The Role of Arginine in Thrombin Receptor Tethered-Ligand Peptide in Intramolecular Receptor Binding and Self-Activation

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Synthetic heptapeptide of the human thrombin receptor tethered-ligand peptide, H–Ser–Phe–Leu–Leu–Arg–Asn–Pro–NH_2 (SFLLRNP), activates fully the thrombin receptor without thrombin. The functional role of Arg-5 was examined using a series of analogs having amino acid substitutions at position 5 in this assays was to assess the abilities to hydrolyze phosphoinositide in human neuroblastoma SH-EP cells and to aggregate the human platelet. The replacement of Arg-5 by Ala reduced the activity (9% activity of the parent peptide) in the PI-turnover assay, and abolished completely the platelet aggregation activity. SFLL/Lys/NP was also active, but moderately: 36% in PI-turnover and 12% in platelet aggregation. These results indicated that the electrostatic interaction of the Arg-guanidino group is important for a peptide to interact with the receptor. When citrulline or glutamine was placed at position 5 instead of arginine, the resulting SFLL/citrulline/NP and SFLL/Gln/NP were found to be potent in both assays. Since citrulline and glutamine possess a side chain which can serve as hydrogen donor and/or acceptor, the receptor activation of these peptides appears to be due to hydrogen bonding at this position. The molecular mechanisms to explain both electrostatic and hydrogen-bonding interactions were postulated based on the structural modeling of seven-transmembrane domain thrombin receptor.

A serine proteinase thrombin is able to evoke various biological responses from a variety of cells.[1—3] In recent years, functional thrombin receptors have been cloned from cells such as human platelet and endothelial cells, rat vascular smooth muscle cell, and hamster lung fibrobrasts.[4—6] These thrombin receptors were found to be a novel type of the seven-transmembrane domain receptors. Their characteristic feature is that the receptor protein contains the ligand segment in the receptor molecule itself.[4—6] When thrombin binds to the receptor N-terminal extracellular extension and cleaves the peptide bond, the receptor is activated spontaneously. Thus, the newly exposed N-terminal moiety starting from SFLLRNP (one-letter amino acid code) acts as a tethered ligand to activate thrombin receptor.[7]

Synthetic peptide SFLLRNP can activate the receptor without proteolytic cleavage by thrombin.[8] Systemic studies on the structure–activity relationships of this tethered ligand peptide have been developed by us and other groups to find out which structural elements are important for receptor binding and activation; several structural essentials were defined. For instance, the N-terminal Ser-1-amino and Phe-2 phenyl groups are the structures most important to elicit a full receptor activation.[4,8—13] We also found that the two consecutive Leu residues at the position 3 and 4 interact with a receptor differently.[14] Leu-3 appeared to behave as a connecting unit to construct a bioactive conformation, while Leu-4 seemed to interact directly with the receptor.

Arginine at position 5 of the tethered ligand is a sole amino acid residue with a charged side chain. This positively-charged Arg residue was supposed to have an important role in receptor interaction due to its ability to produce an electrostatic interaction with the receptor. In the present study, in order to elucidate the functional role of the arginine side chain, analogs with various amino acids at position 5 were synthesized and tested for their ability to activate phospholipase C coupled with phosphoinositide (PI)-turnover in human epithelial-like SH-EP cells. They were also examined for aggregation of human platelets.

Results and Discussion

Peptide syntheses were carried out by the manual solid phase methodology using *t*-butoxycarbonyl (Boc)-amino acids and Boc–Pro–*p*-methylbenzhydrylamine (MBHA) resin as described previously.[14] Peptides were finally liberated by treatment of resins with hydrogen fluoride (HF), and purified by gel filtration (Sephadex G-15, 1.0×100 cm), followed by preparative reversed-phase HPLC (C18, 25×250 mm). The purity was verified by analytical HPLC (C18, 4.0×250 mm) and amino acid analysis.

Along with activation of thrombin receptor by the teth-

ered ligand, the receptor stimulates the G-protein-coupled phospholipase C to release inositol tris-phosphate from phosphatidylinositol polyphosphate.[15] The human platelets possess receptors sensitive to α-thrombin on their surface.[16] Epithelial-like SH-EP cells from human neuroblastoma also contain such a receptor system. We have shown that SH-EP cells respond to synthetic peptide SFLLRNP, stimulating PI-turnover.[8,13,14] SFLLRNP (**1**) elicits a full stimulation of PI-turnover in a dose-dependent manner (Fig. 1).

