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Binding of a Thrombin Receptor Tethered Ligand Analogue to Human Platelet Thrombin Receptor

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SUMMARY

A thrombin receptor-radioligand binding assay was developed using [^{3}H]A(pF-F)R(ChA)(hR)Y-NH_2 ([^{3}H]haTRAP), a high affinity thrombin receptor-activating peptide (TRAP), and human platelet membranes. Scatchard analysis of saturation binding data indicated that [^{3}H]haTRAP bound to platelet membranes with a K_d of 15 nM and a B_{max} of 5.2 pmol/mg of protein. The binding was reduced by GPPNHP, a nonmetabolizable GTP analogue. Various TRAPs and a TRAP antagonist, but not other receptor agonists, displaced [^{3}H]haTRAP from the binding sites. SFLLRN-NH_2, a thrombin receptor-tethered ligand analogue, and [^{3}H]haTRAP exhibited competitive binding for the same binding sites. The relative affinity of these peptides for the binding site paralleled their EC_{50} or IC_{50} values for platelet aggregation. These data indicate that [^{3}H]haTRAP binds specifically and saturably to the functioning G protein-linked thrombin (tethered ligand) receptor in human platelet membranes.

Thrombin, a serine protease, activates target tissues through proteolytic action on the heterotrimeric G protein-coupled thrombin receptor (1–3). By cleaving the receptor at Arg41 of the human receptor amino-terminal sequence, thrombin exposes a new amino-terminal domain that acts as a tethered ligand to stimulate receptor function. TRAPs containing 5 to 14 amino acid residues, which correspond to the newly exposed amino-terminus, mimic many of the actions of thrombin, including aggregation of platelets (1, 4, 5), modulation of vascular smooth muscle contractility (6, 7), and mesangial cell proliferation (8; see 9 and 10 for review). In platelets, TRAPs induce all the known second messenger responses observed upon thrombin activation, including stimulation of phospholipase C, accumulation of phosphoinositides, calcium mobilization (11–13), and tyrosine phosphorylation of cytoskeletal proteins (14).

Several lines of evidence indicate that thrombin and the thrombin receptor play a central role in thrombosis and vascular lesion formation (15–17). Increased levels of thrombin receptor expression have been demonstrated in human atheroma (15) and in vascular lesions in baboon carotid arteries after surgical endarterectomy (16). TRAP antagonists and an antibody to the thrombin receptor inhibit thrombin- or TRAP-stimulated platelet aggregation *in vitro* (5, 14, 18–20) as well as experimental arterial thrombosis in primates (17, 20). These results underscore the therapeutic utility of inhibiting the thrombin receptor in atherosclerosis and thrombosis. Little is known about receptor-binding characteristics of TRAPs and their analogues in platelets because of the lack of availability of a high affinity, radiolabeled TRAP suitable for a receptor ligand binding assay. Recently, through systematic removal of carboxyl-terminal amino acid residues and modification of the 14-amino-acid TRAP (SFLLRNPNDKYEPF-OH), Feng *et al*. (21, 22) synthesized a high affinity peptide, A(pF-F)R(ChA)(hR)Y-NH_2, which exhibited an EC_{50} value of 10 nM for stimulation of human platelet aggregation. In this report, we describe a high-volume thrombin receptor binding assay using [^{3}H]haTRAP and human platelet membranes.

Experimental Procedures

Preparation of [^{3}H]haTRAP. A(pF-F)R(ChA)(hR)(I_2-Y)-NH_2 (1.03 mg) and 10% Pd/C (5.07 mg) were suspended in DMF (250 μl) and diisopropylethylamine (10 μl). The vessel was attached to the tritium line, frozen in liquid nitrogen, and evacuated. Tritium gas (342 mCi) was then added to the flask, which was stirred at room temperature for 2 hr. At the completion of the reaction, the excess

ABBREVIATIONS: TRAP, thrombin receptor-activating peptide; [^{3}H]haTRAP, alanine-parafluorophenylalanine-arginine-cyclohexylalanine-homoarginine-[^{3}H]phenylalanine amide; monoiodo-haTRAP, alanine-parafluorophenylalanine-arginine-cyclohexylalanine-homoarginine-paraiodophenylalanine amide; pF-F, parafluorophenylalanine; ChA, cyclohexylalanine; hR, homoarginine; Mpa, 3-mercaptopropionyl-phenylalanine-cyclohexylalanine-cyclohexylalanine-arginine-lysine-proline-asparagine-aspartate-lysine amide; GPPNHP, 5′-guanylyl imidodiphosphate; DMSO, dimethylsulfoxide; DMF, dimethylformamide; TFA, trifluoroacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HPLC, high performance liquid chromatography.

