Differentiation of the Two Forms of GPIb Functioning as Receptors for α-Thrombin and von Willebrand Factor: Ca^{2+} Responses of Protease-Treated Human Platelets Activated with α-Thrombin and the Tethered Ligand Peptide

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ABSTRACT: Previous results have shown that both GPIb and the seven transmembrane domain receptor (STDR) are required for optimal thrombin-induced platelet activation (Greco et al., 1996). Limited degradation (∼10%) of GPIb and the STDR by elastase reduced the Ca^{2+} response to 0.5 nM α-thrombin by only 10% whereas *Serratia marcescens* metalloprotease reduced the Ca^{2+} response by 80% and fully abrogated high-affinity thrombin binding and aggregation. vWF/ristocetin-induced agglutination was only slightly reduced (20%) while Ca^{2+} and aggregation responses to higher thrombin concentrations were retained. At increasing elastase and *Serratia* protease concentrations, degradation of the STDR proceeded from the amino-terminal domain, but Ca^{2+} responses to the tethered ligand peptide SFLLRNPNDKYEPF were not affected by either protease. These results show that both putative thrombin receptors are susceptible to protease degradation and suggest that *Serratia* protease is able to differentiate the GPIb-mediated events associated with thrombin activation from those associated with ristocetin-induced agglutination.

Glycoprotein Ib (GPIb) has two major functions in blood platelets; the first is mediating the initial adhesion of platelets to von Willebrand factor while the second is functioning as the high-affinity receptor for α-thrombin (Lopez, 1994). In the preceding paper (Greco et al., 1996), we have provided evidence that both GPIb and the STDR[1] are required for optimal thrombin-induced platelet activation.

In those studies, we examined platelets in which the thrombin binding site on GPIbα was blocked with monoclonal antibodies or when GPIb was absent, as is the case with Bernard-Soulier platelets. In its classical form, Bernard-Soulier syndrome is characterized by the absence of the GPIb complex (Nurden & Caen, 1975; Berndt et al., 1985), lack of interaction with von Willebrand factor (Howard et al., 1973; Caen & Levy-Toledano, 1973), and desensitization of the platelet response to α-thrombin (Jamieson & Okumura, 1978; De Marco et al., 1991). Because of the sensitivity of GPIb to proteolysis, and the small number of patients exhibiting this syndrome, numerous attempts have been made to induce Bernard-Soulier-like responses in normal platelets by selective cleavage of GPIbα with exogenous proteases such as chymotrypsin (Okumura et al., 1978; McGowan & Detwiler, 1986), leukocyte elastase (Brower et al., 1985; Wicki & Clemetson, 1985), cathepsin G (Wicki & Clemetson, 1985), plasmin (Adelman et al., 1986), a snake venom protease, kistomin (Huang et al., 1993), and a metalloprotease purified from *Serratia marcescens* (Cooper et al., 1982; McGowan & Detwiler, 1985; Harmon & Jamieson, 1988; Yamamoto et al., 1991). This treatment generally yielded platelets which did not interact with von Willebrand factor and which showed a reduced binding and desensitized response to α-thrombin.

Recognition of the roles of GPIb and the STDR in thrombin-induced platelet activation has permitted a reevaluation of the significance of changes in thrombin responsiveness following platelet proteolysis. In the present studies, we have evaluated changes in $[Ca^{2+}]_i$ in platelets stimulated with α-thrombin or the activating tethered ligand peptide SFLLRNPNDKYEPF following treatment either with human polymorphonuclear leukocyte elastase or with *Serratia marcescens* protease: these two proteases were chosen because neither of them activates platelets, suggesting that the $R^{41}-S^{42}$ bond of the STDR is not cleaved. Elastase can reach concentrations of 100 nM during inflammation and may play a role in modifying the hemostatic response under these conditions (Brower et al., 1985). *Serratia marcescens* protease was chosen because of its apparent relative selectivity for GPIbα in intact platelets (Brower et al., 1985; Wicki & Clemetson, 1985; Cooper et al., 1982; McGowan & Detwiler, 1985; Harmon & Jamieson, 1988; Yamamoto et al., 1991) although it has a rather low degree of peptide bond specificity against soluble substrates (McQuade & Crewther, 1969; Decedue et al., 1979).

Our data delineate the patterns of degradation of these two putative thrombin receptors with these two proteases and suggest that the form of GPIb acting as a high-affinity receptor for α-thrombin exhibits increased sensitivity to *Serratia* protease digestion as compared with the form that functions as a receptor for vWF.

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[1] Abbreviations: STDR, seven transmembrane domain thrombin receptor; MoAb, monoclonal antibody; PRP, platelet-rich plasma; rt, room temperature.

