Different Roles of Two Consecutive Leucine Residues in a Receptor-Tethered Ligand Peptide (SFLLRNP) in Thrombin Receptor Activation

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A synthetic peptide, H–Ser–Phe–Leu–Leu–Arg–Asn–Pro–NH_2, which corresponds to a ligand peptide tethered to a human thrombin receptor, was able to activate the thrombin receptor with no thrombin. In order to inspect the structural requisites of two consecutive leucines (Leu-3 and Leu-4) in receptor activation, two sets of analogs with substitutions at either position 3 or 4 were synthesized and evaluated for their ability to hydrolyze phosphoinositide in human neuroblastoma SH-EP cells. The replacement of Leu-4 by Ala drastically decreased the activity of the parent peptide (only about 4% activity), while that of Leu-3 retained about 50% activity. A similar result was obtained when replaced by Gly. Substitution by Phe for Leu-3 sustained full activity. Although the Leu-4/Phe substitution exhibited a slight reduction in activity, Leu-4/Ile substitution unexpectedly diminished the activity (20%). These results suggested that two consecutive leucines have different roles in receptor activation; i.e., Leu-3 behaves something like a connection of peptide units to construct a bioactive conformation and Leu-4 acts like a structural element essential for interactions with receptors.

In addition to performing a central role in hemostasis and blood coagulation, the serine proteinase thrombin is able to evoke biological responses from a variety of cells, such as platelets, endothelial cells, and fibroblasts.[1] These responses are mediated by the cell-surface thrombin-specific receptor, which was first cloned by Vu et al. in 1991.[2] Based upon the clarified cDNA sequence, the human thrombin receptor was thought to be a member of receptors with seven transmembrane domains. The thrombin receptor was classified into a novel type of receptors with a unique activation mechanism. Thrombin binds to the binding site present at the extracellular N-terminal portion of the receptor, and then cleaves the peptide bond between Arg-41 and Ser-42. The newly exposed N-terminal portion, starting with the sequence of SFLLRNP[3] (one-letter amino acid code), functions as a ligand to activate the receptor. This type of receptor has not been found for any other receptors clarified to date.

Although the ligand is tethered to the receptor, it was found that exogenously added synthetic peptides corresponding to this ligand (number of residues: $n=5$—17) can activate the receptor with no thrombin.[2,4—10] On the basis of this initial finding, studies on the structure-activity relationships of a tethered ligand peptide have been developed in order to discover the structural elements that are important for receptor activation. The minimal size of the peptide length was found to be $n=5$—7, depending upon the types of cells or tissues used for the assays. The several structural essentials for receptor activation were defined. These includes, for instance, the N-terminal Ser-1-amino and Phe-2-phenyl groups.[2,4—6,9—12]

We recently reported that a highly specific aromatic π–π interaction exists between the Phe-2-phenyl group and the receptor binding site. At the adjacent position to this Phe-2, the tethered ligand SFLLRNP possesses two consecutive hydrophobic leucine residues. Such a consecutive Leu–Leu residue is present in some bioactive peptides (for example, galanin,[13] leiurotoxin I,[14] and secretin[15]), though their specific roles in biological responses have not yet been noticed. In the present study, in order to evaluate the structural importance of each Leu residue, Leu-3 and Leu-4 of SFLLRNP were replaced by a series of amino acids, respectively. Synthetic peptides were tested for their ability to activate phospholipase C coupled with phosphoinositide (PI)-turnover using human epithelial-like SH–EP cells. The

results have indicated that two consecutive Leu residues have different roles in receptor activation.

Results and Discussion

Thrombin receptors couple with G-protein(s),[16] and thus the ability of tethered ligand-related peptides to produce the second messengers, would reflect their intrinsic biological activity. In epithelial-like SH-EP cells from human neuroblastoma, thrombin is responsible for stimulating PI-turnover to elicit various cellular responses.[17] We have shown that the SH-EP cells can be used for an activity assessment of SFLLRNP analogs, evaluating the effectiveness in stimulating PI-turnover.[11,12] In this assay thrombin was very potent, and its half-maximal effective concentration (EC_{50}) was estimated to be only 25 pM. As previously reported, synthetic heptapeptide SFLLRNP (native form) elicited a full stimulation of PI-turnover in a dose-dependent manner (Fig. 1a).