tritium was removed and the reacted peptide solution was diluted with DMF (0.5 ml) and filtered to remove the catalyst. The collected DMF solution of the crude peptide was diluted with water and freeze-dried to remove the labile tritium. The solid peptide was redissolved in water and the freeze-drying process repeated. The tritiated peptide ([^{3}H]haTRAP) was dissolved in 0.5 ml of 0.1% aqueous TFA and purified by HPLC using the following conditions: column, Vydac C18, 25 cm × 9.4 mm i.d.; mobile phase, (A) 0.1% TFA in water, (B) 0.1% TFA in CH_3CN; gradient, (A/B) from 100/0 to 40/60 over 30 min; flow rate, 5 ml/min; detection, UV at 215 nm. The radiochemical purity of [^{3}H]haTRAP was 99% as analyzed by HPLC. A batch of 14.9 mCi at a specific activity of 18.4 Ci/mmol was obtained.

Preparation of platelet membranes. Platelet membranes were prepared using a modification of the method of Natarajan *et al.* (23) from 20 units of platelet concentrates obtained from the North Jersey Blood Center (East Orange, NJ) within 48 hr of collection. All steps were carried out at 4° under approved biohazard safety conditions. Platelets were centrifuged at 100 × *g* for 20 min at 4° to remove red cells. The supernatants were decanted and centrifuged at 3,000 × *g* for 15 min to pellet platelets. Platelets were resuspended in 10 mM Tris·HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA (buffer A), to a total volume of 200 ml and centrifuged at 4,400 × *g* for 10 min. This step was repeated two times. Platelets were resuspended in 5 mM Tris·HCl, pH 7.5, 5 mM EDTA to a final volume of approximately 30 ml and were homogenized with 20 strokes in a Dounce homogenizer. Membranes were pelleted at 41,000 × *g*, resuspended in 40–50 ml of 20 mM Tris·HCl, pH 7.5, 1 mM EDTA, 0.1 mM dithiothreitol, and 10-ml aliquots were frozen in liquid N_2 and stored at −80°. To complete membrane preparation, aliquots were thawed, pooled, and homogenized with five strokes of a Dounce homogenizer. Membranes were pelleted and washed three times in 10 mM triethanolamine-HCl, pH 7.4, 5 mM EDTA, and resuspended in 20–25 ml of 50 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, and 1% DMSO. Aliquots of membranes were frozen in liquid N_2 and stored at −80°C. Membranes were stable for at least 3 months. Twenty units of platelet concentrates typically yielded 250 mg of membrane protein. Protein concentration was determined by a Lowry assay (24).

High volume binding assay. The assay was performed in 96-well Nunc plates (No. 269620; Naperville, CT) at a final assay volume of 200 μl. Platelet membranes and [^{3}H]haTRAP were diluted to 0.4 mg/ml and 25 nM, respectively, in binding buffer (50 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.1% bovine serum albumin). DMSO (100%) and peptide (10 mM in 100% DMSO) solutions were diluted in binding buffer containing 20% DMSO. Unless otherwise indicated, 20 μl of this diluted solution of DMSO or peptide and 80 μl of ligand were added to each well, and the reaction was started by the addition of 100 μl of membranes. The plates were covered and vortex-mixed gently on a Lab-Line Titer Plate Shaker (Melrose Park, IL) for 1 hr at room temperature. Packard UniFilter GF/C filter plates (Meriden, CT) were soaked for at least 1 hr in 0.1% polyethyleneimine. Inclusion of 0.1% bovine serum albumin in the incubation buffer as well as presoaking of the filter plate in 0.1% polyethyleneimine were necessary to reduce binding of [^{3}H]haTRAP to tubes, pipette tips, and the filter plate. The incubated membranes were harvested using a Packard FilterMate Universal Harvester and were rapidly washed four times with 300 μl of ice-cold 50 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA. MicroScint 20 scintillation cocktail (25 μl) was added to each well, and the plates were counted in a Packard TopCount Microplate Scintillation Counter. The specific binding was defined as the total binding minus the nonspecific binding observed in the presence of excess (50 μM) unlabeled haTRAP.

Membrane-[^{3}H]haTRAP binding assay after preincubation of platelets with thrombin. Intact platelets (4 mg/4 ml) were incubated at 30° for 20 min with vehicle (binding buffer) or 2 units/ml thrombin in buffer A containing 10 mM glucose. At the end of incubation, 5 units/ml hirudin was added to the incubated platelets, and platelets were homogenized with 15 strokes in all-glass dual tissue homogenizers (Kontes) and centrifuged at 41,000 × *g* for 15 min. The pellets were suspended in binding buffer and centrifuged again. The final pellets were resuspended in binding buffer at 1 mg/ml protein. Membranes (170 μg/0.2 ml reaction mixture) were incubated with 10 nM [^{3}H]haTRAP at room temperature for 1 hr.

