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Essential Groups in Synthetic Agonist Peptides for Activation of the Platelet Thrombin Receptor†

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ABSTRACT: Thrombin appears to activate platelets by a novel mechanism that involves the cleavage of its receptor, and it has been proposed that the newly generated N-terminal region of the receptor then acts as a tethered ligand [Vu, T. H., Hung, D. T., Wheaton, V. I., & Coughlin, S. R. (1991) *Cell 64*, 1057–1068]. Peptides with sequences corresponding to those of the tethered ligand are capable of activating the receptor. In the present study, groups within this tethered ligand peptide that are important for activation of the receptor have been identified by synthesizing a series of peptides. A 14-residue peptide based on the tethered ligand stimulated the aggregation of gel-filtered platelets with an EC_{50} of 7 μM, and a concentration of 10 μM was the minimum concentration necessary to yield a full aggregation response in platelet-rich plasma. Truncation of the peptide from the C-terminus to nine residues did not markedly affect the response to the peptide. Shorter peptides of five, six, and eight amino acids retained their agonist activity, but the minimal concentration necessary to achieve a full aggregation response in platelet-rich plasma was 2–5-fold higher. Side chains within the tethered ligand peptide that are important for receptor activation were identified by synthesizing a series of peptides in which residues were sequentially replaced by alanine. The results indicated that the side chains of phenylalanine, leucine, and arginine in positions 2, 4, and 5, respectively, are essential for full activity. Most notably, substitution of phenylalanine in the second position resulted in complete loss of agonist activity at concentrations up to 800 μM. The α-amino group of the peptide also appears to be essential since acetylation of this group completely abolished the activity of the peptide. The side chains of the residues in positions 1, 3, and 6–9 do not appear to be involved in interactions that are essential for receptor activation.

Thrombin activation of platelets is essential to normal hemostasis, and the recent work by Coughlin and co-workers (Vu et al., 1991a,b) has resulted in a significant advance in our understanding of the mechanism of this important process. These workers have isolated a cDNA clone encoding the thrombin receptor from platelets (Vu et al., 1991a). The deduced amino acid sequence of this receptor showed it to be a member of the family of G-protein-coupled receptors. Other members of this family include rhodopsin and adrenergic and muscarinic receptors (Kerlavage, 1991). In addition to the seven helical transmembrane domains common to all members of the family, the thrombin receptor contains a large N-terminal extracellular domain. A thrombin cleavage site is found within this extracellular domain, and it has been shown that thrombin activates the receptor by cleavage at this site (Vu et al., 1991a,b). A peptide corresponding to the newly created N-terminal region of the receptor was found to activate the receptor, and it was proposed that the newly created N-terminus is a "tethered" ligand for the receptor. Subsequently, it has been shown that the tethered ligand peptide can induce the aggregation and secretion reactions of platelets (Vu et al., 1991a) and all the known second messenger responses induced by thrombin activation (Huang et al., 1991).

Vu et al. (1991a) have shown that the sequence of the two N-terminal residues of the tethered ligand peptide is critical for its activity. In addition, data of Vouret-Craviari et al. (1992) indicate that at least five N-terminal residues are

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required for stimulation of serotonin release. However, there is no other information regarding residues within the tethered ligand peptide that are essential for its activity. In order to address this problem, the following classes of peptides have been synthesized and tested as agonists in platelet aggregation assays: (1) Peptides of varying lengths were used to establish the minimum length required for efficient activation of the receptor. Truncation of the agonist peptide to the nine N-terminal residues also allowed specific modification of the α-amino group. (2) Peptides in which each of the first nine N-terminal residues were sequentially replaced by alanine were used to evaluate the importance of individual side chains. The results obtained indicate that peptides as short as five amino acids are capable of efficiently initiating platelet aggregation. Furthermore, the α-amino group of the first residue and the side chains of the second, fourth, and fifth residues are the only groups important for the activity of the tethered ligand peptide.

Materials and Methods

Synthesis and Characterization of Peptides. Peptides were synthesized by using an Applied Biosystems Model 430A or 431A synthesizer together with *t*-Boc[1]-protected amino acids. The peptides were purified to greater than 95% homogeneity by HPLC as previously described (Maraganore et al., 1990). The identity and purity of the peptides were established by amino acid analysis and FAB mass spectroscopy.

