The platelet high affinity binding site for thrombin appears to be described by a classical receptor-ligand interaction that is distinct from the platelet thrombin receptor/substrate, PAR-1. However, the identification and function of the high affinity binding site with respect to its physiological importance have continued to elude investigators. Prior studies using two mutant thrombins suggested that thrombin interaction with the platelet high affinity binding site is mediated through an extensive portion of the thrombin molecule involving residues within the substrate binding pocket and the anion binding exosite (Leong, L., Henriksen, R. A., Kermodoe, J. C., Rittenhouse, S. E., and Tracy, P. B. (1992) Biochemistry 31, 2567–2575) and may mimic a thrombin-hirudin interaction. To test this hypothesis, an anti-hirudin peptide antibody (anti-hirpeptide Ab) was raised against a peptide mimicking the COOH terminus of hirudin. The Ab recognized adherent platelets and those in suspension as determined by enzyme-linked immunosorbent assay and immunofluorescence microscopy, respectively. 125I-Thrombin binding to platelets was inhibited in the presence of the anti-hirpeptide Ab in a dose-dependent manner with maximal inhibition >90%. Analyses of data from binding studies of 125I-thrombin to platelets at a fixed Ab concentration indicated that the anti-hirpeptide Ab inhibited the high affinity binding interaction exclusively. In addition, thrombin-induced increases in platelet [Ca2+]i, were enhanced by blocking the high affinity binding site with the Ab due to redistribution of the agonist to PAR-1. Thrombin Quick I-induced platelet calcium mobilization was unaffected by the presence of the Ab, consistent with the inability of thrombin Quick I to bind to the high affinity site. Even though glycoprotein (GP) Ib contains a hirudin-like region within the α subunit, the postulated high affinity binding site, direct binding of 125I-thrombin could not be demonstrated to transfected Chinese hamster ovary and L cells expressing the GP Ib-IX-V complex. Furthermore, an anti-GP Ib Ab, raised to the peptide region proposed as the thrombin high affinity site, did not enhance thrombin-induced platelet calcium mobilization. The anti-hirpeptide Ab recognized a population of platelet membrane proteins distinct from PAR-1 and GP Ib by three-color immunofluorescence using confocal microscopy. These combined studies demonstrate that the high affinity binding site for thrombin is a unique platelet protein distinct from GP Ib which modulates the effective thrombin concentration localized at the human platelet surface.

High affinity, dissociable binding of thrombin to human platelets has been demonstrated and described by several laboratories (1–5). Data from several investigators have led to the postulate that the glycoprotein (GP) Ib-IX-V complex is a high affinity binding site for thrombin on the platelet surface which may facilitate platelet activation (6–8). In addition, it has been proposed that kininogen binding to GP Ib-IX-V modulates thrombin binding to platelets and subsequent aggregation (9). To date, definitive isolation of the high affinity binding site for thrombin on platelets has not been accomplished. The focus of this investigation is to characterize the identity and function of the platelet high affinity binding site for thrombin.

Thrombin interaction with the high affinity binding site on platelets is governed by a $K_d \sim 0.1–4.0 \text{ nM}$ at 50–1,200 sites as determined by dissociable, equilibrium binding techniques (1, 3, 4). Additional studies from our laboratory with native human thrombin and two mutant thrombins, thrombin Quick I and thrombin Quick II, demonstrate that thrombin binding to the platelet surface is mediated through an extensive portion of the thrombin surface, involving residues near the substrate binding pocket and the anion binding exosite (1). Studies characterizing thrombin Quick I and thrombin Quick II binding to platelets (1) together with studies examining the binding of hirudin to these mutant thrombins (10) led to our hypothesis that the conformation of the platelet high affinity binding site for thrombin resembles hirudin.

The amino acid substitution of Val for Gly at position 558 (226)2 in thrombin Quick II falls within the primary substrate binding pocket (13). Thrombin Quick II has decreased binding affinity for hirudin, suggesting that the conformation of the active site cleft is not optimal for binding hirudin (10). Decreased affinity of thrombin Quick II for a hirudin mutant with substitutions in the amino terminus implies that this region of hirudin interacts near the active site of thrombin (10). Interestingly, thrombin Quick II binding to platelets ($K_d \sim 3.3–9.5$)

* This work was supported by National Institutes of Health Grant HL-46703 (Project 4) (to F. B. T.) and by National Institutes of Health National Research Service Award F32 HL-09376 (to K. L. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Biochemistry, College of Medicine, University of Vermont, Given Bldg. C409, Burlington, VT 05405. Tel.: 802-656-1995; Fax: 802-862-8229; E-mail: ptracy@salus.med.uvm.edu.

‡ The abbreviations used are: GP, glycoprotein; Ab, antibody(ies); BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; MEM, minimal essential medium; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; CHO, Chinese hamster ovary; FBS, fetal bovine serum; PAR, protease-activated receptor; PFR, i-Phe-Pro-Arg chloromethyl ketone.

2 Human thrombin residues are numbered according to the human prothrombin sequence (11). The corresponding chymotrypsin-based numbering is given in square brackets (12).
Platelet High Affinity Binding Site for Thrombin

Platelet activation responses. In addition, we explored the re-conformation of the COOH terminus of hirudin. We generated the platelet high affinity binding site for thrombin mimics the site blocked thrombin increase its agonist activity (1). Platelet activation does not require the high affinity binding site because saturation of the high affinity binding site with FPR-thrombin, although not inducing platelet activation, enhances the activation response to thrombin stimulation (1). Platelet activation induced by thrombin Quick I proceeds through PAR-1 and requires 10-fold more enzyme to yield responses equal in magnitude to thrombin stimulation (1, 14). Protein purity was assessed by SDS-polyacrylamide gel electrophoresis before and after disulphide bond reduction according to the method of Laemmli (19). Proteins were visualized by Coomassie Brilliant Blue staining.