Platelet aggregation assay. Human platelet aggregation in response to TRAPs was measured according to the procedure of Bednar *et al.* (25). Measurements were made at room temperature using a Spectromax platelet reader (Molecular Devices, Menlo Park, CA). Plates were vortexed at speed 7 on a plate shaker. The EC_{50} values of TRAPs were determined using platelet aggregation data obtained at 2.5 min after the addition of TRAPs.

HPLC procedure. Proteolytic breakdown of the [^{3}H]haTRAP ligand during the binding assay was analyzed on a Beckman gold liquid chromatographer (Beckman Instruments, Fullerton, CA). Aliquots (100 μl) of 42,000 × *g* supernate of the reaction mixtures were injected onto a C8 reverse phase column and eluted at 1.3 ml/min over 20 min with a gradient from 18.15% to 56.25% acetonitrile in 0.1% TFA. Fractions (0.3 ml) were collected, and radioactivity was determined by counting 50-μl aliquots with 200 liters of OptiPhase Supermix (Wallack, Gaithersberg, MD) in a Wallack Microbeta Scintillation Counter. Before incubation, the tritiated ligand eluted as a single peak. Percent hydrolysis was calculated as the ratio of dpm eluting outside the major peak to the total dpm recovered from the column.

Materials. SFLLRNPNDKYEPF-OH, SFLLRN-NH_2, SFLLRN-OH, SFLLRN-NH_2, and SFLLR-OH were purchased from Bachem Bioscience Inc. (King of Prussia, PA). Human angiotensin II, bradykinin, α-neurokinin, substance P, and vasoactive intestinal peptide were obtained from Sigma Chemical (St. Louis, MO). A(pF-F)R(ChA)(hR)Y-NH_2, A(pF-F)R(ChA)(hR)(I-Y)-NH_2, A(pF-F)R-(ChA)(hR)(I_2-Y)-NH_2, Mpr-F(ChA)(ChA)RKPNDK-NH_2, SLIGKV-NH_2, FSLLRN-NH_2, and human galanin were custom synthesized by AnaSpec (San Jose, CA). The purity of these peptides was >95%. Human thrombin was purchased from Research Genetics (Huntsville, AL). Tritium gas (97%) was purchased from EG&G Mound (Miamisburg, OH). The gas was subsequently loaded and stored on an IN/US Systems Trisorber (Tampa, FL). All reagents and solvents were reagent grade and were used without further purification. All chemicals, unless otherwise indicated, were purchased from Sigma Chemical.

Results

Stability of [^{3}H]haTRAP during binding assay. During 1-hr incubation with platelet membranes under the standard binding conditions, 10 nM of [^{3}H]haTRAP was degraded approximately 18% in the absence of protease inhibitors and 3% in the presence of protease inhibitors (0.1% bacitracin, 10 g/ml *N*-tosyl-L-phenylalanine chloromethyl ketone, 100 μM phenylmethyl sulfonyl fluoride and 10 μg/ml leupeptin). However, no significant differences in [^{3}H]haTRAP binding were noted in the presence and absence of the protease inhibitors: the total and nonspecific bindings of [^{3}H]haTRAP were 1800 ± 120 and 300 ± 30 cpm, respectively, in the absence of protease inhibitors and 1680 ± 240 and 250 ± 30 cpm in the presence of protease inhibitors (four replicate determinations). The lack of effect of [^{3}H]haTRAP degradation on the [^{3}H]haTRAP binding may simply reflect that the most binding (approximately 80% of the maximal equilibrium binding) occurs within 30 min of incubation and the bound [^{3}H]haTRAP is somehow protected from proteases.

Kinetics of [^{3}H]haTRAP binding to human platelet membranes. The specific binding of [^{3}H]haTRAP to human platelet membranes rapidly increased with incubation up to

