The activation of human platelets by α-thrombin is mediated at least in part by cleavage of protease-activated G-protein-coupled receptors, PAR-1 and PAR-4. Platelet glycoprotein Ibα also has a high affinity binding site for α-thrombin, and this interaction contributes to platelet activation through a still unknown mechanism. In the present study the hypothesis that GpIbα may contribute to platelet activation by modulating the hydrolysis of PAR-1 on the platelet membrane was investigated. Gel-filtered platelets from normal individuals were stimulated by α-thrombin, and the kinetics of PAR-1 hydrolysis by enzyme was followed with flow cytometry using an anti-PAR-1 monoclonal antibody (SPAN 12) that recognizes only intact PAR-1 molecules. This strategy allowed measurement of the apparent $k_{\text{cat}}/K_m$ value for thrombin hydrolysis of PAR-1 on intact platelets, which was equal to $1.5 \pm 0.1 \times 10^7$ M$^{-1}$ sec$^{-1}$. The hydrolysis rate of PAR-1 by thrombin was measured under conditions in which thrombin binding to GpIb was inhibited by different strategies, with the following results. 1) Elimination of GpIbα on platelet membranes by moccarhadin treatment reduced the $k_{\text{cat}}/K_m$ value by about 6-fold. 2) A monoclonal anti-GpIb antibody reduced the apparent $k_{\text{cat}}/K_m$ value by about 5-fold. 3) An oligonucleotide DNA aptamer, HD22, which binds to the thrombin heparin-binding site (HBS) and inhibits thrombin interaction with GpIb, reduced the apparent $k_{\text{cat}}/K_m$ value by about 5-fold. 4) Displacement of α-thrombin from the binding site on GpIb using PPACK-thrombin reduced the apparent $k_{\text{cat}}/K_m$ value by about 5-fold, and 5) mutation at the HBS of thrombin (R98A) caused a 5-fold reduction of the apparent $k_{\text{cat}}/K_m$ value of PAR-1 hydrolysis. Altogether these results show that thrombin interaction with GpIb enhances the specificity of thrombin cleavage of PAR-1 on intact platelets, suggesting that GpIb may function as a "cofactor" for PAR-1 activation by thrombin.

Platelet activation by the coagulation protease α-thrombin plays a crucial role in physiologic hemostatic processes and in thrombotic diseases. The activation of human platelets by thrombin is mediated by at least two receptors belonging to the family of protease-activated receptors (PARs), and PAR-1 and PAR-4 (1–2). These receptors are activated upon cleavage by thrombin and mediate transmembrane signaling by coupling to G-proteins (1–2). α-Thrombin also binds with high affinity to the platelet glycoprotein Ib (GpIb) that belongs to the leucine-rich repeat family of proteins (3). Thrombin binding to GpIb contributes to platelet activation by the enzyme, as demonstrated by the finding that Bernard-Soulier platelets, which lack the GpIb-IX-V complex, have a delayed response to thrombin stimulation (4). In addition, several in vitro studies have demonstrated that the inhibition of thrombin binding to GpIb by different strategies causes a reduction in thrombin-induced platelet activation (5–9).

The mechanism by which the binding of α-thrombin to GpIb contributes to platelet activation is not clear. Numerous studies have shown that a proteolytic-active enzyme is required to activate platelets. PPACK-thrombin, which retains its ability to bind to GpIb, does not induce platelet aggregation (10). Moreover, GpIb does not undergo cleavage by thrombin. On the other hand, the cleavage of G-protein-coupled PARs seems to be essential in platelet activation and transmembrane signaling. The finding that thrombin binding to GpIb involves a distinct thrombin domain, the HBS, which is far from the thrombin catalytic site and the fibrinogen recognition site (7–9), would suggest that a ternary complex thrombin-GpIb-PAR-1 may form on the platelet membrane that could be responsible for optimal hydrolysis and signal transduction.

In the present study, the hypothesis that thrombin binding to GpIb may affect the hydrolysis of PAR-1 by the enzyme on intact platelets was investigated. The hydrolysis of PAR-1 was evaluated as a paradigm to construct a model where GpIb acts as a cofactor for PAR(s) cleavage.