EXPERIMENTAL PROCEDURES

Materials—Taipan snake venom, potato apyrase, Sephadex G-25–150, prostaglandin E2, o-phenylenediamine dihydrochloride, 1,3,4,6-tetrachloro-3-a-6-diphenylglycouril, and digitonin (50% purity) were purchased from Sigma. Digitonin was purified further by recrystallization from 100% ethanol. Fura 2-AM was obtained from Molecular Probes, Inc. Spectrozyme TH was purchased from American Diagnostica. Hirudin was purchased from Genentech. Crystallized bovine serum albumin (BSA) was purchased from ICN Immunobiologicals. Na2592 was purchased from Amersham Pharmacia Biotech. n-Phenyl-Arg chloromethyl ketone was obtained from Calbiochem. Amino acid derivatives for peptide synthesis were purchased from Peninsula Laboratories Inc. 4-Methylbenzhydrazine resin was purchased from Advanced ChemTech. Anisole, 1,3-diisopropylcarbodiimide, disopropylamine, and dimethyl sulfoxide were obtained from Aldrich and used without further purification. Fluorescein isothiocyanate (FITC) goat anti-rabbit IgG (heavy and light chains) was purchased from Vector Laboratories, and goat anti-rabbit IgG horseradish peroxidase was obtained from Southern Biotechnology Associates. Apiezon A was purchased from James G. Bidwell, Ltd., and n-butyl plasma was from Fisher. Dulbecco’s modified minimal essential medium (MEM), α-MEM, low endothelin bovine serum, G418, HAT supplement, and methotrexate were obtained from Life Technologies, Inc.

Plates—Platelet reagents included human platelets (1–2 x 109 platelets/mL), bovine thrombin (Novo, 30 U/mL), and the anti-hirpeptide, anti-TR52–69, and anti-GP Ib269–287 Abs recognized IgG were 150,000 and 14.0, respectively. Specificity of the anti-peptide Ab was assessed by solid phase enzyme-linked immunosassay (ELISA) against antigen peptides and an irrelevant peptide, CVPDGRGQYQGR, as described previously (22). The anti-hirpeptide, anti-TR52–69, and anti-GP Ib269–287 Abs recognized their respective antigen peptides. Binding to the irrelevant peptide was not detected.

Extended Characterization of the Anti-hirpeptide Immunoglobulin—Having demonstrated that the anti-hirpeptide Ab recognized the COOH terminus of hirudin, we evaluated its ability to recognize full-length hirudin. Hirudin inhibition of thrombin-catalyzed chromogenic substrate hydrolysis was examined in the presence of anti-hirpeptide Ab. Hirudin (6 μM) was preincubated with 0–500 nM anti-hirpeptide Ab for

Based on these combined observations, we hypothesized that the platelet high affinity binding site for thrombin mimics the formation of the COOH terminus of hirudin. We generated an anti-hirudin peptide (anti-hirpeptide Ab) and demonstrated that it recognizes human platelets. The anti-hirpeptide Ab was used as a tool to characterize the interaction between thrombin and its high affinity binding site on the platelet surface. We further described the functional role of the high affinity binding site with respect to thrombin-induced platelet activation responses. In addition, we explored the relationship of the high affinity binding site with GP Ib and PAR-1.

Materials—Taipan snake venom, potato apyrase, Sephadex G-25–150, prostaglandin E2, O-phenylenediamine dihydrochloride, 1,3,4,6-tetrachloro-3-a-6-diphenylglycouril, and digitonin (50% purity) were purchased from Sigma. Digitonin was purified further by recrystallization from 100% ethanol. Fura 2-AM was obtained from Molecular Probes, Inc. Spectrozyme TH was purchased from American Diagnostica. Hirudin was purchased from Genentech. Crystallized bovine serum albumin (BSA) was purchased from ICN Immunobiologicals. Na2592 was purchased from Amersham Pharmacia Biotech. n-Phenyl-Arg chloromethyl ketone was obtained from Calbiochem. Amino acid derivatives for peptide synthesis were purchased from Peninsula Laboratories Inc. 4-Methylbenzhydrazine resin was purchased from Advanced ChemTech. Anisole, 1,3-diisopropylcarbodiimide, disopropylamine, and dimethyl sulfoxide were obtained from Aldrich and used without further purification. Fluorescein isothiocyanate (FITC) goat anti-rabbit IgG (heavy and light chains) was purchased from Vector Laboratories, and goat anti-rabbit IgG horseradish peroxidase was obtained from Southern Biotechnology Associates. Apiezon A was purchased from James G. Bidwell, Ltd., and n-butyl plasma was from Fisher. Dulbecco’s modified minimal essential medium (MEM), α-MEM, low endothelin bovine serum, G418, HAT supplement, and methotrexate were obtained from Life Technologies, Inc.

Platelet High Affinity Binding Site for Thrombin

Platelet activation induced by thrombin Quick I proceeds through PAR-1, which mediates thrombin-induced platelet activation events described by the formation of a Michaelis complex (14, 15). We have shown previously that in the presence of anti-TR52–69, a polyclonal antibody raised against a peptide spanning the thrombin cleavage site within PAR-1, the platelet intracellular calcium mobilization induced by thrombin and thrombin Quick I is inhibited >90% (14). Platelet activation induced by thrombin Quick I proceeds through PAR-1 and requires 10-fold more enzyme to yield responses equal in magnitude to thrombin stimulation (1, 14). In addition, platelet activation does not require the high affinity binding site because saturation of the high affinity binding site with FPR-thrombin, although not inducing platelet activation, enhances the activation response to thrombin stimulation (1, 14). It is important to note that thrombin Quick I does not demonstrate high affinity binding to the platelet surface at concentrations that effect platelet activation, nor does active site-blocked thrombin (FPR-thrombin) was prepared using d-Ph-Pro-Arg chloromethyl ketone as described previously (1). The labeled thrombins were >95% precipitable with 10% trichloroacetic acid, with specific radioactivities of 2,200–2,500 cpm/ng. Radioiodinated thrombin retained full clotting activity (~3,000 NIH units/mg).

