Immunologic Analysis of the Cloned Platelet Thrombin Receptor Activation Mechanism: Evidence Supporting Receptor Cleavage, Release of the N-Terminal Peptide, and Insertion of the Tethered Ligand Into a Protected Environment

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The recently cloned functional thrombin receptor is thought to be activated by thrombin cleavage of the bond between R41 and S42, followed by the insertion of the new N-terminal region ("tethered ligand") into an unknown site in the receptor. Antibodies to peptides at or near the cleavage site have been reported to inhibit thrombin-induced platelet activation to varying extents, but the precise mechanism(s) of their inhibition is unknown. We have produced: (1) a polyclonal antibody in rabbits to a peptide containing amino acids 34 to 52 (anti-TR34-52); enzyme-linked immunosorbent assays (ELISA) indicate that anti-TR34-52 contains antibodies to regions on both sides of the thrombin cleavage site; (2) two murine monoclonal antibodies (MoAbs) to a peptide containing amino acids 29 to 68; one antibody reacts primarily with residues N-terminal to the thrombin cleavage site, and the other reacts primarily with residues C-terminal to the cleavage site; and (3) a polyclonal rabbit antibody to a peptide containing amino acids 83 to 94 (anti-TR83-94). Anti-TR34-52 binds to platelets as judged by flow cytometry, and pretreating platelets with a thrombin receptor peptide ligand does not lead to loss of antibody reactivity, suggesting that platelet activation does not initiate redistribution or internalization of surface thrombin receptors. In contrast, pretreating platelets with thrombin leads to complete loss of anti-TR34-52 binding. Similarly, the binding of both MoAbs to platelets is dramatically reduced by pretreatment with thrombin. However, the binding of anti-TR34-52 is not decreased by thrombin activation, confirming that the receptor is not internalized. Anti-TR34-52 profoundly inhibits low dose thrombin-induced platelet shape change and aggregation, but the inhibition can be overcome with higher thrombin doses. However, anti-TR34-52 does not inhibit platelet aggregation induced by tethered ligand peptides. The TR34-52 peptide is a thrombin substrate, with cleavage occurring at the R41-S42 bond as judged by high performance liquid chromatography (HPLC) and platelet aggregation analysis. Anti-TR34-52 prevented cleavage of the TR34-52 peptide, suggesting that the antibody prevents platelet activation, at least in part, by preventing cleavage of the thrombin receptor. These data, although indirect, provide additional support for a thrombin activation mechanism involving thrombin cleavage of the receptor; in addition, they provide new evidence indicating that receptor cleavage is followed by loss of the N-terminal peptide, and insertion of the tethered ligand into a protected domain.

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the cleavage site, and an additional cysteine. Two of three such antisera caused partial inhibition of thrombin-induced platelet activation, and the third had no inhibitory activity. They speculated that the partial inhibition was caused by prevention of agonist peptide function and/or inhibition of receptor cleavage, but no direct data were reported.

Brass et al.16 most recently prepared monoclonal antibodies (MoAbs) to the 14 amino acid peptide ligand (amino acids 42 to 55). Three of the antibodies (ATAP2, ATAP120, and ATAP138) inhibited platelet aggregation induced by α-thrombin, γ-thrombin, and trypsin, but not adenosine diphosphate (ADP), epinephrine, or the thromboxane analogue U46619. The inhibition of thrombin-induced platelet activation could be overcome by increasing the thrombin concentration. One of the antibodies (ATAP138) was tested for its ability to inhibit aggregation induced by a 6 amino acid receptor peptide ligand (SFLLRN; amino acids 42 to 47); no inhibition was observed. Approximately 1,800 molecules of ATAP138 bound per platelet at saturation. Studies were not conducted to assess whether the antibodies interfered with the cleavage of the receptor, interfered with the binding of the tethered ligand into its proposed receptor site, or interfered with both processes.

We have recently, briefly described a polyclonal rabbit antibody to a 20 amino acid synthetic peptide corresponding to amino acids 34 to 52 of the thrombin receptor (with the addition of an N-terminal cysteine for purposes of cross-linking) that contains eight amino acids N-terminal, and 11 amino acids C-terminal, to the thrombin cleavage site (TR34-52) (Fig 1).17 This antibody (anti-TR34-52) profoundly inhibits platelet aggregation induced by low concentrations of thrombin and partially inhibits thrombin-induced shape change, but has little or no effect on aggregation induced by the thrombin peptide.17 In the present study, we have localized the regions of antibody reactivity with an enzyme-linked immunosorbent assay (ELISA), assessed the thrombin inhibition dose-response relationship, analyzed the antibody’s reactivity with untreated and thrombin-treated platelets by flow cytometry, and directly determined that the antibody can inhibit thrombin-induced cleavage of the TR34-52 peptide. In addition, we report on the binding of two murine MoAbs that react primarily with epitopes on either side of the thrombin cleavage site, and a polyclonal antibody directed against a peptide containing amino acids 83 to 94 of the thrombin receptor. The binding patterns of these antibodies to resting and thrombin-activated platelets provide insights into the mechanism by which the thrombin receptor is activated.

MATERIALS AND METHODS

Polyclonal Antibody Studies

All chemicals were of reagent grade from either Sigma Chemical (St Louis, MO) or Fisher Scientific (Fairlawn, NJ) unless otherwise specified. Hirulog-1, a high affinity inhibitor of thrombin,18 was a gift from Dr John Maraganore of Biogen (Cambridge, MA). Purified human thrombin (3,500 U/mg) was a gift from Dr Jolyon Jesty, State University of New York at Stony Brook.19 Partially purified human thrombin was obtained from Sigma (St Louis, MO). Antibody S-12, a murine MoAb directed against P-selectin (GMP-140)20 was a gift of Dr Rodger Mcever, University of Oklahoma. Antibody 10E5, a murine MoAb to glycoprotein Ilb/IIIa (GPIIb/IIIa), has been previously described.21

Blood preparation. Blood was drawn from healthy volunteers into 0.37 volume of EDTA (269 mmol/L) for flow cytometry studies, or either 0.14 volume of acid-citrate dextrose (ACD-A; 0.12 mol/L dextrose, 0.075 mol/L Na3 citrate, 0.038 mol/L citrate, pH 5.0 to 5.1) or 0.01 volume 40% Na, citrate for platelet aggregation studies. Platelet-rich plasma (PRP) was prepared by centrifugation at 700g for 4 minutes at 22°C. Gel-filtered platelets (GFP) for platelet aggregation were prepared by: adding 0.1 volume ACD-A to the PRP and centrifuging at 1,000g for 10 minutes at 22°C; resuspending the pellet in 600 μL of Hepes-buffered modified Tyrode’s solution (HBMT); 10 mmol/L HEPES, 138 mmol/L NaCl, 2.7 mmol/L KCl, 12 mmol/L NaHCO3, 0.4 mmol/L Na2PO4, 0.1% wt/vol dextrose, 0.2% wt/vol bovine serum albumin (BSA), pH 7.4; and gel-filtering through a 20-mL Sepharose-2B (Pharmacia, Piscataway, NJ) column. The cloudy, white platelet peak was collected and the platelet count determined using a Coulter Counter (Model ZM, Hialeah, FL). MgCl2 was added to the platelet eluate at 2.0 mmol/L final concentration.

