Binding of Thrombin to the G-protein-linked Receptor, and Not to Glycoprotein Ib, Precedes Thrombin-mediated Platelet Activation*

(Received for publication, December 22, 1995, and in revised form, September 27, 1996)

Longbin Liu‡§¶, John Freedman¶, Adriana Hornstein¶, John W. Fenton II**, Yingqi Song§, and Frederick A. Ofosu‡‡‡

From the §Canadian Red Cross Society, Blood Services, Hamilton, Ontario, L8N 1H8 Canada, the ¶Department of Pathology, McMaster University, Hamilton, Ontario L8N 3Z5, Canada, the ¶¶Department of Medicine, St. Michael's Hospital, University of Toronto, Toronto, M5B 1W8 Ontario, Canada, and the **New York Department of Health, Albany, New York 12201-0509

The roles of the G-protein-linked thrombin receptor and platelet glycoprotein Ib (GPIb) as α-thrombin-binding sites on platelets remain controversial. α-Thrombin has been proposed to bind to both GPIb and the hirudin-like domain of the G-protein-linked receptor (from which it cleaves the NH₂-terminal extracellular domain to release a 41-mer peptide (TR-(1–41), where TR is α-thrombin receptor)) to initiate platelet activation. Using affinity-purified rabbit anti-human TR-(1–41) IgG and immunoblotting, we demonstrated TR-(1–41) release from platelets suspended in Tyrode's buffer containing 2 mM CaCl₂ and incubated with 0.5 nM α-thrombin for 10–60 s at 37 °C. As quantified by enzyme-linked immunosorbdent assay, 0.32–0.59 nM TR-(1–41) was released from washed platelets (5 × 10¹¹ platelets/liter) after their incubation with 10 nM α-thrombin for 10 s. Parallel binding of α-thrombin to and activation of the platelets were confirmed by flow cytometry. A monoclonal antibody against the hirudin-like domain of the G-protein-linked receptor abrogated α-thrombin binding to platelets, cleavage of TR-(1–41), and platelet activation by 1.0 nM (but not 10 nM) α-thrombin. Proteolysis of platelet GPIb with Serratia marcescens protease or O-sialoglycoprotein endopeptidase had no effect on α-thrombin binding to platelets or their subsequent activation. In contrast, chymotrypsin, which cleaves both GPIb and the G-protein-linked receptor, abrogated α-thrombin binding to platelets, TR-(1–41) release, and platelet activation. Furthermore, monoclonal antibodies directed against the reported α-thrombin-binding site on GPIb inhibited neither α-thrombin binding nor activation of the platelets. Thus, α-thrombin binds to and cleaves the G-protein-linked receptor when it activates platelets, and GPIb does not appear to serve as an important binding site when α-thrombin activates platelets.

Binding of α-thrombin to platelets precedes platelet activation by this enzyme and two platelet membrane glycoproteins have been identified as thrombin-binding sites (1–12). Based on the results of studies estimating α-125I-thrombin binding to platelets, ~50 high-affinity sites (Kd ~ 1 nM) involving GPIb and ~2000 GPIb-independent binding sites with moderate affinity (Kd ~ 10 nM) for α-thrombin on platelets have been reported (3, 4, 7). GPIb is a disulfide-linked, two-chain protein consisting of a heavy (α) chain (Mr 140,000) and a light (β) chain (Mr 24,000). Distinct sites on GPIb for α-thrombin and von Willebrand factor binding are located within the Mr 45,000 NH₂-terminal domain of GPIbα (5, 7, 8, 11, 12). Support for GPIb as a high-affinity binding site for α-thrombin arises from observations that Bernard-Soulier platelets (congenitally deficient in platelet GPIb) are poorly activable by α-thrombin (1). Additionally, cleavage of GPIb by chymotrypsin, elastase, or Serratia marcescens protease impairs the responses of platelets to subnanomolar (but not higher) concentrations of α-thrombin (13–16). Furthermore, monoclonal antibodies recognizing epitopes in the Mr 45,000 NH₂-terminal domain of GPIbα inhibit the responses of platelets to ≤1.0 nM α-thrombin (17–19).

