Structure-Function Relationships in the Activation of Platelet Thrombin Receptors by Receptor-derived Peptides*

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According to present models, thrombin activates platelets by cleaving its receptors after Arg41, creating a new N terminus which acts as a tethered ligand. In support of this model, a peptide (SFLLRN(P)NDKYPF or TRP(42/55) corresponding to residues 42–55 has been shown to activate the receptor. In the present studies, the structural basis for thrombin receptor activation was examined using fragments of this peptide, as well as variants of the peptide with selected amino acid substitutions. The results show that the features of SFLLRN(P)NDKYPF required to mimic the effects of thrombin reside within the first 6 residues, SFLLRN.

A hexapeptide comprised of these residues was approximately 5 times more potent than the parent peptide in assays of platelet aggregation and, in addition, caused tyrosine phosphorylation, inhibition of CAMP formation, and an increase in cytosolic Ca2+. Omission of either the Ser residue or the Arg and Asn residues greatly diminished peptide activity, as did the substitution of Ala for Phe or Arg. Substitution of Ala for Ser or the initial Leu, on the other hand, had little adverse effect. The inactive peptides SALLRN and NPNDKYPF had no effect on platelet activation initiated by SFLLRN, but FLLRN inhibited platelet aggregation in response to both SFLLRN and thrombin. These results suggest that within SFLLRN the Phe and Arg residues are particularly important and that Phe must be preceded by another amino acid, the identity of which is not tightly constrained. This observation and comparisons with the homologous domains of proteins whose tertiary structure is known were used to predict the conformation of the SFLLR sequence. The model which emerged suggests that the SFLLR domain may be part of an extended β structure in the intact receptor and that cleavage by thrombin causes it to contract and assume a modified helical configuration.

In this predicted conformation the side chains of Phe and Arg point in the same direction, potentially into a pocket formed by the remainder of the receptor.

Formation of multicellular platelet aggregates, all of which play a necessary role in hemostasis. Most, if not all, of these effects are thought to be mediated by cell-surface receptors coupled via G proteins1 to intracellular enzymes such as phospholipase C and phospholipase A2 (1). These in turn generate the second messengers that regulate platelet activation.

Thrombin has also been shown to stimulate tyrosine kinases in platelets, leading to the phosphorylation on tyrosine of multiple platelet proteins (2, 3). The manner in which tyrosine kinases are coupled to thrombin receptor activation is unknown.

The structure of the thrombin receptor present in the human megakaryoblastic DAMI cell line has recently been deduced by expression cloning in Xenopus laevis oocytes (4). Key features of the receptor that are predicted from the cDNA sequence include a single polypeptide chain with seven membrane-spanning domains and an extracellular N terminus that contains a thrombin cleavage site between Arg41 and Ser42. Notably, a peptide corresponding to receptor residues Ser42 through Phe55 (TRP42/55 or SFLLRN(P)NDKYPF) has been shown to cause platelet aggregation and to activate oocytes expressing the receptor (4). This has led to the suggestion that the newly created N terminus of the receptor following cleavage by thrombin may be a tethered ligand for the receptor (4). Additional support for this model has been obtained by the cloning of the thrombin receptor from hamster fibroblasts. Although only 79% homologous overall, the hamster and human clones are essentially identical in the domains thought to be involved in thrombin binding and receptor activation, and a peptide corresponding to the one which activates the human receptor has been shown to activate the hamster receptor (5).

Thus it appears that the thrombin receptor resembles other receptors that interact with G proteins but is activated by a novel mechanism involving proteolysis and generation of a tethered ligand. Recent studies from this laboratory have shown that the activation peptide TRP42/55 can evoke the entire range of G protein-dependent responses observed when thrombin is added to the megakaryoblastic HEL cell line (6, 7).

The peptide also causes homologous desensitization of the HEL cell thrombin receptor (7), activates phospholipase C and phosphatidylinositol 3-kinase in platelets (8), and stimulates prostaglandin I2 formation while raising the cytosolic free Ca2+ concentration in endothelial cells (9). In the present studies, we have examined the structural basis for the interaction of TRP42/55 with the thrombin receptor. The results show that the activity of the peptide resides within its first 6 amino acids and suggests which residues are particularly critical for receptor activation. Comparisons with other pro-

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† The abbreviation used is: G protein, GTP-binding regulatory protein.
proteins suggest a three-dimensional model for the interaction of the receptor N terminus with the remainder of the receptor that is compatible with the observed effects of amino acid substitutions on platelet activation by the peptide.