Peptide 5 (SFLLR) was prepared by trypsin cleavage of the Arg–Asn bond of peptide 2 (SFLLRNPND). Cleavage of peptide 2 was performed in 0.1 M ammonium bicarbonate with 2% (w/w) trypsin. Peptide 5 was isolated by HPLC using the system described below for the determination of peptide concentrations.

Peptide concentrations were determined by HPLC peak height analysis using a standard of known concentration. Aliquots of unknown and standard peptides were mixed and applied to an C_8 (Aquapore RP-300) reverse-phase column equilibrated with 0.1% trifluoroacetic acid. The peptides were eluted with a linear gradient of 0–50% buffer B (70% acetonitrile in 0.085% trifluoroacetic acid) over 45 min at a flow rate of 1.0 mL/min. The effluent stream was monitored at 214 nm, and the molar concentration of the unknown peptide was calculated from its peak height relative to that of the standard peptide.

Platelet aggregation measurements were performed as previously described (Chao et al., 1989; Jakubowski & Maraganore, 1990) using a PAP-4 four-channel platelet aggregation profiler. Platelet-rich plasma (PRP) or gel-filtered platelets (GFP) in a buffered, Ca^{2+}/Mg^{2+} supplemented suspension were prepared from citrated whole blood of healthy volunteers (Chao et al., 1989; Jakubowski & Maraganore, 1990). Aggregation was followed for 5–10 min. With 20 nM thrombin or 10 μM peptide 1 (SFLLRNPNDKYEPF), the aggregation response was maximal after 2 min.

Derivatives of the tethered ligand peptide were evaluated for their effect on PRP or GFP. The concentration range used varied with the peptide. Four to six peptide concentrations were used with PRP to establish the value of $[\text{agonist}]_{100\%}$; this parameter is defined as the minimum concentration of the peptide required to achieve a maximal aggregation response using PRP. Maximal aggregation was established by using 20 μM peptide 1. Values for $[\text{agonist}]_{100\%}$ are given together with the difference (in parentheses) between the $[\text{agonist}]_{100\%}$ value and the next lowest concentration that was without effect.

[1] Abbreviations: *t*-Boc, *tert*-butoxycarbonyl; HPLC, high-performance liquid chromatography; PRP, platelet-rich plasma; GFP, gel-filtered platelets; TMS, transmembrane segment.

Table I: Effect of Agonist Peptide Length on the Minimum Concentration Required To Achieve a Maximal Aggregation Response with Platelet-Rich Plasma[a]

peptide no.	sequence	$[\text{agonist}]_{100\%}$ (μM)
1	SerPheLeuLeuArgAsnProAsnAspLysTyrGluProPhe	10 (5)
2	SerPheLeuLeuArgAsnProAsnAsp	10 (5)
3	SerPheLeuLeuArgAsnProAsn	20 (5)
4	SerPheLeuLeuArgAsn	25 (5)
5	SerPheLeuLeuArg	50 (10)

[a] Assays were performed in PRP at 37 °C under continuously stirred conditions, and the parameter $[\text{agonist}]_{100\%}$ was estimated as described under Materials and Methods. At least four concentrations of peptide were tested, and the lowest concentration of peptide that yielded a maximal aggregation response was defined as $[\text{agonist}]_{100\%}$. The values in parentheses after those for $[\text{agonist}]_{100\%}$ represent the difference between the $[\text{agonist}]_{100\%}$ value and the next lowest concentration that was without effect.

Results and Discussion

Vu et al. (1991a) have shown that the micromolar concentrations of the tethered ligand peptide SFLLRNPNDKYEPF (peptide 1) are effective in stimulating the aggregation of platelets. In the current study, it was found that a maximum platelet aggregation response was obtained in platelet-rich plasma (PRP) with this peptide at a concentration of 10 μM. In PRP, a very steep dose–response curve was obtained; at a concentration of 5 μM SFLLRNPNDKYEPF, a response less than 10% of the maximal response was observed. In fact, agonist peptide responses in PRP were either maximal or reversible under stirred conditions; no intermediate responses were observed. With gel-filtered platelets (GFP), a steep dose–response curve was also observed (Figure 1). However, in this case intermediate responses were observed, and it was possible to obtain estimates for EC_{50} values by fitting the data to the Hill equation (Stryer, 1988); an EC_{50} value of 6.7 ± 0.2 μM was obtained with peptide 1 (Table III).