Platelet Preparations—Washed platelets were prepared as described previously (14). For most experiments, washed platelets were suspended in 5 mM HEPES-buffered Tyrode’s solution (0.137 M NaCl, 2.7 mM KCl, 12 mM NaHCO3, 0.36 mM NaH2PO4, 1 mM MgCl2, 2 mM CaCl2, 5 mM dextrose), pH 7.4, with 0.35% bovine serum albumin (HEPES Tyrode’s albumin) at 37 °C. CaCl2 was omitted from the wash solutions for experiments measuring intraplatelet [Ca2+]i. In some experiments, platelets were pretreated with 1 μM acetylsalicylic acid and 0.5 μM prostaglandin E2, in platelet-rich plasma for 30 min before washing.

Peptide Synthesis and Preparation of Antibodies Directed against the COOH-terminal Region of Hirudin—Based on binding studies with the mutant thrombins, the most significant interactions involved in thrombin binding to hirudin occur between its anion binding exosite and the COOH-terminal region of hirudin. Therefore, a peptide mimicking the COOH-terminal region of full-length hirudin was designed. The peptide RNPNDGDFEPEEYLQNE was synthesized manually as a peptide amide using 4-methylbenzhydramine and t-butyloxyacarbonyl-blocked amino acids with an additional COOH-terminal cysteine for coupling as described previously (22). In addition, peptides representing the hirudin-like regions found in PAR-1 and platelet GP Ib were synthesized according to the same procedure. The PAR-1 peptide sequence was YLQNE REKNESSGLTER representing the human PAR-1 sequence. The GP Ib peptide sequence was DEGDTDDLYYDPPEDETDGP, representing residues 269–287. All peptides were purified by HPLC. Peptide compositions and concentrations were determined by amino acid analysis performed by the Protein Chemistry Laboratory at the University of Texas Medical Branch Cancer Center. The peptides are designated as Cilow: the COOH-terminal hirudin peptide, hirpeptide; the PAR-1 peptide, TR52–69; and the GP Ib peptide, GP Ib269–287.

Three rabbit anti-peptide antisera were prepared by Cocalico Biologicals (Reamstown, PA) according to our supplied protocol (14). Immunoglobulins, both nonimmune and specific, were purified using protein A immobilized to Sepharose 4B. Protein purity was assessed by SDS-polyacrylamide gel electrophoresis. Molecular weight and the extinction coefficient, ε280 nm, 1 cm, for IgG were 150,000 and 14.0, respectively.

Specificity of the anti-peptide Abs was assessed by solid phase enzyme-linked immunosassay (ELISA) against antigen peptides and an irrelevant peptide, CVPDGRGQYQGR, as described previously (22). The anti-hirpeptide, anti-TR52–69, and anti-GP Ib269–287 Abs recognized their respective antigen peptides. Binding to the irrelevant peptide was not detected.
suspensions, and aliquots were removed over time (0–30 min). Bound 10% FBS, 400 mM NaCl, 0.01% Tween 80, pH 7.4. Thrombin activity was monitored by the adhered platelets. Horseradish peroxidase-coupled goat anti-rabbit IgG was added as secondary Ab. Reactivity was determined at 490 nm. Anti-hirpeptide Ab (■) recognized platelets compared with nonimmune control IgG (○). Results are the mean ± S.E. and are representative of three donors.

Platelet Responses in the Presence of Anti-hirpeptide Ab—For studies that examined the effect of the anti-hirpeptide Ab on thrombin interaction with platelets, the platelets (2–4 × 10^9/ml) were preincubated with anti-hirpeptide Ab or nonimmune Ig for 10 min at ambient temperature. Measurements of dissociable, equilibrium binding of 125I-thrombin to platelets and thrombin-induced platelet intracellular calcium flux were performed as described previously (14).

Thrombin Binding to GP Ib-IX-V-expressing Cell Lines—Cell lines, CHO and fibroblast-like L cells, transfected with GP Ibα, Ibβ, IX, and/or V supergenic gift from Dr. Jose Lopez, Baylor College of Medicine. The stable CHO and L cell lines have been characterized extensively (23). Untransfected CHO cells were maintained in α-MEM containing 10% heat-inactivated fetal bovine serum (FBS). CHO βX cells expressing GP Ibβ and IX subunits were maintained in α-MEM supplemented with 10% FBS and 400 μg/ml G418. CHO αV cells, expressing Ibα, Ibβ, and IX subunits, were maintained in α-MEM with 10% FBS, 400 μg/ml G418, and 80 μM methotrexate (23). The L cell lines were grown in Dulbecco’s modified MEM supplemented with 10% FBS. L2H cells expressing Ibα, Ibβ, and IX were maintained in Dulbecco’s modified MEM supplemented with 10% FBS, 1 mM sodium hyponxanthine, 4 μM aminopterin, and 160 μM thymidine. L2H cells expressing GP V were supplemented further with 960 μM thymidine. All cells were maintained at 37 °C in an atmosphere of 5% CO2 and 99% humidity. Expression of the GP Ib/IX/V protein subunits was verified using specific monoclonal Ab for each glycoprotein by flow cytometric techniques as described previously (24). Subunits of the complex were labeled with the following antibodies: GP Ibα-AN51 (Dako Corp.), GP IX-FMC-25 (provided by Dr. Jose Lopez), and GP V-CD 42b (Dako). Direct binding of 125I-thrombin to the CHO and L cell lines was performed as described above with minor modifications. Cells were lifted at confluence with 0.54 mM EDTA. Cells were washed three times in phosphate-buffered saline, 2 mM EDTA, 1% BSA. Cells were counted and adjusted to 1 × 10^7/ml. 125I-Thrombin (1 nM) was added to the cell suspensions, and aliquots were removed over time (0–30 min). Bound and free fractions were separated by centrifugation over oil as described previously (1). In other experiments, the binding was determined over a range of thrombin concentrations (0–25 nM). Nonspecific binding was quantified by the addition of unlabeled FPR-thrombin (50 nM) to the 125I-thrombin-cell suspension.