Production of antibodies. The TR34-52 peptide was synthesized on a solid phase synthesizer (Model 430A; Applied Biosystems, Foster City, CA) using Fmoc chemistry; a 4-methylbenzhydrylamine (4-MBHA) resin was used to produce a C-terminal amide after cleavage. The amino acids T, N, R, and Y were double-coupled to ensure incorporation. The peptide was cleaved from the...
resin with hydrofluoric acid in the presence of anisol, dimethysul-
hide, and p-thiocresol (10:1:1:0.2 by volume, respectively). The re-
sin was washed with ether and dichloromethane, and then extracted
in acetic acid and lyophilized. The pH of the peptide was raised to
10 and then lowered to 3 to reverse any N → O shift of the serine or
threonine residues and then purified on a C8 reverse-phase high
performance liquid chromatography (HPLC) column with a
water/acetonitrile buffer system containing 0.1% trifluoroacetic
acid, using a gradient from 0% to 60% acetonitrile over 40 minutes;
analytical C8 reverse-phase HPLC showed that 79% of the optical
density was in the major peak. In a likewise manner, the following
peptides were synthesized: a 12 amino acid peptide (QLPAFISE-
DASI, TR34-52), as well as the same peptide with an N-terminal
cysteine, whose sequence was derived from a region in the first
extracellular domain of the thrombin receptor, near the membrane
spanning domain; and an 11 amino acid peptide (SFLLRNPNDKY,
DASG; TR83-94), as well as the same peptide with an N-terminal
cysteine, and pthiocresol (10:1:1:0.2 by volume, respectively). The re-
action of 20 mg/mL and a trace amount of '251-TR34-52 (see below) was
terminated by dialysis against PBS, pH 7.4. As judged by the incor-
poration of the radioactive peptide, 70% of the peptide was coupled
to the KLH.

The two TR34-52-KLH antigens were first mixed 1:1 by peptide
mass and then mixed 1:2 (vol:vol) with either complete Freund’s adju-
vant (for initial immunization) or incomplete Freund’s adju-
vant (for subsequent immunizations); the TR42-52 mixtures
were then sonicated for 1 minute at 4°C in 15- to 20-second bursts (Wave
Energy Systems model MT-1; Lab-line Ultratop Labsonic System
#9100, Melrose Park, IL); the TR34-52 peptide was manually homog-
ized 1:1 (vol:vol) in complete Freund’s adjuvant (first immuniza-
tion) or incomplete Freund’s adjuvant (subsequent immuniza-
tions) by rapid transfer between two 10-mL syringes. New Zealand
white rabbits were injected on day 1 with 1 mg of peptide and on
days 8 and 15 with 0.5 mg of peptide. On day 22, sera were obtained
for analysis and on day 29 the rabbits were exsanguinated. The sera
with the highest activities, anti-TR42-52 and anti-TR34-52, were
chosen for further investigation.

Peptide ELISA assays. The wells of microtiter plates (#3915;
Bio Rad Laboratories, Richmond, CA) were coated with TR42-52
peptide, TR34-52 peptide (100 μg/mL; 100 μL/well) or BSA (0.5%;
100 μL/well) in 0.05 mol/L carbonate buffer, pH 9.5, for 1 hour
at 37°C. Any remaining binding sites on the plates were blocked
with 0.5% BSA in Tris-saline-azide (TSA, 0.01 mol/L Tris/HCl, 0.15
mol/L NaCl, 0.05% NaN3, pH 7.4) for at least 1 hour at 22°C or
overnight at 4°C. Serum or purified antibody was added to the wells
at various dilutions and allowed to incubate for 2 hours at 22°C.
After extensive washing with TSA containing 0.05% Tween-20,
guant peptide-phosphatase-conjugated antibody (100 μL/
wells/1/5,000 to 1/10,000 dilution in TSA; Tago, Burlingame, CA)
was added and allowed to incubate 2 hours at 22°C. The plate was
washed extensively with TSA containing 0.05% Tween-20 and then the
phosphatase substrate (100 μL/well; 2 mg/mL p-nitrophenyl
phosphate disodium in 0.1 mol/L NaHCO3, 0.01 mol/L MgCl2, pH 9.5;
Sigma Chemical Co, St Louis, MO) was added. The optical density
was measured at 405 nm in a microplate reader (Vmax, Molecular
Devices, Menlo Park, CA). In some experiments, anti-TR42-52
TR34-52, or the unrelated peptide Arg-Gly-Asp-Phe (RGDF) (20
nmol/L to 200 μmol/L), for 1 hour at 22°C before adding it to the
plate. The ability of the free peptide to inhibit antibody binding to
the immobilized peptide was measured by the decrease in optical
density compared to a control in which buffer was added to anti-
obeads instead of peptide.

