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Interaction Mode of the Phe-Phenyl Group of Thrombin Receptor-Tethered Ligand SFLLRNP in Receptor Activation

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Phenylalanine at position 2 of thrombin receptor-tethered ligand peptide (SFLLRNP) is crucially important for the activation of thrombin receptor. Its substitution by *para*-fluorophenylalanine [(*p*-F)Phe] enhanced several times the activity in human epithelial-like SH-EP cells [Nose *et al.* (1993) *Biochem. Biophys. Res. Commun.* 193, 694-699]. To clarify the interaction mode of Phe-2-phenyl in receptor activation, a series of analogs having chemical modifications on the benzene ring of Phe-2 were synthesized and examined for their ability to induce the aggregation of human platelets. When the fluorine atom was placed at the *meta* or *ortho* position, the resulting analogs exhibited considerably diminished activity (about 10-20% of *para*-derivative), indicating that the substitution is allowed only at the *para* position. The derivative with pentafluorophenylalanine was totally devoid of activity. These results suggested that Phe-2 requires hydrogen atom(s) on the benzene ring presumably for interaction with the receptor. No activity enhancement was observed for analogs with *para*-chloro-, bromo-, or iodophenylalanine, indicating the importance of the high electronegativity of fluorine to intensify the dipole of CH(s) remaining in the Phe-2-benzene ring. Inactivity of analogs having *para*-iodophenylalanine and homophenylalanine indicated the importance of the size of *para* substituents, and the placement of hydroxyl, nitro, and trifluoromethyl groups at the *para* position led to no activity. The interaction of Phe-2 of SFLLRNP appeared to be structurally restricted to a limited space in the receptor. The results suggested the presence of face-to-edge π-π interaction based upon the CH/π interaction between the ligand Phe-2-phenyl group and the receptor aromatic group.

Key words: *para*-fluorophenylalanine, receptor recognition, tethered ligand peptide, thrombin, thrombin receptor.

Biologically active peptides usually contain a crucially important amino acid residue(s), and the aromatic amino acid phenylalanine often acts to elicit such intrinsic activity (*1, 2*). Despite the appreciation of such an important role of phenylalanine, the molecular mechanism of the residue, particularly its phenyl group or benzene ring in the side chain, has never been elucidated in detail in the ligand-receptor interaction. One of reason for this is that phenylalanines are usually involved in so-called *hydrophobic* interactions, the nature of which are not explicit.

The ligand peptide of thrombin receptor contains a critical phenylalanine essential for receptor recognition and activation. This peptide originates within the receptor molecule *per se* and is exposed by serine proteinase thrombin. Once thrombin binds to the receptor and cleaves the peptide bond between Arg^{41} and Ser^{42}, a newly exposed N-terminal fragment Ser-Phe-Leu-Leu-Arg-Asn-Pro (SFLLRNP) functions as a tethered ligand (*3*). The remarkable feature of thrombin receptor is that it can be activated with synthetic peptide SFLLRNP without thrombin. This has allowed extensive structure-activity studies for exploration of the structural elements important to receptor activation (*4-10*). The importance of Phe-2 (phenylalanine at position 2) was immediately apparent from examinations of analogs having amino acids other than Phe. The replacement of Phe-2 with Ala, which implies the elimination of β-phenyl group, resulted in complete inactivation (*11*). Also, the substitution by cyclohexylalanine reduced the activity drastically, indicating that the aromaticity is

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Abbreviations: Boc, *tert*-butoxycarbonyl; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HPTLC, high-performance thin layer chromatography; MBHA, *p*-methylbenzhydrylamine; (*m*-F)Phe, *meta*-fluorophenylalanine; (*o*-F)Phe, *ortho*-fluorophenylalanine; (*p*-F)Phe, *para*-fluorophenylalanine; (*p*-Cl)Phe, *para*-chlorophenylalanine; (*p*-Br)Phe, *para*-bromophenylalanine; (*p*-I)Phe, *para*-iodophenylalanine; (*p*-CF_3)Phe, *para*-trifluoromethylphenylalanine; (F_5)Phe, pentafluorophenylalanine; PPP, platelet-poor plasma; PRP, platelet-rich plasma; RP-HPLC, reversed-phase high performance liquid chromatography; SFLLRNP, Ser-Phe-Leu-Leu-Arg-Asn-Pro; and TFA, trifluoroacetic acid.

important for interaction with the receptor (*12*).