Another α-thrombin receptor on platelets, a member of the superfamily of G-protein-linked receptors and also found on endothelial cells, smooth muscle cells, and fibroblasts, has been cloned (9, 17, 20–22). α-Thrombin binds to and cleaves this receptor at Arg-41/Ser-42, releasing a 41-mer activation peptide (called TR-(1–41) in this study, where TR is α-thrombin receptor) and exposing a new NH₂-terminal domain, which then binds to an undefined part of the same receptor to activate the platelets (9, 20, 21). Whether interactions of α-thrombin with this G-protein-linked thrombin receptor, GPIb, or both are required for platelet activation by α-thrombin remains an unresolved question. Some investigators consider the G-protein-linked α-thrombin receptor to be the moderate-affinity binding site since there are 1700 copies of this α-thrombin receptor/platelet (23, 24), and Bernard-Soulier platelets have normal numbers of this receptor (24). However, monoclonal antibodies that bind to the hirudin-like domain of the G-protein-linked thrombin receptor abrogate the responses of platelets to ≤1.0 nM α-thrombin (25–28). This level of α-thrombin would be expected to bind preferentially to its high-affinity binding sites on platelets. It is possible that GPIb, by initiating α-thrombin binding to platelets, could localize α-thrombin to sites on platelets where the cleavage of the G-protein-linked α-thrombin receptor would be facilitated to cause platelet activation (19, 23).

This study examined whether the cleavage of the G-protein-linked thrombin receptor necessarily occurs when platelets are activated with 0.5, 1.0, and 10 nM α-thrombin. Affinity-purified polyclonal antibodies against the 41-mer activation peptide

---

*This work was supported in part by a grant-in-aid from the Heart and Stroke Foundation of Ontario. 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.

†Recipient of a career development fellowship award from the Canadian Red Cross Society, Blood Services.

‡‡To whom correspondence should be addressed: Dep't. of Pathology, McMaster University, 1200 Main St. West, Hamilton, Ontario L8N 3Z5, Canada. Tel.: 905-525-9140 (ext. 22263); Fax: 905-521-2613.

1The abbreviations used are: GP, glycoprotein; TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.
Centrifugation at 2500 g to remove erythrocytes from whole blood and 1 volume of ACD. Platelet-rich plasma was isolated by centrifugation at 10,000 g for 10 min at 22°C. The washed platelets were resuspended in the modified Tyrode's buffer containing 1 mM CaCl₂ and were kept at 37°C.

Platelet Activation by α-Thrombin—Washed control platelets were incubated with a protease (2 × 10⁹ platelets/liter) that had been preincubated with 0, 0.5, 1, or 10 nM α-thrombin at 37°C. In some experiments, the washed platelets were resuspended in the modified Tyrode's buffer without 2 mM CaCl₂ and incubated with α-thrombin for up to 30 min at 37°C. Periodic aliquots were fixed in 10 g/liter paraformaldehyde for flow cytometric analysis or were added to the modified Tyrode's buffer containing 1 μM hirudin to inactivate the added α-thrombin, followed by detection of TR-(1–41) release from the platelets as detailed below. In other experiments, the following monoclonal anti-platelet glycoproteins (at the final concentrations shown in parentheses) were added to control washed platelets resuspended in the modified Tyrode's buffer for 10 min at 37°C prior to the addition of α-thrombin in flow cytometric analysis: TM60 (100 μg/liter), LJ-B10 (120 μg/liter), 6D1 (100 μg/liter), and ATAP-138 (150 μg/liter). These experiments determined how each monoclonal antibody influenced the binding of α-thrombin to platelets and the subsequent responsiveness of the platelets to the bound α-thrombin.

Immunoblotting Analysis—Washed platelets preincubated with a protease were centrifuged at 15,500 × g for 1 min at 37°C to determine the cleavage and release of TR-(1–41) from the extracellular domain of the G-protein-linked thrombin receptor into the supernatants. The supernatants were recovered and subjected to dot blotting by loading 10 μl of each supernatant onto strips of nitrocellulose membrane, which were then air-dried at room temperature and incubated in 10 g/liter gelatin dissolved in a buffer containing 20 mM Tris, 500 mM NaCl, 0.2 g/liter trisodium azide, and 0.5 g/liter Tween 20, pH 7.4 (TBS-Tween) overnight. After washing twice with TBS-Tween, the membranes were incubated with 2 mg/liter biotinylated rabbit anti-human TR-(1–41) IgG (in TBS-Tween containing 1 g/liter gelatin) for 2 h. After washing the membrane four times with the above buffer, blots containing TR-(1–41) were identified using alkaline phosphatase-conjugated streptavidin, followed by color development with 5-bromo-4-chloro-3-indolyl phosphate, p-toluidine, and nitro blue tetrazolium.