MATERIALS AND METHODS

Platelet Aggregation and PAC1 Binding—Blood was obtained from healthy donors and anticoagulated with citrate (1 part 3.8% sodium citrate to 9 parts blood). Platelet-rich plasma was prepared by centrifugation at 1000 x g for 3 min. Platelet aggregation was measured in a Chrono-Log (Havertown, PA) aggregometer using platelet-rich plasma in all cases except when thrombin was used as the agonist. For the studies with thrombin, platelets were isolated by gel filtration. Gel-filtered platelets were also used for the PAC1 binding studies, in which case the binding of fluorescein isothiocyanate-labeled PAC1 was detected by flow cytometry (10, 11).

cAMP Measurements—The formation of cAMP by platelet membranes was measured with [α-32P]ATP as the substrate using the method of Solomon (12) as previously described (13).

Tyrosine Phosphorylation—Platelets (7 x 10^6/ml) isolated by gel filtration were incubated with the specified agonists for 2 min at 37 °C while stirring at 1000 rpm (3). Afterward, the cells were lysed with 4 x sample buffer (14), boiled for 5 min, and centrifuged for 10 min at 15,000 x g. The supernatants were subjected to electrophoresis on 7.5% polyacrylamide, transferred to nitrocellulose, and probed with a mixture of two anti-phosphotyrosine antibodies: 4G10 (15) and PY20 (1 mg/ml, ICN Immunobiologics, Costa Mesa, CA). Horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) was used as a secondary antibody, and immunoreactivity was detected using the ECL chemiluminescence reaction (Amersham Corp.).

Analysis of Structure—A comparative modeling approach was used to develop molecular models for the receptor by searching the Brookhaven crystallographic data base (16) for proteins with sequence similarities to the region of interest within the receptor (17-19) as previously described (20-22). Identified structures were used as starting templates for construction of the hexapeptide model and its analogs. The models identified in this manner were energy-minimized using the program Discover (Biosym Technologies) and its supplied consistent valence forcefield. Lacking an explicit receptor model with which to interact, the objective was to identify any conformations accessible to the hexapeptide regardless of the environment. The calculations were performed in vacuum employing a distance-dependent dielectric model. Side chains and N- and C-terminal groups were defined to be in their neutral state to prevent unscreened interactions between charged groups from dominating the structural search. Typical convergence criteria were assumed for complete optimization. Identification of sequence motifs was performed using the program Creator (Wistar Institute). Model building was performed using the program Insight II (Biosym Technologies).

Other Materials—Human α-thrombin (3000 units/mg) was a gift from Dr. John Fenton (New York State Department of Health, Albany, NY). Peptides were synthesized by the Protein and Nucleic Acid Core Facility of Children’s Hospital of Philadelphia, purified by high pressure liquid chromatography, and confirmed by amino acid analysis and protein sequencing.

RESULTS AND DISCUSSION

One of the hallmarks of platelet activation by thrombin is the conformational change in the plasma membrane glycoprotein Ib-IIIa complex which allows fibrinogen binding and platelet aggregation to occur. This conformational change can be detected using PAC1, a monoclonal antibody that binds only to the activated form of the Ib-IIIa complex (10). In the studies shown in Fig. 1, fibrinogen receptor expression and platelet aggregation were initiated by a series of peptides derived from the thrombin receptor sequence Ser-Arg-Glu-Pro-Thr-Arg (TRP42/5s). As predicted by earlier observations (4), TRP42/5s (SFLLRNPNKDYEFP) stimulated PAC1 binding and platelet aggregation, while the same peptide with Ser and Phe reversed, FSLLRNPNKDYEFP, did not. The concentration of TRP42/5s at which aggregation was half-maximal was approximately 4 μM (Table 1). A peptide comprised of the first nine amino acids of TRP42/5s, SFLLRNPNPD, was equally active, while a peptide comprised of the last nine amino acids, NPNDKYEF, was inactive. Of the peptides tested, the hexapeptide SFLLRN was the most potent, causing half-maximal platelet aggregation at approximately 0.8 μM. The tetrapeptide SFLL was more than 2 orders of magnitude less potent than SFLLRN with half-maximal platelet aggregation occurring at 187 μM. Restoration of the arginine residue that normally precedes Ser in the intact receptor (RSFLLRNPNPD) did not abolish activity but increased the peptide concentration required for platelet aggregation 10-fold to 45 μM.

