receptors. The phenyl group of Phe-3 seems to have no specific binding site. Perhaps, Phe-3 is important in maintaining a bioactive conformation of the ligand peptide when bound to the receptors.

It should be noted that SFLRNP (**5**) was not a stimulant of PI-turnover (Table 1). The structure of SFLRNP represents two types of deletion, namely S–FLRNP lacking Phe-2 and SF–LRNP lacking Phe-3. Inactivity of this SFLRNP further demonstrate the respective importance of Phe-2 and Phe-3 in the activation of thrombin receptors.

The present results showed the structural importance of Phe-2 and Phe-3 for the intrinsic activation of thrombin receptors and indicated their roles different from each other. Substance P, a neuropeptide with eleven amino acids and a ligand specific for NK-1 tachykinin receptor, possesses also the two consecutive Phe residues at positions 7 and 8. It has been clarified that these residues have apparently different functions in receptor interaction; *i.e.*, Phe-7 is critical for the receptor activation (22), while Phe-8 is important to recognize the NK-1 receptor from three similar receptor subtypes (23). These discriminatory functional roles of Phe residues should of course be attributable to the complementary structures of receptors and ensured by structural properties underlying in phenylalanines. In the ligand binding site present in thrombin receptors, a highly specific aromatic portion is expected for Phe-2 to elicit a strong π-π aromatic interaction. Phe-3 seems to occupy a kind of openspace between tight binding sites and may be important to retain a conformation for receptor binding.

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