Quantification of TR-(1–41) Release by α-Thrombin and Chymotrypsin—Release of TR-(1–41) from platelet-rich plasma resulting from the stimulation of platelets resuspended in Tyrode's buffer with 1.0, 10.0, and 50 nM α-thrombin or with 10 and 50 nM chymotrypsin was quantified by an ELISA for TR-(1–41). In this ELISA, 200 μl of affinity-purified chicken anti-human TR-(1–41) (in TBS-Tween containing 1 g/liter gelatin) containing 10 μg of IgG/IgG was added to each well of microtiter platelets and incubated at 4°C for 16 h. The free IgG was removed by suction, and the washed platelets were blocked with 1 g/liter fatty acid-free bovine serum albumin in a buffer containing 0.01 M Tris-HCl, 0.15 M NaCl, and 0.5 g/liter Tween 20, pH 8.0 (TBS-T). After four washes with a buffer containing 0.01 M Na₂HPO₄, 0.145 M NaCl, and 0.5 g/liter Tween 20, pH 7.4 (PBS-T), a standard curve for estimating the concentration of TR-(1–41) was constructed as follows: 100 μl of increasing concentrations of TR-(1–41) (20 pg to 50 ng) in PBS-T containing 10 g/liter bovine serum albumin were added to microtiter wells and incubated at 37°C for 60 min. Each well then received four washes with PBS-T, followed by the addition of 100 μl of biotinylated chicken anti-human TR-(1–41) (100 μg/liter) for a 60-min incubation at 37°C. After four washes with PBS-T, 100 μl of alkaline phosphatase-conjugated streptavidin diluted 1:10,000 were then added, and the plates were incubated at 37°C for 1 h. After another four washes with PBS-T, 100 μl of 1 g/liter p-nitrophenyl phosphate were added for a 40-min incubation at 37°C, and the color yield at 405 nm was quantified.

To quantify the release of TR-(1–41) from platelets, washed platelets (5 × 10⁹/liter) were resuspended in the modified Tyrode's buffer supplemented with bovine serum albumin to a final concentration of 10 g/liter. α-Thrombin (1.0 or 10.0 nM) or chymotrypsin (10 or 50 nM) was then added to the platelet suspension. Periodic aliquots were fixed and added to 0.02 volume of 50 μl D-Phe-Pro-ArgCH₂Cl (for platelets incubated with α-thrombin) or 1.5 mM phenolphthalein sulfon fluoride (for platelets incubated with chymotrypsin). The concentrations of TR-(1–41) released from the platelets were estimated by ELISA immediately after the platelets had been centrifuged at 10,000 × g for 10 min at 22°C.
Flow Cytometric Analysis of Platelets—The procedures described previously were used to estimate α-thrombin binding to platelets (28). Briefly, platelets fixed in 10 g/liter paraformaldehyde for 10 min at 22 °C were centrifuged at 1175 g for 1 min at 22 °C and then washed twice with 154 mM NaCl containing 1 g/liter bovine serum albumin. After resuspension in FACSFlow fluid (Becton Dickinson, Mississauga, Canada), the platelets were incubated with phycoerythrin-conjugated Z-avidin for 30 min, washed twice with 154 mM NaCl containing 1 g/liter bovine serum albumin, and finally resuspended in FACSFlow fluid. The percentage of 10,000 platelets that had bound α-thrombin and the associated fluorescence intensity were determined using a FACSscan argon ion flow cytometer operating at 488 nm and at 15-milliwatt power using LysisII software. The instrument was set up to measure the size (forward scatter), granularity (side scatter), and platelet fluorescence. All parameters were collected using a 4-decade logarithmic amplification. The data are reported as thrombin fluorescence intensity on the platelets (mean channel fluorescence in arbitrary units).