It has not yet been established whether all of the cellular responses to thrombin are mediated by a single set of receptors or whether different classes of receptors are involved, perhaps with different structures and different mechanisms of acti-
Thrombin Receptor Activation

Platelet and HEL cell responses to thrombin receptor-derived peptides

| Human receptor residues | Peptide sequence       | Aggregation* | Inhibition of adenylylcyclase | Increased Ca2+ | Tyrosine phosphorylation* |
|-------------------------|------------------------|--------------|------------------------------|---------------|---------------------------|
|                         |                        | µM           |                              |               |                           |
| 42–55                   | SFLLRNPNKDYEPF         | 4 ± 1        | +                            | +             | +                         |
| 47–55                   | - - - - - PNKDYEPF     | >200         | -                            | -             | -                         |
| 42–50                   | SFLLRNPNPD            | 4 ± 1        | +                            | +             | +                         |
| 42–47                   | SFLLRN                | 0.8 ± 0.2    | +                            | +             | +                         |
| 42–45                   | SFLL                  | 187 ± 13     | -                            | -             | ±                         |
| 41–50                   | FSSLRNPNKDYEPF        | >200         | -                            | -             | -                         |
| 42–47                   | RSSLRNPNPD            | 45 ± 5       | ND*                          | ND            | ND                        |
| 43–47                   | SFLLRN                | 0.8 ± 0.2    | -                            | -             | -                         |
| 42–45                   | SFLL                  | 187 ± 13     | -                            | -             | -                         |

* The mean peptide concentrations (±S.M.) required for half-maximal platelet aggregation are derived from two to six determinations. In the cases indicated as ">200," there was no response at 200 µM. Amino acid residues that vary from those predicted to be present in the human thrombin receptor (4) are shown in boldface.

** and ND, not determined.

This table shows the agonistic activities of various thrombin receptor-derived peptides. The table includes the human receptor residues, the peptide sequence, and the respective responses such as platelet aggregation, inhibition of adenylylcyclase, increased calcium, and tyrosine phosphorylation.

Fig. 2. Peptide-induced tyrosine phosphorylation of platelet proteins. Platelets were incubated with thrombin (10 nM) or one of the peptides shown (50 µM) for 2 min. Total cell lysates were then prepared and probed with antibodies to phosphotyrosine. The results shown are representative of three studies.

In all three cases the "activation sequence" is followed by a series of acidic residues which are thought to play a role in the binding of thrombin to the receptor (23, 24). Since the heptapeptide SFLLRNPNDKYEPEF reported ability to evoke thrombin-like responses from hamster CCL39 fibroblasts (5), substitution of Phe for Leu in the peptide or the receptor does not appear to affect activity.

Structural Features of the Receptor-derived Hexapeptide—A second series of receptor-related peptides was constructed to examine the structural elements of the peptide SFLLRN that are required for thrombin receptor activation. The results are summarized in Table I. Starting at the N terminus of the peptide, replacing the Ser with Ala (AFLLRN) or Thr (TFLLRN) had a modest effect on peptide activity, increasing the peptide concentration required for half-maximal platelet aggregation from 0.8 to 2–3 µM. Omission of the serine residue (FLLRN), on the other hand, abolished activity, as did replacing Phe with Ala (SALLRN). As already described, switching Ser and Phe also abolished activity.

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required for half-maximal platelet aggregation from 0.8 μM to approximately 2 μM. Replacement of the second Leu with Ala (SFLARN) caused a further increase to 19 μM. Substitution of Ala for Arg (SFLLAN) increased the concentration required for half-maximal platelet aggregation to approximately 60 μM, as did switching Arg and Asn (SFLLNH). Omission of Arg and Asp (SFLL) resulted in a peptide which was half-maximally active at approximately 187 μM.

These results focus particular attention on the identity and placement of the initial residues of SFLLRNPNNDKYEPF. Therefore, the ability of the inactive peptides FLLRN, SALLRN, and NPNDKYEPF to inhibit SFLLRN-induced platelet aggregation was tested (Fig. 3). Of the three, only FLLRN was an inhibitor. Notably, FLLRN was also able to inhibit platelet activation by thrombin (Fig. 3D), suggesting that it can also interfere with the proposed interaction of the N terminus of the receptor (after proteolysis by thrombin) with the remainder of the receptor. Parenthetically, FLLRN had no effect on the ability of thrombin to clot fibrinogen.