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EXPERIMENTAL METHODS

Unless otherwise stated, platelet preparation methods, reagents, peptide syntheses, measurements of $[Ca^{2+}]_i$ mobilization, antibody preparation, flow cytometry procedures, and data analysis were as described in the preceding paper (Greco et al., 1996). Purified *Serratia marcescens* protease was kindly provided by Dr. Arnold Kreger, Musculoskeletal Sciences Research Institute, Herndon, VA. Human neutrophil elastase (22 units/mg with a purity of >95% as determined by SDS-PAGE) was purchased from Calbiochem (San Diego, CA), diluted in phosphate-buffered saline to an 8 μM stock solution, and stored as frozen aliquots. Soybean trypsin inhibitor with a specific activity such that 1 mg inhibits 1.6 mg of trypsin with 10 000 BAEE units/mL protein was obtained from Sigma Chemical Co. (St. Louis, MO).

Epitope Mapping. The synthetic peptides TNATLDPRSFLLRNP, LLRNPNDKYEPF, and SFLLRNPNDKYEPF (2 μg in 0.1 M $NaHCO_3$, pH 9.5) derived from STDR sequences were coated on Immulon wells (Dynatech Laboratories, Chantilly, VA) before the individual addition of the rabbit polyclonal antibodies anti-TNA (anti-TNATLDPRSFLLRNP) and anti-LLR (anti-LLRNPNDKYEPF) or the mouse monoclonal ATAP138 antibody used as a control. After extensive washing, bound antibodies were visualized by color development using alkaline phosphatase-conjugated anti-species antibodies.

Platelet Proteolysis. The effects of elastase or *Serratia marcescens* protease on GPIb and the STDR as well as on platelet responses to α-thrombin, TLP, or other agonists were conducted concurrently on platelets from the same donor. In all cases, platelet-rich plasma from each donor was shown to respond maximally to 750 μM arachidonic acid, confirming the presence of an active cyclooxygenase pathway. PGE_1 (1 μg/mL) and citric acid (4.2 mM) were added to the PRP at this point to prevent activation during subsequent washing procedures. Also, when required for determination of $[Ca^{2+}]_i$, the PRP at this point was incubated with Fura-2/AM (2.5 μM) for 30 min at 37 °C.

Washed control or Fura-2-loaded platelets (1×10^9/mL) were incubated for 30 min with various concentrations of *Serratia marcescens* protease at room temperature, diluted with approximately 5 volumes of wash buffer, centrifuged (1400*g*, 5 min), and resuspended in Tyrode's-HEPES buffer (2×10^8 platelets/mL).

In the case of treatment with leukocyte elastase, the incubation was for 2 min at 37 °C prior to the addition of α-thrombin (Brower et al., 1985). To ascertain that elastase did not directly affect the activity of α-thrombin, both α-thrombin (0.5 nM) and elastase (50 nM) were mixed and incubated for 2 min at 37 °C prior to the addition of a 20-fold molar excess of soybean trypsin inhibitor followed by further incubation for 1 min. When this mixture was added to a platelet suspension, Ca^{2+} mobilization was identical to that in experiments using 0.5 nM α-thrombin alone. In samples to be used for flow cytometry, elastase was blocked by the addition of soybean trypsin inhibitor (1 μM final concentration) for 1 min prior to dilution with platelet wash buffer. These elastase-treated platelets were recovered by centrifugation, washed once with 1.5 mL of platelet wash buffer, and finally resuspended in 100 μL of Tyrode's-HEPES buffer containing 1 mM each of EGTA and EDTA.

Thrombin Binding. Twenty-two triplicate-point competition binding isotherms were developed for evaluating the binding of PPACK-thrombin to control and *Serratia* protease-treated platelets. These experiments were carried out in paired fashion: washed platelets from three different donors were divided into two portions, one portion being used as control and the other portion being treated with *Serratia* protease (0.1 μg/mL; 30 min/rt) as described above. PPACK-thrombin was used to avoid possible complications arising with α-thrombin, binding was measured over the concentration range 0.1 nM−1 μM, and the data from the three binding isotherms were subjected to coanalysis using the LIGAND program.

Ristocetin-Induced Agglutination. Control and *Serratia* protease-treated platelets were washed and fixed immediately with paraformaldehyde (1%/30 min/rt). Following further washing, the suspension was adjusted to 4×10^8/mL, and a 200 μL aliquot was diluted with an equal volume of autologous platelet-poor plasma prior to measuring agglutination in a Payton Aggregometer over a range of ristocetin concentrations (0.9−1.5 mg/mL) (Adelman et al., 1986).