Epitope mapping. To assess the reactivity of the anti-TR42-52
with different regions of the external sequence of the thrombin
receptor, epitope mapping was performed by an ELISA technique
using peptides whose sequences were derived from the thrombin
receptor, beginning at the N-terminus of the protein after cleavage
of the proposed signal peptide, and including all three extracellular
loops (amino acids 26 to 99, 161 to 176, 240 to 265 and 336 to 347,
respectively). In all, 49 overlapping decapeptides, each offset by two
amino acids, were synthesized by t-Boc chemistry onto solid-phase
resin and then purified on a C8 reverse-phase high performance
liquid chromatography (HPLC) column with a
water/acetonitrile buffer system containing 0.1% trifluoroacetic
acid, using a gradient from 0% to 60% acetonitrile over 40 minutes;
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loops (amino acids 26 to 99, 161 to 176, 240 to 265 and 336 to 347,
respectively). In all, 49 overlapping decapeptides, each offset by two
amino acids, were synthesized by t-Boc chemistry onto solid-phase
ing 200 μL of precoate buffer (2.0% wt/vol BSA [Sigma], 0.1% Tween-20 [Bio-Rad, Richmond, CA]; in PBS, pH 7.2), for 60 minutes at 22°C on an orbital shaker table (Lab-line Industries, Melrose Park, IL) at 100 rpm. The pins were removed from the precoate buffer, excess buffer was flicked off, and the pins were placed into wells containing 175 μL of either purified anti-TRβ452 IgG or NRS IgG diluted to 67.0 μg/mL in precoate buffer. After 1 hour of shaking at 100 rpm at 22°C, the pins were washed three times, each for 10 minutes in PBS, pH 7.2. The pins were then placed into wells of a microtiter plate containing 175 μL of the peroxidase-labeled goat antirabbit IgG diluted 1:5,000 in sheep serum buffer (1.0% vol/vol sheep serum [Sigma], 0.1% vol/vol Tween 20 in PBS, pH 7.2) and the plate was rotated at 22°C for 60 minutes at 100 rpm. The pins were washed three times at 22°C for 10 minutes per wash and then placed in the wells of a microtiter plate containing 150 μL of substrates solution (0.5% mg/mL diammonium 2,2-azino-bis[3-ethyl-benzthiazoline-6-sulfonate] [ABTS] [Sigma], 0.01% wt/vol hydrogen peroxide [Sigma] in 0.1 mol/L Na2HPO4, 0.08 mol/L citric acid, pH 4.0). The plate, with the pins remaining in the wells, was rotated at 22°C for 3 minutes at 100 rpm. The optical density was read at 405 nm and 490 nm in an ELISA plate reader (Molecular Devices), and the 490-nm value was subtracted from the 405-nm value to correct for background. Data were calculated as the difference in optical density between the anti-TRβ452 and the NRS for each peptide.

Removal of the antibody from the pin peptides. To reuse the peptides on the pins, the previous reactants had to be removed. The pins were placed in a sonicator bath (Sonicor, Copagie, NY) containing 0.1 mol/L sodium phosphate with 1% wt/vol sodium deoxychloride sulfate (SDS) [Fisher Scientific] and 0.1% 2-mercaptoethanol (Pharmacia LKB Biotechnology), pH 7.2, at 60°C. The pins were sonicated (3 kW/m2 at 60 kHz) for 10 minutes and rinsed twice with distilled water preheated to 60°C. The pins were then washed in a bath of distilled water at an initial temperature of 60°C for 30 minutes with agitation. After shaking off excess water, the pins were immersed in boiling methanol (Fisher) for 15 to 30 seconds and allowed to air dry for 15 minutes. The pins were then placed into a solution containing 20% with starting buffer and 100 μL of serum and centrifuging at 10,000 rpm at 22°C, the pins were washed three times, each for 10 minutes in PBS, pH 7.2. The pins were then placed into wells of a microtiter plate containing 175 μL of the peroxidase-labeled goat antirabbit IgG diluted 1:5,000 in sheep serum buffer (1.0% vol/vol sheep serum [Sigma], 0.1% vol/vol Tween 20 in PBS, pH 7.2) and the plate was rotated at 22°C for 60 minutes at 100 rpm. The pins were washed three times at 22°C for 10 minutes per wash and then placed in the wells of a microtiter plate containing 150 μL of substrate solution (0.5% mg/mL diammonium 2,2-azino-bis[3-ethyl-benzthiazoline-6-sulfonate] [ABTS] [Sigma], 0.01% wt/vol hydrogen peroxide [Sigma] in 0.1 mol/L Na2HPO4, 0.08 mol/L citric acid, pH 4.0). The plate, with the pins remaining in the wells, was rotated at 22°C for 3 minutes at 100 rpm. The optical density was read at 405 nm and 490 nm in an ELISA plate reader (Molecular Devices), and the 490-nm value was subtracted from the 405-nm value to correct for background. Data were calculated as the difference in optical density between the anti-TRβ452 and the NRS for each peptide.

Purification of antibody. Control and immune sera were heated to 56°C for 1 hour and delipidated by adding 1 volume of Sorecleat (Calbiochem Inc) to 1.5 volume of serum and centrifuging at 1,000g for 15 minutes at 22°C. The supernatant was filtered through a 0.45 μ filter (Genex Inc, Gaithersberg, MD), diluted to 20% with starting buffer (10 mmol/L Na2HPO4, 1.5 mol/L NaCl, pH 8.5) and applied to a 10 mL Protein A Sepharose column (BioProbe International, Tusin, CA). After washing the column in starting buffer, IgG was eluted either in two steps (pH 5 and pH 3) or one step (pH 3.5) using a 0.1 mol/L citrate buffer. The eluting fractions were collected in tubes containing sufficient 1 mol/L Tris/HCl (pH 9.5) to immediately neutralize the pH. Fractions containing IgG were dialyzed against TSE. Purified IgG was assayed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli23, protein concentration was estimated by optical density at 280 nm using A280 = 14.

F(ab)2 fragments of normal rabbit serum IgG (NRS) and anti-TRβ452 IgG were produced by overnight incubation of IgG with 0.5% (wt/vol) pepsin (Worthington Biochemical, Freehold, NJ) in 0.2 mol/L NaCl, 0.2 mol/L NaCl, pH 4.0 at 37°C. The reaction was terminated by dialysis against TSA.

Flow cytometry analysis. Aliquots of EDTA PRP or GFP (Sephacore 2B; 0.15 mol/L NaCl, 0.01 mol/L Tris/HCl, 0.01 mol/L EDTA, pH 7.4 (TSE); 3 × 106 platelets/mL) were incubated with buffer or various concentrations (100 to 500 μg/mL) of purified IgG or F(ab)2 fragments of NRS, anti-TRβ452, or anti-TRβ54 (primary antibodies); a control polyclonal rabbit antithrombocyte serum (final dilution 1:200; Accurate Chemical, Westbury, NY) was also used in some experiments as a positive control. After 20 to 30 minutes at 22°C, the platelets were washed twice in 1.8 mL TSE and resuspended to 400 μL in TSE. Each sample was then incubated with affinity-purified, fluorescein isothiocyanate (FITC)-conjugated, F(ab)2 fragments of goat antirabbit F(ab)2 fragment (Jackson ImmunoReserch) so that the final dilution was 1:100. After 20 to 30 minutes at 22°C, the platelets were washed once in 1.8 mL TSE buffer, resuspended to 1.0 mL in TSE, and then evaluated by flow cytometry.

