Structural Essentials of Ser-1 in Tethered Peptide Ligand of Human Thrombin Receptor for Phosphoinositide Hydrolysis

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In order to inspect the structural elements of Ser-1 in receptor activation by SFLLRNP (one-letter amino acid code), a ligand peptide tethered to the thrombin receptor, a series of analogs with such replacements as D-Ser, Ala, Thr, and Ac-Ser have been synthesized. These analogs were evaluated for their ability to hydrolyze the phosphoinositide in human neuroblastoma SH-EP cells. It was found that the α-amino group and L-configuration of Ser-1 are very important in the activation of receptors. N-Acetylation or deletion of Ser-1 completely eliminated the activity of SFLLRNP (a half-maximal effective concentration, $EC_{50}=0.89$ μM (1 M=1 mol dm^{-3})), and these modifications induced no antagonist activity. Incorporation of D-Ser also drastically diminished the activity, but retained about 50% activity of the maximal response by 100 μM SFLLRNP. The Ser/Ala substitution sustained 30% of the activity of SFLLRNP to elicit a full stimulation. The Ser/Thr substitution, however, enhanced the activity (20%) in spite of its decreased activity (60%) reported for platelet aggregation. These results indicated that the β-hydroxyl group of Ser-1 is important to receptor activation, but not essential. The effect of chemical modifications on the receptor activities of the tethered ligand is discussed with regard to the efficacy between phosphoinositide hydrolysis and biological activities.

The serine proteinase thrombin plays a central role in blood coagulation and platelet aggregation.[1,2] Thrombin is also mitogenic for several types of cells[3] and causes chemotaxis of monocytes.[4] Some of these biological effects are mediated through specific receptors coupled with G protein(s)[5] and thrombin receptors have recently been cloned and found to be a novel type of receptor with seven transmembrane domains.[6—8] A characteristic feature of thrombin receptor is that the receptor contains in itself the ligand segment.[6] This segment in the extracellular portion has two sites specific for thrombin; i.e., the cleavage site of LDPR/SFLL (38-45) (one-letter amino acid code) and the binding site of DKYEPFWEDEE (50-60). Once thrombin binds to the receptor and cleaves the peptide bond between Arg-41 and Ser-42, the newly exposed N-terminal portion which starts from the sequence of SFLLRNP functions as a ligand to activate the receptor.

The ligand moiety of thrombin receptors is masked by an N-terminal extension ($n=41$) in the intact form. Vu et al.[6] found that the synthetic peptide SFLLRNPNDKYEPF, which corresponds to the N-terminal tetradecapeptide (residues 42-55) of thrombin receptor, can activate the thrombin receptor by stimulating Ca^{2+} release even with no thrombin. Based on this initial finding, studies on the structure-activity relationships of tethered ligand have been started to find out the structures important to receptor recognition and activation.[9—14] It was found that the elicitation of receptor responses requires peptides with the first five to seven amino acid residues.

Recently, we have found that the Phe-phenyl group at position 2 is crucial for receptor activation.[14] In this study we have synthesized the N-terminal heptapeptide SFLLRNP and its analogs with modification of Ser-1 (Fig. 1) in order to clarify the structural essentials of this residue for receptor interaction, especially for activation of second messengers coupled with G proteins. Peptides were assayed for their ability to stimulate the hydrolysis of phosphoinositide (PI) mediated through thrombin receptor in epithelial-like neuroblastoma SH-EP cells. SFLLRNP has been reported to be the minimum peptide required to activate phospholipase C coupled with PI-turnover.[7] Since some recent reports have suggested diversity in the potency of synthetic ligand peptides examined in different assays,[12,13] a careful activity assessment of peptides in PI-turnover would clarify their intrinsic ability to impel the receptor to function.