RESULTS

Epitope Mapping. When the polyclonal anti-TNA antibody against the peptide TNATLDPRSFLLRNP was tested by ELISA against the overlapping sequences SFLLRNPNDKYEPF and LLRNPNDKYEPF, the reactivity was essentially the same as against nonimmune IgG_1, suggesting that the anti-TNA antibody was directed mainly against the 8-mer amino terminal domain TNATLDPR. Similar studies with the polyclonal anti-LLR antibody raised against LLRNPNDKYEPF showed that the anti-LLR antibody was directed mainly against the carboxyl-terminal sequence NDKYEPF of the immunizing peptide. As positive control, we confirmed that MoAb ATAP138 was directed against a sequence contained in the peptide, NPNDKY, as previously reported (Brass et al., 1992).

Platelet Proteolysis. The anti-GPIb MoAbs TM60 and LJ-Ib10 react strongly with intact platelets as measured by flow cytometry (Yamamoto et al., 1991; De Marco et al., 1994), and this was confirmed in the present study.

(i) Serratia Protease. At the lowest concentration of *Serratia* protease used (0.1 μg/mL), the mean content ($n = 3$) of GPIb showed a 10% decrease when measured with TM60 and a similar increase when measured with LJ-Ib10 (Figure 1A), but this difference was not significant since variations of ±15% in absolute values were seen with different donors. At increasing concentrations of *Serratia* protease, the amount of immunoreactive GPIb was progressively reduced, and >90% of the antigen was lost at a *Serratia* protease concentration of 2.5 μg/mL, consistent with values previously obtained by autoradiography or flow cytometry (Harmon & Jamieson, 1988; Yamamoto et al., 1991).

The STDR also was susceptible to digestion with *Serratia marcescens* protease. In particular, reactivity with the anti-TNA antibody was reduced by 5−10% at 0.1 μg/mL *Serratia* protease and by about 80% at 2.5 μg/mL *Serratia* protease. Reactivity with anti-LLR was unaffected at lower Serratia protease concentrations (0.1 and 0.5 μg/mL) but at 2.5 μg/mL was also reduced by 80%. Reactivity with anti-EPF was