For thrombin digestion studies, GFP (3 to 4 × 106 platelets/mL) in TSE was incubated at 37°C for 5 minutes with 0.1, 1.0, or 10 μL human purified thrombin. The reaction was terminated by addition of 50-fold molar excess of Hirulog for 5 minutes at 37°C; primary antibody was then added and the samples were processed as above. In other studies, GFP was incubated with TRβ55 (50 μg/mL) to activate the platelets for 5 minutes at 37°C before adding the primary antibody and processing as above. In some experiments, aliquots of GFP were incubated 30 minutes with primary antibody before the addition of thrombin or TRβ55.

Flow cytometry analysis was performed on a FACStar analyzer (Becton-Dickinson, San Jose, CA) using a 70 μ aperture and an argon laser at 200 mW (Innova 90; Coherent Laser Products, Palo Alto, CA). Data analysis was performed with the Lysys II program supplied by the manufacturer and each analysis consisted of 106 platelets. Before analysis, platelets that were treated identically to the experimental samples except for receiving buffer instead of either antibody were analyzed by forward angle light scattering in order to gate 90% of the single platelet population.

Cleavage of TRβ452 and 125I-TRβ452 by thrombin. Reduced and alkylated TRβ452 (see below) (1 mg/mL) was incubated with thrombin (1 U/mL) for 1 hour at 37°C and then Hirulog (50 μg/mL final concentration) was added to stop the reaction. Trichloroacetic acid (10% final concentration) was then added at 4°C to precipitate the thrombin but not the peptides, and after 60 minutes the solution was centrifuged at 12,000 g for 3.5 minutes at 22°C. Cleavage of TRβ452 was assessed by reverse-phase HPLC as above, using uncleaved TRβ452 and TRβ55 as standards. To test the effect of the anti-TRβ452 antibody on thrombin cleavage of TRβ452, a trace dose of 125I-TRβ452 was incubated with either anti-TRβ452 F(ab)2, or NRS F(ab)2 (1 mg/mL) and the cleavage was performed as above. The HPLC fractions were counted in a gamma spectrometer (Auto-gamma model 5550; Packard Instrument Co, Meriden, CT) and the identification of the 125I-TRβ452 cleavage products was facilitated by adding some of the unlabeled TRβ452 cleavage products so that they could be identified readily on the HPLC optical density profile.

Platelet aggregation and shape change. Platelet aggregation was performed as previously described using PRP or GFP.21 To avoid the confounding effects of platelet aggregation, studies of platelet shape change were performed on platelets pretreated for 15 minutes with either 10 mmol/L EDTA or a saturating (25 μg/mL) dose of the murine MoAb 10E5, which binds to the platelet GPIIIb/IIa receptor and prevents platelet aggregation.21

Induction of TRβ452 and reduced and alkylated TRβ452 peptide and TRβ452 peptide that had been reduced (0.35% 2-mercaptoethanol for 60 minutes at 22°C) and alkylated (60 mmol/L iodoacetamide, pH 8.0, for 60 minutes followed by gel filtration.
over a Sephadex G-25 column) to prevent dimerization, were radiolabeled with 

$^{125}$I using glass vials coated with 50 μL of 5 mg/mL iodogen (Pierce). Radiolabeled peptide was separated from unincorporated $^{125}$I by gel filtration over a Sepharose G-15 column with TSA, $^{125}$I-TR3′52 was used to monitor the efficiency of peptide coupling to KLH and the reduced and alkylated $^{125}$I-TR3′52 was used to follow the cleavage of the peptide in solution by thrombin.

**Monoclonal Antibody Studies**

**Synthetic peptides.** Thrombin receptor peptides, PESKAT-NATLDPRSF, TR28-43, SFLIRNPNDKYEP, TR3′46, PFWEDEKNEGL, TR3′66, and PESKAT-NATLDPR-SFLIRNPNDKYEFPWEDEKNEGLTEC, TR29-68 (with an added C-terminal cysteine) were prepared using standard solid-phase t-BOC chemistry using an Applied Biosystems Model 431A peptide synthesizer using the manufacturers instructions (TR29-43, TR42-54, and TR54-66) or with standard FMOC chemistry using an Applied Biosystems Model 430A peptide synthesizer (TR29-68). The peptides were prepared with free carboxyl groups and the N-terminal amino groups were not acetylated. Completed peptides were released from their diolabeled with TSA. $^{125}$I-TR3′52 was used to monitor the efficiency of peptide coupling to KLH and the reduced and alkylated $^{125}$I-TR3′52 was used to follow the cleavage of the peptide in solution by thrombin.

**Antibody screening was done by ELISA using the**

oxidase, diluted 1:2,000 in washing buffer; after incubation at room temperature for 1 hour, the wells were washed and the assay developed with ABTS substrate (1 mg/mL in 0.1 mol/L citric acid, 0.1 mol/L NaPO₄, pH 4.0), followed by the addition of H₂O₂ (1:10,000 dilution) for 15 minutes. The reaction was stopped with 5% SDS and read in an ELISA reader at 560 nm. The same methods were used to determine the immunoreactivity of hybridomas or purified antibodies with the peptide fragments TR29-43, TR42-54, and TR54-66.

**Ascites production, antibody purification, and antibody iodination.** Ascites enriched in MoAbs was prepared by intraperitoneal injection of Pristane-pretreated BALB/c mice. Antibody was purified from the ascites by affinity chromatography on protein A-Sepharose. Iodination of antibody with $^{125}$I was performed with immobilized chloramine-T (IODO-Beads; Pierce). The specific activity was ~1,180 cpm/μg.