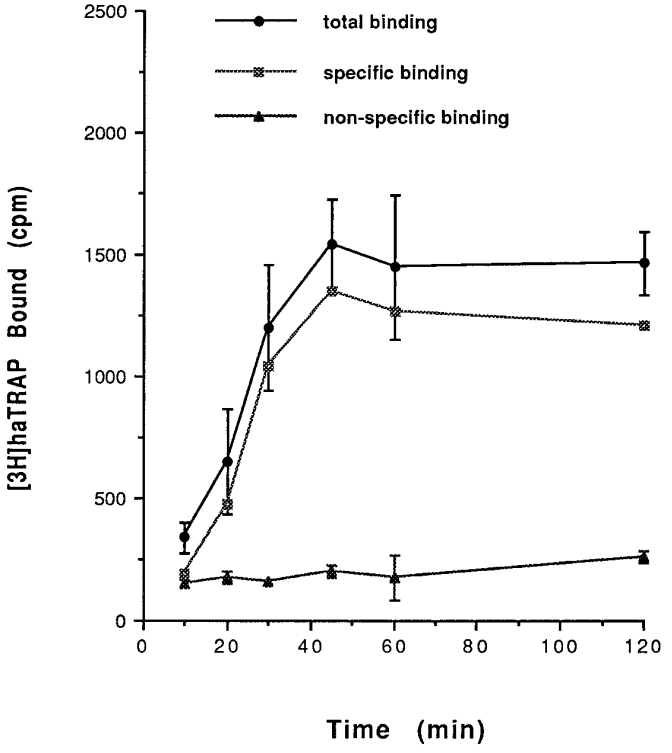

Fig. 1. Time course of the specific [^{3}H]haTRAP binding to human platelet membranes at room temperature (20°). Ten nanomolar [^{3}H]haTRAP and 40 μg of membranes were used. These results are representative of at least two independent experiments. *Points and bars,* mean ± standard error of three determinations.

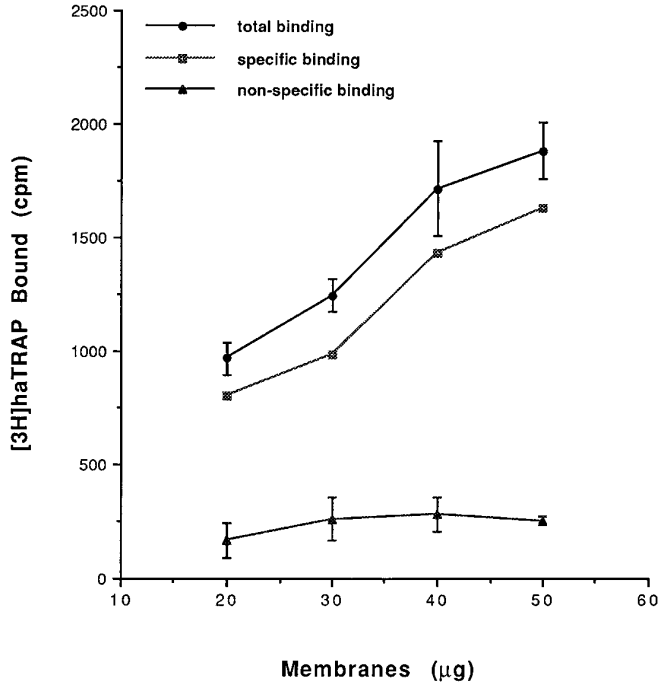

Fig. 2. Specific binding of [^{3}H]haTRAP to human platelet membranes as a function of platelet membrane concentrations. Membranes were incubated with 10 nM [^{3}H]haTRAP. *Points*, mean of four determinations. These results are representative of three separate experiments.

40 min, reaching a plateau 40–120 min (Fig. 1). [^{3}H]haTRAP binding increased with increasing concentrations of membrane protein and appeared maximal at 40 μg/0.2 ml (Fig. 2).

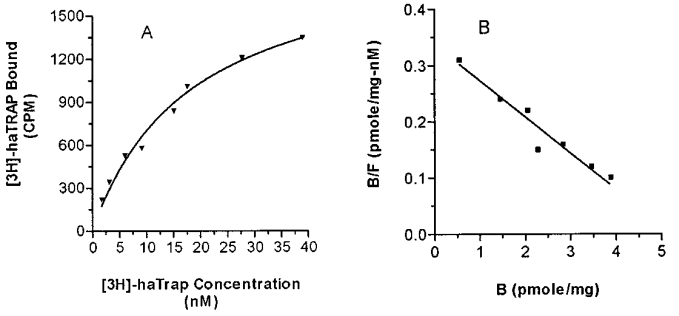

Fig. 3. A, Saturation binding of [^{3}H]haTRAP. B, Scatchard plot of saturation binding of [^{3}H]haTRAP. Forty micrograms of membrane was used. *Points*, mean of three determinations. Unlabeled haTRAP (100 M) was used to determine nonspecific binding. These results are representative of three separate experiments.