Immunofluorescence and Confocal Microscopy—Washed platelets (1 × 10^7/ml) were triple labeled with Ab directed against platelet proteins as described previously (14). The Ab directed toward PAR-1 and GP Ib were chosen as such because they recognize epitopes other than the hirudin-like regions found within these platelet membrane proteins. To platelets suspended in HEPES-Tyrode’s buffer with 20 mM EDTA and 5 μM prostaglandin E1, the following antibodies were added: 2 μM rabbit anti-hirpeptide Ab followed by Texas Red-conjugated donkey anti-rabbit IgG (Jackson Immunologicals, Inc.); Cy5-conjugated monoclonal anti-TR32–46 (2 μM), specific for PAR-1 (14); and FITC-conjugated monoclonal AN51 (10 μM), specific for platelet GP Ib (Dako). Control platelet samples were labeled with FITC or Cy5-conjugated nonspecific mouse IgG and Texas Red-conjugated donkey anti-rabbit IgG. Platelets were visualized using the Bio-Rad MRC 1000 confocal system connected to a Olympus BX50 upright microscope in the Cell Imaging Facility, University of Vermont College of Medicine. The FITC, Texas Red, and Cy5 fluorophores were excited at 488, 568, and 647 nm, respectively, with a krypton/argon laser. Image analysis was performed using Adobe Photoshop software.

RESULTS

Effect of Anti-hirpeptide Ab on High Affinity Binding of Thrombin to Platelets—The anti-hirpeptide Ab was generated to test the hypothesis that the high affinity binding site for thrombin mimics the conformation of hirudin. Having shown that the anti-hirpeptide Ab recognized platelets, we examined its ability to inhibit the dissociable equilibrium and high affinity binding of 125I-thrombin to platelets. 125I-Thrombin (1 nM) binding to platelets (3 × 10^7/ml) was assessed over a range of anti-hirpeptide Ab concentrations (0–2 μM) (Fig. 2). Specific binding was quantified and expressed as the percent of binding in the presence of anti-hirpeptide Ab compared with the binding in the presence of nonimmune rabbit IgG, which was without effect on 125I-thrombin binding to platelets. The anti-hirpeptide Ab inhibited the specific binding of 125I-thrombin to platelets in a dose-dependent manner with maximal inhibition >90% at 2 μM Ab. 125I-FPR-thrombin binding to platelets was inhibited by anti-hirpeptide Ab to the same extent as native thrombin (data not shown).

To determine whether the inhibitory activity of anti-hirpeptide Ab was specific for the high affinity binding site, thrombin binding studies were performed in the presence of a fixed Ab concentration. The anti-hirpeptide Ab (2 μM) was preincubated with platelets (3 × 10^7/ml) for 10 min followed by the addition of 125I-thrombin (0.025–50 nM) for 2 min. Thrombin binding to platelets was reduced substantially in the presence of the anti-
hirpeptide Ab (Fig. 3A). LIGAND analyses of the binding data obtained in the absence of the anti-hirpeptide Ab indicated that thrombin binding to the platelet surface was best described by a one-site model with a nonsaturable component. Thrombin interaction with this high affinity site was characterized by a $K_d = 0.34 \pm 0.23 \times 10^{-9} \text{ M}$ with 70 ± 38 binding sites (five donors). These binding parameters are consistent with values reported previously from our laboratory and others (1, 3, 4).

There was no improvement in the fit when a two-site model for thrombin binding was employed, consistent with our previous studies (1). Scatchard analyses of the binding data clearly demonstrate that the high affinity binding interaction between thrombin and platelets is completely inhibited by the presence of the anti-hirpeptide Ab (Fig. 3B). Furthermore, the analyses of the binding data obtained in the presence of the anti-hirpeptide Ab demonstrated a slight decrease in the apparent binding affinity ($K'_d = 0.53 \pm 0.10 \times 10^{-9} \text{ M}$, five donors), suggesting that the anti-hirpeptide Ab is a competitive inhibitor of thrombin.

**Effect of Anti-hirpeptide Ab on Agonist-induced Calcium Responses**—The contribution of high affinity, dissociable binding to agonist-induced platelet activation has been characterized previously in studies from our laboratory (1). We demonstrated that in the presence of FPR-thrombin, at concentrations that saturate the high affinity binding site, thrombin-induced platelet activation results in an enhanced response compared with the response in the absence of FPR-thrombin. These data were interpreted to indicate that the high affinity binding site sequesters low concentrations of thrombin at the platelet surface, thereby regulating the effective thrombin concentration available for platelet activation. Because the anti-hirpeptide Ab recognized the high affinity site, it should function in a similar manner when present during agonist-induced platelet activation.

