0026-895X/96/010190-08$3.00/0

MOLECULAR PHARMACOLOGY, 49:190–197 (1996).

Thrombin Receptor Activation by Thrombin and Receptor-Derived Peptides in Platelet and CHRF-288 Cell Membranes: Receptor-Stimulated GTPase and Evaluation of Agonists and Partial Agonists

STEVEN M. SEILER, MARIANNE PELUSO, JEFFREY G. TUTTLE, KELLY PRYOR, CLIFFORD KLIMAS, GARY R. MATSUEDA, and MICHAEL S. BERNATOWICZ

Departments of Cardiovascular Biochemistry (S.M.S., M.P., J.G.T.) and Macromolecular Structure (K.P., C.K., G.R.M., M.S.B.), Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543

Received August 3, 1995; Accepted October 13, 1995

SUMMARY

Thrombin receptor activation, by thrombin or SFLLR-containing peptides, stimulates GTPase activity in platelet and CHRF-288 membranes. Polyclonal antibodies to peptides derived from the thrombin receptor (anti-TR_{52-69} and anti-TR_{36-49}), which block many of thrombin's actions on platelets and endothelial cells, also block thrombin activation of membrane GTPase (as does thrombin active site and anion-binding exosite inhibitors). Most of the receptor-activated GTPase, stimulated by both thrombin and SFLLRNP in platelet membranes, was inhibited by prior treatment with pertussis toxin or *N*-ethylmaleimide, suggesting that under these conditions much of the thrombin receptor-stimulated GTPase in platelet membranes is a member of the pertussis toxin-sensitive $G_{\alpha i}$ family. In platelet membrane preparations, the peptide agonists stimulated approximately twice as much GTPase activity as stimulated by α-thrombin. In contrast, the membranes prepared from CHRF-288 cells showed similar maximal SFLLRNP- and α-thrombin-stimulated GTPase activity. Stimulation of the platelet membrane GTPase by a variety of different peptide agonists correlated with their ability to stimulate platelet aggregation. Several peptide-based agonists were more potent than the wild-type sequence. The most potent was Ser-(*p*-fluoro-Phe)-(2-Napthyl-Ala)-Leu-Arg-NH_2, which stimulated platelet aggregation (EC_{50} = 80 nM) and GTPase activity (EC_{50} = 110 nM). The peptide YFLLRN stimulated GTPase activity but only to ~40% of the activity observed with optimal concentrations of other receptor agonists. YFLLRN also limited the stimulation observed with SFLLRNP in a competitive fashion, indicating that YFLLRN is a competitive partial agonist at the thrombin receptor. These studies show that the tethered-ligand receptor mediates the GTPase activation by thrombin in platelet and CHRF-288 cell membranes, and this provides a specific, reliable, and convenient cell-free assay system with which one can evaluate agonists and partial agonists.

The clotting protease thrombin has been implicated in thrombotic, atherosclerotic, and inflammatory pathologies, and, the proteolytically activated tethered-ligand receptor appears to mediate many of thrombin's actions on the cell types involved (1). This receptor has been cloned (2–5) and is present on human platelets (2), endothelial cells (4), fibroblasts (3), vascular smooth muscle cells (5), CHRF-288 cells (6), and others. The tethered-ligand thrombin receptor has seven putative transmembrane-spanning domains and belongs to a family of G protein-coupled receptors (2). α-Thrombin recognizes this receptor by binding to the receptor's hirudin-like domain and activating the receptor by limited proteolytic cleavage, generating a new amino terminus. The new amino terminus, through intramolecular interaction, activates the receptor (2). Thrombin activation of the receptor can be mimicked by peptides, homologous to the newly generated amino terminus (TR_{42-46}). These peptides can activate the receptor directly, without prior thrombin cleavage (2, 7, 8). The activated receptor stimulates several different heterotrimeric G proteins in different cell types, the α subunits of which include G_i (9), G_{i2} (10), G_q (10–12), G_o (13), and G_{12} and G_{13} (11).

The involvement of the thrombin tethered-ligand receptor in human platelets has been implicated by the ability of SFLLR-containing peptides to mimic thrombin's activation of platelets (2, 14–19, 20, 21). Furthermore, blocking antibodies to the proteolytically activated thrombin receptor (22, 23) as well as thrombin receptor antagonists (16) are capable of preventing thrombin-stimulated human platelet aggregation. However, the tethered-ligand receptor is not the sole

ABBREVIATIONS: BSA, bovine serum albumin; HUVEC, human umbilical vein endothelial cell; M199, Medium-199; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHRF-288, NEM, *N*-ethylmaleimide; KLH, keyhole limpet hemocyanin; GYKI-14766 (LY 294468), *N*-methyl-D-Phe-Pro-Arg-aldehyde; PG, prostaglandin.

thrombin binding site on platelets and other cells (24). Thrombin binds to glycoprotein Ib and cleaves glycoprotein V. In platelets from nonprimate species, e.g., rat and dog, the involvement of the tethered-ligand receptor is not clear. Although these platelets are responsive to α-thrombin, they are not responsive to activating peptides; however, other cell types (e.g., smooth muscle cells and fibroblasts) from these same species respond through thrombin receptor-mediated activation (25, 26).

Previous studies showed that α-thrombin caused activation of GTPase in membranes derived from human platelets (27, 28). The megakaryoblastic CHRF-288 cell line is also known to abundantly express the thrombin receptor (~200,000 receptors/cell (6)). We wanted to determine whether the thrombin-activated GTPase could also be observed in CHRF-288 cell membranes and whether this activity was mediated by the tethered-ligand receptor in both cell types. If the GTPase activity in these membrane systems was activated by the tethered-ligand receptor, then it might provide a useful cell-free assay system independent of downstream effector systems that could be used to evaluate agonists, partial agonists, and antagonists. This system would have the added advantage of using native cell membrane preparations and avoiding problems associated with transfected cells such as variations in receptor number and host-cell G protein availability.

Materials and Methods

Peptides and thrombin. All of the peptides used in these studies were prepared as carboxyl-terminal amides. The heptapeptide (SFLLRNP) was obtained from Bachem. The thrombin receptor-derived peptides TR_{52-69} (YEPFWEDEEKNESGLTEY), TR_{42-52} (SFLLRNPNDKYC), and TR_{36-49} (CATLDPRSFLLRNPNPN) were synthesized using manufacturer-specified Fmoc/*t*-butyl protocols on a Milligen/Biosearch 9050 peptide synthesizer. The peptides were purified and characterized by high performance liquid chromatography, amino acid analysis, and mass spectometry/fast atom bombardment. Other SFLLR analogues were prepared as previously described (8). Human α-thrombin (2000–3000 units/mg) was kindly provided by Dr. J. Fenton II (New York State Department of Health, Albany, NY).

Platelet membrane preparation. For most of the studies reported here using platelet membranes, human platelets were obtained from concentrates (Interstate Blood Bank, Philadelphia, PA). The platelet concentrates were pooled and centrifuged at 132 × *g* for 10 min at 4° to remove remaining red blood cells. The platelet-rich plasma was collected and supplemented with 5 mM EDTA, and the platelets were recovered by centrifugation at 2200 × *g* for 20 min at room temperature. The platelet pellets were resuspended in wash buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris·HCl, pH 7.5) and gently washed three times. The platelets were then homogenized manually with 20 strokes of a glass/glass homogenizer in ice-cold 5 mM EDTA and 5 mM Tris·HCl, pH 7.5. The homogenate was centrifuged at 31,000 × *g* for 20 min at 4°. The membrane pellet was resuspended in 20 mM Tris·HCl, pH 7.5, 1 mM EDTA, and 0.1 mM dithiothreitol and distributed in 1-ml aliquots at a concentration of 2 mg/ml and stored at −70°. Protein content was determined according to the method of Bradford (29).