**Binding of $^{125}$I-antibodies to GFP.** Platelets were isolated from human blood on the day of use by gel filtration. In a typical experiment, 60 μL of blood was collected into 8.7 mL of ACD (85 mmol/L sodium citrate, 111 mmol/L dextrose, 71 mmol/L citric acid) containing 50 ng/mL PGI₂, and centrifuged for 20 minutes at 160 g. PRP was removed and centrifuged for 10 minutes at 730g at room temperature to sediment the platelets. The resulting platelet pellet was resuspended in 5 mL of Buffer A (12 mmol/L sodium bicarbonate, 10 mmol/L HEPES, 138 mmol/L NaCl, 5.5 mmol/L glucose, 2.9 mmol/L KCl, and 0.35% human serum albumin, pH 7.4) and applied to a 40-mL Sepharose Cl-2B column (1.5 × 23 cm; Pharmacia) in the same buffer. Platelets were collected from the column in the void volume to remove plasma proteins. In some experiments, the platelets were incubated with EDTA (1 mmol/L), EDTA + ADP (20 μmol/L), or EDTA + thrombin (0.05 U/mL; Hematologic Technologies, Inc. Essex Junction, VT) for 10 minutes at 37°C. For the antibody binding experiments, platelets (0.5 to 1.0 × 10⁸ platelets/mL) were incubated with $^{125}$I-antibodies for 45 minutes at room temperature. After binding, 100 μL aliquots of each sample were layered onto 0.5 mL of 20% sucrose in buffer A containing 2 mmol/L CaCl₂ and 2 mmol/L MgCl₂ and centrifuged for 2 minutes at 12,000g in a microfuge to separate free $^{125}$I-antibody from $^{125}$I-antibody bound to platelets. The $^{125}$I-antibody associated with the platelets was determined by clamping off the tip of the microfuge tube and counting in a γ counter. Data from binding experiments were used to determine the dissociation constant ($K_d$) as determined by the method of Scatchard using the computer program LIGAND.

**RESULTS**

**Polyclonal Antibodies**

**Characterization of anti-TR3′43 by ELISA.** Anti-TR3′43 serum reacted positively (OD₄₉₀ ≥ 0.2) in the ELISA assay using immobilized TR3′43 at dilutions of serum as high as 1/12,800 (corresponding to ~0.78 μg/mL IgG), whereas similar dilutions of control preimmune serum failed to produce any positive signal. Anti-TR3′43 did not react with a BSA substrate. The specificity of the interaction of anti-TR3′43 with immobilized TR3′43 was tested by preincubating the antisera with TR34-52, TR42-52, or an unrelated RGDF peptide. TR34-52 at 200 μmol/L final concentration reduced the optical density produced by the 1/1,600 serum dilution by 83%; dose-response inhibition was seen at lower peptide concentrations. In contrast, TR32-52 was able to block only 50% of the reactivity even at 200 μmol/L, and a similar dose-dependency was noted at lower concentrations. This suggests that anti-TR3′43 contains antibodies directed at sequences on TR3′43 that are both N-terminal and C-terminal to the thrombin cleavage site. The unrelated RGDF peptide at identical concentrations failed to show any inhibitory activity. In further specificity testing, antisera prepared against the protein vitronectin did not bind to immobilized TR3′43 and neither TR3′43 nor TR42-52 in-
Table 1. Immunoreactivity of Anti-TR34-52 With Thrombin Receptor Pin Peptides

<table>
<thead>
<tr>
<th>Amino Acids*</th>
<th>ODex*</th>
</tr>
</thead>
<tbody>
<tr>
<td>26-34 (+ cysteine 33a)†</td>
<td>0.00</td>
</tr>
<tr>
<td>28-36 (+ cysteine 33a)</td>
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</tr>
<tr>
<td>30-38 (+ cysteine 33a)</td>
<td>0.02</td>
</tr>
<tr>
<td>32-40 (+ cysteine 33a)</td>
<td>0.78</td>
</tr>
<tr>
<td>34-42 (+ cysteine 33a)</td>
<td>1.36</td>
</tr>
<tr>
<td>35-44</td>
<td>1.28</td>
</tr>
<tr>
<td>37-46</td>
<td>0.73</td>
</tr>
<tr>
<td>39-48</td>
<td>1.04</td>
</tr>
<tr>
<td>41-50</td>
<td>0.91</td>
</tr>
<tr>
<td>43-52</td>
<td>1.04</td>
</tr>
<tr>
<td>45-54</td>
<td>1.04</td>
</tr>
<tr>
<td>47-56</td>
<td>0.85</td>
</tr>
<tr>
<td>49-58 through 91-100</td>
<td>0.00</td>
</tr>
<tr>
<td>161-170 through 167-176</td>
<td>0.00</td>
</tr>
<tr>
<td>240-249</td>
<td>0.00</td>
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<tr>
<td>242-251</td>
<td>0.28</td>
</tr>
<tr>
<td>244-252, 246-255</td>
<td>0.00</td>
</tr>
<tr>
<td>248-251</td>
<td>0.74</td>
</tr>
<tr>
<td>250-259 through 254-263</td>
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</tr>
<tr>
<td>255-265</td>
<td>0.38</td>
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<tr>
<td>336-345</td>
<td>0.00</td>
</tr>
<tr>
<td>338-347</td>
<td>0.16</td>
</tr>
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</table>

* Amino acids 29-99, 1st extracellular domain; 161-176, 1st extracellular loop; 240-265, 2nd extracellular loop; 336-347, 3rd extracellular loop.
† The difference in optical densities (ODex - ODex0) between anti-TR34-52 and normal rabbit IgG for each peptide (negative values were taken as 0.00).
† A cysteine was inserted between amino acids 33 and 34 (cys 33a) to facilitate comparison with the TR34-52 peptide that contained an added cysteine N-terminal to amino acid 34.

Hindered antivitronectin serum from binding to purified vitronectin. F(ab′), fragments of anti-TR34-52 gave ELISA results that were similar to those observed with the intact antibody.

Epitope mapping. Anti-TR34-52 gave strong signals (ODex > 0.70) in the pin ELISA with the decapetides containing the sequences 32 to 40 through 47 to 56 (Table 1). All of these peptides contain amino acid sequences in the immunizing peptide (TR34-52). Only one of the peptides containing sequences from the other external domains of the thrombin receptor bound enough anti-TR34-52 to give a strong positive signal, and three other isolated peptides gave weak signals. It is unlikely that any of these four positives represent sequence-specific binding because the peptides immediately preceding and succeeding the positives did not give positive responses, despite having the same sequence in 8 of 10 amino acids. Because some of the peptides giving a positive ELISA response contain amino acids that are only N-terminal to the thrombin cleavage site at R41-S42 (eg, amino acids 32 to 40), and others contain sequences that are only C-terminal to the thrombin cleavage site (eg, amino acids 43 to 52), these data provide additional support for the presence of antibodies to regions on both sides of the thrombin cleavage site in anti-TR34-52.