Peptides corresponding to the tethered ligand sequence of the receptors from mouse (peptide 6) and hamster (peptide 7) were also tested for their ability to aggregate platelets in PRP. These peptides were almost as effective in initiating aggregation as the human receptor peptide (Table II). The values of $[\text{agonist}]_{100\%}$ for the peptides based on the mouse and hamster sequences were respectively 2- and 5-fold higher than the value for the human agonist peptide. These results suggest that the residues that are essential for the activity of the human peptide may be conserved in the mouse and hamster peptides.

Removal of two amino acids from the N-terminus of the tethered ligand peptide results in complete loss of its agonist activity (Vu et al., 1991a; Hung et al., 1992). In contrast, removal of up to five amino acids from the C-terminus of the peptide did not affect its agonist activity in PRP (peptide 2, Table I). The EC_{50} of peptide 2 with GFP was 12.2 ± 0.5 μM, which is about 2-fold higher than that obtained with peptide 1 (Table III). Further shortening of the peptide to five amino acids (peptide 5) led to a 5-fold increase in the $[\text{agonist}]_{100\%}$ value in PRP; smaller increases were seen with peptides of six and eight residues (Table I). Interestingly, the side chains of the residues between 6 and 9 were found not to be important for the agonist activity of the tethered ligand peptide (see below, Table III). Thus, it seems possible that although the side chains of these residues do not make any interactions with the receptor, the peptide chain between residues 6 and 9 may assist in maintaining the active conformation of the N-terminal five residues. The effect of C-terminal truncation of the hamster peptide on its agonist ac-

Table II: Structure–Function Relationships for the Agonist Activity of Tethered Ligand Peptides with Platelet-Rich Plasma[a]

peptide no.	sequence	$[agonist]_{100\%}$ (μM)
1	SerPheLeuLeuArgAsnProAsnAspLysTyrGluProPhe	10 (5)
2	SerPheLeuLeuArgAsnProAsnAsp	10 (5)
4	SerPheLeuLeuArgAsn	25 (10)
6	SerPhePheLeuArgAsnProSerGluAsnThrPheGluLeuValProLeu	20 (5)
7	SerPhePheLeuArgAsnProGlyGluAsnThrPheGluLeuIleProLeu	50 (25)
8	Ac-SerPheLeuLeuArgAsnProAsnAsp	>400
9	**Ala**PheLeuLeuArgAsnProAsnAspLysTyrGluProPhe	25 (5)
10	Ser**Ala**LeuLeuArgAsnProAsnAspLysTyrGluProPhe	>400
11	SerPhe**Ala**LeuArgAsnProAsnAspLysTyrGluProPhe	25 (13)
12	SerPheLeu**Ala**ArgAsnProAsnAspLysTyrGluProPhe	200 (100)
13	SerPheLeuLeu**Ala**AsnProAsnAspLysTyrGluProPhe	400 (100)
14	SerPheLeuLeuArg**Ala**ProAsnAspLysTyrGluProPhe	10 (5)
15	SerPheLeuLeuArgAsn**Ala**AsnAspLysTyrGluProPhe	5 (3)
16	SerPheLeuLeuArgAsnPro**Ala**AspLysTyrGluProPhe	25 (13)
17	SerPheLeuLeuArgAsnProAsn**Ala**LysTyrGluProPhe	10 (5)
18	Ser**Tyr**LeuLeuArgAsn	>400
19	Ser**Trp**LeuLeuArgAsn	200 (100)
20	Ser**Trp**LeuLeuArgAsnProAsnAsp	200 (100)

[a] Assays were performed in PRP at 37 °C, and the parameter $[agonist]_{100\%}$ was estimated as described in the legend to Table I. The values in parentheses after those for $[agonist]_{100\%}$ represent the difference between the $[agonist]_{100\%}$ value and the next lowest concentration that was without effect. Peptides 6 and 7 represent the mouse and hamster sequences, respectively. Substituted residues are shown in boldface type.