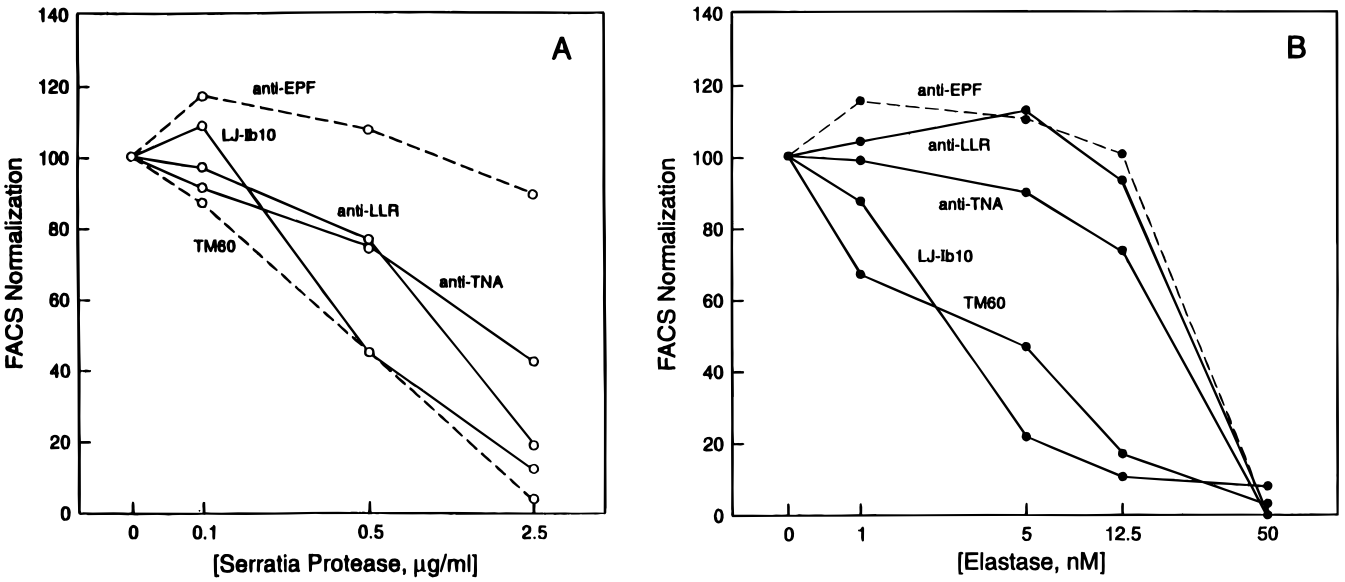

FIGURE 1: Proteolysis. Platelets from individual donors were treated with *Serratia marcescens* protease (panel A; 30 min, rt) or with elastase (panel B; 2 min, 37 °C), washed, and utilized for flow cytometry. For each experiment, the fluorescence shift of control platelets on addition of antibody was taken as 100% and compared with the mean fluorescence shift of protease-treated platelets in paired experiments. The results shown are the mean values from 2−5 determinations on platelets from each of 3 donors with TM60 and 9 donors with LJ-Ib10. Results obtained with other anti-GPIb antibodies are discussed in the text. Standard deviations did not exceed ±15%, but error bars are not shown so as to simplify the figure. Dashed lines are used in some cases to improve clarity.

essentially unchanged at all *Serratia* protease concentrations examined.

(ii) Elastase. As expected from previous studies (Brower et al., 1985; Wicki & Clemetson, 1985), GPIb was readily degraded by elastase. At 5 nM elastase, GPIb was degraded by an average of 53% as measured with TM60 (range 49−67%) in 2−5 replicate studies using platelets from 5 different donors and by 78% as measured by LJ-Ib10 (range 61−93%) with 9 different donors. Degradation by 5 nM elastase was 74% as measured in a single donor with LJ-P3 and 60% and 70% in two donors with LJ-Ib1.

The STDR was essentially unaffected by up to 5 nM elastase as recognized by flow cytometry using the anti-TNA, anti-LLR, and anti-EPF antibodies but was completely degraded at 50 nM elastase (Figure 1B). Similar results to those obtained with these polyclonal anti-peptide antibodies were obtained with the MoAb ATAP138 (data not shown).

[Ca^{2+}] Dynamics. $[Ca^{2+}]_i$ in resting platelets was determined to be 121 ± 17 nM. This basal value was increased to 230 ± 56 nM in control platelets treated with 0.5 nM α-thrombin but to only 150 ± 24 nM in platelets that had been previously treated with 0.1 μg/mL *Serratia* protease. At 10 nM α-thrombin, $[Ca^{2+}]_i$ rose to 800 nM in control platelets but to only 340 nM if the platelets had received prior treatment with 0.1 μg/mL *Serratia* protease. If peak $[Ca^{2+}]_i$ values of control platelets at different thrombin concentrations were normalized, it could be seen that their ability to mobilize Ca^{2+} in response to low concentrations of α-thrombin (0.5 nM) was reduced by about 80% at 0.1 μg/mL *Serratia* protease, although substantial amounts of GPIb and the STDR were still detectable, and there was little further reduction in Ca^{2+} response at higher protease concentrations (Figure 2A). In contrast, Ca^{2+} response to 0.5 nM α-thrombin was reduced only 10% following treatment with 1 nM elastase and by 50% at 5 nM elastase, and no response was detectable after treatment at 50 nM (Figure 2B). Treatment of platelets with *Serratia* protease (2.5 μg/mL) or elastase (50 nM) did not affect their Ca^{2+} response to 15 μM TLP (Figure 2) or up to 100 μM (not shown).

Taken together, these results demonstrated that only minor losses of reactivity with antibodies to GPIb or the STDR were detectable by flow cytometry of platelets treated with the low concentrations of *Serratia* protease (0.1 μg/mL) or elastase (1 nM) examined here (Figure 1) although there was a highly significant loss of Ca^{2+} response to thrombin seen with *Serratia* protease-treated platelets which was not seen with elastase (Figure 2). Because of this differential effect, subsequent studies focused on the effects of *Serratia* protease treatment on thrombin binding and associated platelet functions.

Thrombin Binding. Coanalysis of three 22 triplicate-point competition isotherms for the binding of PPACK-thrombin to control platelets conformed to a three-site model of high-, moderate-, and low-affinity binding (Figure 3, filled circles). However, when binding isotherms for platelets treated with a low concentration of *Serratia* protease (0.1 μg/mL) were similarly examined, the loss of the high-affinity binding component for low concentrations ($<10^{-9}$ M) of PPACK-thrombin was clearly seen (Figure 3, open circles). Scatchard coanalysis of binding data from three different donors using the LIGAND program in paired studies showed that the best fit for *Serratia*-proteolyzed platelets was a two-site model (Table 1) with a moderate affinity binding component (K_d 2.6 nM; 180 sites/platelet) and a low-affinity binding component (K_d 1600 nM; 84 300 sites/platelet) but lacking high-affinity binding (Table 2). Nonspecific binding was negligible in both cases (∼2%).