Synthetic SFLLRNP analogs with modifications at position 5 were first evaluated for their ability to stimulate the PI-turnover (Fig. 1A and B). The EC_{50} values, a half-maximal effective concentration of full stimulation, were computed by program ALLFIT (Table 1).[17] The replacement by the configurational isomer (D-Arg) resulted in complete inactivity (Table 1). Similar results were reported also for other residues (residues 1 to 4).[8,13,14] When Arg-5 was substituted by Ala, the resulting SFLL/Ala/NP (**3**) showed a drop in PI-turnover activity (EC_{50} = 17 μM, 9% activity of compound **1**) (1 M = 1 mol dm^{-3}). Although Ala-5 derivative still exhibited a full activation (Fig. 1), the result clearly indicates the structural importance of Arg side chain in receptor activation. Gly-5 derivative **4** was only weakly active (EC_{50} > 100 μM) (Table 1). Although the Arg/Lys substitution caused a slight drop in activity (**5**, EC_{50} = 4.2 μM) (Table 1), the Arg/citrulline substitution almost sustained a full activity of compound **1** (**6**, EC_{50} = 1.7 μM) (Table 1). Citrulline has a side chain of $-CH_2CH_2CH_2NHCONH_2$, the urea structure of which is a strong hydrogen-bonding donor and/or acceptor. The Gln-5 derivative **8** was also potent (EC_{50} = 4.0 μM) (Table 1), while Glu-5 derivative **7** was almost completely inactive.

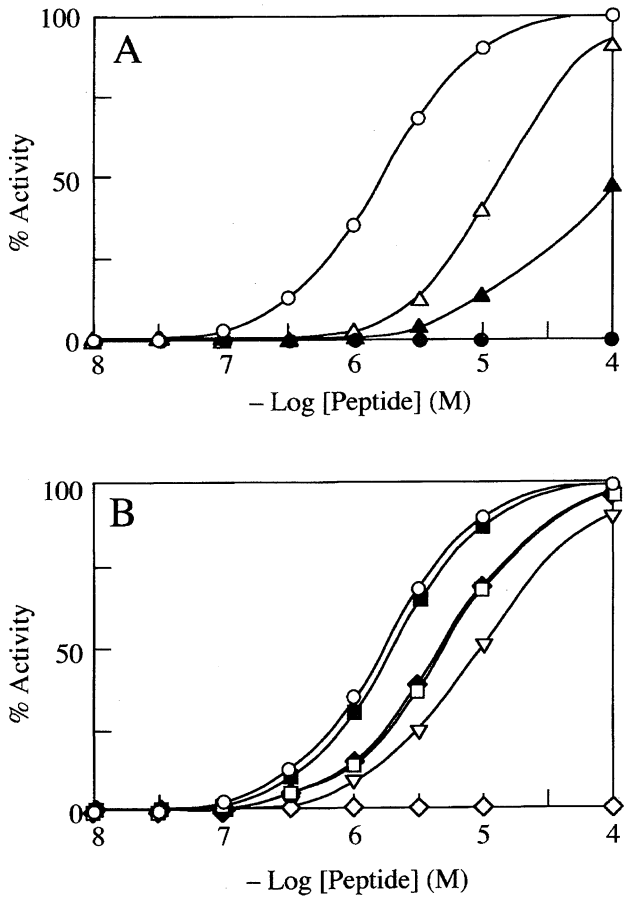

Fig. 1. Concentration dependent curves of ligand peptide of thrombin receptor and its analogs substituted at position 5 (Arg-5) in phosphoinositide turnover in SH-EP cells. A: Wild type (SFLLRNP, **1**, ○—○), D-Arg-5 (SFLL/D-Arg/NP, **2**, ●—●), Ala-5 (SFLL/Ala/NP, **3**, △—△), and Gly-5 (SFLL/Gly/NP, **4**, ▲—▲). B: Wild type (SFLLRNP, **1**, ○—○), Lys-5 (SFLL/Lys/NP, **5**, □—□), Cit-5 (SFLL/Cit/NP, **6**, ■—■), Glu-5 (SFLL/Glu/NP, **7**, ◇—◇), Gln-5 (SFLL/Gln/NP, **8**, ◆—◆), and Tyr-5 (SFLL/Tyr/NP, **9**, ▽—▽).