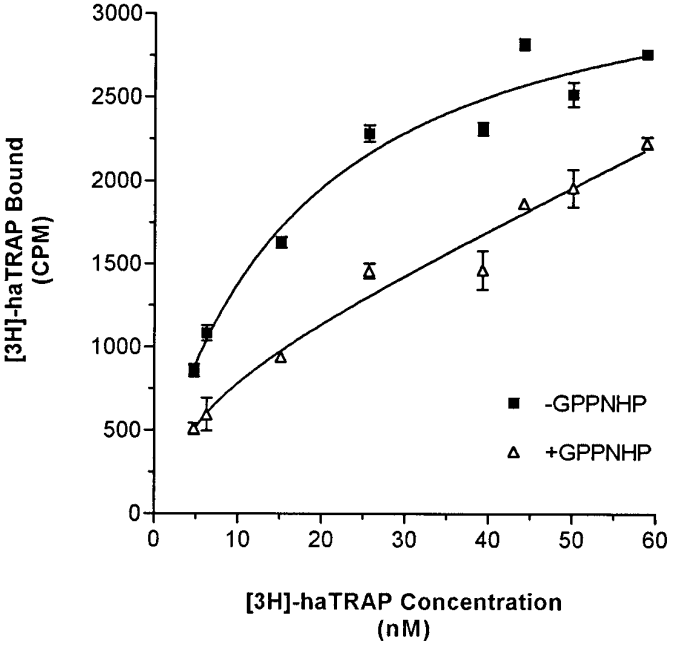

Fig. 4. Effect of GPPNHP on [^{3}H]haTRAP binding. Platelet membranes (40 μg) were incubated with increasing concentrations of [^{3}H]haTRAP in the presence and absence of 100 μM GPPNHP. These results were repeated several times with essentially similar results.

Thus, subsequent binding experiments were performed with 40 μg of membrane protein and a 60-min incubation period. Figure 3A illustrates that [^{3}H]haTRAP binds to platelet membranes in a saturable manner. Scatchard analysis of the data demonstrates that [^{3}H]haTRAP binds to a single population of binding sites with an equilibrium dissociation constant (K_d) of 15 nM and a maximal binding (B_{max}) of 5.2 pmol/mg membrane protein (Fig. 3B). The Hill coefficient was 1.03. The number of binding sites was calculated at 600 sites/platelet.

Decrease of [^{3}H]haTRAP binding by a guanine nucleotide. GPPNHP, a nonmetabolizable analogue of GTP, shifted the [^{3}H]haTRAP saturation binding curve to higher concentrations of radiolabeled peptide (Fig. 4). The decreased affinity of [^{3}H]haTRAP upon addition of a guanine nucleotide is indicative of functional coupling of the [^{3}H]haTRAP binding sites to a heterotrimeric G-protein. Furthermore, haTRAP (0.1 μM) and SFLLRN-NH_2 (1 μM) activated GTPase activity 2.5- and 2-fold, respectively, in the platelet mem-

brane preparations, which provides additional evidence that the [^{3}H]haTRAP binding sites are coupled to G proteins (data not shown).

Effects of various TRAPs and other peptides on [^{3}H]haTRAP binding. To characterize the [^{3}H]haTRAP binding sites, competition binding experiments with a dozen peptides, including TRAPs and a thrombin receptor antagonist, were conducted. SFLLRN-NH_2, SFLLRN-OH, SFLLRNPNDKYEPF-OH, haTRAP, iodo-haTRAP, and a thrombin receptor antagonist, Mpa peptide (13), displaced [^{3}H]haTRAP in a concentration-dependent manner (Fig. 5). The relative potencies of the peptides in inhibiting [^{3}H]haTRAP binding are haTRAP > iodo-haTRAP > SFLLRN-NH_2 > SFLLRN-OH > SFLLRNPNDKYEPF-OH > SFLLR-OH > Mpa peptide (Fig. 5; Table 1). Because [^{3}H]haTRAP was degraded to some extent during incubation in the absence of protease inhibitors, the IC_{50} values of TRAPs were also determined in the presence of protease inhibitors. The IC_{50} values (shown in brackets in Table 1) obtained in the presence of inhibitors did not significantly differ from those obtained in the absence of inhibitors (Table 1). These data, together with the observations (see the first section) of the lack of effect of protease inhibitors on specific binding, indicate that a small amount of degradation of [^{3}H]haTRAP during binding assays in the absence of protease inhibitors has no detectable consequences on the accuracy and reliability of binding data. SLIGKV-NH_2, an agonist for a newly cloned human proteinase-activated receptor 2 (PAR-2) (26), and other receptor agonist peptides such as bradykinin and angiotensin II showed extremely low affinities for the haTRAP binding sites (Table 1). FSLLRN-NH_2, a negative control peptide containing the reversed sequence of the first two amino acids of the receptor-activating (tethered ligand) peptide, also showed no detectable affinity for the binding sites.