Aggregation Studies. This loss of high-affinity binding in platelets that had been treated with a low concentration of *Serratia* protease (0.1 μg/mL) was accompanied by a loss of aggregability in response to 0.5 nM α-thrombin although the shape change was retained and responses to TLP (15 μM) and collagen (2.5 μg/mL) were unaffected (Figure 4A).

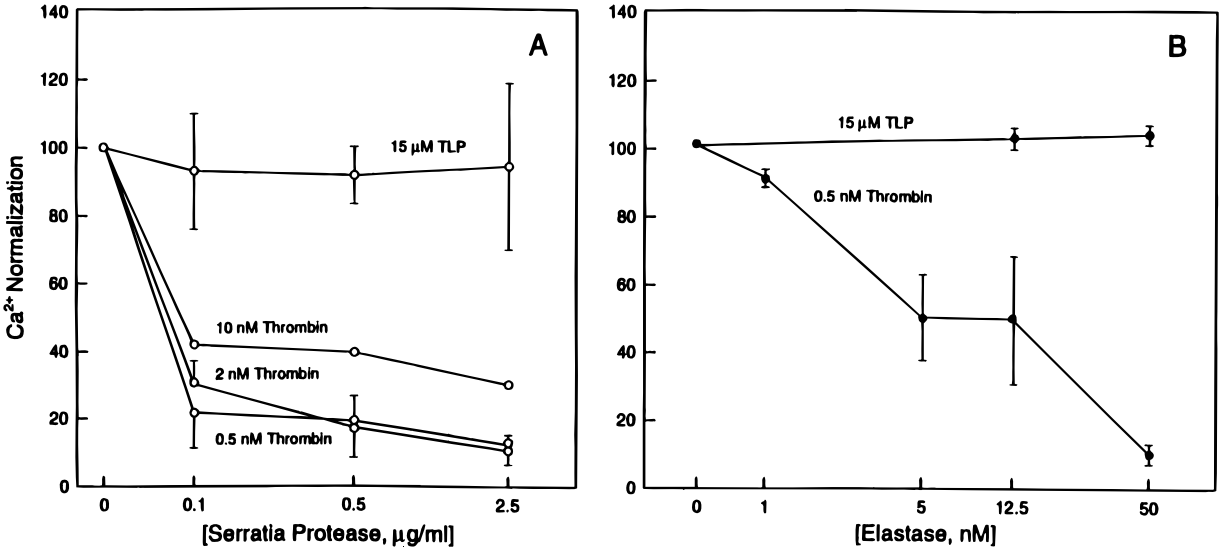

FIGURE 2: Thrombin-induced peak $[Ca^{2+}]_i$ values in protease-treated platelets. Fura-2-loaded platelets were treated with the indicated amounts of *Serratia* protease (panel A; 30 min, rt) or elastase (panel B; 2 min, 37 °C) and then used for the measurement of $[Ca^{2+}]_i$ in the presence of 1 mM external Ca^{2+}. Peak Ca^{2+} values obtained in control platelets at each thrombin concentration (0.5 nM, 150 nM; 2 nM, 370 nM; and 10 nM, 800 nM) were normalized to 100% and compared with $[Ca^{2+}]_i$ values obtained at the same concentrations of α-thrombin with protease-treated platelets. Note that Ca^{2+} mobilization with the agonist peptide, TLP, was unaffected by either protease and this held true even at TLP concentrations of 100 μM. Data are the mean of experiments using platelets from 3−4 different donors except at 10 nM thrombin (panel A) which is the average of data from 2 donors. Both preparations showed similar base-line values for $[Ca^{2+}]_i$ (70−90 nM) in the absence of added agonist, and neither protease alone affected Ca^{2+} levels.

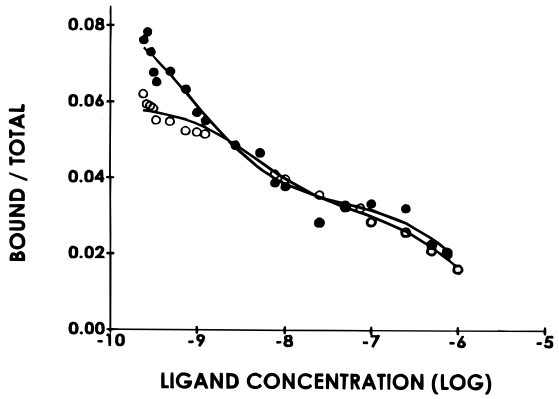

FIGURE 3: Binding of PPACK-thrombin. For clarity, the competition thrombin binding isotherms are shown for a single donor. The lines are the best fit to the data points based on a three-site model for control platelets (solid circles) and a two-site model for platelets treated with *Serratia* protease at low concentration (0.1 μg/mL; open circles). However, the binding parameters given in Table 1 for control and *Serratia*-proteolyzed platelets are calculated by the LIGAND program from coanalysis of 18−22 triplicate data points using platelets from three separate donors in paired experiments.