**EXPERIMENTAL PROCEDURES**

*Materials—Human α-thrombin was purified and characterized as previously reported (11). Mutant thrombin R98A, which contains an alanine substitution at Arg-98 (thrombin B-chain numbering), and recombinant wild-type (WT) human thrombin were obtained and characterized as described previously (12). The mutant form retains its catalytic activity, although its ability to interact with heparin was severely impaired, as demonstrated by *in vitro* studies on heparin.*

*1 The abbreviations used are: PAR, protease-activated receptor; PE, phycoerythrin; PPACK, O-phenylalanine-proline-arginine-chloromethylketone; WT, wild-type; HBS, heparin binding site; mAb, monoclonal antibody; HPLC, high performance liquid chromatography.*

*2 Amino acid residues were numbered according to the thrombin sequence where the first amino acid of the B-chain was designated as number 1. (Arg-98 corresponds to Arg-101 in the chymotrypsin numbering system.)*
Precocin was purified from coffee bean, as described previously (19), at the Bakers Medical Research Institute, Prahran, Australia. HD22, a single-stranded DNA oligonucleotide with the sequence 5'AGTCGGTGTTAGCCGAGGGTGGTTGACT-3', which binds with high affinity to the heparin binding site of thrombin (15), was a kind gift of Prof. J. I. Weitz, (McMaster University, Hamilton, Ontario).

The PAR-1(38–60) peptide, LDFRRLRNPNKDEFWPEDEWE, was synthesized and characterized by mass spectrometry at Promm s. r. l. (Milan, Italy). The monoclonal antibody LiJb10, which is able to selectively inhibit thrombin-GpIb interaction, was a generous gift from Dr. Zavero Ruggieri (The Scripps Research Institute, La Jolla, CA).

Preparation of Platelets—Platelets from normal donors were obtained by gel filtration of platelet rich plasma onto Sepharose 2B columns (25 × 1 cm) equilibrated with HEPES buffer, as detailed above, containing 2 mM CaCl₂ and used at a final count of 200,000/μl. Platelet aggregation by thrombin was studied using a 4-channel PACKS-4 aggregometer (Helena Laboratories, Sunderland, UK), according to the Born method. In some experiments, platelets were stimulated by recombinant human WT thrombin and R98A thrombin at concentrations ranging from 0.39 nM to 50 nM. In other experiments, platelets were stimulated by thrombin at concentrations ranging from 0.625 to 20 nM, or by R98A thrombin at concentrations ranging from 3.125 to 100 nM, in the absence and in the presence of monoclonal antibody LiJb10 (0.15 mg/ml, final concentration). The velocity of absorbance change was measured and expressed as percent/min.

RESULTS

Measurement of Thrombin Hydrolysis of PAR-1 on Intact Platelets—Measurement of PAR-1 hydrolysis by α-thrombin was accomplished by a cytofluorimetric method, using a fluorescent monoclonal antibody, SPAN-12, that recognizes only the intact NH₂-terminal portion of the receptor. After cleavage, the mAb does not interact with the receptor, so that disappearance of the mAb signal reflects the hydrolytic reaction. Internalization of the receptor, which could also lead to loss of signal, has been shown to occur only for cleaved PAR-1 molecules (16–18). Under the conditions of the study, using intact gel-filtered platelets, the concentration of PAR-1 present on the platelet membrane (100–2000 copies of PAR-1/platelet, Refs. 19–20) is much lower (nanomolar) than the Km value of its hydrolysis by thrombin (micromolar range), so that the kinetics of PAR-1 cleavage can be fitted to an exponential equation, whose rate constant, kobs, is proportional to the kcat/Km value of thrombin-PAR-1 hydrolysis, according to the following equation (21).

\[ k_{\text{obs}} = e^{v_{\text{cat}}/K_m} \]  

In the experimental setup, the fluorescent signal given by the SPAN-12 mAb was monitored as a function of time at fixed thrombin concentrations. Although the antibody staining procedure is relatively long compared with PAR-1 cleavage kinetics at the concentrations of thrombin used, this limitation was overcome by performing the staining on ice and using high concentrations of hirudin to completely inhibit thrombin activity. Fluorescence signals measured as a function of time were fitted to the following equation,

\[ F(t) = F_0 \exp\left(-k_{\text{obs}} t\right) + F_\infty \]  

where F₀ is the fluorescence at time t, F₀ is the initial, F_∞ is the final fluorescence value, and k_{obs} is the observed rate constant for the single exponential decay. Knowing the enzyme concentration and using Eq. 2, the k_{cat}/K_m value could be calculated. Additional experiments were carried out to confirm the validity of this experimental approach. From Eq. 2 it follows that if the k_{obs} value actually reflects the k_{cat}/K_m value of the hydrolytic reaction, then it must depend on the enzyme concentration.