In some studies, platelets and platelet membranes were prepared with all of the solutions containing protease inhibitors (including 10 mM benzamidine, 20 μg/ml leupeptin, 0.1% aprotinin, and 1 unit/ml hirudin) in the anticoagulant, platelet wash buffers, homogenization buffer, and storage buffer.

CHRF-288 cell membranes. The megakaryoblastic CHRF-288 cells were obtained from Dr. M. Lieberman (University of Cincinnati College of Medicine, Cincinnati, OH) and cultured in the presence of 1 unit/ml hirudin. The cells were recovered by centrifugation (700 × *g* for 15 at 4°) and washed with Hank's balanced salt solution without Ca^{2+} or Mg^{2+}. The cells were resuspended in homogenization medium containing 10 mM Tris, pH 8.0, 1 mM $MgCl_2$, 5 mM EDTA, 1 mM dithiothreitol, 10 mM benzamidine, 20 μg/ml leupeptin, 0.1% aprotinin, and 1 unit/ml hirudin and homogenized with 15 strokes with a precooled Dounce-type homogenizer. The homogenate was sedimented at 250 × *g* for 5 min at 4°. The loosely packed material and supernatant were centrifuged at 31,400 × *g* for 15 at 4°. The membranes obtained were suspended at 2 mg/ml in 20 mM Tris·HCl, pH 7.5, 1 mM EDTA, and 0.1 mM dithiothreitol containing the above-mentioned protease inhibitors and stored frozen in 1-ml aliquots at −70°.

GTPase measurements. Membranes were diluted with ice-cold 10 mM triethanolamine HCl and 5 mM EDTA, pH 7.4, and collected by centrifugation for 10 min at 39,000 × *g* and 4°. The supernatant was removed, and the pellet was resuspended by trituration in the same medium. The membranes were centrifuged and then resuspended in ice-cold 100 mM NaCl and 10 mM triethanolamine HCl, pH 7.4. The GTPase reaction was started by the addition of the membranes (30 μg in a volume of 50 μl) to 50 μl of reaction media (30). The final reaction contained 0.4 μM GTP ([γ-^{32}P]GTP; 5 × 10^5 dpm/ml), 100 mM NaCl, 0.1 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM ATP, 5 mM phosphocreatine (Tris salt), 100 units/ml creatine phosphokinase, 0.2% BSA, 50 mM imidazole, pH 7.3, and the indicated compounds. The reaction was carried out for 10 min at 22–24° and stopped with 750 μl of cold 5% (w/v) activated charcoal (powder) in 20 mM phosphoric acid. The tubes were centrifuged for 20 min at maximum speed at 4° in an Eppendorf 5415C Microcentrifuge, and 450 μl of the supernatant was added to 5 ml scintillation fluid (Eco-Lite) and counted in a scintillation counter.

Pertussis toxin treatment. Human platelet membranes were washed twice with 30 ml ice-cold 10 mM triethanolamine HCl, pH 7.4, and 5 mM EDTA followed by centrifugation at 25,000 × *g* for 10 min at 4°. The membranes were incubated at ~5 mg/ml with Pertussis Toxin A Protomer (20 μg/ml) in a solution containing 25 mM HEPES, pH 8, 1 mM EDTA, 1 mM ATP, 3 mM potassium phosphoenolpyruvate, 10 μg/ml pyruvate kinase, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM thymidine, 2.5 mM NAD, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μM phenylmethylsulfonyl fluoride (31). A control tube of membranes was incubated as above with an equivalent volume of buffer containing 50 mM Tris·HCl, pH 8, 0.5 mM EDTA, and 0.25% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate. The incubation was terminated with the addition of 10 volumes of ice-cold 25 mM HEPES, pH 8, 1 mM EDTA, and 0.1 mM dithiothreitol, followed by centrifugation at 25,000 × *g* for 20 min at 4°. The membranes were then resuspended in 10 mM triethanolamine HCl, pH 7.4, and 10 mM NaCl to a protein concentration of 0.6 mg/ml.

Treatment of membranes with NEM. The platelet membranes were centrifuged and washed twice with 10 mM triethanolamine HCl, and 5 mM EDTA, pH 7.4. The membranes were centrifuged again, and the pellet was resuspended in 1.0 ml of 50 mM triethanolamine HCl, pH 7.4, 1 mM EDTA, and 10 mM NEM. The samples were diluted 9-fold, and the reaction was incubated on ice for 30 min. (Control membranes were similarly treated without the NEM.) The reaction was stopped with the addition of 1/10th volume of 150 mM 2-mercaptoethanol, followed by 30 ml of 10 mM triethanolamine HCl, pH 7.4. The membranes were centrifuged for 10 min at 30,000 × *g* at 4°, and the pellet was resuspended in 100 mM NaCl and 10 mM triethanolamine HCl, pH 7.4.

HUVECs. HUVEC were purchased from Cell Systems at passage 1 and were serially cultured in M199 with 25 mM HEPES and 16.6 mM $NaHCO_3$ containing 20% fetal calf serum, 2 mM L-glutamine, 125 units/ml penicillin, 125 μg/ml streptomycin, 100 μg/ml porcine heparin, and 50 μg/ml endothelial cell growth supplement in a 95%

air/5% CO_2 humidified atmosphere at 37°. The HUVEC were grown on gelatin-coated 12-well plates and were used in their third or fourth passage at or near confluence. Each well was washed four times with M199 plus gelatin (0.1%) and then with 500 μl of the same buffer containing 10 μg/ml affinity purified antibody and/or 2 μg/ml of the competing antigen peptide. The cells were incubated for 10 min on a slide warmer at 37°. At the end of the incubation, the cells were stimulated with α-thrombin and incubated for 30 min at 37° with 5% CO_2. The conditioned media were collected, and EDTA was added (1 mM final concentration) to each sample. Measurement of 6-keto-$PGF_{1\alpha}$ was performed using a commercially available radioimmunoassay kit (DuPont-NEN). A cell count was performed for each experiment. HUVECs were detached from a total of 10 wells using 1.0 ml of trypsin (0.5%)/EDTA (0.02%)/well. A 1:50 dilution was prepared for each well and counted on a model ZM Coulter Counter. The cell counts were averaged and used to calculate the 6-keto-$PGF_{1\alpha}$ produced/10^5 cells.

Platelet aggregation. Blood was collected from human volunteers into syringes containing trisodium citrate (1/10th volume) and centrifuged at 200 × *g* for 10 min at 22°. The supernatant, platelet-rich plasma, was collected and gently applied to a Sepharose 2B-CL column and eluted with 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3 mM NaH_2PO_4, 3 mM HEPES, pH 7.35, and 3.5 mg/ml bovine serum albumin. The platelets were collected, counted on a Baker System 9000 Cell Counter, pooled, and diluted to 2 × 10^8 platelets/ml with elution buffer. Then, 50 μl containing either saline (control), peptide, or antibody was added to individual wells of a Nunc 96-well microtiter plate. Each well received 150 μl of GFP, and the microtiter plate was vortexed at room temperature for 10 min on a Sarstedt TPM-2 vortex platform shaker at a setting of 700. The optical density at 405 nm was measured with a Molecular Devices UV_{max} microplate reader.