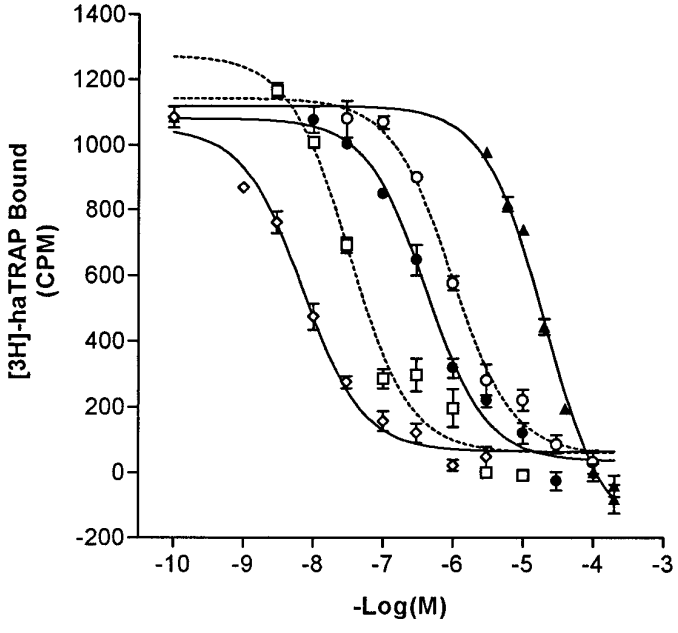

Fig. 5. Displacement of [^{3}H]haTRAP from the binding sites by various TRAPs and a thrombin receptor antagonist. Peptides: ◇, haTRAP or A(pF-F)R(ChA)(hR)Y-NH_2; □, SFLLRN-NH_2; ●, SFLLRN-OH; ○, SFLLRNPNDKYEPF-OH; ▲, Mpr-F(ChA)(ChA)RKPNDK-NH_2 (Mpa peptide). Membranes (40 μg) were incubated with 10 nM [^{3}H]haTRAP and varying concentrations of peptides. *Points and bars*, mean ± standard error of three determinations.

Effects of SFLLRN-NH_2 on saturation binding of [^{3}H]haTRAP. To determine whether TRAPs and [^{3}H]haTRAP compete for the same binding sites, the effect of SFLLRN-NH_2 on saturation binding of [^{3}H]haTRAP was determined. Scatchard plots of the data are consistent with competitive binding of these two peptides for the same binding sites (Fig. 6).

Platelet aggregation assay. Although the EC_{50} values of TRAPs were reported previously, they varied considerably from one laboratory to another (4, 5, 22, 27–30). Furthermore, no single group of investigators obtained platelet aggregation EC_{50} values for the TRAPs listed in Table 1. Thus, it was necessary for us to determine the EC_{50} values of TRAPs to compare the relative affinities of TRAPs for the [^{3}H]haTRAP binding sites and their potencies for activation of platelet aggregation. All six TRAPs caused human platelet aggregation in a concentration-dependent manner, with haTRAP being the most potent and SFLLR-OH the least potent (Fig. 7). Mpa peptide, a known TRAP antagonist (13, 26), showed minimal aggregating activity (Fig. 7). SLIGKV-NH_2, a PAR-2–tethered ligand analogue (26), caused minimal platelet aggregation, which suggests the absence of PAR-2 in platelets. Our EC_{50} values for the TRAPs tended to be higher than the reported values (4, 5, 22, 27–30). These differences could be caused partly by differences in the temperatures at which platelet aggregation experiments were conducted by us (20°) and by others (37°). TRAPs are expected to bind to thrombin receptors more slowly at 20° than at 37°, thus resulting in underestimated potencies of TRAPs in our experiments.

Comparison of [^{3}H]haTRAP-binding inhibitory activities and platelet aggregation for TRAPs. Table 1 compares the IC_{50} values of TRAPs for inhibition of [^{3}H]haTRAP binding with their EC_{50} values for human platelet aggregation (Fig. 7). An excellent correlation ($r = 0.99$; $p < 0.0001$) was observed between inhibition of [^{3}H]haTRAP binding and activation of platelet aggregation for six TRAPs. The binding IC_{50} value (20 μM) of the Mpa peptide lies between the reported IC_{50} values (5 μM and 100 μM) for inhibition of platelet aggregation (13, 30). Taken together, these results indicate that the [^{3}H]haTRAP-binding site is specific, saturable, and represents the functional thrombin receptor.

Effect of preincubation of intact platelet with thrombin on subsequent [^{3}H]haTRAP binding to platelet membranes. Preincubation of intact platelets with 2 units/ml of thrombin decreased [^{3}H]haTRAP binding to platelet membranes by 48% compared with vehicle-preincubated platelets: specific binding of [^{3}H]haTRAP was 1948 ± 38 cpm ($n = 3$) and 1008 ± 56 cpm ($n = 3$), respectively, for the vehicle-preincubated and thrombin-preincubated platelets.

Efficiency and reliability of the [^{3}H]haTRAP binding assay. Incubation, filtration, and counting of thrombin receptor-radioligands were all performed in 96-well plates, which eliminated time-consuming sample transfer steps. The reliability of this binding assay was estimated by determining the IC_{50} of the Mpa peptide in seven separate experiments performed on different days. The IC_{50} of the Mpa peptide (mean ± standard error) was 21 ± 1 μM. The coefficient of variation was low (4.8%), which indicates robustness of the assay in determining effects of a receptor antagonist.