Thus, the observed rate constant was measured at different thrombin concentrations, ranging from 0.5 to 8 nM. The above...
Procedures“ included 60,000 gel-filtered platelets/μl and 0.12 μg/ml of the mAb. Solid lines are drawn according to Eq. 3 with the bestfit \( k_{obs} \) values: (○), 6.6 ± 0.6 × 10^{-3} sec^{-1}; (●), 1.2 ± 0.5 × 10^{-3} sec^{-1}; (●), 2.9 ± 0.6 × 10^{-2} sec^{-1}; (●), 1.2 ± 0.3 × 10^{-2} sec^{-1}; (●), 1.2 ± 0.3 × 10^{-3} sec^{-1}. Data are presented as mean ± S.E. from two different determinations. In the inset, the experimental values of \( k_{obs} \) pertaining to PAR-1 cleavage on intact platelets are plotted as a function of thrombin concentration, according to Eq. 2. The straight line was drawn according to the bestfit \( k_{cat}/K_m \) value of 1.5 ± 0.1 × 10^{-7} M^{-1} sec^{-1}.

analysis assumes that the concentration of the uncleaved receptor does not change during the time course of the experiments. To test this hypothesis, control experiments were carried out with a different mAb, WEDE-15, directed against the COOH-terminal domain of the PAR-1 cleavage site. Using thrombin in the nanomolar range, only a decrease in WEDE-15 binding to gel-filtered platelets was observed, consistent with an internalization process of the hydrolyzed PAR-1 receptor, which has been previously reported (16–18). This implies that over the time course of the experiments, newly exposed receptors (present in the surface connecting system) did not significantly alter the total PAR-1 concentration and that loss of WEDE-15 binding to platelets is likely because of an internalization process involving the cleaved receptor molecules. These findings therefore allow calculation of the apparent \( k_{cat}/K_m \) value pertaining to thrombin-PAR-1 interaction as outlined above.

As shown in Fig. 1, the pseudo first-order rate constant of PAR-1 cleavage increased as a function of α-thrombin concentration ranging from 0.5 to 8 nM, consistent with the canonical Michaelis scheme for serine protease activity that predicts a linear relation between the catalytic rate and the enzyme concentration (Eq. 2). The inset in Fig. 1 demonstrates this relationship with the slope of the straight line expressing the apparent \( k_{cat}/K_m \) value, which is equal to 1.5 ± 0.1 × 10^{-7} M^{-1} sec^{-1}. This value is similar to that for thrombin hydrolysis of the PAR-1(38–60) peptide in solution (see below), supporting the validity of the cytofluorimetric strategy used in this investigation.

Effect of Inhibiting Thrombin-GpIb Interaction on Hydrolysis

of PAR-1 in Intact Platelets—The effect of inhibiting thrombin-GpIb interaction on the enzyme hydrolysis rate of PAR-1 was evaluated by five different experimental strategies: 1) elimination of the thrombin binding site on GpIb by mocularagin treatment of gel-filtered platelets, 2) use of a monoclonal antibody S22, which binds to GpIbα and inhibits thrombin interaction, 3) use of the DNA aptamer HD22, which specifically interacts with the heparin binding site (HBS) of thrombin and inhibits enzyme binding to GpIbα, 4) use of a thrombin mutant, R98A, which bears a perturbation of the HBS structure, and 5) use of PPACK-thrombin in competition experiments with α-thrombin.