Preparation of rabbit polyclonal antibodies. The TR_{52-69} peptide was conjugated to the protein carrier KLH using glutaraldehyde. The new amino-terminal activating sequence 42–52 peptide (TR_{42-52}) was conjugated to KLH using the heterobifunctional linker *N*-succinimidyl bromoacetate (32). For primary immunization, female New Zealand White rabbits were immunized with 100 μg of the immune conjugate with complete Freund's adjuvant and 50 μg with incomplete Freund's adjuvant on subsequent boosts. All animals were immunized subcutaneously four times with ~1 month between immunizations. The rabbits that were immunized with TR_{52-69}-KLH were designated R38 and those immunized with TR_{42-52}-KLH were designated R42. Both the R38 and R42 antisera, which were later affinity purified, were from blood taken 10 days after the third immunization. For affinity purification of the antibodies, the peptide TR_{42-52} (R38) was covalently coupled to BSA-Sepharose using the heterobifunctional linker *N*-succinimidyl bromoacetate, and the peptide TR_{52-69} (R42) was covalently coupled to BSA-Sepharose using glutaraldehyde. Whole antisera (~20 ml from each rabbit) was diluted 1/10 in 10 mM Tris, pH 7.5, before loading onto the columns. Each antiserum was passed over the affinity columns three times to ensure complete binding. The columns were then washed sequentially with 20 column-volumes 10 mM Tris, pH 7.5, and 500 mM NaCl, 10 mM Tris, pH 7.5. Antibodies bound by acid-sensitive interactions were eluted by passing 10 column-volumes of 100 mM glycine, pH 2.5, through the columns in 1-ml fractions. Fractions were collected into tubes containing 1 M Tris, pH 8.0, for neutralization.

GYKI-14766 was obtained from Gedeon Richter (Budapest, Hungary) (33). BMS-180742 was used as a thrombin anion-binding exosite inhibitor. It inhibited thrombin clotting activity and ability of thrombin to stimulate platelet aggregation but had no effect on thrombin active-site cleavage of synthetic substrates (34).

Results

Characterization of thrombin receptor-stimulated GTPase. α-Thrombin and peptides derived from the new amino terminus of the thrombin receptor (TR_{42-48} and related peptides) stimulated GTPase activity in platelet (Fig. 1) and CHRF-288 cell (Fig. 2) membranes. SFLLRNP stimulated GTPase ~2-fold basal activity with similar efficacy for both platelet and CHRF-288 membrane preparations (EC_{50} = 1–3 μM). Unexpectedly, the extent of thrombin's ability to activate platelet membrane GTPase was half of that observed with peptide stimulation using platelet membranes (Fig. 1). In control studies, the GTP cleavage was linear with time, and platelet membranes pretreated with saturating thrombin for 20–30 min before GTPase assay showed activity similar to membranes without preincubation (not shown). Furthermore, the combination of thrombin and SFLLR-peptide stimulate GTPase to the same degree as the peptide alone (Fig. 1), which suggests that thrombin proteolytic activity did not inactivate the platelet GTPase.

Effects of thrombin inhibitors. Characterization of the thrombin-stimulated responses indicated that a functioning thrombin active site was required for GTPase activation. The active-site inhibitor GYKI-14766 completely prevented the

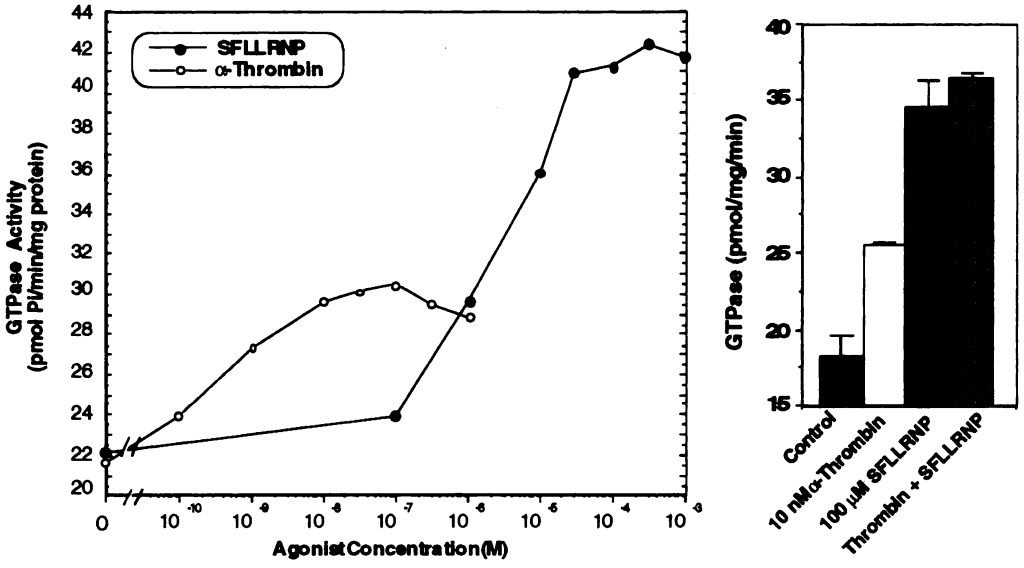

Fig. 1. *Left*, stimulation of platelet membrane GTPase activity by α-thrombin (○) or SFLLRNP (●). Human platelets were isolated and platelet membranes were prepared in the presence of hirudin and other protease inhibitors. The membranes were washed free of the protease inhibitors and treated with the indicated concentrations of α-thrombin or SFLLRNP. A representative experiment is shown (average of duplicate determinations). *Right*, effect of 10 nM human α-thrombin and 100 μM SFLLRNP alone and in combination on the platelet membrane GTPase activity. The mean ± standard deviation of triplicate determinations are shown for a representative experiment.

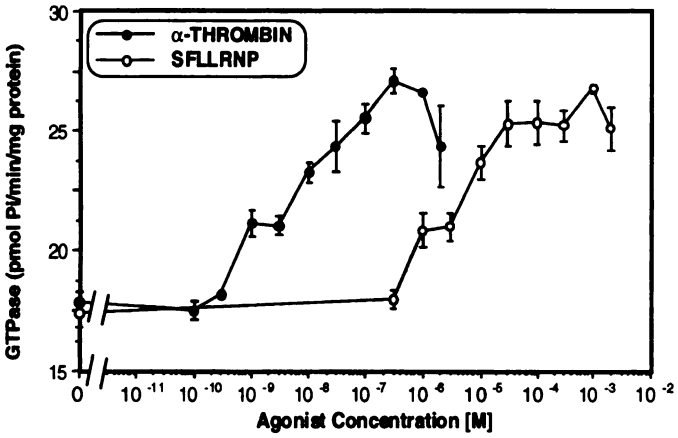

Fig. 2. Stimulation of CHRF-288 membrane GTPase by human α-thrombin (●) and SFLLRNP (○). CHRF-288 cells were grown in the presence of hirudin, and the membranes were prepared in the presence of protease inhibitors. The membranes were washed free of the protease inhibitors and treated with the indicated concentrations of the reagents. Each point represents the mean ± standard deviation of data from two to four experiments.

thrombin-stimulated GTPase activity in CHRF-288 cell membranes (Fig. 3) and platelet membranes (not shown) while having no effect on SFLLRNP-stimulated GTPase or basal GTPase activity (Fig. 3).

BMS 180742, which inhibits thrombin by binding to its anion-binding exosite, also blocked thrombin's ability to stimulate GTPase while exerting no effect on SFLLRNP-stimulated or basal activity (Fig. 3) similar results were obtained using platelet membranes (not shown). γ-Thrombin also stimulated the GTPase activity, but 50-fold higher concentrations were required for comparable activation (not shown).