Results and Discussion

Peptide synthesis was carried out by the automated solid phase methodology using *t*-butoxycarbonyl(Boc)-amino acids and Boc–Pro–Pam resin. As a coupling reagent, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU)[15] was used in the presence of 1-hydroxybenzotriazole (HOBt). Ac-SFLLRNP was prepared by acetylation of the resin-liked peptide after removal of Boc. Peptides were liberated by treatment of resins with hydrogen fluoride (HF) and were routinely purified by gel filtration (Sephadex G-15) followed by preparative reversed-phase HPLC (C4, 20×250 mm). The purity of the peptides was verified by analytical HPLC and amino acid analysis

Table 1. Physical Properties of Ligand Peptide of Thrombin Receptor and Its Analogs

Peptides		Yield[a] (%)	Amino acid analysis							
			Asp[b]	Ser	Thr	Ala	Pro	Leu	Phe	Arg
SFLLRNP	**1**	47	0.97	0.87	—	—	1.01	2.00	1.08	0.97
FLLRNP	**2**	49	0.95	—	—	—	1.00	2.00	0.89	0.90
Ac-SFLLRNP	**3**	44	0.97	0.88	—	—	0.93	2.00	1.02	1.05
sFLLRNP[c]	**4**	52	0.93	0.95	—	—	0.99	2.00	1.01	1.03
AFLLRNP	**5**	53	0.95	—	—	1.08	0.89	2.00	1.05	0.98
TFLLRNP	**6**	39	0.92	—	0.87	—	0.92	2.00	1.02	1.08

a) The yield of peptides was calculated for the products obtained after HPLC purification on the basis of the amount of Boc-amino acid resign. b) Asp is the acid degradation product of Asn. c) Small capital "s" indicates D-Ser in this table.

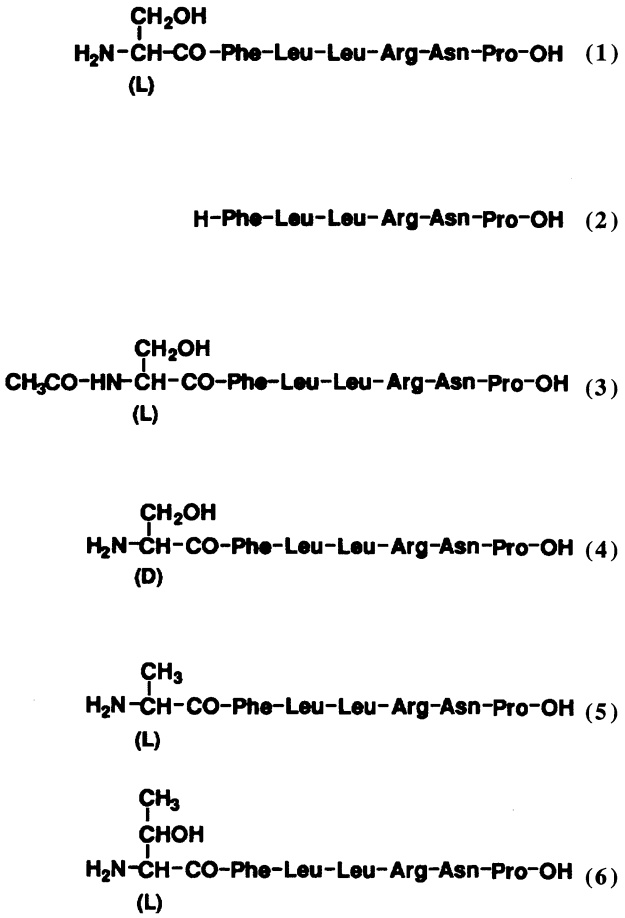

Fig. 1. Amino acid sequences of synthetic peptide analogs of tethered ligand of human thrombin receptor.

(Table 1).