Platelet intracellular calcium mobilization induced by thrombin was determined in the presence of anti-hirpeptide Ab. Platelets ($2 \times 10^9/\text{ml}$) were incubated with anti-hirpeptide Ab (0–500 nM) for 5 min before the addition of 0.5 nM thrombin and monitored for changes in intracellular calcium mobilization (14). In the presence of anti-hirpeptide Ab, thrombin-induced platelet calcium mobilization was increased substantially compared with the response in the presence of nonimmune Ig (Fig. 4). Similar platelet activation profiles were observed for all seven donors; however, the maximal enhancement of the calcium response was donor-dependent (range 120–240%).

To characterize further the role of the high affinity binding site with respect to platelet activation, we utilized the mutant thrombin, thrombin Quick I, which also mediates platelet activation through cleavage of PAR-1 (14). However, thrombin Quick I does not demonstrate high affinity, dissociable binding and therefore would not distribute between the high affinity binding site and PAR-1 (1). Thus, we anticipate that thrombin Quick I-induced platelet calcium mobilization would be unaffected by the anti-hirpeptide Ab. Platelet intracellular calcium flux was assessed using thrombin Quick I or thrombin as the agonist. Platelets, washed with acetylsalicylic acid and prostaglandin E1 ($2 \times 10^{-5}\text{ M}$), were preincubated with anti-hirpeptide Ab (5–100 nM) followed by stimulation with 2 nM thrombin Quick I or 0.2 nM thrombin, concentrations that will induce near equal activation responses. The presence of anti-hirpeptide Ab did not enhance the thrombin Quick I-induced platelet calcium flux compared with that induced by thrombin (Fig. 5). These data demonstrate further that the anti-hirpeptide Ab recognizes the high affinity binding site. Interestingly, thrombin- and thrombin Quick I-induced intraplatelet calcium mobilization could be inhibited by higher anti-hirpeptide Ab concentrations (Fig. 5), consistent with previous results using FPR-thrombin (1). These data suggest the anti-hirpeptide Ab may recognize the hirudin-like region of PAR-1 in addition to the hypothesized hirudin-like region in the high affinity binding site.

**The Anti-hirpeptide Ab Recognizes Hirudin-like Regions within PAR-1 and GP Ib**—The identification of the platelet high affinity binding site for thrombin has eluded investigators. Studies have demonstrated that thrombin binding to platelets is inhibited in the presence of an anti-GP Ib Ab (6). The amino-terminal domain of the GP Ibα chain is postulated to contain a high affinity binding site for thrombin. This amino acid domain, comprised of several negatively charged amino acids, has been compared with similar domains within PAR-1 and the carboxyl-terminal region of hirudin. Because PAR-1, GP Ib, and the high affinity binding site are all expressed on the platelet surface, we hypothesized that an Ab directed against a peptide rich in anionic amino acids could potentially recognize any or all of these sites. To address this hypothesis, we characterized the specificity of the anti-hirpeptide Ab for its antigen peptide compared with synthetic peptides of hirudin-
like regions found in PAR-1 (peptide TR52-69) and GP Ib (peptide GPIb269-287) in a solution phase ELISA.

Briefly, anti-hirpeptide Ab was incubated with increasing concentrations of the HPLC-purified peptides (10^{-8} to 10^{-4} M) for 1 h in solution at ambient temperature. After the solution phase incubation, aliquots of each reaction were transferred onto hirpeptide-coated ELISA plates. Any anti-hirpeptide Ab unbound to peptide from the competitive solution phase reaction could then react with hirpeptide coated on the plate. The results indicate that the anti-hirpeptide Ab is more reactive for the hirpeptide, EC_{50} = 2 \times 10^{-6} M, compared with TR52-69, EC_{50} = 6 \times 10^{-5} M; or GPIb269-287, EC_{50} = 7 \times 10^{-5} M (Fig. 6).

EC_{50} is defined in this context as the concentration of peptide required to bind 50% of the anti-hirpeptide Ab from the solution phase. These data indicate that the anti-hirpeptide Ab interacts with the TR52-69, consistent with its ability to inhibit thrombin-induced intraplatelet [Ca^{2+}] mobilization at high Ab concentrations. The anti-hirpeptide Ab also interacts with GPIb269-287, consistent with the presence of a hirudin-like domain in GP Ibα.

**GP Ib Is Not the High Affinity Binding Site for Thrombin**—To investigate the possibility that GP Ib may be the high affinity binding site for thrombin found on platelets, we characterized thrombin binding to CHO and L cell lines expressing GP Ibα-Ibβ-IX or Ibβ-IX-V subunit as described previously (24).

Radiolabeled thrombin binding to CHO and L cell lines expressing the GP Ibα-Ibβ-IX or Ibβ-IX-V subunit was performed as described previously for platelets (14). Cells (1 \times 10^6/ml) were incubated with ^{125}I-thrombin (1 nM) for 0–30 min or with varying concentrations of thrombin (0–25 nM)

---

**Fig. 3. Inhibition of thrombin binding to the platelet high affinity binding site.** Platelets (3 \times 10^8/ml) were incubated with a fixed amount of anti-hirpeptide Ab or nonimmune Ig (2 μM) in HEPES Tyrode’s buffer, 0.35% BSA, pH 7.4, for 10 min. ^{125}I-Thrombin (0.05–50 nM) was added to the platelet suspension for 2 min. Nonspecific binding was determined at each thrombin concentration by the addition of unlabeled FPR-thrombin (50-fold molar excess). Panel A, thrombin binding to platelets in the presence of nonimmune Ig (●) or anti-hirpeptide Ab (○) is expressed as molecules of thrombin bound per platelet as a function of labeled ligand added. The inset is an enlargement of the graph at the lower thrombin concentrations. Data are representative of five experiments using different donors. Panel B, Scatchard plot of the data presented in panel A.
for 2 min. There was no detectable time- or concentration-depen-
dent binding of \(^{125}\text{I}\)-thrombin to any of the cell lines (data
not shown). The CHO and L cell lines expressing the Ib-IX, Ib-IX-III, or Ib-IX-IX-V complex demonstrated no specific throm-
bin binding compared with nontransfected CHO or L cells.
Nonspecific binding was measured in the presence of excess
unlabeled PPBR-thrombin (50-fold molar excess). Because non-
specific binding was \(< 5\%\) of the added \(^{125}\text{I}\)-thrombin, specific
binding, if present, could have been quantified easily. These
data suggest that the glycoprotein Ib-IX-V complex is not the
high affinity binding site for thrombin found on platelets.