**Peptide Modeling**—In an attempt to correlate these results with predictions about the tertiary structure of the N terminus of the receptor, the Brookhaven crystallographic data base (16) was searched for proteins containing all or part of SFLLRNPN and its hamster homolog, SFFLRNP. The approach used is described under “Materials and Methods” and was previously applied by one of us (T. K.-E.) in correlating peptide structure and function in the development of bioactive peptides capable of modulating virus-receptor interactions (20–22, 25, 26). Although complete matches were not found, partial matches spanning 3–4 residues were identified representing segments of the hexapeptide or its analogs. SFPL was present in the crystal structure of the Fc fragment, 1fc1 (Brookhaven data base entry designation), where it forms part of a β-strand structure. In addition, 13 proteins were found with the LLR sequence and 10 with the FLR sequence. In each of these, the identified sequence was part of a β-strand structure, suggesting that in a larger protein SFPLL might assume a β configuration. A search for proteins containing the RNP sequence produced seven matches. In each of these RNP was part of a turn within the protein structure. One of the identified proteins was bacteriophage T4 lysozyme (1l24). In lysozyme the RNP turn is preceded by a helix structure. In searching for a structure for the SALL analog of the hexapeptide, we observed this sequence type in the crystal structure of human lysozyme (1l21), where it also forms a helix. Superpositioning this helical region of the two lysozyme molecules indicates that they are similar.

The results of this analysis suggest that when part of a larger protein, the SFLLRNPN sequence would be predicted to form a β-strand followed by a turn, but the free hexapeptide SFLLRN or the thrombin-cleaved N terminus might form either a β structure or a helix. Both of these possibilities were tested using the program Insight II (Biosym Technologies). Starting templates were extracted from the identified crystal structures and the side chains replaced with the SFLLRN sequence. Side chain angles were adopted from established side chain rotomer libraries (27) and by side chain conformational scans (28). The models were energy-minimized to convergence, followed by molecular dynamics calculations to sample the conformational population around the modeled structures. After each dynamics calculation the conformers were again minimized to convergence looking for conformers with the lowest energy. This approach led to an extended helical structure for the peptide as a low energy form in which the side chains of the Arg and Phe residues are on the same side looking down the axis of a helix. This configuration was 11 kcal more preferred over the lowest energy structure derived from the β-strand starting geometry. Substitution of Ala or Thr for the Ser residue or Ala for either of the two Leu residues had little effect on this conformation, just as these substitutions had relatively little effect on platelet activation by the peptide (Table I). Substitution of Ala for Phe (SALLRN), on the other hand, produced a helical conformation which, if anything, was somewhat less distorted than SFLLRN. Since SALLRN proved to be inactive as either an agonist or an antagonist, this again points to the role of the phenylalanine side chain, rather than the helical conformation per se.

**Conclusions**—These results suggest several conclusions about the mechanism of thrombin receptor activation and the interaction between the peptide and receptor. First, all of the responses to thrombin that have been tested to date which are known to occur in platelets can be evoked by the receptor-derived peptides, suggesting that they are mediated either by a single class of receptors or by two or more classes of receptors sharing a common external structure. This includes responses known to involve G proteins, as well as responses to thrombin that do not have a defined G protein dependence, such as tyrosine phosphorylation. Second, all of the structural
features of the peptide, SFLLRNPNDKYEPF, and by implication the N terminus of the receptor, required for activation are present within the first 6 residues, SFLLRN. This suggests that only the tip of the thrombin-cleaved N terminus is needed for activation. Portions of the remainder of the N terminus proximal to the first transmembrane domain are thought to help to bind and orient thrombin toward its cleavage site in the receptor (23, 24). Presumably they also help to retain and orient the SFLLRN sequence.

Finally, molecular modeling suggests that in the intact receptor SFLLRN may be part of an extended $\beta$-structure but that proteolysis by thrombin causes it to assume a modified $\alpha$-helical configuration in which the side chains of the critical Phe and Arg residues point in the same direction, potentially facing into a pocket formed by the remainder of the receptor, although this remains to be established. Although this model can only be thought of as a working hypothesis, it does fit with the observed consequences of amino acid substitutions within the SFLLRN sequence and provides a basis for future experimental design.

Addendum—While this paper was in preparation, a report by Vouret-Craviari et al. (29) appeared describing comparable studies with peptides derived from the hamster thrombin receptor sequence. Like us, they found that the initial 5–6 residues distal to the site of receptor cleavage are sufficient for activation of the thrombin receptor on hamster CCL39 fibroblasts. Notably, however, they found that the receptor-derived peptides failed to stimulate thymidine incorporation in the fibroblasts unless added in the presence of fibroblast growth factor. The possibility was raised that this is due to the inability of receptor-derived peptides to stimulate thrombin toward its cleavage site in the intact receptor. Additionally, molecular modeling suggests that in the intact receptor SFLLRN sequence provides a basis for future experimental design.

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