Biological responses induced by thrombin are, for instance, platelet aggregation, serotonin secretion, and smooth muscle contraction. It has not yet been establishied whether all of these cellular responses to thrombin are mediated by a single set of receptors or whether different subclasses of receptors are involved. Since thrombin receptors couple with G protein(s),[16] it is important to evaluate the ability of tethered ligand for production of the second messengers. Epithelial-like SH-EP cells from human neuroblastoma exhibit a basal increase of DNA synthesis dependent upon the concentration of exogenously added thrombin.[17] In this cell line, thrombin is also responsible for stimulation of PI-turnover,[17] which produces the second messengers to elicit various cellular responses. In the present study, synthetic peptides were tested for their effectiveness in stimulating PI-turnover, since it is useful in measuring the intrinsic ability of peptides for receptor activation and thus for assessing the receptor efficacy between PI-turnover and various terminal biological responses.

Thrombin was very potent in stimulating PI-turnover and full stimulation was achieved at a concentration of 0.32 nM (1 M=1 mol dm^{-3}). From the analysis of the thrombin dose-response curves, the half-maximal effective concentration (EC_{50}) was estimated to be only 25 pM. This extremely high activity, which is approximately 100—1000-fold more effective than ordinary hormone-receptor activation, appears to be attributable to the facts that thrombin is an enzyme and the ligand is tethered to the receptor molecule. When the synthetic heptapeptide SFLLRNP (**1**) was tested for activation of SH-EP cells, it was found that **1** can elicit the full stimulation of PI-turnover in a dose-dependent manner (EC_{50}=0.89 μM) (Fig. 2, Table 2). This confirms the reported result that a synthetic peptide corresponding to the ligand moiety of thrombin receptor can activate the receptor without thrombin.[6] Although **1** was considerably weaker than thrombin, the potency as a receptor ligand seemed to be moderate. In contrast, peptide FLLRNP (**2**) which lacks the N-terminal Ser residue was completely inactive, being devoid of production of any inositol phosphates (Fig. 2). These results clearly indicated that the tethered ligand latent in the receptor molecule is extremely specific for self-activation and the Ser residue at position 1 is crucial to this activation.

It was found that the N-terminal amino group of the tethered ligand is critical for interaction with the receptor binding site, and the L-configuration of Ser-1 is also important. When the amino group of Ser-1 was acetylated, the resulting Ac-SFLLRNP (**3**) was completely inactive (Table 2). Ac-SFLLRNP exhibited no stimulation of PI-turnover even at a concentration of 100 μM (Fig. 2). D-Ser^1-containing analog **4**, on the other hand, showed a very weak stimulatory activity. There

Table 2. 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
SFLLRNP	**1**	0.89± 0.05	100
FLLRNP	**2**	(Inactive)	0
Ac-SFLLRNP	**3**	(Inactive)	0
sFLLRNP[a)]	**4**	113 ±14	0.79
AFLLRNP	**5**	2.8 ± 0.31	32
TFLLRNP	**6**	0.75± 0.02	120

a) Small capital "s" indicates D-Ser in this table.

was a dose-dependent stimulation of PI-turnover, but only up to 50% of the maximal response at the higher concentrations (10—100 μM) (Fig. 2).

In order to examine the structural importance of the β-hydroxyl group of Ser-1 in the stimulation of PI-turnover, Ser-1 was replaced by Ala which lacks the β-hydroxyl group of Ser. It was found that Ala-1-containing analog **5** was fairly active (Fig. 2), showing a dose-response curve with full stimulation of SH-EP cells. It retained (EC_{50}=2.8 μM) about 30% of the activity of SFLLRNP. These results implied that the hydroxyl group of Ser-1 is important, but not essential to activate thrombin receptor and Ser-1 may be substituted by other amino acids. In fact, Thr-1-containing analog **6** (EC_{50}=0.75 μM) was more active than **1**. The dose-response curve of **6** is shifted slightly and distinctly to a lower concentration (Fig. 2). The methyl group on the Ser-1 β-carbon appeared not to interfere but rather to reinforce the interaction of ligand with receptors.