The argument could be made that the GP Ib-IX-V complex
expressed on these cells does not mimic that expressed by
platelets. If the hirudin-like region found within GP Ibo,
expressed at the platelet surface is a high affinity binding site for
thrombin, then an Ab raised against this region should induce
an enhanced platelet activation response in a manner identical
to that observed with the anti-hirpeptide Ab. We examined
thrombin-induced platelet calcium mobilization in the presence
of anti-GP Ib\(269-287\) Ab. Thrombin-induced platelet calcium
mobilization was not enhanced in the presence of the anti-GP
Ib\(269-287\) in contrast to the enhancement seen in the presence
of the anti-hirpeptide Ab (Fig. 7). Presumably, the inhibition of
platelet calcium flux by the anti-GP Ib\(269-287\) Ab can be ex-
plained by its reactivity for the hirudin-like region of PAR-1
(peptide TR\(52-69\)). We determined that the reactivity (EC\(50\)
as described above) of anti-GP Ib\(269-287\) Ab for peptide GP Ib\(269-287\) \(\approx\) 0.45 \(\mu\)M and for peptide TR\(52-69\) \(\approx\) 2.11 \(\mu\)M by ELISA. The
inability of the anti- GP Ib\(269-287\) Ab to enhance thrombin-
duced platelet calcium mobilization coupled with our inability
to demonstrate direct thrombin binding to the cell lines
strongly argues against the concept that GP Ib is the high
affinity binding site for thrombin.

The Platelet High Affinity Binding Site Is Distinct from GP
Ib—These collective data suggest that the high affinity binding
site for thrombin is a unique platelet protein resembling hiru-
din. To establish the unique character of this platelet mem-
brane protein, we utilized confocal microscopy and three fluo-
rescent-tagged antibodies to label surface-expressed high
affinity binding sites, GP Ib and PAR-1. We chose Ab to GP Ib
and PAR-1 which do not recognize the hirudin-like regions
found within these two platelet proteins. Thus, platelets were
labeled with rabbit anti-hirpeptide Ab followed by Texas Red-
conjugated donkey anti-rabbit IgG, Cy5-conjugated monoclonal
anti-TR\(52-69\), specific for PAR-1 (14); and FITC-conjugated
monoclonal AN51, specific for platelet GP Ib. Control platelet
samples were labeled with Texas Red-conjugated donkey anti-
rabbit IgG alone, Cy5 nonimmune mouse IgG, or FITC-nonim-
une mouse IgG. The anti-hirpeptide Ab recognized a popula-
tion of membrane sites (Fig. 8, A and D, shown in red) which is
distinct from PAR-1 (Fig. 8, B and D, shown in blue) and from
platelet GP Ib (Fig. 8, C and D, shown in green). There is some
colocalization of the anti-hirpeptide Ab with the anti-GP Ib Ab
and the anti-TR\(52-69\) Ab as anticipated because of the ability of
the anti-hirpeptide Ab to recognize the hirudin-like regions of
GP Ib and PAR-1. Control platelet samples stained negatively

---

**FIG. 4.** Effect of anti-hirpeptide Ab on thrombin-induced
platelet calcium response. Platelets (2 \(\times\) \(10^8\)ml) were incubated
with varying concentrations of anti-hirpeptide Ab (0–500 \(\mu\)M) before
stimulation by thrombin. Intraplatelet calcium mobilization in re-
sponse to thrombin (0.5 nM, ●) is shown. The change in intraplatelet
[Ca\(^{2+}\)] is expressed as a percent of the response induced by thrombin
alone. Results are the mean ± range from duplicate determinations.
Some error bars fall within the limits of the symbol. Data are repre-
sentative platelet activation responses from 12 individual donors.

**FIG. 5.** Platelet activation in the presence of increased anti-
hirpeptide Ab. Platelets (2 \(\times\) \(10^8\)ml) were incubated with increasing
concentrations of anti-hirpeptide Ab (0–2 \(\mu\)M) for 10 min at ambient
temperature. Platelets were activated with either thrombin (0.2 nM, ▲)
or thrombin Quick I (2 nM, ▼). The change in intracellular [Ca\(^{2+}\)] is
expressed as a percent of the response induced by the agonist in the
presence of nonimmune rabbit Ig, which had no effect on that observed
with thrombin alone. Results are the mean ± range from duplicate
determinations. Data are representative of four different donors.

---

**FIG. 6.** Reactivity of anti-hirpeptide Ab for hirudin-like pep-
tides. Anti-hirpeptide Ab (66 \(\mu\)M) was incubated with various concen-
trations of each of the following peptides: hirpeptide (■), peptide
TR\(52-69\) (▲), peptide GPIb\(269-287\) (●), or buffer alone (○) for 1 h. Aliquots
of the anti-hirpeptide Ab/peptide suspensions were added to 4.14 \(\mu\)M
hirpeptide-coated 96-well plates. By ELISA, the remaining unbound
anti-hirpeptide Ab reactivity was assessed. The anti-hirpeptide Ab
demonstrated greater reactivity for hirpeptide compared with peptide
TR\(52-69\) or peptide GP Ib\(269–287\). Data are expressed as mean ± S.D. All
error bars fall within the limits of the symbol.
These data are consistent with the results obtained from the platelet activation (Figs. 5 & 7) and ELISA (Fig. 6) experiments. The data clearly indicate that the anti-hirpeptide Ab recognizes a unique platelet membrane protein.