Effects of antireceptor antibodies. Antipeptide antibodies directed at domains of the thrombin receptor have been found to prevent thrombin activation of the receptor and subsequent cellular actions (4, 22, 23). We made a series of rabbit polyclonal antibodies to the human thrombin receptor sequence TR_{52-69} (YEPFWEDEEKNESGLTEY; R38), the new amino-terminal-activating sequence TR_{42-52} (SFLLRNPNDKYC; R42), and TR_{36-49} (CATLDPRSFLLRNPNPN; R49). These antibodies prevented thrombin-induced platelet aggregation, which could be prevented by excess peptide antigen to which the antibodies were made (not shown). The receptor antibodies also blocked thrombin-stimulated prostacyclin production in HUVECs [an example is the use of anti-TR_{52-69} (YEPFWEDEEKNESGLTEY; R38) is shown in Fig. 4]. Similar results were obtained with anti-TR_{36-49} (CATLDPRSFLLRNPNPN; R49). For both antibodies, the inhibition could be prevented by excess of the antigen peptide to which the antibodies were elicited (Fig. 4).

These immune-purified antibodies inhibited thrombin but not SFLLRNP-stimulated GTPase in both platelet (Fig. 5) and CHRF-288 cell membranes (Fig. 6). Although representative data with anti-TR_{52-69} (R38) and anti-TR_{36-49} (R49) are shown, similar results were obtained with anti-TR_{42-52} (R42).

Inhibition of thrombin receptor-stimulated GTPase by pertussis toxin and NEM. To characterize the thrombin receptor-activated GTPase in the platelet membranes, the effect of several G protein inhibitors was tested. Both pertussis toxin and NEM treatment dramatically prevented most of the thrombin receptor stimulation of GTPase activity by both thrombin and SFLLRNP in platelet membranes (Fig. 7). In control studies, NEM treatment had very little effect on prostacyclin receptor-stimulated GTPase.[1]

Peptide activation. In previous studies, we tested SFLLR and analogues for their ability to activate the thrombin receptor using platelet aggregation (8). From those studies, structurally different peptides with a range of potencies, both greater and weaker than the native SFLLR sequence, were tested for their ability to stimulate GTPase activity. Peptides that stimulated platelet aggregation [SFRLR, SFLLR, and SFA(2-Nap)LR] also stimulated GTPase activity, whereas peptides that did not stimulate platelet aggre-

[1] S. M. Seiler, C. L. Brassard, M. E. Federici, J. Romine, and N. A. Meanwell. [3-[4-(4,5-diphenyl-2-oxazolyl)-5-oxazolyl]phenoxy]acetic acid (BMY-45778) is a potent prostacyclin partial agonist. Manuscript in preparation.

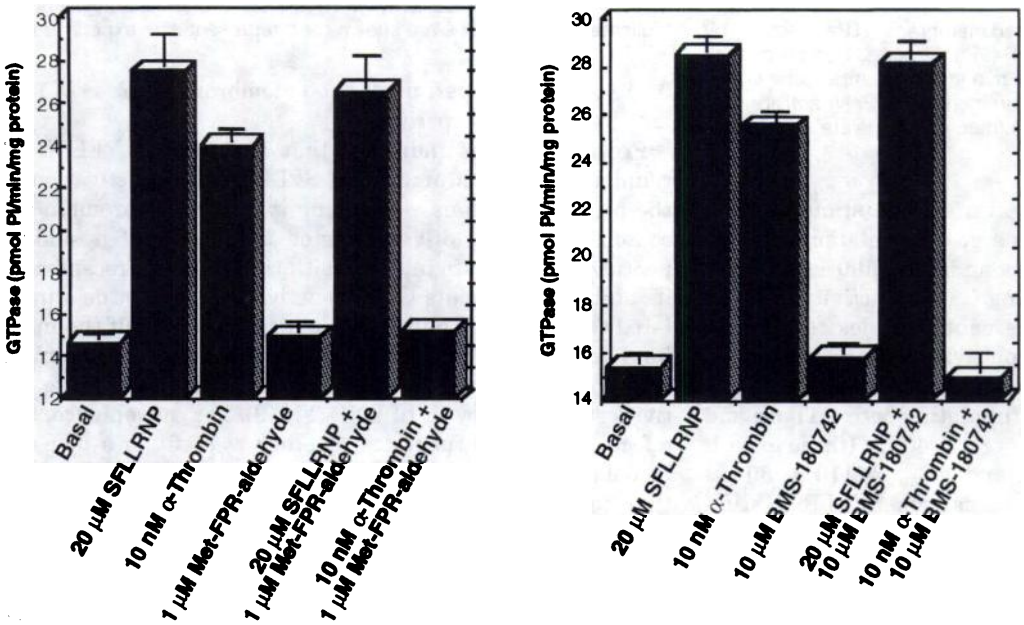

Fig. 3. Inhibition of human α-thrombin- but not SFLLRNP-stimulated GTPase by the thrombin active-site inhibitor GYKI-14766 (*left*) or the thrombin exosite inhibitor BMS-180742 (*right*). CHRF-288 cell membranes were treated with the indicated concentrations of the thrombin inhibitors in the reaction media. The mean ± standard deviation of triplicate determinations are shown for a representative experiment.

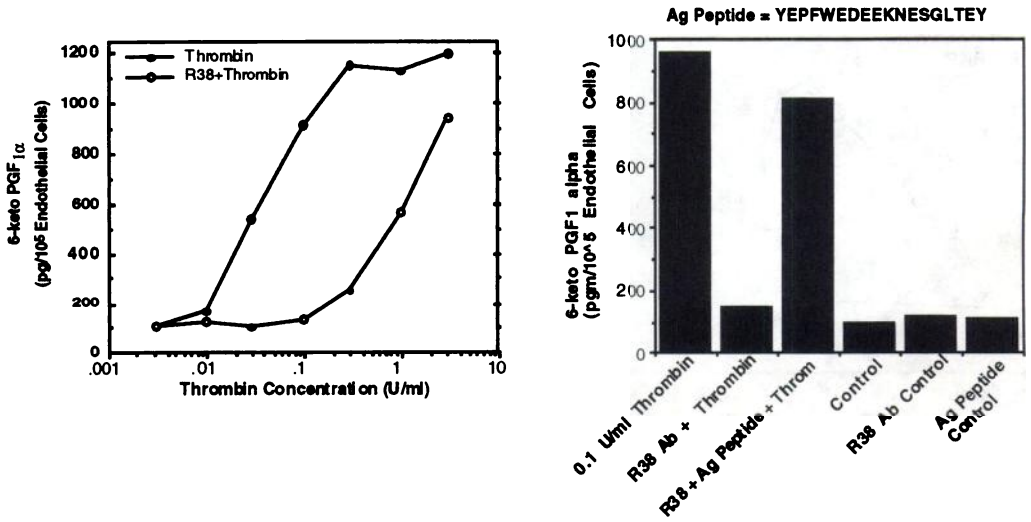

Fig. 4. Inhibition of prostacyclin production by antibodies (anti-TR_{52-69}) to the human thrombin tethered-ligand receptor. HUVECs were grown in gelatin-coated 12-well plates to confluence. Each well was washed with M199 plus gelatin (0.1%); 500 μl of the same buffer with and without 10 μg/ml of affinity-purified anti-TR_{52-69} (R38) antibody was added; and the cells were incubated for 10 min at 37°. α-Thrombin was added, and the cells were incubated for 30 min at 37° with 5% CO_2. The media were collected for measurement of 6-keto-$PGF_{1\alpha}$ using a commercially available radioimmunoassay kit. A cell count was also performed for each experiment. *Right*, inhibition of thrombin-stimulated prostaglandin 6-keto-$PGF_{1\alpha}$ production by 10 μg/ml anti-TR_{52-69} and reversal of the inhibition by excess peptide to which the antiserum was evoked (2 μg/ml). Each point represents the mean of duplicate determinations for a representative experiment performed several times with similar results.