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

Peptides[a]		EC_{50} μM	Relative potency
SFLL/**Arg**/NP–NH_2	**1**	1.5±0.14	100
SFLL/**D-Arg**/NP–NH_2	**2**	Inactive	—
SFLL/**Ala**/NP–NH_2	**3**	17±9.8	8.8
SFLL/**Gly**/NP–NH_2	**4**	> 100	<1.5
SFLL/**Lys**/NP–NH_2	**5**	4.2±1.1	36
SFLL/**Cit**/NP–NH_2	**6**	1.7±0.18	88
SFLL/**Glu**/NP–NH_2	**7**	Inactive	—
SFLL/**Gln**/NP–NH_2	**8**	4.0±1.5	37
SFLL/**Tyr**/NP–NH_2	**9**	12±6.7	12

a) Cit, citrulline (N^5-carbamoyl ornithine).

The results of platelet aggregation assays are shown in Table 2. The half-maximal effective concentration values (EC_{50}) in platelet aggregation were also calculated by ALLFIT (Table 2). Lys-5, citrulline-5, and Gln-5 derivatives (**5**, **6**, and **8**) exhibited weak activity (12—14%) of parent peptide **1**. On the other hand, D-Arg-5, Ala-5, Gly-5, Glu-5, and Tyr-5 (**2**, **3**, **4**, **7**, and **9**) derivatives were almost completely inactive even at the high concentration of 100 μM (Fig. 2). Comparing these results with those from the PI-turnover assay, it is clear that all the peptides are more sensitive (2—15-fold) in the PI-turnover assay than in the platelet assay. For instance, SFLL/citrulline/NP is about 15-fold more active in

Table 2. Biological Activity of Ligand Peptide of Thrombin Receptor and Its Analogs in Human Platelet Aggregation

Peptides		EC_{50} μM	Relative potency
SFLL/**Arg**/NP–NH_2	**1**	3.4±0.67	100
SFLL/**D-Arg**/NP–NH_2	**2**	Inactive	—
SFLL/**Ala**/NP–NH_2	**3**	Inactive	—
SFLL/**Gly**/NP–NH_2	**4**	Inactive	—
SFLL/**Lys**/NP–NH_2	**5**	29±8.2	12
SFLL/**Cit**/NP–NH_2	**6**	25±9.8	14
SFLL/**Glu**/NP–NH_2	**7**	Inactive	—
SFLL/**Gln**/NP–NH_2	**8**	28±7.8	14
SFLL/**Tyr**/NP–NH_2	**9**	Inactive	—