Similar procedures were used to quantify the expression of GMP-140 (P-selectin), CD63, and the resting and activated conformers of GPIb-IIIa on platelets using the appropriate monoclonal antibodies, except that the data are reported as the percentage of platelets expressing the marker under study. The panel of monoclonal anti-GP antibodies (TM60, LJ-JB10, and 6D1) was also used to detect GPIb on control washed platelets and washed platelets preincubated with chymotrypsin, S. marcescens protease, or O-sialoglycoprotein endopeptidase.

RESULTS

Cleavage of the G-protein-linked Thrombin Receptor by α-Thrombin—To determine whether activation of platelets by α-thrombin necessarily coincided with cleavage of the G-protein-linked thrombin receptor, washed platelets resuspended in the modified Tyrode’s buffer were incubated with up to 10 nM α-thrombin for up to 1 min. Both the binding of α-thrombin to the platelets and activation of the same platelets were estimated. Platelet activation was estimated by quantifying GMP-140 (Fig. 1), CD63, and the activated conformer of GPIb-IIIa expression on platelets by flow cytometry. As shown in Fig. 1, dose-dependent binding of α-thrombin to the platelets and expression of GMP-140 on the activated platelets were observed, beginning 10 s after ≥0.5 nM α-thrombin addition. The fluorescence intensity of GMP-140 associated with each concentration of added α-thrombin remained unchanged during the next 50 s of incubation. However, both α-thrombin binding to platelets and expression of GMP-140 thereon had decreased by ~20% when the incubation of platelets with α-thrombin was increased to 30 min (Table I).

We also explored the response of platelets to a second addition of α-thrombin. In these experiments, washed platelets were incubated with 0.5 or 1.0 nM α-thrombin for 60 s, followed by the addition of 10 nM α-thrombin for a 10-s incubation. The percentage of platelets expressing P-selectin was used to estimate the responses of the platelets to the first and subsequent α-thrombin additions. The addition of 0.5 and 1.0 nM α-thrombin to these platelets for 60 s resulted in 31 and 76% of the platelets expressing P-selectin. The subsequent addition of 10 nM α-thrombin to these platelets resulted in >95% of the platelets expressing P-selectin. Thus, platelets unactivated following the addition of suboptimal concentrations of α-thrombin respond to a second addition of α-thrombin.

Similar dose-dependent expression of CD63 and the activated conformer of GPIb-IIIa on the platelets was also observed after α-thrombin addition (data not shown). Using affinity-purified rabbit anti-human TR-(1–41) IgG and dot blotting, release of TR-(1–41) from platelets incubated with the
The monoclonal antibody or saline was added to washed platelets (5 × 10^7/mliter) resuspended in Tyrode's buffer, with (first four columns) or without (last four columns) CaCl_2 for incubation at 37 °C. α-Thrombin (0.5 nM) was then added to each platelet suspension. Aliquots were withdrawn at 10 s or 30 min into 1% paraformaldehyde, fixed for 10 min, and subjected to flow cytometry to estimate the percentage of platelets that had bound α-thrombin and the percentage of platelets that had become activated (by expressing GMP-140 or P-selectin). The percentage of control platelets not exposed to α-thrombin but expressing thrombin at 10 s and 30 min was 5.1%; the percentage of control platelets expressing GMP-140 at 10 s and 30 min were 5.0 and 4.9%, respectively. Each result was obtained after the platelet suspensions from three to four experiments had been pooled prior to centrifugation and flow cytometry.

### Table I

Effects of TM60, LJ-IB10, and ATAP-138 on α-thrombin binding to washed platelets and their subsequent activation

| Antibody    | 10 s          | 30 min        | 10 s          | 30 min        |
|-------------|---------------|---------------|---------------|---------------|
| None        | 36.0          | 38.0          | 29.6          | 30.4          |
| TM60        | 35.4          | 36.0          | 28.7          | 29.5          |
| LJ-IB10     | 35.0          | 35.5          | 29.0          | 28.3          |
| ATAP-138    | 4.5           | 5.0           | 4.8           | 6.0           |

* GMP, GMP-140 (P-selectin).