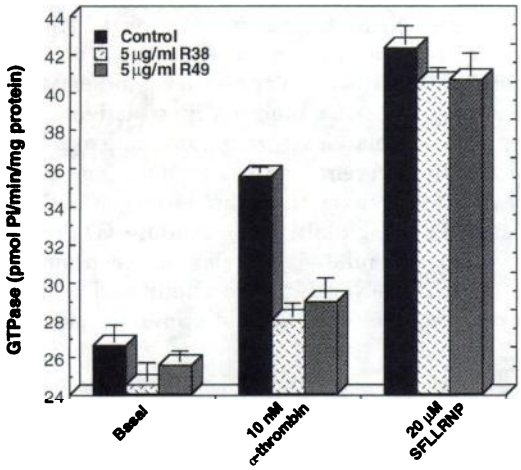

Fig. 5. Inhibition of thrombin-stimulated membrane GTPase activity by anti-TR_{52-69} (R38) and anti-TR_{36-49} (R49) antibodies. Human platelet membranes were preincubated for 20 min at room temperature with 5 μg/ml of the antibodies before measurement of GTPase activity. The mean ± standard deviation of triplicate determinations are shown for a representative experiment.

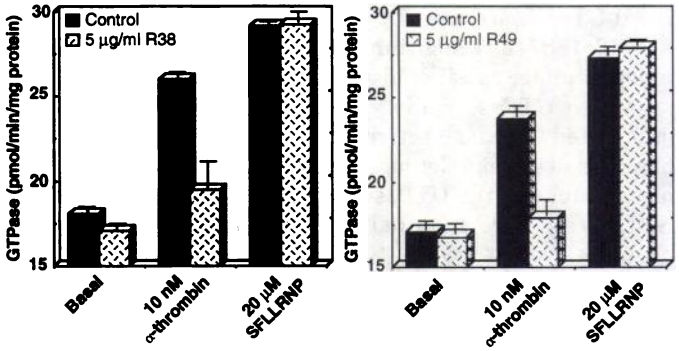

Fig. 6. Inhibition of thrombin-stimulated membrane GTPase activity by anti-TR_{52-69} (R38) and anti-TR_{36-49} (R49) antibodies. CHRF-288 membranes were preincubated for 20 min at room temperature with 5 μg/ml of anti-TR_{52-69} antibodies (*left*, R38) or anti-TR_{36-49} (*right*, R49) before measurement of GTPase activity. The mean ± standard deviation of triplicate determinations are shown for a representative experiment.

gation (e.g., acetylated SFLLR) also did not stimulate GTPase activity (Fig. 8). There was a good correlation between the relative potency of the compounds stimulating platelet aggregation and those stimulating GTPase activity (Fig. 8). The most potent example of the pentapeptides tested contained *para*-fluoro-Phe and β-naphthyl-alanine in the second and third positions. Ser-(*p*-fluoro-Phe)-(2-naphthyl-Ala)-Leu-Arg-NH_2 was the most potent, stimulating both GTPase and platelet aggregation with EC_{50} = 80 ± 40 nM (three experiments) for platelet aggregation and EC_{50} = 110 ± 30 nM GTPase (three experiments). In comparison, SFLLRNP stimulated platelet aggregation with an EC_{50} = 460 ± 220 nM (186 experiments) and membrane GTPase with an EC_{50} = 1.25 ± 0.53 μM (10 experiments). [(SFLLR was comparable and stimulated platelet aggregation with an EC_{50} of 0.400 ± 0.140 μM (three experiments) and membrane GTPase of 1.93 ± 0.46 μM (three experiments)].

Previous studies indicated that the peptide YFLLRNP could antagonize thrombin and SFLLR-peptide activation of the platelet functions, suggesting it may be a thrombin receptor antagonist (35). We tested the ability of a similar peptide, YFLLRN, to interact with the thrombin receptor by its ability to stimulate GTPase activity. This peptide stimulated GTPase alone but to only approximately half the maximal activity observed with SFLLRNP or other full agonists (Fig. 9). Furthermore, YFLLRN could limit the GTPase activity stimulated by SFLLRNP (Fig. 9). For convenience, the effects of the both peptides together were fit to a one-site model of receptor activation (Fig. 9). Curve fitting suggested that both SFLLRNP and YFLLRN interact with the same receptor, with SFLLRNP acting as a full agonist and YFLLRN acting as a partial agonist. The values obtained from the curve fit showed that ϵ_p = 6.5 pmol/mg/min, which is ~40% of the intrinsic efficacy of the reference full agonist

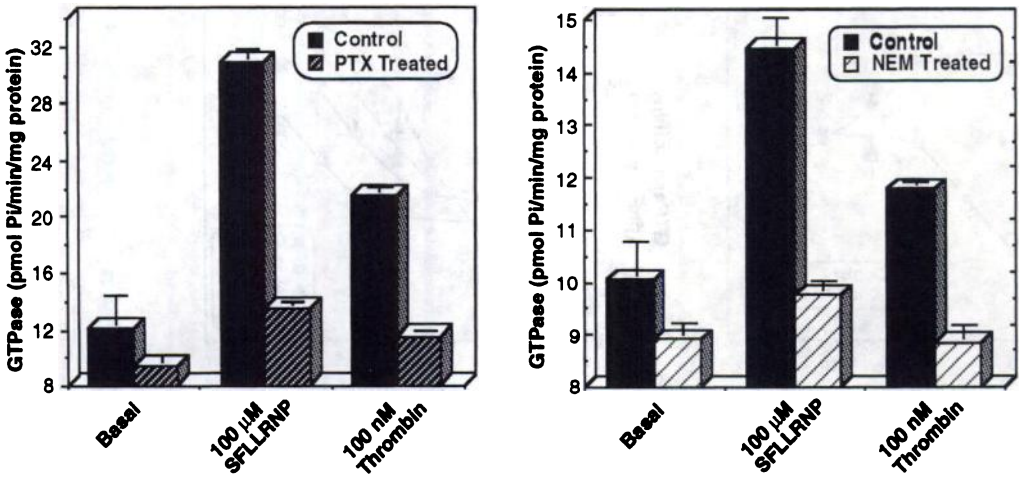

Fig. 7. Inhibition of α-thrombin- and SFLLRNP-stimulated platelet membrane GTPase by pertussis toxin (*left*) and NEM (*right*). Platelet membranes were pretreated with pertussis toxin A protomer or NEM as described in Materials and Methods before measurement of receptor-stimulated GTPase activity.