We have previously reported that *para*-fluorophenylalanine [(*p*-F)Phe] at position 2 of SFLLRNP enhances the activity about 6-fold in the assay of phosphoinositide (PI)-turnover in human epithelial-like SH-EP cells (*11*). Since then, (*p*-F)Phe has been utilized for almost all the synthetic peptide analogs of thrombin receptor-tethered ligands to substantiate this high level of potency (*12-15*). However, the reason why the placement of the fluorine atom on the Phe-2 benzene ring reinforces the receptor interaction is not well understood, although a possible π-π interaction was suggested between the ligand peptide and thrombin receptor (*11*).

In the present study, in order to elucidate the interaction mode of S/(*p*-F)Phe/LLRNP with thrombin receptor, a systemic substitution of *para*-fluorine has been carried out. If the role of *para*-fluorine in the ligand-receptor interaction can be clarified, the functional significance of the original Phe-phenyl group should become apparent. Peptides were evaluated in the assay using human platelets, and the structure-activity relationships were discussed in terms of their potency to aggregate the platelets.

MATERIALS AND METHODS

Materials—A series of fluorinated phenylalanines were purchased from Asahi Glass (Tokyo). Those include monofluorophenylalanines such as *para*-fluorophenylalanine (*p*-F)Phe, *meta*-fluorophenylalanine (*m*-F)Phe, *ortho*-fluorophenylalanine (*o*-F)Phe, and *para*-trifluoromethylphenylalanine (*p*-CF_3)Phe. (*p*-Br)Phe was purchased from Fluka (Buchs, Switzerland). All these amino acids were in the L-configuration. Boc-(*p*-Cl)Phe-OH, Boc-(*p*-I)Phe-OH, Boc-(*p*-NO_2)Phe-OH, and Boc-(F_5)Phe-OH were obtained from Watanabe Chemical Industries (Hiroshima), and Boc-homoPhe-OH was from BACHEM (Bubendorf, Switzerland). Boc-derivatives of ordinary amino acids and *p*-methylbenzhydrylamine (MBHA) resin were obtained from Watanabe. Pre-coated silica gel plates for high-performance thin layer chromatography (HPTLC) were purchased from Merck (Darmstadt, Germany). All other chemicals were of the best grade available.

Syntheses of Boc-Phenylalanine Derivatives—Boc-(*p*-F)-Phe-OH, Boc-(*m*-F)Phe-OH, Boc-(*o*-F)Phe-OH, and Boc-(*p*-Br)Phe-OH were prepared from each corresponding amino acid by using di-*t*-butyl dicarbonate according to the method reported (*16*). Boc-(*p*-CF_3)Phe-OH was also prepared by the same method. Physical constants of these compounds are listed in Table I.

Peptide Synthesis—Analogs of SFLLRNP were synthesized by the manual solid phase synthesis method (*10*). The side chain protecting groups of Boc-amino acids were benzyl for Ser and *p*-tolylsulfonyl (Tos) for Arg. To obtain C-terminal peptide amides, Boc-Pro-MBHA resin was utilized. Coupling reactions were carried out by using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in the presence of 1-hydroxybenzotriazole (HOBt) in a mixed solvent 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 reversed-phase high performance liquid chromatography (RP-HPLC) [Cica-Merck, LiChrospher RP-18 (e) (5 μ): 25×250 mm]. The elution conditions employed were as follows: solvent system, 0.1% aqueous trifluoroacetic acid (TFA)-(A solution) and acetonitrile containing 20% A solution-(B solution); flow rate 5 ml·min^{-1}; temperature, 25°C; 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 μ): 4.0×250 mm], using the same conditions except for a flow rate of 0.75 ml·min^{-1}. For amino acid analyses, peptide samples were hydrolyzed in constant-boiling hydrochloric acid (110°C, 24 h), then applied to a Hitachi (model 835) amino acid analyzer.

Platelet Aggregation Assay—Blood was obtained from healthy donors who denied taking any medications for the previous two weeks. Collected blood was anticoagulated with citrate (1 part 3.8% sodium citrate to 9 parts blood), and platelet-rich plasma (PRP) was obtained by centrifugation at 1,300 rpm for 10 min. Platelet-poor plasma (PPP) was also prepared by centrifugation at 12,000 rpm for 5 min. Prepared PRP was used within 4 h, and the aggregation test was carried out at 37°C by the standard turbidometric procedure using an NBS hema tracer 601 (Niko Bioscience, Tokyo), with PPP as a reference. The peptide concentrations required for half-maximal platelet aggregation were derived from three determinations.