*Fig. 2. Release of TR-(1–41) from washed platelets after their incubation at 37 °C for 10 s with α-thrombin. The supernatants from control platelets incubated for 10 s at 37 °C with α-thrombin (0, 0.5, 1.0, and 10 nM in lanes 1–4, respectively) for 10 s at 37 °C; or from platelets incubated with 50 nM chymotrypsin for 30 s (lane 8), 5 min (lane 9), and 30 min (lane 10) for 10 s at 37 °C; or from platelets preincubated with GMP-140 for 10 s at 37 °C to activate washed platelets (data not shown). The supernatants of platelets incubated with ATAP-138 (150 nM) for 30 min at 37 °C to activate washed platelets (data not shown).

S. marcescens protease specifically cleave GPIb, while chymotrypsin probably cleaves other platelet glycoproteins (13–16). None of these three proteases directly activated the platelets or altered the initial fluorescence of the resting and activated GPIIIa-IIIa formers on platelets (data not shown). However, each protease completely removed GPIb from the platelets or markedly altered the tertiary structure of GPIb since none of the monoclonal anti-GPIb antibodies (TM60, LJ-IB10, or 6D1) bound to platelets preincubated with any of these three proteases (data not shown).

In spite of this observation, 0.5, 1, or 10 nM α-thrombin normally bound to and activated platelets preincubated with S. marcescens protease and O-sialoglycoprotein endopeptidase (Fig. 1). Additionally, neither protease inhibited the expression of CD63 or the activated GPIIIa-IIIa formers on platelets following 0.5, 1.0, or 10 nM α-thrombin addition (data not shown). In contrast to platelets incubated with these two proteases, α-thrombin neither bound to nor activated platelets preincubated with chymotrypsin (Fig. 1). Immunoblotting confirmed the release of fragment(s) of the G-protein-linked thrombin receptor that reacted with anti-human TR-(1–41) IgG from platelets preincubated with chymotrypsin for 30 s (Fig. 2). Cleavage of this receptor by chymotrypsin abrogated the activation of the platelets by α-thrombin.

Effects of Monoclonal Anti-GPIb Antibodies on α-Thrombin Binding to Platelets—In additional experiments exploring the role of GPIb in α-thrombin-mediated platelet activation, the effects of the three monoclonal anti-GPIb antibodies on...
a-thrombin binding to and activation of platelets were also determined. TM60 and LJ-IB10 are monoclonal antibodies against the high-affinity a-thrombin-binding domain on GPIb (7, 18), while 6D1 is directed against the von Willebrand factor-binding domain of GPIb (11, 12). Thus, unlike TM60 and LJ-IB10, 6D1 was not expected to inhibit the interactions of a-thrombin with platelets. The binding of each monoclonal antibody to the platelets was verified by the positive and maximal staining of the platelets with either fluorescein isothiocyanate- or phycoerythrin-conjugated goat anti-mouse antibodies (data not shown). In spite of the above observation, none of the three anti-GPIb antibodies inhibited a-thrombin binding to or the subsequent activation of washed platelets (Fig. 3) or washed platelets resuspended in pooled normal plasma (Fig. 4).

The binding of a-thrombin to and the subsequent activation of washed platelets resuspended in Ca\(^{2+}\)-free Tyrode's buffer were also investigated using 0.5 nM a-thrombin. This concentration of a-thrombin was chosen to ensure that only the high-affinity binding sites for a-thrombin on platelets would be occupied by the enzyme. As reported previously (28), a-thrombin bound to 20% fewer platelets in the absence than in the presence of Ca\(^{2+}\) (Table I). In the absence of Ca\(^{2+}\), LJ-IB10 significantly inhibited a-thrombin binding to platelets and their activation 10 s and 30 min after 0.5 nM a-thrombin had been added to the washed platelets. However, TM60 did not inhibit a-thrombin binding to platelets or their activation as effectively as LJ-IB10. Thus, Ca\(^{2+}\) enhances the binding of a-thrombin to platelets and in a manner that decreases any requirement for GPIb for directing the initial binding of a-thrombin to platelets and their subsequent activation.