Functional characterization of anti-TR34-52. As previously described, 17 intact anti-TR34-52 IgG at 63 μg/mL and anti-TR34-52 F(ab′), fragments at 125 μg/mL completely inhibited platelet aggregation induced by 0.4 U/mL thrombin in citrated PRP and 0.1 U/mL thrombin in GFP (Fig 2A). Control NRS IgG or F(ab′), fragments at similar concentrations either did not inhibit platelet aggregation at all or produced only minimal inhibition. The inhibitory effects of anti-TR34-52 on aggregation could be partially overcome by increasing the thrombin concentration (Fig 2B). Doubling the antibody concentration did not further inhibit the response induced by the higher thrombin concentrations. The inhibition of platelet aggregation produced by anti-TR34-52 was thrombin-specific, as neither intact anti-TR34-52 nor F(ab′), fragments of anti-TR34-52 had any effect on ADP-induced platelet aggregation (15 μmol/L) (data not shown). Anti-TR34-52 at concentrations that inhibited thrombin-induced platelet aggregation did not inhibit shape change or aggregation induced by the thrombin receptor peptide ligand TR42-52 (data not shown).

Platelet shape change induced by thrombin was assessed in PRP in the presence of 10 mmol/L EDTA or saturating doses of 10E5 to prevent platelet aggregation. Anti-TR34-52 F(ab′), partially inhibited thrombin-induced shape change when compared with the NRS F(ab′), control (Fig 3). This inhibition could also be partially overcome by increasing the concentration of thrombin.

At least two potential mechanisms could account for the ability of anti-TR34-52 to inhibit thrombin-induced platelet aggregation: (1) inhibition of thrombin-induced cleavage of the thrombin receptor, and/or (2) inhibition of the tethered ligand's insertion into its presumed binding site on the thrombin receptor as a result of antibody binding to the tethered ligand region. To assess whether anti-TR34-52 could inhibit thrombin-induced cleavage of the thrombin receptor, we tested its ability to prevent cleavage of the TR34-52 peptide by thrombin. The reduced and alkylated TR34-52 peptide (1 mg/mL) functioned as a thrombin (1 U/mL) substrate; it was nearly completely cleaved into two fragments after 1 hour at 37°C, one of which eluted on HPLC in a position essentially identical to the TR42-52 peptide, whereas the other, presumably representing the nine amino acid N-terminal peptide, eluted more rapidly. The TR42-52 generated by thrombin cleavage of TR34-52 was able to aggregate platelets at doses comparable to authentic TR42-52, whereas TR34-52 could not aggregate platelets (data not shown). In the presence of 1 mg/mL control F(ab′), fragments, thrombin was still able to cleave the radiolabeled TR34-52 extensively, with 69% of the radioactivity migrating with the TR32-51 peak instead of the TR34-52 peak (Fig 4). However, in the presence of 1 mg/mL anti-TR34-52 F(ab′), fragments, only ~9% of the radioactivity was found in the TR42-52 peak.

Flow cytometry. The anti-TR34-52 bound to platelets as judged by flow cytometry, with the entire gated platelet population showing more fluorescence with anti-TR34-52 F(ab′), than with NRS F(ab′), (Table 2 and Fig 5). The fluorescence
PLATELET THROMBIN RECEPTOR

Fig 2. Effect of anti-TR34-52 on thrombin-induced platelet aggregation. (A) Blood was drawn into ACD-A anticoagulant, PRP was prepared, and then the latter was supplemented with 0.1 volume of additional ACD-A. After centrifugation, the platelet pellet was resuspended in HBMT buffer (see Materials and Methods) without MgCl₂ and gel-filtered. After adding 2 mmol/L MgCl₂, the GFP (0.4 mL; ~350,000 platelets/mL) was incubated with 100 to 150 µL of anti-TR34-52 F(ab)_2, normal rabbit IgG F(ab)_2, or Tris-saline (TS) buffer to achieve the indicated final concentrations, for ~30 minutes at 22°C. Aggregation was induced with 0.1 U/mL purified human thrombin. The normal rabbit IgG F(ab)_2 (not shown) did not show any inhibition at concentrations up to 124 µg/mL, but partial inhibition was observed at 250 µg/mL and 500 µg/mL. (B) GFP prepared as in Fig 2A (0.45 mL; 334,000 platelets/mL) was incubated with anti-TR34-52 F(ab)_2 or normal rabbit IgG F(ab)_2 (35 µL; 250 µg/mL final concentration) for ~30 to 60 minutes at 22°C. Aggregation was initiated at the indicated time points with purified human thrombin. The inhibition of aggregation was nearly complete at 0.06 and 0.09 U/mL of thrombin, but inhibition was less evident at 0.17 and 0.23 U/mL. However, even at the higher doses the anti-TR34-52 produced a delay in onset of aggregation, a shallower slope of aggregation, and smaller aggregate size (as judged by the width of the deflections).

signal produced by anti-TR34-52 was considerably less than that produced by antibody 10E5, which was included as a positive control (data not shown). Maximal fluorescence was attained with ~100 µg/mL anti-TR34-52, suggesting that this is a near saturating concentration; this is in accord with the platelet-aggregation inhibition data. When platelets were preincubated with thrombin (0.1, 1, or 10 U/mL for 5 minutes at 37°C) before the anti-TR34-52 was added, the fluorescence intensity showed a dose-response reduction; with 10 U/mL thrombin, the fluorescence intensity was actually less than that produced by the NRS F(ab)_2 (Table 2 and Fig 5). The dose response of the thrombin-induced loss of anti-TR34-52 binding was very similar to the dose response expression of P-selectin, an α-granule membrane protein, on the platelet surface as judged by the binding of antibody S-12. Thrombin treatment did not reduce the fluorescence...
Thrombin cleavage, with more than 90% of the anti-TR34-52 F(ab)_2 or control NRS F(ab)_2 (1 mg/mL final concentration) eluted at 24.5 minutes and so it is not depicted in this elution profile. The mixtures were then subjected to reverse phase HPLC and the eluate was monitored by Ultra-Violet absorbance at 220 nm. The C-terminal fragment of TR34-52 was eluted at 24.5 minutes and so it is not depicted in this elution profile.

Fig 4. Inhibition of thrombin-induced cleavage of peptide TR34-52 by anti-TR34-52. A trace dose of 125I-TR34-52 (18,000 cpm) in 0.15 mol/L NaCl, 0.01 mol/L Tris/HCl, pH 7.4 was incubated with anti-TR34-52 F(ab)_2 or control NRS F(ab)_2 (1 mg/mL final concentration) and then purified thrombin (1 U/mL final concentration) was added for 1 hour at 37°C. Hirulog (14 pg/mL) was added for 10 minutes to stop the reaction, and then the samples were made 10% in trichloroacetic acid and placed at 4°C for 1 hour. The precipitated proteins were removed by centrifugation at 12,000g for 3.5 minutes at 22°C. The supernatant was then mixed with ~10 µg of unlabeled TR34-52 that had been partially cleaved with thrombin so that the optical density elution pattern of the partially cleaved TR34-52 could serve to identify the elution of 125I-TR34-52 and its fragments. The mixtures were then subjected to reverse phase HPLC on a C-8 column. Cleavage of the 125I-TR34-52 was extensive in the presence of the NRS F(ab)_2, with 69% of the radioactivity eluting with the TR34-52 peptide. In contrast, the anti-TR34-52 F(ab)_2 prevented thrombin cleavage, with more than 90% of the 125I-TR34-52 remaining intact. Note that the nine amino acid fragment of TR34-52 eluted at ~24.5 minutes and so it is not depicted in this elution profile.