When Leu-3 or Leu-4 was substituted by their stereoisomer, the resulting D-Leu-containing analogs were completely devoid of PI-turnover activity (Figs. 1a and 2a). No antagonist activity was observed for these peptides. The L-configuration at positions 3 and 4 is an absolute requisite for the active conformation of SFLLRNP. Similar results have been shown for positions 1 and 2.[11,12] Substitutions by Ala provided a sharp difference in PI-turnover activity between positions 3 and 4. SF/Ala/LRNP exhibited a moderate activity (54%), while SFL/Ala/RNP exhibited only a limited activity. SFL/Ala/RNP did not show a full stimulation of PI-turnover, even at 100 μM (Fig. 2a). The Leu/Gly substitutions turned out similarly. SF/Gly/LRNP showed considerable activity (19%) (Fig. 1a), while SFL/Gly/RNP showed limited activity (Fig. 2a). These results indicate that the leucine at position 4 is much more important than that at position 3. Although the side chain of Leu-3 is not essential for receptor activation, that of Leu-4 is crucial.

Leu/Phe substitution at position 3 sustained full activity. SF/Phe/LRNP exhibited an EC_{50} value of 1.32 μM, which is almost the same as that (1.50 μM) of SFLLRNP. This implied that the aliphatic leucine residue at position 3 can be replaced by aromatic phen-

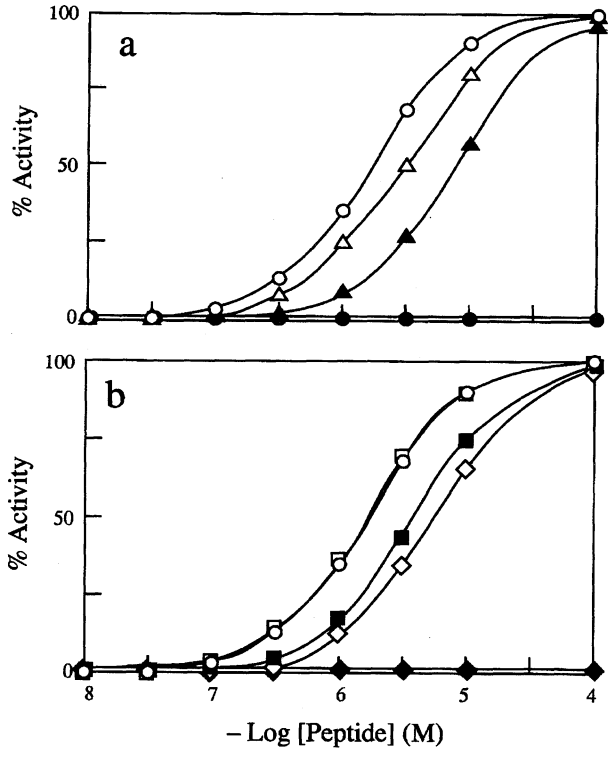

Fig. 1. Concentration dependent curves of ligand peptide of thrombin receptor and its analogs substituted at position 3 (Leu-3) in phosphoinositide turnover in SH-EP cells. a: native form (SFLLRNP, ○—○), D-Leu-3 (SF/D-Leu/LRNP, ●—●), Ala-3 (SF/Ala/LRNP, △—△), and Gly-3 (SF/Gly/LRNP, ▲—▲). b: native form (SFLLRNP, ○—○), Phe-3 (SF/Phe/LRNP, □—□), (*p*-F)Phe-3 (SF/(*p*-F)Phe/LRNP, ■—■), (*p*-Cl)Phe-3 (SF/(*p*-Cl)Phe/LRNP, ◇—◇), and desLeu-3 (SF–LRNP, ◆—◆).