The removal of the NH₂-terminal 1–282 region of GpIbα by mocarhagin severely impaired the PAR-1 hydrolysis by thrombin. As shown in Fig. 2, the \( k_{obs} \) value for PAR-1 hydrolysis by 1 nM α-thrombin was reduced by about 6-fold in GpIbα-depleted platelets compared with untreated platelets. The inset in Fig. 2 shows that the \( k_{cat}/K_m \) value of PAR-1 hydrolysis in mocarhagin-treated platelets, which was 2.8 ± 0.3 × 10^{-7} M^{-1} sec^{-1}, decreased 5.4-fold in comparison with untreated platelets. Similar results were obtained by using two different competitive inhibitors of thrombin binding to GpIbα, the mAb S22, which interacts with the thrombin binding site on GpIbα (5, 22), and the DNA aptamer HD22, which interacts with the thrombin HBS. Fig. 3 clearly shows that in both cases a roughly 5–6-fold reduction in \( k_{obs} \) was obtained using a large excess of the inhibitors. Although the targets of the inhibitors were different, the effect was the same, as in both cases the thrombin-GpIbα interaction was inhibited. Altogether these results show that inhibition of thrombin binding to GpIbα causes a marked reduction of the apparent catalytic specificity constant of the thrombin-PAR-1 interaction.

The findings reported above raise the question of whether thrombin interaction with GpIbα might induce an intracellular
signal that could alter the interaction of thrombin with PAR-1, for instance through liberation of a second messenger molecule acting on the expression/conformation of PAR-1 molecules. PPACK-thrombin, which is catalytically inactive, yet still able to interact with GpIb, did not cause aggregation of gel-filtered platelets, even at 200 nM and did not induce any intracytoplasmic Ca²⁺ flux (data not shown), in accordance with numerous previous reports (6, 10). On the other hand, competition experiments showed that a high concentration of PPACK-thrombin (140 nM), by displacing α-thrombin from GpIbα, reduced by about 3-fold the apparent $k_{cat}/K_m$ value of PAR-1 hydrolysis on intact platelets, as shown in Fig. 4. Again, this experiment is in accord with the other functional experiments described above and corroborates the hypothesis that thrombin-GpIb interaction is able to enhance the specificity of thrombin cleavage of PAR-1 on intact platelets.

This hypothesis was further supported by the finding that a thrombin mutant, R98A, bearing an alanine substitution in the HBS of thrombin at Arg-98, showed a similar reduction of the $k_{obs}$ value of PAR-1 hydrolysis compared with recombinant WT human thrombin, as shown in Fig. 5. In control experiments, this diminished activity of PAR-1 hydrolysis on platelets could not be attributed to an intrinsic reduction of the catalytic efficiency of the thrombin mutant. Solution experiments employing the PAR-1-(38–60) peptide, bearing both the cleavage and the recognition site for thrombin (23), demonstrated that thrombin R98A cleaved the solution peptide with a $k_{cat}/K_m$ that was slightly higher than that of WT thrombin, as shown in Fig. 6. This finding is consistent with the hypothesis that the reduction of the $k_{cat}/K_m$ value of PAR-1 hydrolysis on intact platelets is likely because of a defective interaction of the thrombin mutant with GpIbα. Moreover, the biological effect of this mutation on platelet activation can be seen in Fig. 7, which shows that the EC₅₀ value for platelet aggregation by R98A is roughly 10-fold higher compared with WT thrombin.

**Effect of Anti-GpIb mAb LJ1b10 on Platelet Aggregation by WT Thrombin and R98A Mutant Thrombin**—These experiments were carried out to evaluate whether the thrombin HBS could be involved in the interaction with other thrombin receptors. PAR-1 was already demonstrated not to be involved in HBS ligation (6–9), but PAR-4 could potentially contain a binding site for the thrombin HBS, although a close inspection of the PAR-4 primary sequence (24) does not show a negatively charged domain to support this hypothesis. The mAb LJ1b10 specifically inhibits the interaction of thrombin with GpIbα, without affecting the binding of von Willebrand factor to GpIbα (22, 25). The use of this mAb allows one to rule out a potential inhibitory effect on the aggregation of gel-filtered platelets that could be attributed to an impaired interaction of GpIb with platelet von Willebrand factor, which is released upon thrombin stimulation.