At an intermediate α-thrombin concentration (2 nM), the shape change response and the degree of aggregation varied depending on the concentration of *Serratia* protease to which they had been exposed (Figure 4B) while aggregation of *Serratia*-treated platelets in response to 10 nM α-thrombin was essentially indistinguishable from that of control platelets (Figure 4C).

Rate and Extent Effects. The effects of α-thrombin on the rate and extent of $[Ca^{2+}]_i$ changes in *Serratia* protease-treated platelets were essentially identical with those seen with Bernard-Soulier platelets as demonstrated in Figures 4 and 5 of the preceding paper (Greco et al., 1996).

Control platelets gave a curvilinear response to incremental increases in α-thrombin, and the $[Ca^{2+}]_i$ value was increased by 375 nM over base line at 2 nM α-thrombin whereas *Serratia*-treated platelets gave a $[Ca^{2+}]_i$ value of only 100 nM at the same α-thrombin concentration and with no detectable increase in $[Ca^{2+}]_i$ up to 0.5 nM α-thrombin. Above 2 nM, the $[Ca^{2+}]_i$ responses to increasing concentrations of α-thrombin were essentially parallel in the two platelet preparations; they differed by ∼300 nM at each α-thrombin concentration and increased ∼40 nM per 1 nM increase in α-thrombin up to 10 nM. Similar results were obtained in the absence of external Ca^{2+} (2 mM EGTA), but the difference between parallel portions of the two curves was reduced to ∼250 nM, indicating only a small contribution from Ca^{2+} influx.

When peak $[Ca^{2+}]_i$ values at each α-thrombin concentration were divided by the time to reach the peak, the rate of Ca^{2+} increase in *Serratia* protease-treated platelets at 5 nM α-thrombin was only 10 nM/s whereas control platelets had reached a plateau of 120 ± 22 nM/s at that concentration.

Ristocetin Agglutination. Platelets were washed and fixed with formaldehyde immediately after treatment with *Serratia* protease (0.1 μg/mL) in order to measure only GPIb-dependent effects. Under these conditions, there was a loss of ∼20% in immunoreactive GPIb detected by TM60, and strong agglutination in response to ristocetin continued to be seen with *Serratia* protease-treated platelets although there was a rightward shift in the dose-response curve, indicating that about a 20% higher concentration of ristocetin was required for equivalent agglutination in *Serratia* protease-treated platelets than in controls (Figure 5).

DISCUSSION

The present studies show that limited degradation (∼10%) of both GPIb and the STDR by *Serratia* protease is associated with 80% loss of Ca^{2+} response to low concentrations of α-thrombin (0.5 nM) but only about 10% loss following treatment with elastase. This reduced Ca^{2+} response following treatment with a low concentration of *Serratia* protease suggested that the form of GPIbα constituting the

Table 1: Parameters for Best-Fit Model[a]

	one site				two site				three site			
	sum of squares	Df	*F*	*p*	sum of squares	Df	*F*	*p*	sum of squares	Df	*F*	*p*
controls	10454	73	10.8	0.001	6892	71	2.52	0.088	6428	69	---	---
Serratia (3)	7839	58	12.4	0.001	5408	56	---	---	**	**	**	**

[a] Df, degress of freedom; *F*, *F* ratio test; *p*, level of significance; ---, best-fit model; **, program suggested an unfit model. $n = 3$ in paired experiments for controls and platelets treated with *Serratia* protease (0.1 μg/mL; 30 min, rt).

Table 2: PPACK-Thrombin Binding Parameters of *Serratia* Protease-Treated Platelets[a]

	high affinity		moderate affinity		low affinity		
	K_d (nM)	sites/plt	K_d (nM)	sites/plt	K_d (nM)	sites/plt	NS
control	0.58 ± 0.19	36 ± 19	5.8 ± 4.0	260 ± 155	1395 ± 590	68940 ± 24130	$(2.30 \pm 0.77) \times 10^{-2}$
Serratia-treated	nd	nd	2.6 ± 0.8	180 ± 64	1604 ± 930	84320 ± 32950	$(1.87 \pm 1.05) \times 10^{-2}$

[a] K_d values are in nanomolar, and *R* values in sites per platelet $\pm$ SD. nd = not detectable in best-fit model. $n = 3$ in paired experiments for controls and samples treated with *Serratia* protease (0.1 μg/mL; 30 min; rt). NS = nonspecific binding; that is, the ratio of nonspecifically bound to free ligand.