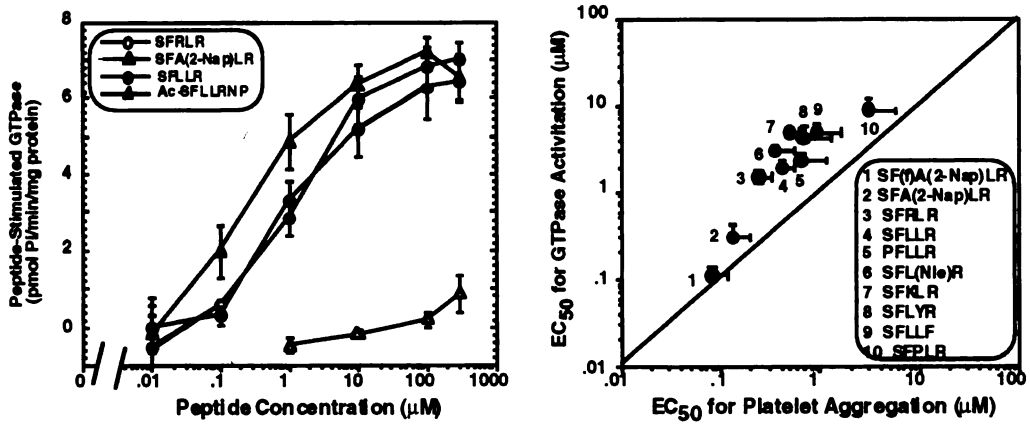

Fig. 8. Stimulation of platelet membrane GTPase by selected pentapeptides activating the human thrombin receptor. Each point is the average of three separate experiments ± standard deviation. The data shown describe the stimulation above basal activity. The basal level (zero point on *y*-axis) averaged 9.7 ± 2.6 pmol/mg/min (10 experiments). *Right*, correlation between the ability of peptides to stimulate membrane GTPase activity (*y*-axis values) and stimulate platelet aggregation (*x*-axis values). The data represent the mean ± standard deviation for three different determinations performed on different days.

SFLLRNP, which alone has a value of ϵ_a = 16.5 pmol/mg/min (Fig. 9).

Discussion

Several different blocking antibodies to the tethered-ligand thrombin receptor prevented thrombin activation of platelet as well as CHRF-288 cell membrane GTPase. These studies indicate that most, if not all, of the GTPase activity stimulated by thrombin in human platelet and CHRF-288 cell membranes is mediated through activation of the tethered-ligand thrombin receptor. This is further supported by the sensitivity of thrombin-stimulated GTPase to thrombin active-site as well as exosite inhibitors.

Much of that GTPase activity is also blocked by pertussis toxin or NEM treatment, suggesting that the receptor is coupled to a member of the $G_{\alpha i}$ family of G_α proteins. A number of different G proteins have been identified in platelet membranes, including all three members of the $G_{\alpha i}$ family (36). The finding that the thrombin receptor is coupled to a pertussis toxin-sensitive GTPase is consistent with the receptor activation causing inhibition of membrane adenylyl cyclase (9, 18), as well as with the activation of platelet phospholipase C through a pertussis toxin-sensitive mechanism (37). Furthermore, thrombin receptor activation in platelets has been shown to decrease the pertussis toxin-dependent ADP-ribosylation of the major membrane form of $G_{\alpha i}$ and soluble form of $G_{\alpha i}$(38). However, this does not rule out the possibility that other G proteins present in platelets in smaller amounts, including G_q (10, 11) and G_{12} and G_{13} (11), are activated by thrombin receptors.

Surprisingly, we observed that the SFLLR-containing peptides and analogues activate nearly twice as much GTPase as does thrombin in the platelet (but not CHRF-288 cell) membrane preparations. One possible explanation is that thrombin's proteolytic activity somehow inactivates the platelet GTPase stimulation. However, thrombin and SFLLR-peptide added together stimulated GTPase to the same degree as the peptide alone. Furthermore, membranes taken from CHRF-288 cells showed that thrombin stimulates GTPase to the same degree as receptor-activating peptides. This suggests that the lack of full thrombin response is not due to thrombin inhibition of the GTPase in platelet membranes. The possibility that the platelets contained a SFLLRNP-activated receptor in addition to the thrombin receptor was also considered. A possible candidate would be the protease activated receptor-2, which can be activated by peptides homologous to the new amino terminus of that receptor. However, peptides homologous to the new amino terminus of the human or mouse protease activated receptor-2

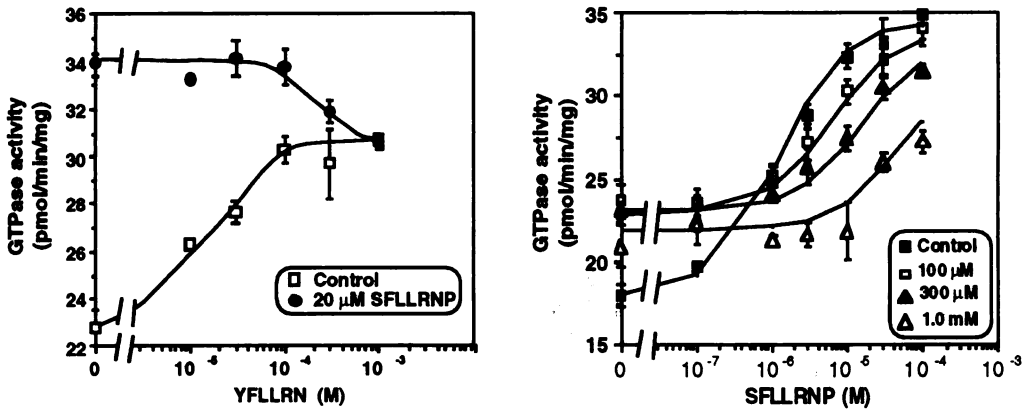

Fig. 9. Stimulation of platelet membrane GTPase and inhibition of SFLLRNP-stimulated GTPase by YFLLRN. Platelet membranes were incubated with (●) or without (○) 20 μM SFLLRNP at increasing concentrations of YFLLRN. The mean ± standard deviation for triplicate determinations is shown. *Right*, SFLLRNP stimulation of platelet membrane GTPase in the presence of increasing concentrations of YFLLRN. *Inset*, concentration of the added YFLLRN (control, ■; 100 μM YFLLRN, □; 300 μM YFLLRN, ; △1 mM YFLLRN, △). The data was globally fit using Sigma Plot (data obtained and then replotted using Cricket graph) simulating a one-site fit (43) to the following equation:

$$S = \frac{\epsilon_a [A]}{([A] + K_a(1 + [P]/K_p)} + \frac{\epsilon_p [P]}{([P] + K_a(1 + [A]/K_a)} + d$$

where S is the stimulus recorded (GTPase activity), ϵ is the intrinsic efficacy of the agonist (SFLLRNP), ϵ_p is the intrinsic efficacy of the partial agonist (YFLLRN), $[A]$ is the concentration of the agonist (SFLLRNP), $[P]$ is the concentration of the partial agonist (YFLLRN), K_a is the relative affinity constant for the agonist (SFLLRNP), K_p is the relative affinity constant for the partial agonist (YFLLRN), and d is a scale displacement value equal to the unstimulated (basal) GTPase activity. The values obtained from the curve fit are ϵ_p = 6.5 pmol/mg/min, which is ~40% of the reference full agonist SFLLRNP, which alone has a value of ϵ_a = 16.5 pmol/mg/min. The relative affinity of the partial agonist YFLLRN (K_p) is 26 μM relative to that of SFLLRNP (K_a = 1.27 μM).