DISCUSSION

The current investigation characterizes and clarifies the functional role of the platelet high affinity binding site for thrombin. In this study, we have presented data that demonstrate that the platelet high affinity binding site is a unique platelet membrane protein. The anti-hirudin peptide Ab, generated to the carboxyl-terminal domain of hirudin, recognizes platelets and competes with thrombin for hirudin. In addition, we demonstrate that the anti-hirpeptide Ab recognizes a specific platelet protein by confocal microscopy (Fig. 8). Together with the functional studies, these results indicate that the protein identified with the anti-hirpeptide Ab is distinct from PAR-1 and GP Ib.

The anti-hirpeptide Ab also provided a means for characterizing the high affinity binding site with respect to platelet function. The processes of binding to and activation of platelets are distinct events occurring at unique sites on the platelet surface. Whereas dissociable, equilibrium binding of thrombin to the platelet surface is inhibited when the high affinity binding site is occupied by the anti-hirpeptide Ab, thrombin-induced platelet calcium mobilization is enhanced, indicating the presence of anti-hirpeptide Ab binding to PAR-1. Based on these results, we hypothesize that the high affinity binding site sequesters low concentrations of thrombin at the platelet surface thereby decreasing the local thrombin concentration available for interaction with PAR-1.

PAR-1 and GP Ib contain amino acid sequences similar to that found in the COOH-terminal region of hirudin, and they are postulated to interact with thrombin (25, 26). Peptides mimicking the hirudin-like region of PAR-1 bind to thrombin and inhibit thrombin hydrolytic activity toward chromogenic substrates (26, 27). Crystal structure data and platelet studies have demonstrated that this region of PAR-1 interacts with the anion binding exosite of thrombin (15, 28, 29). Based on the observations that the anion binding exosite of thrombin binds to the hirudin-like region of PAR-1 and recognizes the COOH terminus of hirudin, we hypothesized that the anti-hirpeptide Ab would recognize the hirudin-like domain of PAR-1. The anti-hirpeptide Ab was 8-fold less reactive toward the TR 52–69 peptide compared with the COOH-terminal hirudin peptide in a solution phase ELISA. These data support our observation that higher concentrations of the anti-hirpeptide Ab inhibit thrombin- and thrombin Quick I-induced platelet calcium mobilization mediated by PAR-1.

GP Ib contains a hirudin-like sequence within the amino-terminal domain of the α chain which interacts with thrombin under select conditions (6, 25). Synthetic mimic peptides to...
the hirudin-like region within GP Ib inhibited thrombin binding to purified GP Ib and platelets, suggesting that this region of GP Ib may be a binding site for thrombin (25). If a hirudin peptide motif is present in platelet membrane proteins, it is possible an Ab against the hirudin-like peptide in GP Ib would also recognize PAR-1. We demonstrate an anti-GP269–287 Ab, directed against the hirudin-like peptide in GP Ib, inhibits thrombin-induced calcium mobilization (Fig. 7). There is no enhanced platelet activation response, suggesting that this Ab does not recognize the high affinity binding site for thrombin which we hypothesize also contains a hirudin-like peptide. Based on studies of thrombin interactions with platelets and GP Ib using anti-GP Ib antibodies, several investigators propose that GP Ib is a high affinity binding site for thrombin on platelets (25, 30, 31). An alternative explanation for these data includes the possibility that the anti-GP Ib Ab used in these experiments cross-reacts with hirudin-like regions found on other platelet membrane proteins including PAR-1 and the unique high affinity binding site identified in this study.

The functional role of glycoprotein Ib in thrombin high affinity binding to platelets and thrombin-induced platelet activation remains unclear. Surface expression of GP Ib molecules on platelets, ~25,000, far outnumbers high affinity binding sites, ~50–1,200 (1, 3, 4, 25). If GP Ib were the high affinity binding site, then only a subset of the GP Ib molecules would be involved in thrombin binding, and such a subset has not been identified. Furthermore, we are unable to demonstrate dissociable, equilibrium thrombin binding to CHO and L cell lines expressing GP Ibβ-IX-V complexes. These results contradict those obtained by Lopez and colleagues whereby they demonstrate thrombin (1 nM) binding to L2H/V cells that express the GP Ib-IX-V complex by flow cytometry. An explanation for the discrepancy between our results includes the different techniques used: direct radiolabeled thrombin binding versus indirect immunofluorescence detection of thrombin. Based on studies with the transfected cell lines, Lopez has proposed that a macromolecular complex of glycoproteins Ib, IX, and V forms a high affinity binding site for thrombin (8).

Thrombin binding to the platelet high affinity site can be compared with the thrombin-hirudin binding interaction, both of which are governed by high affinity dissociation constants (1, 32, 33). The carboxyl-terminal domain of hirudin binds the anion binding exosite of thrombin, an extension of the active site cleft dominated by positively charged side chains (34). The importance of the anion binding exosite of thrombin in high affinity, dissociable binding to platelets can be demonstrated from studies with thrombin Quick I.