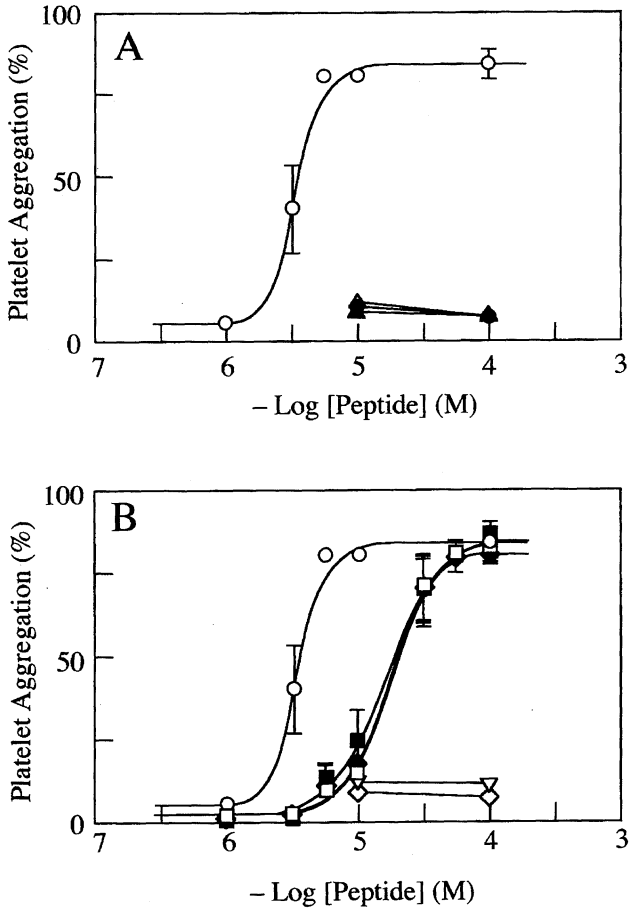

Fig. 2. Concentration dependent curves of ligand peptide of thrombin receptor and its analogs substituted at position 5 (Arg-5) in platelet aggregation. A: Wild type (SFLLRNP, **1**, ○—○), D-Arg-5 (SFLL/D-Arg/NP, **2**, ●—●), Ala-5 (SFLL/Ala/NP, **3**, △—△), and Gly-5 (SFLL/Gly/NP, **4**, ▲—▲). B: Wild type (SFLLRNP, **1**, ○—○), Lys-5 (SFLL/Lys/NP, **5**, □—□), Cit-5 (SFLL/Cit/NP, **6**, ■—■), Glu-5 (SFLL/Glu/NP, **7**, ◇—◇), Gln-5 (SFLL/Gln/NP, **8**, ◆—◆), and Tyr-5 (SFLL/Tyr/NP, **9**, ▽—▽).

the PI-turnover assay than in the platelet aggregation assay. This activity discrepancy may be explained by the fact that PI-turnover is the biological activity directly coupled to receptor binding of the ligand, whereas platelet aggregation is a cellular response mediated through several other intracellular reactions.

The guanidino group of arginine is strongly positively charged, and in most of the cases it makes the electrostatic interaction with acidic amino acids. Since SFLL/Arg/NP, a native form of tethered-ligand, was most potent in both PI-turnover and platelet aggregation, it is apparent that the electrostatic interaction is responsible for interaction of this peptide with the receptor. The analog having another basic amino acid lysine at position 5, SFLL/Lys/NP, was also active but moderately, in both PI-turnover (36% of SFLL/Arg/NP) and platelet aggregation (12%) assays. The potency difference in receptor activation between SFLL/Arg/NP and SFLL/Lys/NP appears to be due to the difference in the strengths of their negative charges.

The electrostatic interaction of Arg-5 of SFLL/Arg/N with human thrombin receptor has been proven by elegant mutagenesis experiments by Coughlin et al.[18] The human and Xenopus thrombin receptors respond selectively to their respective peptide agonists, SFLLRN and TFRIFD. Coughlin et al. constructed a human/Xenopus thrombin receptor chimera with a human-for-Xenopus amino acid substitution, Glu for Leu at position 260. This chimera receptor conferred human receptor-like specificity to the Xenopus receptor, strongly suggesting that Arg-5 in the human agonist peptide might interact with Glu-260 in the human thrombin receptor. They then, made a mutant ligand of SFLLEN and a mutant receptor in which Glu-260 was converted to Arg.[19] It was found that SFLLEN is an effective agonist at this mutant human receptor, implying that Glu-260 is the site of ligand Arg-5 for the electrostatic interaction.

It should be noted, on the other hand, that the analogs containing citrulline or glutamine are rather potent in the PI-turnover assay. Citrulline and glutamine are amino acids with neutral characteristics, but possess the groups serving as a hydrogen donor or acceptor. These might be relevant to the fact that human thrombin receptor consists of two hydrophobic amino acid residues adjacent to Glu-260, namely Asn-259 and Thr-261. When the three-dimensional structure of thrombin receptor was constructed in the computer, these residues were found in the second extracellular loop of thrombin receptor with seven transmembrane domains. It is feasible that citrulline-5, Gln-5 and even Arg-5 in ligand peptides analogs interact with these residues, and such interaction might also be able to cause an activation of the receptor. Alternatively, citrulline-5 and Gln-5 still interact with Glu-260.

Since inactive peptides (**2**, **3**, **4**, **7**, and **9**) were a possible candidate of antagonist, they were assayed for the inhibitory activity. 100 μM sample peptides were tested against 6 μM of compound **1**. It was found that Ala-5 derivative **3** suppresses weakly (about 10% activity of SFLLRNP). Since compound **3** was a weak agonist in the PI-turnover assay, it is not clear that this extremely weak antagonist activity is meaningful in the receptor interaction. No other peptides exhibited any inhibition.