Monoclonal Antibodies

Immunoreactivity. Approximately 120 IgG-positive hybridomas were identified in the initial ELISA screen of hybridoma supernatants with the immunizing antigen. Positive hybridomas were also screened by ELISA using the TR29-43, TR42-54, and TR54-66 synthetic peptides coated to the wells of 96-well plates to more precisely determine the epitope recognition for each of the secreting clones (Table 3). Antibody 33-1 reacted with TR42-54, but failed to react with TR29-43 or TR54-66, indicating that it recognized an epitope C-terminal to the thrombin cleavage site. Antibody 61-1, in contrast, reacted strongly with TR29-43 and only weakly with TR42-54, suggesting that its dominant epitope was N-terminal to the thrombin cleavage site.

Binding of antibodies to GFP. GFP was reacted with 125I-labeled antibodies under various conditions to determine the rate and extent of antibody binding. The time
Table 2. Immunoreactivity of Anti-TR42-52, Anti-TR43-93, and Anti-P-Selectin Antibody S-12 With Thrombin- and/or Peptide Ligand-Activated Platelets as Judged by Flow Cytometry

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Control F(ab')2</th>
<th>Anti-TR42-52 F(ab')2</th>
<th>S-12 IgG</th>
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<tr>
<td>Buffer</td>
<td>10.4*</td>
<td>19.9 (0)†</td>
<td>70 (0)‡</td>
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<td>Thrombin (U/mL)</td>
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<td>11.3</td>
<td>12.5 (78)</td>
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<td></td>
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<td>11.3</td>
<td>8.1 (113)</td>
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<tr>
<td></td>
<td>10.0</td>
<td>9.7</td>
<td>9.5 (100)</td>
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<tr>
<td>TR43-96 (μmol/L)</td>
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<td>14.4</td>
<td>21.1 (0)</td>
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<table>
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<th>Experiment 2</th>
<th>Control F(ab')2</th>
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<th>S-12 IgG</th>
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<tr>
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<td>19.6</td>
<td>30.4 (0)</td>
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<td>Thrombin (U/mL)</td>
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<td>20.1</td>
<td>22.0 (45)</td>
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<tr>
<td></td>
<td>1.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>19.3</td>
<td>11.6 (100)</td>
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<tr>
<td>TR43-96 (μmol/L)</td>
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<th>Experiment 3</th>
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<th>S-12 IgG</th>
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<td>15.6 (0)</td>
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<tr>
<td>Thrombin (U/mL)</td>
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<td>8.3 (69)</td>
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<td>1.0</td>
<td>8.0</td>
<td>4.8 (103)</td>
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<td>10.0</td>
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<tr>
<td>TR43-96 (μmol/L)</td>
<td>51.0</td>
<td>6.2</td>
<td>14.9 (6)</td>
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<th>Experiment 4</th>
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<th>S-12 IgG</th>
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<td>15.8 (68)</td>
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<td>15.7</td>
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<tr>
<td>Thrombin (U/mL)</td>
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<td>16.8</td>
<td>11.8 (67)</td>
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<tr>
<td></td>
<td>10.0</td>
<td>13.1</td>
<td>7.2 (100)</td>
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* Mean fluorescence in arbitrary units.
† Percentage reduction from buffer control in anti-TR42-52 binding compared with reduction produced by 10.0 U/mL thrombin, which is taken as 100%.
‡ Percentage increase from control in S-12 binding compared with increase produced by 10 U/mL thrombin. S-12 is an antibody to P-selectin (GMP-140).

course of binding of 125I-antibody to GFP was measured to determine optimal conditions for equilibrium binding experiments. Subsequently, saturation binding experiments were conducted with increasing amounts of labeled antibody and GFP. The binding of each of the antibodies was found to be specific and saturable. Analysis of the binding data by the method of Scatchard showed that approximately 1,000 to 1,100 antibody molecules bound per platelet at saturation. The Kd determined for MoAbs 61-1 and 33-1 were found to be 8.9 and 1.0 nmol/L, respectively. As shown in Fig 6, the addition of EDTA did not affect the binding of the antibodies, nor did the subsequent addition of ADP (20 μmol/L) or collagen (not shown). However, as with the polyclonal anti-TR42-52, there was a dramatic loss of binding of antibodies 61-1 and 33-1 when platelets were pretreated with thrombin at 0.05 U/mL.

**DISCUSSION**

Anti-TR42-52, a polyclonal rabbit antibody directed against a 20 amino acid peptide encompassing the putative thrombin cleavage site on the thrombin receptor, inhibits thrombin-induced platelet shape change and aggregation, but the inhibition can be overcome with higher concentrations of thrombin. The ability of higher concentrations of thrombin to overcome the inhibition could be consistent with the presence of another thrombin receptor-mediated activation process, but differences in affinity of the receptor for anti-TR42-52 and thrombin might also account for the phenomenon. Anti-TR42-52 does not inhibit platelet shape change and aggregation induced by TR42-52, a peptide derived from the putative new amino terminus after thrombin cleavage. This suggests that anti-TR42-52 does not block the site on the receptor into which the tethered ligand inserts.