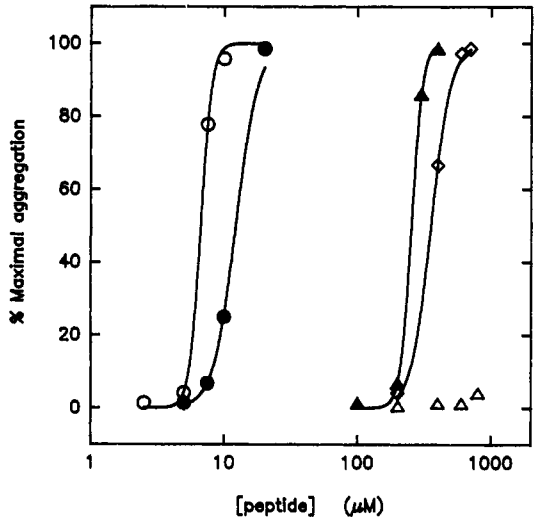

FIGURE 1: Concentration dependence of the stimulation of the gel-filtered platelet aggregation by tethered ligand peptide analogs. Gel-filtered platelets were prepared as described under Materials and Methods. Aggregation assays were performed at 37 °C with continuous stirring. The effects of peptides 1 (○), 2 (●), 10 (△), 12 (▲), and 13 (◆) were examined. The results are expressed as the percentage of maximum aggregation at 5 min. Maximum aggregation was defined as that observed with 20 μM peptide 1 at 5 min; 20 nM thrombin caused an identical aggregation response. The data were fitted by nonlinear regression to the Hill equation, and the lines drawn represent the fit of the data to this equation.

tivity with human platelets has also been investigated (Vouret-Craviari et al., 1992). Shortening of the hamster peptide (no. 7, Table II) from 14 to 7 residues decreased its EC_{50} for the release of serotonin from human platelets from 10 to 3 μM. This result indicates that the region of the hamster peptide between residues 7 and 14 is actually detrimental to agonist activity with human platelets. It can be noted that while the first seven residues of the hamster, mouse, and human tethered ligand peptides have six identical amino acids, the sequences diverge between residues 7 and 14 (Table II). Although further truncation of the hamster peptide to six residues did not affect its activity with human platelets, shortening of the peptide to five amino acids increased the EC_{50} by about 10-fold, and removal of an additional amino acid abolished its agonist activity (Vouret-Craviari et al., 1992).

Table III: Structure–Function Relationships for the Agonist Activity of Tethered Ligand Peptides with Gel-Filtered Platelets[a]

peptide no.	sequence	EC_{50} (μM)
1	SerPheLeuLeuArgAsnProAsnAspLysTyrGluProPhe	6.7 ± 0.2
2	SerPheLeuLeuArgAsnProAsnAsp	12.2 ± 0.5
10	Ser**Ala**LeuLeuArgAsnProAsnAspLysTyrGluProPhe	>800
12	SerPheLeu**Ala**ArgAsnProAsnAspLysTyrGluProPhe	253 ± 10
13	SerPheLeuLeu**Ala**AsnProAsnAspLysTyrGluProPhe	355 ± 5

[a] Aggregation assays were performed, and data were analyzed as described in the legend to Figure 1. EC_{50} values were calculated by nonlinear regression fitting of the data shown in Figure 1 to the Hill equation. Substituted residues are shown in boldface type.

In order to assess which residues in the tethered ligand peptide are essential for activity, alanine was sequentially substituted for each of the first nine residues, and the ability of these peptides to induce aggregation in PRP was tested. The results presented in Table II indicate that phenylalanine, leucine, and arginine in positions 2, 4, and 5, respectively, were the only residues whose side chains are essential for activity (peptides 10, 12, and 13, Table II). These residues are conserved in the hamster and mouse peptides (Table II). Three of the residues (Leu3′, Asn8′, and Asp9′)[2] that were not essential for activity are not conserved between the human, mouse, and hamster sequences (Table II). In contrast, the side chain of Ser1′, which is conserved, was not essential for activity. The EC_{50} values of peptides 10, 12, and 13 were also determined with GFP (Figure 1, Table III). The replacement of Phe2′ by alanine increased the EC_{50} value by over 100-fold from 7 to over 800 μM. Substitution of Leu4′ increased the EC_{50} by 38-fold to 253 μM, while the replacement of Arg5′ led to a 53-fold increase (EC_{50} = 355 μM, Table III). Thus, the lack of activity observed with the four-residue hamster agonist peptide (Vouret-Craviari et al., 1992) can be ascribed to the absence of Arg5′ in this peptide.