**DISCUSSION**

Platelets have ~25,000 copies of GPIb, the platelet glycoprotein proposed to provide ~50 high-affinity binding sites for a-thrombin (K\(_d\) ~ 1 nM) since platelets of Bernard-Soulier patients (and thus congenitally deficient in GPIb) aggregate slowly, but demonstrate normal dense body release in response to subnanomolar a-thrombin. Additionally, cleavage of GPIb or occupancy of GPIb by some monoclonal anti-GPIb antibodies inhibits platelet aggregation and release by ≤1.0 nM a-thrombin, but not by 10 nM a-thrombin (1, 5, 7, 8, 11, 12–16). A G-protein-linked thrombin receptor on platelets to which a-thrombin binds (probably via the hirudin-like domain of this receptor) and cleaves off the first 41 amino acid residues (called TR-(1–41) in this study) has been described (9, 10, 19–21). There are ~1700 copies of this receptor/platelet (27), and some investigators have assigned the moderate-affinity a-thrombin-binding sites (K\(_d\) ~ 10 nM) on platelets to this receptor (11, 23, 24). Antibodies against the hirudin-like domain of this G-protein-linked receptor inhibit the responsiveness of platelets to a-thrombin (27, 28, 33). Thus, the primary site on platelets to which a-thrombin binds to initiate platelet activation remains unclear.

In this study, cleavage of platelet TR-(1–41) by a-thrombin was directly monitored, as were a-thrombin binding to platelets and the subsequent activation of the same platelets. No attempt was made in this study to quantify the number of a-thrombin molecules/platelet or the concentrations of markers of platelet activation that became expressed on activated platelets. Rather, the percentages of platelets that rapidly bound a-thrombin and subsequently expressed surface P-selection, CD63, and the activated conformer of GPIIb-IIIa for each concentration of the enzyme were quantified. We have presented data demonstrating the parallel binding of a-thrombin to platelets, cleavage and release of TR-(1–41) from the platelets, and activation of the same platelets with each concentration of a-thrombin. There was a similar (–1:1) relationship between the binding of a-thrombin to platelets and the expression of each of the three markers of platelet activation within 60 s of a-thrombin addition. This study also confirmed the observation by Norton et al. (33) that a-thrombin releases TR-(1–41) from platelets. It is unclear why 1.0 nM a-thrombin did not release TR-(1–41) as effectively as 10 nM a-thrombin when both concentrations of the enzyme activated ≥75% of the washed platelets (Figs. 1 and 3). We eliminated the possibility that this a-thrombin receptor became inaccessible to a-thrombin following the exposure of platelets to suboptimal concentrations of a-thrombin. Specifically, we demonstrated that platelets preincubated with 0.5 or 1 nM a-thrombin responded appropriately to a subsequent addition of a-thrombin. Thus, the fraction of the thrombin receptor not previously occupied by suboptimal concentrations of a-thrombin remained accessible to added a-thrombin. Since ~2.0 nM TR-(1–41) could be theoretically released from platelets (27), the fact that 10 nM a-thrombin fully activated the platelets but released only ≤0.6 nM TR-(1–41) suggests that complete cleavage of the receptor is not required for maximum platelet activation. Nonetheless, partial cleavage of this a-thrombin receptor is required to initiate platelet activation since abrogation of thrombin-mediated cleavage of this receptor by ATAP-138 also abrogated platelet activation.

A likely reason for the failure of a-thrombin to quantitatively cleave all available TR-(1–41) from platelets may reside in the ability of a-thrombin to induce endocytosis of this receptor, as demonstrated for two megakaryoblastic cell lines, namely human erythroleukemia cells and Children's Hospital Research Foundation cell line 288 (34–36). This failure of up to 10 nM a-thrombin to fully cleave the G-protein-linked thrombin receptor and to release TR-(1–41) from platelets parallels the effects a-thrombin has on fibrinogen and fibrin has on the enzymatic activity of a-thrombin. Similar to the release of TR-(1–41), a-thrombin cleaves fibrinogen in a dose-dependent manner, with fibrinopeptide A release proceeding to the maximum extent achievable with each a-thrombin concentration within 60 s (37). a-Thrombin binding to fibrin also clearly impairs the ability of this enzyme to release fibrinopeptide A from fibrinogen (37). Binding of a-thrombin to the cleaved receptor (which