(SLIGKVD or SLIGRS) had no effect on platelet or CHRF-288 cell membrane GTPase. Another possibility is the human platelet contains a processed thrombin receptor capable of activation by peptide analogues but not by thrombin. Such a peptide-sensitive, thrombin-insensitive receptor could be the consequence of proteolytic action on the receptor (6, 39) and has been observed in thrombin receptors treated with chymotrypsin (40), neutrophil-derived cathepsin G (41), and edopeptidase Glu C.[2] Platelet membranes were isolated using hirudin, benzamidine, and leupeptin and aprotinin throughout the blood collection, platelet, and membrane isolation procedures. This procedure yielded membranes preparations that showed the same differences between thrombin and peptide activation, which suggests that if proteolytic processing of the thrombin receptor is responsible, then human platelets have the observed differences before blood collection; however, a definitive explanation for the differences observed remains to be elucidated.

The GTPase assay system described here using either platelets or CHRF-288 cell membranes has proved to be a very sensitive, specific, and reliable cell-free assay system with which to evaluate thrombin receptor agonists, partial agonists, and antagonists. The ability of peptides to stimulate GTPase correlates with the ability of the peptides to stimulate platelet aggregation with a similar rank order of potency. Previous amino acid substitution studies have determined the specific requirements for each amino acid in the native SFLLR sequence (7, 8, 19). Replacement of individual amino acids with Ala suggested the importance of Phe as the second amino acid and Arg in the fifth position for activating the receptor (19, 7); however, *para*-fluoro-Phe in the second position of the heptapeptide SFLLRNP increased the thrombin receptor activating activity 3–5-fold (42). Independently, 2-naphthyl-Ala increased potency when placed in the third position (8). Combining these substitutions gave a more potent peptide agonist as indicated by the ability of the new peptide analogue Ser-(*p*-fluoro-Phe)-(2-naphthyl-Ala)-Leu-Arg-NH_2 to stimulate platelet aggregation as well as GTPase activity.

Previous studies indicated the peptide YFLLRNP can inhibit thrombin and SFLLR-peptide-induced platelet aggregation (35). However, it also stimulated platelet shape change and potentiated platelet aggregation by ADP, platelet-activating factor, and epinephrine (35). Although by itself it reportedly did not cause platelet aggregation, Ca^{2+} mobilization, or secretion (35), our studies have indicated that this and truncated peptides containing the Tyr substituted for Ser in the first position caused platelet aggregation measured in gel-filtered platelets when we made the measurements. Furthermore, these peptides are partial agonists, stimulating thrombin receptor-activated GTPase to ~40% of the activity observed with SFLLRNP and with the ability to prevent SFLLRNP from fully activating the receptor. The antagonists and partial agonists generated thus far have been useful in determining roles of the thrombin tethered-ligand receptor in initiating signal transduction in platelets and other cells; however, the antagonists described thus far are only weakly potent at best. These limitations are important to overcome in the refinement of thrombin receptor antagonists before serious evaluation of their efficacy can be determined.

Acknowledgments

We would like to thank Harold Goldenberg, Karen Hartl, Daniel Roberts, and Nick Allegretto for technical help, especially in the early portion of these studies.

[2] S. M. Seiler, unpublished observations.