Antagonist Activity in Platelet Aggregation—Inactive peptides in the platelet aggregation test were further examined for their ability to suppress the aggregation activity of SFLLRNP amide. Concentrations were 100 μM for test peptides and 3 μM for agonist SFLLRNP amide. Prior to addition of agonist, test peptides were incubated with PRP for 2 min and the extent of aggregation was assessed with and without test peptides.

RESULTS AND DISCUSSION

Although we have prepared several Boc-protected amino acids for solid-phase peptide syntheses (Table I), various halogenized amino acid derivatives are now commercially available, because halogen-containing peptides are often found to have unexpectedly high biological activity (*11, 17-21*).

Thrombin causes blood coagulation, aggregation of platelets, and chemotaxis of monocytes (*22-24*). These biological activities are mediated through a specific receptor, which is a novel type of seven transmembrane domain receptor coupled with G-protein (*3, 25, 26*). A characteris-

TABLE I. **Analytical data for synthesized Boc-amino acids.**

Compound	Yield (%)	Mp (T/°C)	$[\alpha]_D^{20}$	R_f (HPTLC)*
Boc-(*p*-F)Phe-OH	94	81-83	+24.3 (*c*1, EtOAc)	0.75
Boc-(*m*-F)Phe-OH	80	76-78	+23.3 (*c*1, EtOAc)	0.75
Boc-(*o*-F)Phe-OH	96	92-95	+10.6 (*c*1, EtOAc)	0.75
Boc-(*p*-Br)Phe-OH	75	115-117	+24.4 (*c*1, EtOH)	0.77
Boc-(*p*-CF_3)Phe-OH	81	117-119	+15.1 (*c*1, EtOH)	0.72

*Solvent system: $CHCl_3$-MeOH-AcOH=50:10:2 (v/v).

tic feature of thrombin receptor is that the receptor protein molecule itself contains a ligand Ser-Phe-Leu-Leu-Arg-Asn-Pro (SFLLRNP) in the N-terminal extracellular portion (*3*). Since the synthetic heptapeptide having this sequence activates the receptor without thrombin reaction, studies on the structure-activity relationships of tethered ligand have been performed with a number of peptide analogs to find the structural essentials for receptor recognition and activation. The most striking finding was the activity enhancement by incorporation of *para*-fluorophenylalanine at position 2. This activity enhancement was assumed to be due to a reinforced π-π interaction between Phe-2-phenyl and receptor aromatic residues (*11*). Our attempt at molecular modeling of thrombin receptor to depict a seven transmembrane domain receptor revealed a dense disposition of aromatic amino acid residues at the portion proximal to the extracellular side of the fifth transmembrane, the amino acid sequence of which is **YYAYYFSAFSAVFFFVPLIISTVCYVSII**. The first 15 amino acid residues include 9 aromatic amino acids (five phenylalanines and four tyrosines, in bold letters in the sequence).

The efficacy of the fluorine atom at the *para* position became immediately apparent upon synthesis of analogs with *meta*- or *ortho*-fluorophenylalanine (Fig. 1). As shown in Table II, S/(*m*-F)Phe/LLRNP and S/(*o*-F)Phe/LLRNP were weak in the induction of human platelet aggregation, showing 20 and 12% activity of S/(*p*-F)Phe/LLRNP, respectively. They were also weaker (30-51%) than parent SFLLRNP. These results suggested that the fluorine atom itself might be involved in the interaction with the receptor at the right position, namely, at the *para* position, whereas its placement at either the *meta* or *ortho* position would be disadvantageous in the receptor interaction. However, the total inability of the analog containing pentafluorophenylalanine (F_5)Phe (Fig. 1) to elicit platelet aggregation negated the possibility that fluorine itself is involved in the interaction with the receptor (Table II and Fig. 2).

Although the high hydrophobicity of (F_5)Phe seemed to be favorable for receptor interaction, the result clearly indicates that the replacement by fluorine of all of five hydrogen atoms in the benzene ring totally eliminates the ability of ligand peptide to bind to the receptor. Apparently, fluorine is not involved in the receptor interaction. This implies that one or more hydrogen atoms are essential for interaction with the receptor. Such an interaction involving a hydrogen atom in the Phe-benzene ring would be a hydrogen-bonding with the receptor aromatic group, but not an interaction with the receptor alkyl group. It is known that two phenylalanines in the protein form a so-called edge-to-face π-π interaction (*27-29*), and that phenylalanine interacts similarly with other aromatic amino acids in proteins (*30*). If this is the case, the intensification of the dipole of benzene CHs by substitution with fluorine would reinforce the π-π interaction and subsequently the biological activity. The effect of fluorine substitution should be maximized at the *para* and *ortho* positions of the particular fluorine. *para*-Fluorophenylalanine contains the fluorine atom at position 4 in the numbering system of the Phe-benzene ring. The *para* and *ortho* positions of this fluorine atom correspond to positions 1 for *para* and 3 and 5 for *ortho*. Since there is no CH at the *para* position, where the Phe-β-methylene group is present, CHs at the *ortho* position corresponding to positions 3 and 5 of Phe benzene