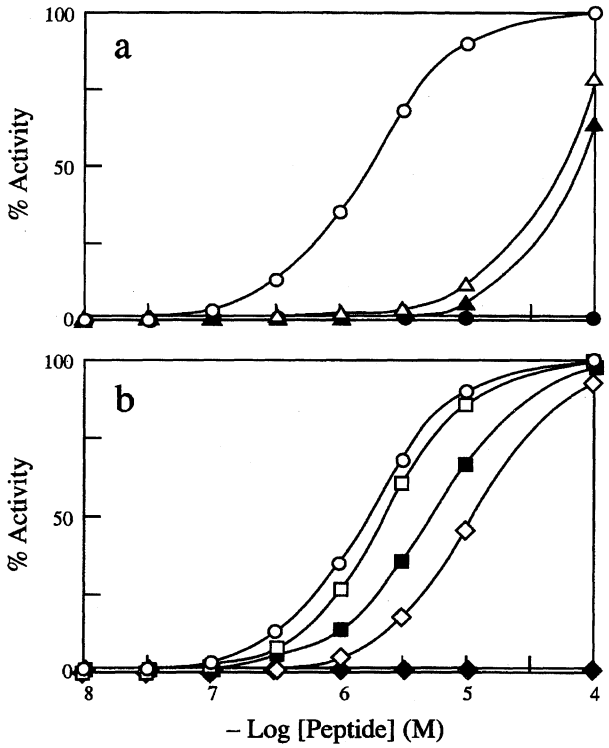

Fig. 2. Concentration dependent curves of ligand peptide of thrombin receptor and its analogs substituted at position 4 (Leu-4) in phosphoinositide turnover in SH-EP cells. a: native form (SFLLRNP, ○—○), D-Leu-4 (SFL/D-Leu/RNP, ●—●), Ala-4 (SFL/Ala/RNP, △—△), and Gly-4 (SFL/Gly/RNP, ▲—▲). b: native form (SFLLRNP, ○—○), Phe-4 (SFL/Phe/RNP, □—□), Ile-4 (SFL/Ile/RNP, ■—■), Val-4 (SFL/Val/RNP, ◇—◇), and desLeu-4 (SFL–RNP, ◆—◆).

ylalanine. However, SF/(*p*-F)Phe/LRNP and SF/(*p*-Cl)Phe/LRNP, in which Leu-3 was replaced by *para*-fluoro- or chlorophenylalanine, did not enhance the activity at all; the EC_{50} values were 4.18 and 6.46 μM for SF/(*p*-F)Phe/LRNP and SF/(*p*-Cl)Phe/LRNP, respectively (Fig. 1b). These results are in great contrast to the considerable activity enhancement (5-fold) observed for S/(*p*-F)Phe/LLRNP. Even S/(*p*-Cl)Phe/LLRNP retained the full activity of SFLLRNP (unpublished data). Although the aromaticity of Phe-2 is essential for receptor activation,[12] it is apparently disadvantageous at position 3. Collectively, at position 3 the amino acid backbone in the L-configuration appears to be most important.

The substitution of leucine by phenylalanine at position 4 caused a slight reduction in activity (Table 1). Thus, Leu/Phe substitutions at both positions 3 and 4 resulted in almost no change in receptor activation. This suggests that the receptor sites adopting Leu–Leu residues can tolerate a structural change from the isobutyl (Leu) to the benzyl (Phe) groups. It is apparent that the site for Leu-3 is not essential for receptor activation, since Ala-3- and Gly-3-derivatives sufficiently retain the activity. However, as a consequence of a drastic activity drop of Ala-4- and Gly-4-derivatives, the hydrophobic receptor site for Leu-4 is essential.

On the other hand, when Leu-4 was replaced by Ile, this substitution was found to considerably decrease the activity ($EC_{50}=7.34$ μM, 20% activity of SFLLRNP) (Fig. 2b). A similar result has been reported for hexapeptide SFLLRN.[18] The analog SFL/Ile/RN elicited only 10% activity of its parent SFLLRN in PI-turnover. We further found that Leu/Val substitution at the same position further diminished the activity (10% of SFLLRNP) (Fig. 2b). These results indicate that the thrombin receptor has a quite strict structural requirement for binding Leu-4 in SFLLRNP. The isobutyl group in the L-configuration seems to be an absolute requisite.

SFLRNP represents the derivatives of desLeu-3 (SF–LRNP) and/or desLeu-4 (SFL–RNP). This analog did not in the least stimulate and antagonize the PI-turnover (Figs. 1b and 2b). The inactivity of SFLRNP demonstrates the importance of both Leu-3 and Leu-4 in the activation of the thrombin receptor.

Hui et al. has reported that N-terminal tetrapeptide SFLL is almost one-hundred times less active than pentapeptide SFLLR in platelet aggregation.[8] The N-terminal SF is essential (as described above). In this study, it was found that the desLeu derivative SFLRNP is inactive. All of these results indicate that, in addition to N-terminal Ser–Phe segment, the C-terminal Leu–Arg segment is important; also, Leu-3 is important as their connecting unit. In conclusion, two consecutive leucines (Leu-3 and Leu-4) have different roles in receptor activation.