References

1. Coughlin, S. R. Thrombin receptor function and cardiovascular disease. *Trends Cardiovasc. Med.* **4**:77–83 (1994).
2. Vu, T.-K. H., D. T. Hung, V. I. Wheaton, and S. R. Coughlin. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64**:1057–1068 (1991).
3. Rasmussen, U. B., V. Vouret-Craviari, S. Jallat, Y. Schlesinger, G. Pagés, A. Pavirani, J.-P. Lecocq, J. Pouysségur, and E. Van Obberghen-Schilling. cDNA cloning and expression of a hamster α-thrombin receptor coupled to Ca^{2+} mobilization. *FEBS Lett.* **288**:123–128 (1991).
4. Bahou, W. F., B. S. Coller, C. L. Potter, K. J. Norton, J. L. Kutok, and M. S. Goligorsky. The thrombin receptor extracellular domain contains sites crucial for peptide ligand-induced activation. *J. Clin. Invest.* **91**:1405–1413 (1993).
5. Zhong, C., D. J. Hayzer, M. A. Corson, and M. S. Runge. Molecular cloning of the rat vascular smooth muscle thrombin receptor: evidence for *in vivo* regulation by basic fibroblast growth factor. *J. Biol. Chem.* **267**:16975–16979 (1992).
6. Hoxie, J. A., M. Ahuja, E. Belmonte, S. Pizarro, R. Parton, and L. F. Brass. Internalization and recycling of activated thrombin receptors. *J. Biol. Chem.* **268**:13756–13763 (1993).
7. Scarborough, R. M., M. A. Naughton, W. Teng, D. T. Hung, J. W. Rose, T.-K. H. Vu, V. I. Wheaton, C. W. Turck, and S. R. Coughlin. Tethered ligand agonist peptides: structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. *J. Biol. Chem.* **267**:13146–13149 (1992).
8. Natarajan, S., D. Riexinger, M. Peluso, and S. Seiler. Tethered ligand derived penta peptide agonists of thrombin receptor: a study of side chain requirements for platelet activation. *Int. J. Peptide Protein. Res.* **45**:145–151 (1995).
9. Hung, D. T., Y. H. Wong, T.-K. H. Vu, and S. R. Coughlin. The cloned platelet thrombin receptor couples to at least two distinct effectors to stimulate phosphoinositide hydrolysis and inhibit adenylyl cyclase. *J. Biol. Chem.* **267**:20831–20834 (1992).
10. LaMorte, V. J., A. T. Harootunian, A. M. Spiegel, R. Y. Tsein, and J. R. Feramisco. Mediation of growth factor induced DNA synthesis and calcium mobilization by G_q and G_{12}. *J. Cell Biol.* **121**:91–99 (1993).
11. Offermanns, S., K.-L. Laugwitz, K. Spicher, and G. Schultz. G proteins of the G_{12} family are activated via thromboxane A_2 and thrombin receptors in human platelets. *Proc. Natl. Acad. Sci. USA* **91**:504–508 (1994).
12. Benka, M. L., M. Lee, G. R. Wang, S. Buckman, A. Burlacu, L. Cole, A. DePina, P. Dias, A. Granger, B. Grant, A. Hayward-Lester, S. Karki, S. Mann, O. Marcu, A. Nussenzweig, P. Piepenhagen, M. Raje, F. Roegiers, S. Rybak, A. Salic, J. Smith-Hall, J. Waters, N. Yamamoto, J. Yanowitz, K. Yeow, W. B. Busa, and M. E. Mendelson. The thrombin receptor in human platelets is coupled to a GTP binding protein of the $G_{\alpha q}$ family. *FEBS Lett.* **363**:49–52 (1995).
13. Baffy, G., L. Yang, S. Raj, D. R. Manning, and J. R. Williamson. G protein coupling to the thrombin receptor in Chinese hamster lung fibroblasts. *J. Biol. Chem.* **269**:8483–8487 (1994).
14. Brass, L. F., M. Ahuja, E. Belmonte, S. Pizarro, A. Tarver, and J. A. Hoxie. The human platelet thrombin receptor: turning it on and turning it off. *Ann. N. Y. Acad. Sci.* **714**:1–12 (1994).
15. Kinlough-Rathbone, R. L., D. W. Perry, M. A. Guccione, M. L. Rand, and M. A. Packman. Degranulation of the human platelets by the thrombin receptor peptide SFLLRN: comparison with degranulation by thrombin. *Thromb. Haemost.* **70**:1091–1023 (1994).
16. Seiler, S. M., M. Peluso, I. M. Michel, H. Goldenberg, J. W. Fenton II, D. Riexinger, and S. Natarajan. Inhibition of thrombin and SFLLR-peptide stimulation of platelet aggregation, phospholipase A_2 and Na^+/H^+ exchange by a thrombin receptor antagonist. *Biochem. Pharm.* **49**:519–528 (1995).
17. Huang, R.-S., A. Sorisky, W. R. Church, E. R. Simons, and S. E. Rittenhouse. Thrombin receptor-directed ligand accounts for activation by thrombin of platelet phospholipase C and accumulation of 3-phosphorylated phosphoinositides. *J. Biol. Chem.* **266**:18435–18438 (1991).
18. Seiler, S. M., I. M. Michel, and J. W. Fenton II. Involvement of the tethered-ligand receptor in thrombin inhibition of platelet adenylate cyclase. *Biochem. Biophys. Res. Commun.* **182**:1296–1302 (1992).
19. Vassallo, R. R. J., T. Kieber-Emmons, K. Cichowski, and L. F. Brass. Structure-function relationships in the activation of platelet thrombin receptors by receptor-derived peptides. *J. Biol. Chem.* **267**:6081–6085 (1992).
20. Pumiglia, K. M., and M. B. Feinstein. Thrombin and thrombin receptor agonist peptide induce tyrosine phosphorylation and tyrosine kinases in the platelet cytoskeleton. *Biochem. J.* **294**:253–260 (1993).
21. Lau, L.-F., K. Pumiglia, Y. P. Côté, and M. Feinstein. Thrombin-receptor agonist peptides, in contrast to thrombin itself, are not full agonists for activation and signal transduction in human platelets in the absence of platelet-derived secondary mediators. *Biochem. J.* **303**:391–400 (1994).
22. Brass, L. F., R. R. J. Vassallo, E. Belmonte, M. Ahuja, K. Cichowski, and J. A. Hoxie. Structure and function of the human platelet thrombin receptor: studies using monoclonal antibodies directed against a defined domain within the receptor N terminus. *J Biol. Chem.* **267**:13795–13798 (1992).
23. Hung, D. T., T.-K. H. Vu, V. I. Wheaton, K. Ishii, and S. R. Coughlin. Cloned platelet thrombin receptor is necessary for thrombin-induced platelet activation. *J. Clin. Invest.* **89**:1350–1353 (1992).
24. Tapparelli, C., R. Metternich, and N. S. Cook. Structure and function of thrombin receptors. *Trend. Pharmacol. Sci.* **14**:426–428 (1993).
25. Kinlough-Rathbone, R. L., M. L. Rand, and M. A. Packman. Rabbit and rat platelet do not respond to thrombin receptor peptides that activate human platelets. *Blood* **82**:103–106 (1993).
26. Connolly, T. M., C. Condra, D.-M. Feng, J. J. Cook, M. T. Stranieri, C. F. Reilly, R. F. Nutt, and R. J. Gould. Species variability in platelet and other cellular responsiveness to thrombin receptor-derived peptides. *Thromb. Haemost.* **72**:627–633 (1994).
27. Grandt, R., K. Aktories, and K. H. Jakobs, Evidence for two GTPases activated by thrombin in membranes of human platelets. *Biochem. J.* **237**:669–674 (1986).
28. Houslay, M., D. Bojanic, S. O'Hagan, and A. Wilson, Thrombin, unlike vasopressin, appears to stimulate two distinct guanine nucleotide regulatory proteins in human platelets. *Biochem. J.* **238**:109–113 (1986).
29. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
30. Shenker, A., P. Goldsmith, C. G. Unson, and A. M. Spiegel. The G protein coupled to the thromboxane A_2 receptor in human platelets is a member of the novel G_q family. *J. Biol. Chem* **266**:9309–9313 (1991).
31. Katada, T., J. K. Northup, G. M. Bokoch, M. Ui, and A. Gilman. The inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. *J. Biol. Chem.* **259**:3568–3577 (1984).
32. Bernatowicz, M. S., and G. R. Matsueda. Preparation of peptide-protein immunogens using N-succinimidyl bromoacetate as a heterobifunctional crosslinking reagent. *Anal. Biochem.* **155**:95–102 (1986).
33. Bajusz, S., E. Szell, D. Bagdy, E. Barabas, G. Horvath, M. Dioszegi, Z. Fittler, G. Szabo, A. Juhasz, E. Tomori, and G. Szilagyi. Highly active and selective anticoagulants: D-Phe-Pro-Arg-H, a free tripeptide aldehyde prone to spontaneous Inactivation, and its stable N-methyl derivative, D-MePhe-Pro-Arg-H. *J. Med. Chem.* **33**:1729–1735 (1990).
34. Seiler, S. M., H. J. Goldenberg, I. M. Michel, J. T. Hunt, and G. B. Zavoico. Multiple pathways of thrombin-induced platelet activation differentiated by peptide desensitization and by thrombin exosite inhibitors. *Biochem. Biophys. Res. Commun.* **181**:636–643 (1991).
35. Rasmussen, U. B., C. Gachet, Y. Schlesinger, D. Hanau, P. Ohlmann, E. Van Obberghen-Schilling, J. Pouyssegur, J.-P. Cazenave, and A. Pavirani. A peptide ligand of the human thrombin receptor antagonizes thrombin and partially activates platelets. *J. Biol. Chem* **268**:14322–14328 (1993).
36. Brass, L. F., J. A.. Hoxie, and D. R. Manning. Signaling through G proteins and G protein-coupled receptors during platelet activation. *Thromb. Haemost.* **70**:217–223 (1993).
37. Brass, L. F., M. Laposata, H. S. Banga, and S. E. Rittenhouse. Regulation of the phosphoinositide hydrolysis pathway in thrombin-stimulated platelets by a pertussis toxin-sensitive guanine nucleotide-binding protein: evaluation of its contribution to platelet activation and comparisons with the adenylate cyclase inhibitory protein, G_i. *J. Biol. Chem.* **261**:16838–16847 (1986).
38. Wilhelm, B., and W. Siess. Activation of the cloned platelet thrombin receptor decreases the pertussis-toxin-dependent ADP-ribosylation of the membrane and soluble inhibitory guanine-nucleotide-binding-α proteins: inhibition by the prostacyclin analog, iloprost. *Eur. J. Biochem.* **216**:81–88 (1993).
39. Brass, L. F. Homologous desensitization of HEL cell thrombin receptors: distinguishable roles for proteolysis and phosphorylation. *J. Biol. Chem.* **267**:6044–6050 (1992).
40. Vouret-Craviari, V., D. Grall, J.-C. Chambard, U. B. Rasmussen, J. Pouyssegur, and E. Van Obberghen-Schilling. Post-translational and activation-dependent modifications of the G protein-coupled thrombin receptor. *J. Biol. Chem.* **270**:8367–8372 (1995).
41. Molino, M., N. Blanchard, E. Belmonte, A. P. Tarver, C. Abrams, J. A. Hoxi, C. Cerletti, and L. F. Brass. Proteolysis of the human platelet and endothelial cell thrombin receptor by neutrophil-derived cathepsin G. *J. Biol. Chem.* **270**:11168–11175 (1995).
42. Nose, T., Y. Shimohigashi, M. Ohno, T. Costa, N. Shimizu, and Y. Ogino. Enhancement of thrombin receptor activation by thrombin receptor-derived heptapeptide with para-fluorophenylalanine in place of phenylalanine. *Biochem. Biophys. Res. Commun.* **193**:694–699 (1993).
43. Kenikin, T. *Pharmacologic Analysis of Drug-Receptor Interaction*, Ed. 2. Raven Press, New York, 1993.

Send reprint requests to: Steven Seiler, Ph.D., Department of Pharmacology, K3119A, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543.