Thrombin Quick I contains an Arg to Cys substitution within the anion binding exosite which alters the function and conformation of this thrombin molecule (10, 13). Thrombin Quick I demonstrates decreased fibrinogen clotting activity and platelet aggregation ability but nearly normal activity toward chromogenic substrates (18, 35). Thrombin Quick I equilibrium binding to the platelet surface is undetectable and competes weakly with thrombin, highlighting the importance of the anion binding exosite in high affinity binding to platelets. Consistent with this observation, thrombin Quick I binding to hirudin is 4 orders of magnitude less than native thrombin–hirudin binding (10). Thrombin Quick I induces increases in intraplatelet [Ca²⁺] less effectively than thrombin at equivalent concentrations, such that 10-fold more thrombin Quick I is required to produce a response equal to that of thrombin (1). Thrombin Quick I-induced platelet intracellular [Ca²⁺] mobilization was unaffected by low concentrations of anti-hirupetide Ab, consistent with previous studies from our laboratory in which the presence of low concentrations of FPR-thrombin had no effect on thrombin Quick I-induced intraplatelet [Ca²⁺] (1).

In contrast, thrombin-induced platelet activation was enhanced by saturation of the high affinity binding site with FPR-thrombin, suggesting that the high affinity binding site is regulating the effective concentration of thrombin available at the platelet surface (1).

In conclusion, we propose that the platelet high affinity binding site for thrombin allows for the regulation of local thrombin concentrations at the platelet surface thus modulating platelet activation. The importance of this regulatory mechanism is yet to be explored fully. Thrombin sequestration at the high affinity binding site on platelets may provide an essential regulatory mechanism for thrombin-induced platelet activation in vivo such that a threshold level of thrombin is required to initiate explosive platelet activation responses via cleavage of PAR-1 and subsequent assembly of prothrombinase, generation of thrombin, and platelet aggregation. The platelet high affinity binding site for thrombin may be most effective in preventing platelet activation and thrombus formation at low thrombin concentrations. By sequestering low levels of thrombin at the platelet surface, the high affinity binding site can prevent unwanted cleavage of PAR-1 and subsequent platelet desensitization. By down-regulating the effective thrombin concentration, platelets modulate the coagulation response within the vasculature, both arterial and venous circulation, at sites of endothelial injury or developing atherosclerotic plaque formation. In effect, the platelet high affinity binding site for thrombin may delay or prevent thrombus formation in pathological conditions such as thrombotic stroke or coronary artery occlusion.

Acknowledgments—We acknowledge the Clinical Research Center at Fletcher Allen Health Care for blood drawing services, Dr. William Church for peptide synthesis, Dr. John Kermode for assistance with binding data analyses, and Dr. Jose Lopez for providing the GP Ib-IX-V transfected cell lines and antibodies to the GP Ib-IX-V subunits. We also acknowledge Dr. Douglas Taatjes and the Cell Imaging Facility at the University of Vermont College of Medicine for assistance with confocal microscopy studies. We thank Dr. Ruth Ann Henriksen for providing the thrombin Quick I.

**References**

1. Leong, L., Henriksen, R. A., Kermode, J. C., Rittenhouse, S. E., and Tracy, P. B. (1992) Biochemistry 31, 2567–2575
2. Shuman, M. A., Tollefsen, D. M., and Majerus, P. W. (1976) Blood 47, 43–54
3. Martin, B. M., Wasiewski, W. W., Fenton, J. W., and Detwiler, T. C. (1976) Biochemistry 15, 4886–4892
4. Tollefsen, D. M., Feagler, J. R., and Majerus, P. W. (1974) J. Biol. Chem. 249, 2646–2651
5. Harmon, J. T., and Jameson, G. A. (1985) Biochemistry 24, 58–64
6. DeMarco, L., Mazzuccato, M., Masotti, A., Fenton, J. W. II, and Ruggeri, Z. M. (1991) J. Biol. Chem. 266, 23776–23783
7. Harmon, J. T., and Jameson, G. A. (1986) J. Biol. Chem. 261, 13224–13229
8. Dong, J. F., Sue-Tung, G., and Lopez, J. A. (1997) Blood 89, 4355–4363
9. Bradford, H. N., Dela Cadena, R. A., Kunapuli, S. P., Dong, J.-F., Lopez, J. A., and Colman, R. W. (1997) Blood 90, 1508–1515
10. Stone, S. R., Schmitz, T., Henriksen, R. A., Hofsteenge, J., and Dott, J. (1991) Biochemistry 30, 6392–6397
11. Degan, S. J. F., MacGillivray, R. T. A., and Davie, E. W. (1983) Biochemistry 23, 2087–2097
12. Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., and Hofsteenge, J. (1989) EMBO J. 8, 3467–3475
13. Henriksen, R. A., and Mann, K. G. (1988) Biochemistry 27, 9160–9165
14. Hayes, K. L., Leong, L., Henriksen, R. A., Bouchard, B. A., Ouellette, L., Church, W. R., and Tracy, P. B. (1994) J. Biol. Chem. 269, 28606–28612
15. Vu, T.-K. H., Hung, D. T., Wheaton, V. L., and Coughlin, S. R. (1991) Cell 64, 1057–1068
16. Bajaj, S. P., Rappaport, S. L., and Prodanos, C. (1981) Prep. Biochem. 11, 391–398
17. Owen, W. G., and Jackson, C. M. (1973) Thromb. Res. 3, 705–714
18. Henriksen, R. A., and Brotherton, A. F. A. (1983) J. Biol. Chem. 258, 13771–13776
19. Laemmli, U. K. (1970) Nature 227, 680–682
20. Mann, R. G. (1976) Methods Enzymol. 45, 123–127
21. Fenton, J. W. II, Landis, B. H., Walz, D. A., and Findlayson, J. S. (1977) in Chemistry and Biology of Thrombin (Landblad, R. L., Fenton, J. W. II, and Mann, K. G., eds) pp. 43–70, Ann Arbor Science Publishers Inc., Ann Arbor, MI
22. Church, W. R., Messier, T., Howard, P. R., Amirai, J., Meyer, D., and Mann, K. G. (1988) J. Biol. Chem. 263, 6259–6267
Platelet High Affinity Binding Site for Thrombin