TABLE 1

Effect of various thrombin receptor tethered ligand analogs and other peptides on [^{3}H]haTRAP binding and human platelet aggregation

IC_{50} and EC_{50} values for respective binding inhibition and platelet aggregation represent the mean ± SE with the number of experiments in parentheses. IC_{50} values (mean ± SE for three to four separate experiments) for inhibition of binding in the presence of protease inhibitors are in brackets. K_i values can be calculated by the following equation: $K_i = IC_{50}/(1 + L/K_d)$ where L is the concentration of [^{3}H]haTRAP used (10 nM) and K_d is 15 nM.

Peptide sequence	[^{3}H]haTRAP binding (IC_{50}, nM)	Platelet aggregation (EC_{50}, nM)
Thrombin receptor activating peptides:		
SFLLR-OH	11,700 ± 1,780 (3) [8,800 ± 510]	57,960 ± 16,830 (3)
SFLLRNPNDKYEPF-OH	1,100 ± 238 (3) [1,710 ± 460]	12,610 ± 2,520 (4)
SFLLRN-OH	540 ± 99 (4) [543 ± 90]	4,510 ± 1,180 (4)
SFLLRN-NH_2	168 ± 42 (3) [92 ± 5]	769 ± 17 (3)
A(pF-F)R(ChA)(hR)(I-Y)-NH_2	33 ± 15 (4) [25 ± 4.2]	302 ± 102 (4)
haTRAP	24 ± 6 (4) [20 ± 3.5]	147 ± 25 (5)
Thrombin receptor inhibiting peptide:		
Mpa	21,000 ± 1,000 (7) [20,700 ± 3560]	≈100,000[a,c] 5,000[b,c]
Other peptides:		
FSLLRN-NH_2	>300,000	nd
SLIGKV-NH_2	>300,000	>300,000
Angiotensin II	>300,000	nd
Bradykinin	>300,000	nd
Galanin	110,000	nd
α-Neurokinin	>100,000	nd
Substance P	>100,000	nd
Vasoactive intestinal peptide	>100,000	nd

[a] Reference 13.
[b] Reference 30.
[c] IC_{50} (nM); nd, not determined.

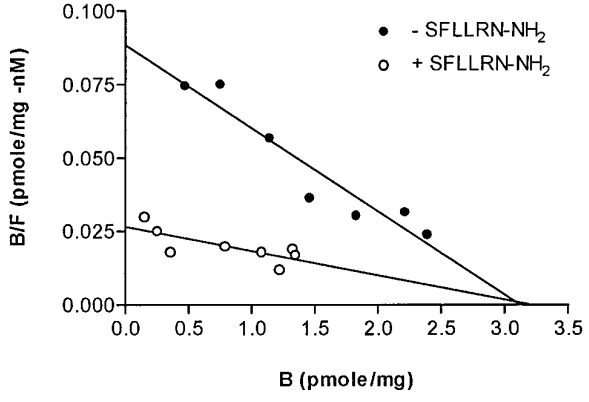

Fig. 6. Scatchard plots of saturation binding of [^{3}H]haTRAP in the presence and absence of SFLLRN-NH_2. Membranes were incubated with increasing concentrations (2–30 nM) [^{3}H]haTRAP in the presence and absence of 0.3 μM SFLLRN-NH_2. *Points*, mean of three determinations. These results are representative of two separate experiments.

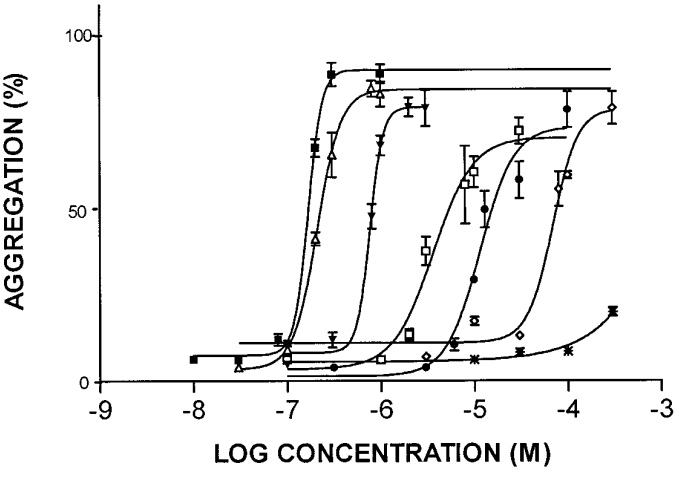

Fig. 7. Platelet aggregation in response to TRAPs and an antagonist. Effect of six to seven concentrations of each peptide on aggregation were determined at 2.5 min after addition of a peptide. Peptides: ■, haTRAP; △, A(pF-F)R(ChA)(hR)(I-Y)-NH_2; ▼, SFLLRN-NH_2; □, SFLLRN-OH; ○, SFLLRNPNDKYEPF-OH; ◇, SFLLR-OH; ∗, Mpa peptide. *Points and bars*, mean ± standard error of four determinations.