Similar results have recently been reported by Van Obberghen-Schilling et al. who used the same set of peptides derived from the human thrombin receptor

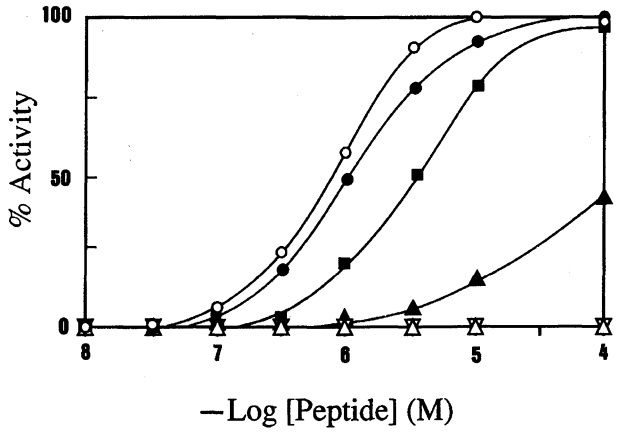

Fig. 2. Concentration dependent curves of ligand peptide of thrombin receptor and its analogs in phosphoinositide turnover in SH-EP cells. SFLLRNP (**1**, ●-●), FLLRNP (**2**, ▽-▽), Ac-SFLLRNP (**3**, △-△), sFLLRNP (**4**, ▲-▲), AFLLRNP (**5**, ■-■), and TFLLRNP (**6**, ○-○).

sequence.[18)] They showed that peptides **5** and **6** are almost equipotent in activation of phospholipase C, inhibition of adenylyl cyclase, and DNA synthesis. The cell utilized for these activities, however, was CCL39 Chinese hamster lung fibroblasts which contains thrombin receptors having about 79% homology to human thrombin receptors. The SH-EP cells used in the present study were cultured cells derived from human neuroblastoma. These suggest that the structure of the binding sites and the activation mechanism of these human and rodent thrombin receptors resemble each other.

Inactive peptides FLLRNP **2** and Ac-SFLLRNP **3** were also tested for their possible antagonist activity in PI-turnover. If they bind to the ligand binding site in the receptor, they would prevent the receptor from binding SFLLRNP and thrombin. Because of the very low affinity of ligand peptides synthesized to date, no radio-labeled peptide ligand has been available for the receptor binding assay. Thus, peptide **2** or **3** was incubated with cells with simultaneous or preceding addition of active SFLLRNP or thrombin in order to examine their direct antagonism on the cells. However, no antagonist activity was found even at higher concentrations. Both SFLLRNP and thrombin exhibited unchanged stimulatory activity in PI-turnover in the presence of peptide **2** or **3** (data not shown). D-Ser-containing peptide **4** also showed no antagonist activity.

Inactivity of acetylated tethered ligand has been shown for platelet aggregation.[12)] Acetylation of dodecapeptide SFLLRNPNDKYE was reported to abolish activity. Inactivity of compound **3** in PI-turnover appears to be compatible with that of Ac-SFLLRNPNDKYE in platelet aggregation. On the other hand, it has recently been reported that Ac-SFLLR retained a little of the activity (about 4%) of the non-acetylated peptide (EC_{50}=2.1 μM) in serotonin secretion in platelets.[13)] For such an activity discrepancy of acetylated peptides between platelet aggregation and serotonin secretion, it is probable that the peptides act upon distinct receptors to elicit different cellular reactions.

Activity discrepancy was also observed between potent derivatives. Vassallo et al. reported that the replacement of Ser-1 of hexapeptide SFLLRN with Thr affected the platelet aggregation activity.[11)] TFLLRN exhibited only 40% of the activity of SFLLRN in human platelet aggregation.[11)] This is in contrast to the result obtained in the present study where TFLLRNP showed a slightly increased (about 20%) potency as compared to SFLLRNP in PI-turnover (Table 1). Since it has been said that platelet aggregation is impelled by thrombin-induced PI hydrolysis,[11)] the disagreement in these activities appears to be eccentric. It is possible that thrombin receptors are expressed specifically in tissues and each receptor may have subtle differences in structure. This was found to be the case for the δ opioid receptors; the one in brain differed from that in the mouse

vas deferens.[19] The thrombin receptor present in SH-EP cells might be different from that in platelet. The recent report by Hollenberg et al. has indeed suggested the existence of distinct thrombin receptor subtypes in the gastric smooth muscle elements and in the arterial endothelial cells.[20] Alternatively, there might be a different mechanism mediated through other types of G proteins and Pro-7 might have cooperative effect on reinforcement of the activity. In order to evaluate such a diversity in receptor efficacy, more detailed biochemical studies are in progress using synthetic peptides.