---

**TABLE II**

**Release of TR-(1–41) from platelets incubated with a-thrombin and chymotrypsin**

| Platelets  | Incubation time(s) | [Enzyme] | [TR-(1–41)] |
|-----------|-------------------|---------|------------|
| A1        | 0                  | 10 nM a-thrombin | 0.48       |
| A6        | 1                  | 1 nM a-thrombin | 0.36       |
| B1        | 0                  | 10 nM a-thrombin | 0.49       |
| B6        | 1                  | 1 nM a-thrombin | 0.22       |
| C1        | 0                  | 10 nM a-thrombin | 0.59       |
| C6        | 1                  | 1 nM a-thrombin | 0.36       |
| A         | 0                  | 10 nM chymotrypsin | ND*       |
| A         | 1                  | 50 nM chymotrypsin | ND        |
| B         | 0                  | 50 nM chymotrypsin | ND        |
| C         | 1                  | 10 nM chymotrypsin | 0.02      |
| C         | 0                  | 50 nM chymotrypsin | 0.01      |

ND, none detected.
then becomes phosphorylated (17, 38) may similarly impair the ability of the bound enzyme to cleave nearby receptors. Continued tight binding of α-thrombin to this site may be important, and one study has reported that continued occupancy of the G-protein-linked receptor by α-thrombin is required to propagate tyrosine phosphorylation. Specifically, Lau et al. (38) have reported that addition of hirudin to platelets preincubated with α-thrombin for 60 s does not deaggregatethe platelets, but inhibits specific tyrosine phosphorylation and simultaneously accelerates specific tyrosine dephosphorylation. Occupancy of this receptor by α-thrombin at the hirudin-like domain of the receptor is clearly crucial for platelet activation since ATAP-138 abrogates the binding of 0.5 or 1 nM α-thrombin to platelets, release of TR-(1–41) from the platelets, and activation of the platelets. As previously reported by Brass et al. (27), we found that 10 nM α-thrombin binds to and activates washed platelets in the presence of a saturating concentration ATAP-138.

The high-affinity binding sites for α-thrombin on GPIb are reportedly located within the Mr 45,000 NH2-terminal domain of GPIba (3, 5, 7, 18, 39–42), and removal of GPIb from platelets by chymotrypsin, S. marcescens protease, or elastase yields platelets with a lower sensitivity to ≤1 nM α-thrombin (13–16). This study has demonstrated that platelets with this putative high-affinity α-thrombin-binding domain on GPIb removed (by protease digestion) bind normally to α-thrombin. In further experiments, two monoclonal antibodies against this putative high-affinity α-thrombin-binding domain on GPIb (TM60 and LJ-1B10) that inhibit the responses of platelets to ≤1 nM α-thrombin (7, 18, 39–42) were used in another attempt to prevent α-thrombin binding to platelets via GPIb. In the presence of 2 mM CaCl2, α-thrombin bound normally to and activated platelets that had been preincubated with either monoclonal anti-GPIb antibody.

Therefore, we conclude that GPIb does not normally participate in the initial interactions of α-thrombin with platelets and that cleavage(s) by chymotrypsin additional to GPIb abrogate the responsiveness of platelets to α-thrombin. Chymotrypsin cleaves the G-protein thrombin-linked receptor at a point distal to Arg-41/Ser-42 (43, 44). This cleavage may explain why only ≤30 ps Tr-(1–41) was detected by the ELISA for Tr-(1–41). Using a chimeric fusion protein consisting of glutathione S-transferase and residues 25–97 corresponding to the NH2-terminal extracellular domain of the G-protein-linked thrombin as the substrate, Bouton et al. (44) reported that the glycocalcin portion of GPIb did not alter the kinetics describing the cleavage of this fusion protein by α-thrombin, whereas fibrinogen fragment E, thrombomodulin, and hirudin fragment 54–65 did. These results suggest minimal rapid binding interactions between α-thrombin and the extracellular domain of GPIb when the enzyme normally cleaves the G-protein-linked thrombin receptor.