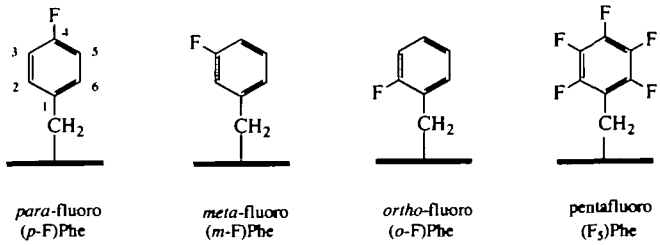

Fig. 1. **Structure of fluorinated phenylalanine side chains.**

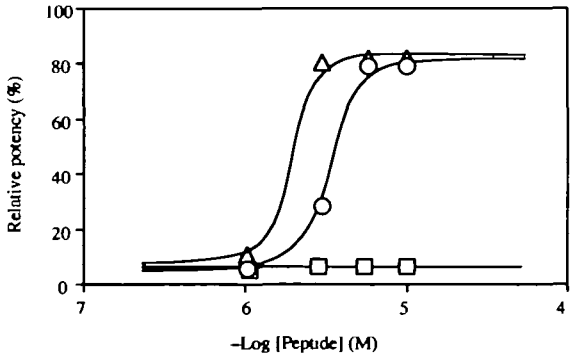

Fig. 2. **Dose-response curves of SFLLRNP and its analogs in human platelet aggregation assay.** ○: SFLLRNP, △: (*p*-F)Phe-2 derivative, □: (F_5)Phe-2 derivative.

TABLE II. **Biological activity of ligand peptide of thrombin receptor and its analogs (SXLLRNP) in human platelet aggregation.**

Side chain structure of residue X	EC_{50} (μM)	Relative potency[a]
$CH_2C_6H_5$	4.3±0.03	100 (40)
$CH_2CH_2C_6H_5$	>100	Inactive
$CH_2C_6H_4$(*p*-F)	1.7±0.44	220 (100)
$CH_2C_6H_4$(*p*-OH)	>100	Inactive
$CH_2C_6H_4$(*p*-NO_2)	>100	Inactive
$CH_2C_6H_4$(*p*-CF_3)	>100	Inactive
$CH_2C_6H_4$(*m*-F)	8.4±0.85	51 (20)
$CH_2C_6H_4$(*o*-F)	14 ±1.11	30 (12)
$CH_2C_6F_5$	>100	Inactive

[a]The relative potencies were calculated using the EC_{50} value (mean± SE) of SFLLRNP (4.3 μM) as a standard, while those in parenthesis were obtained from the value (1.7 μM) of S/(*p*-F)Phe/LLRNP.

TABLE III. **Relative potency of *para*-halogenated phenylalanine-containing thrombin receptor tethered-ligand peptide and elemental properties of halogens.**

S/(*p*-X)Phe/LLRNP	Relative potency[a]	Elemental properties of halogens	
		R (Å)[b]	Electronegativity[c]
X=F	100	1.35	4.0
Cl	16	1.80	3.5
Br	2.9	1.95	2.8
I	0	2.15	2.1

[a]The EC_{50} value of S/(*p*-F)Phe/LLRNP was 1.7 μM (Table II). [b]van der Waals radii of halogen atoms. That of hydrogen is 1.20 Å. [c]These values were derived from Pauling's empirical electronegativity scale.