Experimental

Peptide Synthesis. For automated peptide synthesis, the standard solid-phase technique was use on an Applied Biosystems 430A peptide synthesizer (Foster City, CA, U.S.A.). Boc–Pro–Pam resin (Amino Tech, Ogdensburg, NY, U.S.A.) was used. All amino acids were protected at their amino group with the Boc group and the side chain protecting groups were benzyl for Ser and Thr, and *p*-toluenesulfonyl (Tos) for Arg. All amino acids were coupled twice as performed symmetrical anhydrides, with the exception of Boc–Arg(Tos)–OH and Boc–Asn–OH which were coupled as HOBt active esters using HBTU and HOBt. Acetylation was carried out by 25% acetic anhydride in CH_2Cl_2 for 10 min.

Peptides were cleaved from resin by treatment with HF (7 ml) containing 10% *p*-cresol for 60 min at 0 °C. The crude materials were precipitated by addition of diethyl ether and dried over phosphorus pentaoxide. Peptides were dissolved in 10% acetic acid and purified by gel filtration on a column (2×100 cm) of Sephadex G-15 eluted with 10% acetic acid and then by reversed-phase HPLC on a preparative C4 column (20×250 mm) (Vydac, Hesperia, CA, U.S.A.). HPLC was performed on a Hewlett–Packard 1084B liquid chromatograph (Hewlett–Packard, Geneva, Switzerland), equipped with a processor-controlled sampling and UV monitoring system. The elution conditions employed were as follows: Buffer, a linear gradient of 0.05% aqueous trifluoroacetic acid (TFA) and 0.05% TFA in acetonitrile; flow rate, 3.0 ml min^{-1}; temperature, 25 °C; UV detection, 215 nm. The peptide yields were calculated on the basis of the amount of Boc–Pro–Pam resin utilized for each synthesis; these (39—52%) are listed in Table 1.

The purity was verified by reversed-phase HPLC on an analytical C4 column (4.6×150 mm) (Vydac), using the same flow conditions except for a flow rate of 1 ml min^{-1}. For amino acid analyses, hydrolysis of the peptide samples was carried out in constant-boiling hydrochloric acid (110 °C, 24 h). The amino acids were analyzed on a Beckman Model 121MB analyzer in conjunction with a Beckman Model 126 Data System integrator, and the results are shown in Table 1.

Biological Assay. Synthesized peptides were evaluated in SH-EP cells essentially as reported previously by Ogino and Costa.[17] 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—3×10^4 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^{-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). Reactions were conducted at 37 °C for 30 min, and stopped by the addition of ice-cold methanol (1 ml) containing 60 mM HCl. After centrifugation, the reaction mixture was applied to 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.[21] In one assay, for instance, the maximal stimulation was 7200±150 dpm for thrombin and 7480±180 dpm for TFLLRNP **6** with 270 dpm background. Other active peptides exhibited similar maximal stimulation. As the level of maximal stimulation differed from assay to assay (6500—11500 dpm), Fig. 2 was depicted using normalized % activity from repeated assays.

For antagonist activity, inactive peptides (0.01—1 mM) were incubated with cells for 10 min prior to addition of peptide 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 to estimate the deviation between curves.

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5) 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, [ethylene-bis(oxyethylenenitrilo)]tetraacetic acid; FCS, fetal calf serum; G protein, GTP-binding regulatory protein; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; Pam resin, 1% divinylbenzene-*co*-styrene copolymer with 4-(hydroxymethyl)-phenylacetamidomethyl; 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); TFA, trifluoroacetic acid; and Tos, *p*-toluenesulfonyl.

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