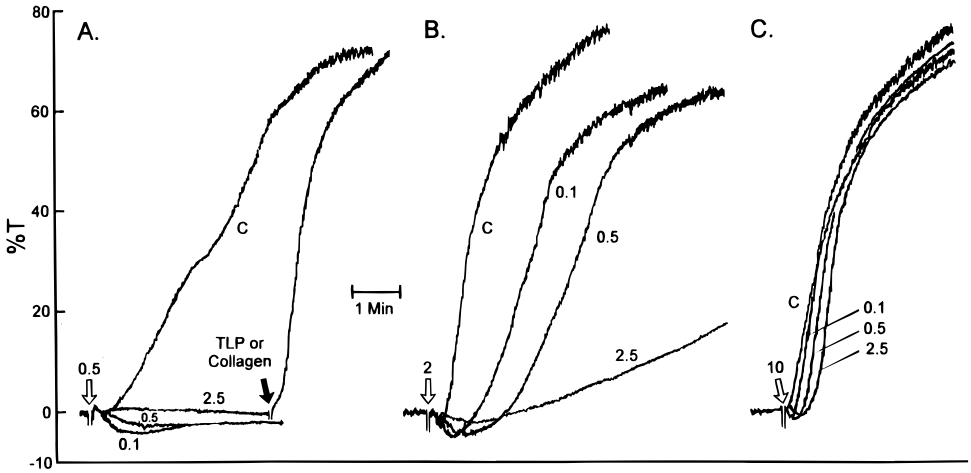

FIGURE 4: Aggregation studies. Low concentrations of α-thrombin (0.5 nM, panel A), intermediate concentrations (2 nM, panel B), and high concentrations (10 nM, panel C) were used to induce aggregation in control platelets (curve C) and platelets treated with *Serratia* protease for 30 min/rt at 0.1 μg/mL, 0.5 μg/mL, and 2.5 μg/mL as indicated. Thrombin was added at the open arrow in each panel. In panel A, TLP (15 μM) or collagen (2.5 μg/mL) was added at the filled arrow, showing that the platelets remained fully responsive.

high-affinity thrombin receptor may have been selectively degraded. This was confirmed by demonstrating a reduced aggregation response to low but not high concentrations of α-thrombin in *Serratia* protease-treated platelets. Using PPACK-thrombin to avoid the possible complications of active α-thrombin, there was a total loss of high-affinity binding sites without significant change in the number of moderate- or low-affinity sites. The dissociation constant at the moderate-affinity binding sites in *Serratia*-treated platelets (2.4 nM) is similar to the value reported for Bernard-Soulier platelets [3.4 nM (De Marco et al., 1991)] although about 25 000 GPIb residues remain on the platelet in the former case. Despite this loss of high-affinity binding and thrombin responsiveness, GPIbα remaining after *Serratia* protease treatment still supported ristocetin-induced agglutination of fixed platelets although with about 20% less sensitivity to ristocetin.

It has recently been shown that there are three sulfated tyrosine residues in GPIbα (Dong et al., 1994); desulfation of any one results in loss of thrombin binding ability although vWF binding is retained (Marchese et al., 1995). These variations in charged groups and in possible hydrogen bonding by the hydroxyl group of a desulfated tyrosine could cause conformational changes in GPIbα, leading to differences in susceptibility to *Serratia* protease. Alternatively, the selective degradation of the high-affinity thrombin response may be due to conformational changes in GPIbα induced by its presence in the multimolecular complex of membrane components that has been proposed as mediating high-affinity thrombin binding (Harmon & Jamieson, 1985).

The ability of *Serratia* protease to degrade GPIbα, but not GPV or GPIX, has been well established in previous studies (McGowan & Detwiler, 1985; Harmon & Jamieson, 1988; Yamamoto et al., 1991) although the preferential loss of the high-affinity binding form and the concomitant decreases in response to low α-thrombin concentrations have not been previously recognized. Elastase cleaves a 45 kDa amino-terminal fragment from GPIbα (Brower et al., 1985; Wicki & Clemetson, 1985). At an intermediate elastase concentration (5 nM), degradation ranged from 50% to 94% depending on which of the four anti-GPIb antibodies was evaluated, but no evidence of susceptible platelet subpopulations was seen by flow cytometry. These differences probably represent different sensitivities of GPIbα to elastase in different donors and different conformational sensitivities of the different antibodies. With either protease, degradation of the STDR appeared to progress from the amino-terminal domain: that is, loss of the TNATLPR sequence recognized by anti-TNA preceded loss of the NDKYEPF sequence recognized by anti-LLR while the domain recognized by anti-