Discussion

In addition to its critical role in the coagulation cascade, thrombin acts on specific receptors in a wide range of target cells, including platelets, endothelial cells, smooth muscle cells, immune cells, fibroblasts, mesangial cells, and brain cells (9, 10, 31). However, because of the unusual way in which thrombin interacts with target cells (32) and the extracellular matrix (33), traditional ligand binding methods have been unsuccessful in identifying functionally relevant thrombin receptors (34). Recent elucidation by Coughlin and other investigators (1–3) of the unique mechanism of thrombin receptor activation provides a basis for developing a conventional thrombin receptor ligand binding assay using a tethered ligand peptide analogue. Although binding studies with intact cells have been reported, no significant correlation has been shown between the binding inhibitory potencies of the TRAP analogues and ability to stimulate or inhibit platelet aggregation. One study reported a specific binding of ^{125}I-SFLLRNPNDKYEPF-OH to bovine pulmonary artery endothelial cells with an affinity constant of 2 μM and a maximal binding of 41 pmol/mg of cell protein (35). A second

study using a biotinylated SFLLRNPNDKYEPF-OH and baby hamster cells overexpressing the human thrombin receptor reported a K_d of 3 μM and a maximal binding of 31 nmol/mg protein for biotinylated SFLLRNPNDKYEPF-OH (36). The receptor density in the latter study was too high, because it was calculated to be higher than 1 mg (1.5 mg) thrombin receptor protein per milligram of protein. It is likely that the biotinylated peptide ligand did not specifically label the thrombin receptor.

In the present study, a high affinity radiolabeled peptide, [^{3}H]haTRAP, binds to platelet membranes with a K_d of 15 nM and a B_{max} of 5.2 pmol/mg of protein. Scatchard analysis of [^{3}H]haTRAP saturation binding data and a Hill coefficient of 1.03 for [^{3}H]haTRAP indicate that [^{3}H]haTRAP bound to a single population of binding sites. TRAPs and an antagonist Mpa peptide inhibited [^{3}H]haTRAP binding, whereas various ligands for other G protein-linked receptors exhibited minimal to absent affinity for the binding sites. These observations indicate the selectivity of [^{3}H]haTRAP binding sites. Additionally, SFLLRN-NH_2 effectively displaced [^{3}H]haTRAP, whereas the same peptide with serine and phenylalanine reversed, FSLLRN-NH_2, did not, which is in line with earlier observations (5) with 14-amino-acid tethered-ligand peptides in platelet aggregation assays. These results again demonstrate selectivity of [^{3}H]haTRAP binding sites for a functionally active tethered ligand analogue and rule out nonspecific binding of [^{3}H]haTRAP to a SF/FS motif. Although haTRAP seems to specifically label thrombin receptor in platelet membranes, it remains to be determined whether haTRAP also has affinity for PAR-2 because human platelets do not seem to have PAR-2. Scatchard analysis of saturation binding of [^{3}H]haTRAP in the presence and absence of SFLLRN-NH_2 indicates that SFLLRN-NH_2 and [^{3}H]haTRAP compete for the same binding sites. The decrease in the binding affinity of [^{3}H]haTRAP in the presence of a guanine nucleotide indicates that the [^{3}H]haTRAP binding sites are linked to a heterotrimeric G protein. Furthermore, the [^{3}H]haTRAP binding site seems to be a functional thrombin receptor based on an excellent correlation between the affinity of various thrombin tethered-ligand analogues and their effects on human platelet aggregation. Additionally, platelet membranes isolated from the thrombin-preincubated platelets bound [^{3}H]haTRAP 48% less than the naive membranes. Platelet thrombin receptors were shown to be desensitized but not internalized (37, 38). Based on these findings, the reduced [^{3}H]haTRAP binding after thrombin pretreatment may have resulted either from thrombin receptor desensitization or competition of the thrombin-generated tethered ligand for the binding sites.

The number of B_{max} was estimated at 5.2 pmol/mg protein or 600 sites/platelet assuming 80% recovery of receptors during platelet membrane preparation. This thrombin receptor number is not much different from the reported numbers (1000–1800/platelet) estimated by Brass *et al.* (39) and Norton *et al.* (38) using a monoclonal antibody to the tethered-ligand domain (TR^{42-55}) and a polyclonal antibody to a region encompassing thrombin cleavage site (TR^{34-52}), respectively.

In summary, the present study demonstrates for the first time a functionally relevant thrombin receptor-radioligand binding assay using platelet membrane preparations and a high affinity TRAP analogue. This assay identifies a G protein-linked, thrombin receptor with which various tethered ligand analogues (TRAPs and a TRAP antagonist) interact to activate or inhibit platelet aggregation. This high volume binding assay should be useful for screening thrombin receptor antagonists as well as for studying the pharmacological properties and regulation of the thrombin receptor in various types of cells.

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