Experimental

Peptides Synthesis. SFLLRNP analogs were synthesized by the manual solid-phase synthesis method. All amino acids were protected at their amino group with Boc group and the side-chain-protecting groups were benzyl for Ser, *p*-tolylsulfonyl (Tos) for Arg, N^{ε}-2-chlorobenzyloxycarbonyl (Cl-Z) for Lys, γ-cyclohexyl ester (OcHex) for Glu, and *O*-2,6-dichlorobenzyl (Cl_2Bzl) for Tyr. To obtain C-terminal amide peptides, Boc–Pro–MBHA resin was utilized. Peptides synthesized are SFLLRNP **1**, SFLL/D-Arg/NP **2**, SFLL/Ala/NP **3**, SFLL/Gly/NP **4**, SFLL/Lys/NP **5**, SFLL/Cit/NP **6**, SFLL/Gln/NP **7**, SFLL/Glu/NP **8**, and SFLL/Tyr/NP **9**. Coupling reactions were carried out with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)[20] in the presence of 1-hydroxy-1*H*-benzotriazole (HOBt) in a mixture of *N*-methylpyrrolidinone and *N*,*N*-dimethylformamide (1 : 2, v/v) for 30 min.

Peptides were liberated from the resin by treatment with anhydrous liquid HF containing 10% *p*-cresol at 0 °C for 1 h, and purified by Sephadex G-15 (1 cm×100 cm) followed by preparative RP-HPLC (Cica–Merck, LiChrospher RP-18 (5 μm): 25×250 mm). The elution conditions employed were as follows: solvent, 0.1% aqueous trifluoroacetic acid (TFA) (A solution) and acetonitrile containing 20% A solution (B solution); flow rate, 4 ml min^{-1}; temperature, 25 °C; and UV detection, 225 nm. Elutions were done with a linear concentration gradient of B solution (20—60%) for 40 min. The purity was verified by analytical RP-HPLC (LiChrospher RP-18 (5 μm): 4.0×250 mm), using the same conditions except for a flow rate of 0.75 ml min^{-1}. For amino acid analysis, peptide samples were hydrolyzed in constant-boiling hydrochloric acid (110 °C, 24 h). The amino acids analyses were carried out on a Hitachi (model 835) amino acid analyzer.

Assessment of Ligand-Stimulated Phosphoinositide-Turnover Hydrolysis. Biological activity of synthetic peptides was evaluated in SH-EP cells essentially as reported previously by Ogino and Costa.[15] The extent of PI-hydrolysis was determined by measuring the accumulation of radiolabeled 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, Buckinghamshire, 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. 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.[17] In one assay, for instance, the maximal stimulation was 8990±270 dpm for SFLLRNP (native form) with 1680 dpm background. Other active peptides exhibited similar maximal stimulation levels. Since the level of maximal stimulation differed from assay to assay (8990—14300 dpm), Fig. 1 was depicted using normalized % activity from reported assays.

Evaluation of Platelet Aggregation. Blood was obtained from healthy donors who denied taking any medications for the previous two weeks. Collected blood was anticoagulated by adding (1 part 3.8% sodium citrate to 9 parts blood), and platelet-rich plasma (PRP) was obtained by centrifugation at 1300 rpm for 10 min. Platelet-poor plasma (PPP) was also prepared by centrifugation at 4000 rpm for 10 min. Prepared PRP was used within 4 h, and the aggregation test was carried out at 37 °C by the standard turbidometric procedure using NBS hema tracer 601 (Niko Bioscience, Tokyo), where PPP was used as a reference. The peptide concentrations required for half-maximal platelet aggregation were derived from three determinations.

Measurement of Antagonist Activity. Antagonist activity was examined for platelet aggregation induced by SFLLRNP. In this assay, 100 μM sample peptides were first incubated with PRP for 5 min at 37 °C, then agonist peptide (6 μM SFLLRNP) was added. Antagonist activity was assessed by comparing the extent of platelet aggregation with and without sample peptides.

Modeling of Thrombin Receptor. The three-dimensional structure of thrombin receptor was constructed on the basis of a multiple amino acid sequences alignment of seven transmembrane domain receptors, after building a structure of β2 adrenergic receptor using the bacteriorhodopsin structure as template.[21] The modeling was performed using the modeling software SYBYL 6.0.3 on a UNIX workstation model indigo 2 (Silicon Graphics, Mt. View, CA, USA).

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