The aromatic amino acids tyrosine and tryptophan were tested for their ability to substitute for Phe2′ (Table II). Marked increases in the $[agonist]_{100\%}$ values were observed

[2] Amino acid residues in the tethered ligand peptides are labeled with primed numbers that indicate their position in the thrombin-cleaved (activated) receptor. The sequence of the activated receptor begins with Ser42 of the amino acid sequence derived from the cDNA clone of the receptor (Vu et al., 1991a). Thus, Ser1′ is equivalent to Ser42.

as a result of both of these substitutions. The six-residue peptide with the Phe2′ → Tyr substitution was unable to induce aggregation in PRP at concentrations up to 400 μM. Thus, the hydrophobicity of the aromatic group of Phe2′ seems to be critical. The size of this aromatic group is also important; replacement of Phe2′ by tryptophan increased the $[\text{agonist}]_{100\%}$ values of the six- and nine-residue peptides to 200 μM.

Although the side chain of residue 1 was not essential for activity, N^{α}-acetylation of the tethered ligand peptide completely abolished its activity (Ac-SFLLRNPND; Table II), which indicates that the α-amino group of the peptide is required for its activity. A primary amine is also required for activity in a number of other peptide and nonpeptide agonists of G-protein-coupled receptors (Savarese & Fraser, 1992).

If marked increases in $[\text{agonist}]_{100\%}$ values were observed, the peptides were tested for antagonist activity at a concentration of 400 μM against the induction of aggregation in PRP by 10 μM peptide 1. In all cases, no antagonist activity was observed.

Studies on other G-protein-coupled receptors indicate that the binding site for the ligand involves residues in several of the transmembrane helices (Savarese & Fraser, 1992). Comparison of the three known amino sequences of the thrombin receptor (Vu et al., 1991a; Rasmussen et al., 1991; Zhong et al., 1992) with those of other members of the G-protein-coupled receptors suggests a number of residues that might be involved in binding the tethered ligand. There is evidence that the formation of a salt bridge between cationic amines and a conserved aspartate in transmembrane segment III (TMS III) is important for ligand binding with β_2-adrenergic receptors (Strader et al., 1991). The results presented in Table II suggest that the α-amino group of the peptide and Arg5′ may be involved in similar electrostatic interactions with the thrombin receptor. The sequences of the putative transmembrane segments of the thrombin receptors contain only two buried acidic groups, Asp148 in TMS II and Glu241 in TMS IV. Asp148 is conserved between all G-protein-coupled receptors, and it seems likely that this residue has a structural role and is not involved in ligand binding (Hibert et al., 1991). Glu241 of the thrombin receptor is found at the junction of TMS IV and the loop that connects TMS IV and TMS V (loop IV–V), and an ionic interaction of this residue with either the α-amino group or Arg5′ would seem possible. Given the length of the side chain of arginine, it is conceivable that the guanidino function of Arg5′ could form a salt bridge with acidic groups in the extracellular loops that connect the transmembrane segments. Loop VI–VII is not well conserved between the three known thrombin receptor sequences and contains no conserved acidic residues (Vu et al., 1991a; Rasmussen et al., 1991; Zhong et al., 1992). There are, however, several conserved acidic residues in loops II–III and IV–V (Asp167, Asp256, and Glu260). Within the transmembrane segments, there are also a number of residues that are conserved in the three sequences and would be able to form hydrogen bonds with the α-amino group, the side chain of Arg5′, and groups in the peptide backbone of the tethered ligand. Site-directed mutagenesis experiments are required to test the importance of these and other residues in the thrombin receptor.

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