Data from epitope mapping studies showed that the antibody reacts with sites on both sides of the putative thrombin cleavage site, making it likely, but not certain, that on intact platelets it also reacts with sites on both sides of the cleavage site. Flow cytometry studies confirmed that anti-TR42-52 bound to intact platelets; the fluorescence intensity was of a relatively low level compared with a MoAb to GPIIb/IIIa.
Fig 5. Immunoreactivity of anti-TR<sup>34-52</sup> to GFP before and after thrombin treatment as judged by flow cytometry. Blood was drawn into EDTA anticoagulant (10 mmol/L final concentration) and PRP was prepared. After centrifugation, the platelet pellet was resuspended in TSE buffer and gel-filtered (see Materials and Methods). In (A), GFP (388,000 platelets/μL) was incubated with either thrombin (10 U/mL final concentration) or TSE for 5 minutes at 37°C, and then Hirulog (50 μg/mL final concentration) for an additional 5 minutes at 37°C. Four-hundred-microliter samples were then incubated with either anti-TR<sup>34-52</sup> F(ab′)<sub>2</sub> or NRS F(ab′)<sub>2</sub> (200 pg/mL final concentrations) for 20 minutes at 22°C, washed twice with TSE, and incubated with 1/100 dilution of FITC-conjugated F(ab′)<sub>2</sub> fragment of goat antirabbit IgG (F[ab′]<sub>2</sub> specific) for 20 minutes at 22°C. The samples were then washed twice with TSE and analyzed by flow cytometry. In (B), GFP was preincubated with either anti-TR<sup>34-52</sup> F(ab′)<sub>2</sub> or NRS F(ab′)<sub>2</sub> at 200 pg/mL for 20 minutes at 22°C, and then digested with thrombin (10 U/mL) and reacted with FITC-conjugated secondary antibody as above. Note that thrombin pretreatment (A) reduces the binding of anti-TR<sup>34-52</sup> to levels below that of control antibody, whereas there is no decrease in anti-TR<sup>34-52</sup> binding when GFP is preincubated with anti-TR<sup>34-52</sup> and then exposed to thrombin (B).

We were surprised to find that pretreating platelets with high concentrations of thrombin (10 U/mL) for 5 minutes at 37°C led to nearly complete loss of anti-TR<sup>34-52</sup> F(ab′)<sub>2</sub> binding, whereas a similar incubation with thrombin did not affect the binding of the control NRS F(ab′)<sub>2</sub>. Because the loss of antibody binding could be caused by thrombin-induced platelet activation and receptor redistribution, as has been shown for thrombin receptors on megakaryocyte-like cells lines, or direct thrombin cleavage of the thrombin receptor, we tried to differentiate between these possibilities by assessing the effect of peptide ligand (TR<sup>42-55</sup>)-induced activation on anti-TR<sup>34-52</sup> binding. The peptide ligand caused as much platelet activation as thrombin, as judged by P-selectin exposure, but did not lead to a loss of anti-TR<sup>34-52</sup> binding, suggesting that activation mediated by the thrombin receptor does not lead to receptor internalization. To exclude the possibility that thrombin induces activation phenomena different from those induced by the peptide ligand, we produced an antibody (anti-TR<sup>83-94</sup>) against a peptide derived from a region of the thrombin receptor's extracellular domain very close to the membrane-spanning region. There was only a minor reduction in anti-TR<sup>83-94</sup> binding after thrombin treatment, further indicating that the receptor is not internalized after thrombin cleavage. These data support the hypothesis that loss of the binding of anti-TR<sup>34-52</sup> is a consequence of thrombin cleavage of the receptor. If anti-TR<sup>34-52</sup> truly binds to sites on both sides of the thrombin cleavage site, the loss of anti-TR<sup>34-52</sup> binding after thrombin activation suggests that the N-terminal fragment of the thrombin receptor, representing amino acids 26 to 41 (amino acids 1 to 25 being a presumed signal peptide), actually leaves the platelet after thrombin cleavage, and that the new tethered ligand region beyond amino acid 42 rapidly enters a protected site in the receptor to which the antibody cannot gain access. Data from our studies with the MoAbs 33-1 (directed at an epitope C-terminal to the thrombin cleavage site) and 61-1 (directed primarily at an epitope N-terminal to the thrombin cleavage site) support this hypothesis, because the binding of both antibodies was dramatically reduced by preincu-

Table 3. Immunoreactivity of Anti-TR<sup>39-69</sup> MoAbs

<table>
<thead>
<tr>
<th>Antibody</th>
<th>TR&lt;sup&gt;39-43&lt;/sup&gt;</th>
<th>TR&lt;sup&gt;42-54&lt;/sup&gt;</th>
<th>TR&lt;sup&gt;44-66&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>33-1</td>
<td>0.050*</td>
<td>0.657</td>
<td>0.051</td>
</tr>
<tr>
<td>61-1</td>
<td>0.727</td>
<td>0.159</td>
<td>0.066</td>
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* OD<sub>490</sub>
bating the platelets with thrombin. However, we would caution, that by their very nature these studies are indirect, and that alternative models may account for these observations.

The relationship between the loss of binding of these antibodies and platelet activation at different thrombin concentrations may provide information on the fraction of receptors that need to be cleaved to achieve different levels of activation. Using anti-TR34-52, we found that 0.1 U/mL thrombin, a dose that initiates significant, but submaximal, activation produced about 45% to 78% of the loss of anti-TR34-52 binding produced by thrombin at either 1 or 10 U/mL. Of interest, the percentage loss of anti-TR34-52 binding induced by 0.1 and 1.0 U/mL thrombin was nearly identical to the percentage gain in surface P-selectin expression induced by the same concentrations of thrombin.

Although indirect, our data also suggest that the inhibition of thrombin-induced platelet activation by anti-TR34-52 is caused, at least in part, by inhibition of thrombin cleavage of the receptor: (1) anti-TR34-52 inhibited the cleavage of radiolabeled TR34-52 by thrombin, and (2) adding thrombin after the anti-TR34-52 was bound to the receptor, did not decrease the amount of anti-TR34-52 on the platelets, as would have been expected if the amino acid 26-41 fragment (with its attached anti-TR34-52 antibody) left the platelet surface. However, we would point out that the ability of anti-TR34-52 to inhibit thrombin cleavage of peptide TR34-52 in solution does not necessarily mean that it inhibits cleavage of the receptor on the platelet surface. It is unlikely that the antibody is blocking the site into which the tethered ligand binds because it did not inhibit aggregation induced by the peptide ligand TR41-52. However, it is possible that some of the antibody's effect is a result of attachment to the tethered ligand region such that even if cleavage occurs, the antibody interferes with the insertion of the tethered ligand into its binding site pocket.

On balance, our data provide support for the mechanism of action proposed by Vu et al. involving thrombin cleavage at the R41-S42 bond of the receptor. In addition, we provide new data that this cleavage does not result in receptor internalization, but does result in loss of the N-terminal peptide and the rapid insertion of the tethered ligand into a protected binding site.

ACKNOWLEDGMENT

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Immunologic analysis of the cloned platelet thrombin receptor activation mechanism: evidence supporting receptor cleavage, release of the N-terminal peptide, and insertion of the tethered ligand into a protected environment

KJ Norton, RM Scarborough, JL Kutok, MA Escobedo, L Nannizzi and BS Coller