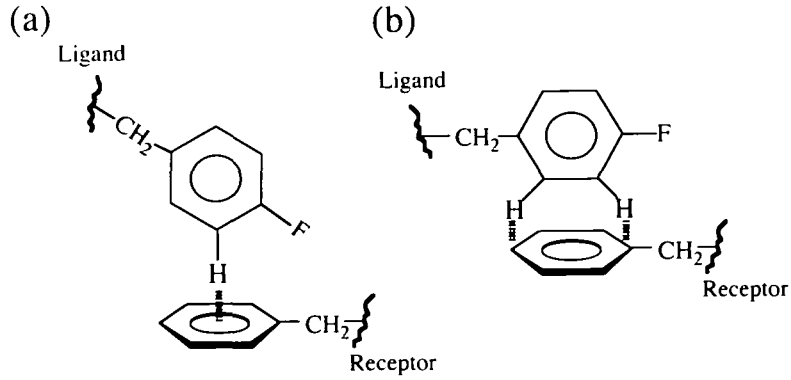

Fig. 3. **Schematic illustration showing putative π-π interactions between ligand (*p*-F)Phe-2-phenyl and receptor Phe-phenyl groups.** (a) Edge-to-face single CH/π interaction, and (b) double CH/π interaction.

ring (Fig. 1) would become more acidic. In other words, these positions are activated in a more acidic state. Thus, it is highly likely that these CHs readily participate in hydrogen-bonding. Such an effect would be maximized by fluorine, the most electronegative halogen, while other halogen atoms would be insufficiently electronegative to be effective. Indeed, as shown in Table III, the substitution of fluorine by chlorine, bromine, or iodine led to a sharp drop in activity.

S/(*p*-I)Phe/LLRNP was completely devoid of platelet aggregation activity. This result raised a question about the size of Phe-phenyl moiety. When a single methylene group was incorporated into Phe-2 between the benzene ring and the β-methylene group, the resulting S/homoPhe/LLRNP was completely inactive (Table II). This implied that the interaction between the peptide and thrombin receptor is structurally severely restricted to a small space for Phe-2, and the ligand peptide is allowed to place only a single methylene between Phe-phenyl and peptide backbone. The inactivity of S/(*p*-I)Phe/LLRNP might be also due to the large atomic size of iodine, the van der Waals distance of which is much larger than that of fluorine (Table III). It should be noted that all the analogs having various substituents at the *para* position, namely, S/(*p*-OH, -NO_2, or -CF_3)Phe/LLRNP, were completely inactive (Table II). These analogs including other inactive peptides exhibited no antagonist activity, indicating that they are completely devoid of receptor-binding activity.

The present study has shown that the *para*-fluorine substitution induces an intensification of the π-π interaction between the ligand peptide and thrombin receptor. This kind of interaction has recently been characterized by Nishio *et al.* as one of the CH/π interactions (*30*). Groups involved in CH/π interactions include methyl, isopropyl, and long-chain alkyl groups for the CH part. It should be noted that CHs in an aromatic ring are also a good source for the CH part. Aromatic groups such as those in amino acids Phe, Tyr, Trp, and His make up the π part (*30*). Although the enthalpy for a single CH/π interaction is small (about 1 kcal/mol), Nishio *et al.* have stressed that this kind of interaction is advantageous entropically in that the chance for interaction is increased by organizing CHs and/or π groups into a discrete chemical structure (*30*). It was noted that the CH/π interaction would bring about a very high specificity or selectivity in the molecular interactions. Phe-2-phenyl of SFLLRNP might be in such a specific CH/π interaction.

CHs at the *meta* and *ortho* positions of Phe-2-phenyl might participate in the CH/π interaction. There are two possible interaction modes for the edge-to-face π-π interaction between Phe-2-phenyl and the receptor aromatic ring. One possibility is that only CH at the *meta* position participates in the CH/π interaction (Fig. 3a), and the other is that both *meta* and *ortho* CHs participate (Fig. 3b). The interaction of a sole *ortho* CH with the receptor-phenyl is unlikely, since the effect of *para*-fluorine should not affect this CH to reinforce the interaction, as mentioned above. This CH is present at the *meta* position of *para*-fluorine, and the substitution by fluorine is only effective for hydrogens at its *ortho* and *para* positions, not at the *meta* position. Although it is not yet clear whether single or double CH/π interaction occurs between the ligand and receptor, the total enthalpy would become sizable in the interaction of multiple CH groups. It should be noted that the multiple binding is preferable, since it is able to bring about a high binding specificity. A difference in free energy of 1 kcal/mol is sufficient to cause a 5:1 specificity.

The results in this study suggest the role of phenylalanine at position 2 of thrombin receptor-tethered ligand in the ligand-receptor interaction. For identification of particular CH(s) in the CH/π interaction, further multiple substitutions of Phe-phenyl by fluorine would be required. Although the syntheses of di-, tri-, or tetrafluorophenylalanines in the L-configuration are chemically quite tedious, these amino acids would clarify the hydrogen atom(s) relevant to the CH/π interaction when incorporated into the peptide.

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