There are three reasons why we could not ascribe a critical role to GPIb for mediating α-thrombin binding to platelets in the time required for α-thrombin to optimally activate platelets. (i) We used 10–60-s incubations to demonstrate optimal binding of α-thrombin to platelets, compared with ≥30-min incubations used in some of the previous studies (7, 18, 24). The incubation times of 10 and 60 s were chosen as activation of platelets by α-thrombin proceeds to the maximum extent...
achievable with each concentration of thrombin in \( \leq 60 \) s (28). This choice was also justified by the demonstration of decreased binding of \( \alpha \)-thrombin to platelets after the enzyme was incubated with platelets for 30 min (compared with 10 s), as shown in Table I. (ii) The platelets used in this study were fixed with 10 g/liter paraformaldehyde after their incubation with \( \alpha \)-thrombin to immobilize the enzyme on platelets. Fixation of the platelets also inactivated \( \alpha \)-thrombin and halted further platelet reactions resulting from \( \alpha \)-thrombin binding to the platelets. Fixation does not alter the binding of \( \alpha \)-thrombin to platelets (40). (iii) We also estimated \( \alpha \)-thrombin binding to platelets resuspended in CaCl\(_2\)-containing media, while the previous studies were without addition of this salt. CaCl\(_2\) enhances the binding of \( \alpha \)-thrombin to platelets and stabilizes the expression of P-selectin on the activated platelets (28), as was confirmed in this study. Additionally, two monoclonal anti-GPIb antibodies (LJ-IB10 and TM60) inhibited \( \alpha \)-thrombin binding to washed platelets and their activation, but only in the absence of added CaCl\(_2\) (Table I). Inhibition of \( \alpha \)-thrombin binding to platelets by these two monoclonal anti-GPIb antibodies (in the absence of Ca\(^{2+}\)) has been reported by many other investigators (11, 12, 39–42).

Previous reports have hypothesized that GPIb and the G-protein-linked thrombin receptor form a functional complex on platelets. Specifically, interactions of \( \alpha \)-thrombin with GPIb localize \( \alpha \)-thrombin to sites that facilitate cleavage of nearby G-protein-linked thrombin receptors during the activation process (18, 23). Our results do not support significant interactions between \( \alpha \)-thrombin and GPIb to effect \( \alpha \)-thrombin binding to platelets, in the presence of Ca\(^{2+}\), to initiate platelet activation. The G-protein-linked thrombin receptor appears to be the primary site to which \( \alpha \)-thrombin binds to initiate platelet activation. Our observations, however, do not exclude GPIb modulating additional signaling events, including changes in extracellular Ca\(^{2+}\) and aggregation resulting from \( \alpha \)-thrombin binding to the platelets (12, 40, 41).

Acknowledgments—We thank Drs. L. F. Brass, B. S. Coller, Z. M. Ruggeri, and N. Yamamoto for generous gifts of the monoclonal anti-platelet antibodies used in this study.

REFERENCES
1. Jamieson, G. A., and Okumura, T. (1978) J. Clin. Invest. 61, 861–864
2. Okumura, T., Hasitz, M., and Jamieson, G. A. (1978) J. Biol. Chem. 253, 3435–3443
3. Harmon, J. T., and Jamieson, G. A. (1988) Biochemistry 27, 2151–2157
4. Harmon, J. T., and Jamieson, G. A. (1986) J. Biol. Chem. 261, 15928–15933
5. Lopez, J. A. (1984) Blood Coagul. & Fibrinolysis 5, 97–119
6. Jandrot-Perrus, M., Didry, D., Guerrin, M. C., and Norden, A. T. (1988) Eur. J. Biochem. 174, 359–367
7. Yamamoto, N., Greco, N. J., Barnard, M. B., Tanoue, K., Yamazaki, H., Jamieson, G. A., and Michelson, A. D. (1991) Blood 77, 1740–1748
8. Ruggieri, Z. M. (1991) Prog. Hemostasis Thromb. 10, 35–68
9. Vu, T. K. H., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991) Cell 64, 1057–1068
10. Rasmussen, U. B., Vouret-Craviari, V., Jallat, S., Schlesinger, Y., Pages, G., Pavirani, A., Lecocq, J. P., Pouyssegur, J., and Van Obberghen-Schilling, E. (1991) FEBS Lett. 288, 123–128
11. De Marco, L., Mazzucato, M., Masotti, A., Fenton, J. W., II, and Ruggeri, Z. M. (1991) J. Biol. Chem. 266, 23776–23783
12. De Marco, L., Mazzucato, M., Masotti, A., and Ruggeri, Z. M. (1994) J. Biol. Chem. 269, 6478–6483
13. Cooper, H. A., Bette, W. P., White, G. C., II, and Wagner, R. H. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 1433–1438
14. Wicki, A. N., and Clemenston, K. J. (1985) Eur. J. Biochem. 153, 1–11
15. Tam, S. W., Fenton, J. W., II, and Detwiler, T. C. (1980) J. Biol. Chem. 255,