Experimental

Peptide Synthesis. Analogs of SFLLRNP were synthesized by the manual solid-phase synthesis method. All of the amino acids were protected at their amino group with a Boc group, and the side-chain-protecting groups were benzyl for Ser and *p*-tolylsulfonyl (Tos) for Arg. Boc–(*p*-F)Phe–OH was prepared from (*p*-F)Phe, which was a generous gift from Asahi Glass Co. (Tokyo). Boc–(*p*–Cl)Phe–OH was purchased from Watanabe Cheminal Ind. (Hiroshima). To obtain C-terminal peptide amides, the Boc–Pro–MBHA resin was utilized. The synthesized peptides were SF/D-Leu/LRNP, SF/Ala/LRNP, SF/Gly/LRNP, SF/Phe/LRNP, SF/(*p*-F)Phe/LRNP, SF/(*p*- Cl)Phe/LRNP, SFL/D- Leu/RNP, SFL/Ala/RNP, SFL/Gly/RNP, SFL/Phe/RNP, SFL/Ile/RNP, SFL/Val/RNP, and SFLRNP. Coupling reactions were carried out by using HBTU–HOBt[19] in a mixture of *N*-methylpyrrolidone and *N*,*N*-dimethylformamide (1 : 2, v/v) for 30 min.

Peptides were liberated from the resin by treatments with anhydrous HF containing 10% *p*-cresol at 0 °C for 1 h, and purified by Sephadex G-15 (1.0×100 cm) eluted with 30% AcOH. Peptides were further purified by a preparative reversed-phase HPLC (Cica-Merck, LiChrospher RP-18 (5 μm): 25×250 mm). The elution conditions employed were as follows: Solvent system, 0.1% aqueous trifluoroacetic acid (A solution) and acetonitrile containing 20% A solution (B solution); flow rate 6 ml min^{-1}; temperature, 25 °C; and UV detection, 225 nm. Elution was performed with a lin-

Table 1. Biological Activity of Ligand Peptide of Thrombin Receptor and Its Analogs in Phosphoinositide (PI)-Turnover in SH–EP Cells

Substituents	PI-turnover activity			
	Substitution at position 3		Substitution at position 4	
	EC_{50} μM	Relative potency	EC_{50} μM	Relative potency
Leu (native)	1.50±0.14	100	1.50±0.14	100
D-Leu	Inactive	0	Inactive	0
Ala	2.77±0.39	54	40.4±13.3	3.7
Gly	8.08±0.86	19	56.3±11.6	2.7
Phe	1.32±0.16	110	1.94±0.30	77
desLeu	Inactive	0	Inactive	0

ear concentration gradient of B solution (40—60%) for 30 min. The purity was verified by analytical reversed-phase HPLC (LiChrospher RP-18 (5 μm): 4.0×250 mm), using the same conditions, except for a flow rate of 0.6 ml min^{-1}. For amino acid analysis, the hydrolysis of peptide samples was carried out in constant-boiling hydrochloric acid (110 °C, 24 h). The amino acid analyses were carried out on a Hitachi (model 835) amino acid analyzer. The purity of the synthesized peptides was thus verified by analytical HPLC and an amino acid analysis.

Biological Assay. The biological activity of synthetic peptides was evaluated in SH-EP cells, essentially as previously reported by Ogino and Costa.[17] The extent of PI hydrolysis was determined by measuring the accumulation of radio-labeled inositol following the incorporation of *myo*-[^{3}H]inositol into cellular phosphoinositides. Briefly, SH-EP cells were first seeded into 24-well culture plates (1—3×10^4 cells/well) and allowed to grow until about 90% confluent. The cells were then labeled in a growth medium containing 1% FCS and 2—4 μCi ml^{-1} of *myo*-[^{3}H]inositol (90 Ci $mmol^{-1}$; 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^{-1} bovine serum albumin, 10 mM LiCl and test peptides or thrombin (human, 3200 U/mg from Dr. J. W. Fenton, II). The 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 1×8, 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 4044±187 dpm for thrombin and 4065±233 dpm for SFLLRNP (native form) with 106 dpm background. Other active peptides exhibited similar maximal stimulation. Since the level of maximal stimulation differed from assay to assay (3300—4900 dpm), Figs. 1 and 2 are depicted using normalized % activity from the reported assays.

Regarding antagonist activity, inactive peptides (0.01—1 mM) were incubated with cells for 10 min prior to the addition of peptides SFLLRNP or thrombin, or incubated together with these agonists. The assays were carried out as described above, and the dose-response curves with and without inactive peptides were compared in order to estimate the deviation between the curves.

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3) The abbreviations according to the biochemical nomenclature of the 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; EGTA, 2, 2′,2″,2‴-[ethylenebis(oxyethylenenitrilo)]tetrakisacetic acid; FCS, fetal calf serum; G protein, GTP-binding regulatory protein; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MBHA resin, 1% divinylbenzene-*co*-styrene copolymer with *p*-methylbenzhydrylamine; PI, phosphoinositide; SFLLRNP, amino acid sequence denoted by the one-letter amino acid code for Ser–Phe–Leu–Leu–Arg–Asn–Pro (all other peptides are also shown in a similar way); and Tos, *p*-tolylsulfonyl.

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