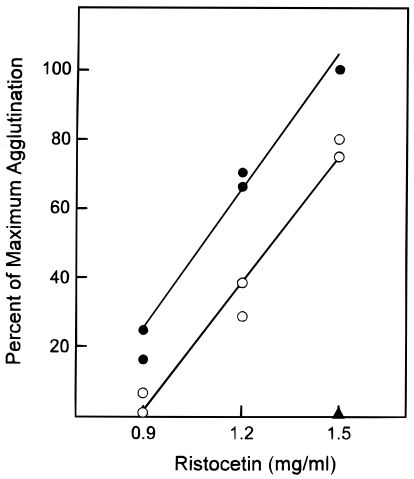

FIGURE 5: Ristocetin-induced agglutination. Control platelets (filled circles) were compared with 0.1 μg/mL *Serratia*-proteolyzed platelets (open circles) from the same donor. Platelets were treated with 50 mM Tris, pH 8.0 (control), or 0.1 μg/mL *Serratia* protease for 30 min at room temperature, washed, and fixed with paraformaldehyde as described under Experimental Methods. Platelet suspensions (4×10^8/mL, 0.2 mL) were mixed with an equal volume of autologous platelet-poor plasma for agglutination studies performed in duplicate. Values are presented after normalization to maximal control values obtained with 1.5 mg/mL ristocetin. A single agglutination response with 1.5 mg/mL ristocetin is shown for platelets treated with 2.5 μg/mL *Serratia* protease (filled triangle).

EPF was not affected by *Serratia* protease and was lost only at the highest elastase concentration. With neither protease did degradation proceed to the point where the platelets became unresponsive to TLP, and this has been shown to be the case even at much higher concentrations (25−100 μg/mL) of *Serratia* protease (Kinlough-Rathbone et al., 1995).

Our studies demonstrate that aggregation alone is not a satisfactory measure of platelet activation since full aggregation can be achieved at high α-thrombin concentrations in *Serratia* protease-treated platelets although $[Ca^{2+}]_i$ remains depressed relative to controls. These results also demonstrate the importance of utilizing a range of α-thrombin concentrations when evaluating the contributions of different receptors to platelet activation: several studies have concluded that the STDR can account for all of the platelet-activating effects of α-thrombin but have reached this conclusion after using thrombin concentrations of 10 nM, or higher, although physiologically-relevant thrombin concentrations are thought to be only about 0.5 nM (De Marco et al., 1991) and not to exceed 2 nM (Shuman & Levine, 1978).

The increased thrombin requirement in the concentration dependence of Ca^{2+} mobilization in *Serratia*-treated platelets and the prolonged time to reach peak $[Ca^{2+}]_i$ in this preparation are similar to those reported in the preceding paper for Bernard-Soulier platelets (Greco et al., 1996). The preceding paper (Greco et al., 1996) also showed that the hypothesis that GPIb sequesters small amounts of α-thrombin and thus reduces sensitivity to activation through the STDR (Hayes et al., 1994) is invalid based on the reduced Ca^{2+} mobilization in Bernard−Soulier and GPIb antibody-blocked platelets. This hypothesis is further compromised by the present demonstration that proteolysis of a minimal amount of GPIb results in essentially a complete loss in platelet sensitivity to low concentrations of α-thrombin, and not a slightly increased sensitivity as would be predicted from that hypothesis.

Antibody-blocked, protease-treated, and Bernard-Soulier platelets are similar with regard to their responses to α-thrombin. Although these three platelet preparations all lack a functional high-affinity receptor, there are important differences between them. The preparations blocked with TM60 or LJ-Ib10 contain all the charged residues of the platelet surface including the carbohydrate-rich (∼60%) glycocalicin moiety of GPIbα. In contrast, the loss of this moiety in the *Serratia* protease-treated preparation results in about 50% reduction in the net negative charge of the platelet surface due to the loss of bound sialic acid. Not only are these charged residues lost in Bernard-Soulier platelets, which are about twice the volume of control platelets, but so are charged groups associated with other components of the GPIb complex (GPIbα, GPIbβ, GPIX, and GPV). Despite these differences, we have found in every case that platelets possessing a functional high-affinity receptor give double the $[Ca^{2+}]_i$ response to incremental increases in α-thrombin concentration and react at a much faster rate than do platelets expressing only the moderate-affinity STDR.

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