As shown by Fig. 8A, platelets stimulated by WT thrombin had a rightward shift in the aggregometric dose-response curve in the presence of 0.15 mg/ml LJ1b10 (with EC₅₀ values of 4.43 ± 0.4 nM and 10.15 ± 0.77 nM, respectively). This result is in agreement with the hypothesis that specific inhibition of thrombin binding to GpIbα causes a reduced platelet response to the agonist. In contrast, the dose-response curve obtained by stimulating platelets with thrombin mutated at the HBS (Fig. 8B) was not affected at all by the presence of the mAb (with EC₅₀ values of 23.5 ± 2.8 nM and 22.1 ± 5 nM, respectively). This result is in agreement with the concept that the thrombin-GpIbα interaction involves the enzyme HBS, as the LJ1b10 mAb could not induce any inhibition of the R98A thrombin-induced platelet activation. This result would also suggest that the
thrombin mutant. Gel-filtered platelets used at 200,000/thrombin mutant (○) and R98A thrombin mutant (●) and 0.5 μM peptide. The experimental points are the mean of two determinations. Solid lines were drawn by nonlinear regression according to Eq. 1, with the bestfit $k_{cat}$ values: (○), 4.3 ± 0.3 × 10⁻² sec⁻¹; (●), 7 ± 0.8 × 10⁻² sec⁻¹.

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FIG. 7. Platelet aggregation capacity of the wild-type and the R98A thrombin mutant. Gel-filtered platelets used at 200,000/μl were exposed to different concentrations of wild-type (○) or R98A thrombin mutant (●). Data are presented as mean ± S.E. from two different determinations. The solid lines were drawn by nonlinear regression with the bestfit $EC_{50}$ values equal to 1.1 ± 0.2 and 10 ± 2 nM for the wild-type and the R98A thrombin mutant, respectively.

thrombin HBS is involved only in GpIb binding and not in the interaction with other platelet receptors, such as PAR-4.

DISCUSSION

In this study, the role of GpIbα in the hydrolysis of PAR-1 by thrombin and in thrombin-induced platelet activation was examined. GpIbα binds thrombin with high affinity and contributes to platelet activation by the enzyme; however, there is no evidence that thrombin ligation to GpIb per se is able to trigger platelet activation. Results from the present study show that GpIbα can function as a cofactor for PAR-1 cleavage and activation in human platelets. In fact, by directly measuring the hydrolysis of PAR-1 on intact platelets, it was possible to evaluate the effect of the inhibition of thrombin binding to GpIbα on PAR-1 cleavage. The cytofluorimetric strategy used to detect PAR-1 hydrolysis on platelets allowed measurement of the apparent specificity constant, $k_{cat}/K_m$, pertaining to thrombin hydrolysis of the receptor. It is noteworthy that the value measured in intact cells is similar to that for hydrolysis of the PAR-1-(38–60) peptide in solution, as confirmation of the validity of the experimental method. Interestingly, the inhibition of thrombin interaction with GpIbα on platelets, obtained by different experimental strategies, consistently showed a 5- to 6-fold reduction of the specificity rate constant of PAR-1 hydrolysis by thrombin. This finding might suggest that in the absence of GpIbα on the platelet membrane, the thrombin-PAR-1 interaction is partially hampered or less productive, given that the $k_{cat}/K_m$ value is much lower than that measured with the PAR-1-(38–60) peptide in solution.

Thrombin bound to GpIbα via its HBS, whose blockade caused a reduction in both PAR-1 hydrolysis and platelet aggregation. That the HBS is not directly involved in the interaction with PAR-1 was demonstrated by the experiment showing that the HBS mutant thrombin, R98A, cleaves the PAR-1-(38–60) peptide in solution with an apparent $k_{cat}/K_m$ similar to or even higher than that pertaining to WT thrombin. Platelet aggregation by R98A thrombin was not sensitive to the presence of the anti-GpIbα mAb LJ-Ib10, whereas the aggregation by WT thrombin showed a rightward-shift of the dose-response curve (Fig. 8). In the former case, in fact, thrombin binding to GpIbα was already impaired by HBS perturbation, whereas in the latter case use of the mAb blocked the GpIb contribution to platelet aggregation. The effect of the anti-GpIb mAb on the aggregation by WT thrombin is in agreement with the inhibitory effect of the mAb on PAR-1 activation by thrombin via the inhibition of enzyme binding to GpIbα.

Because inhibition of the thrombin-GpIbα interaction, obtained through different methods, caused in all cases a reduction of the PAR-1 hydrolysis rate, this finding was interpreted as a consequence to a positive linkage between thrombin binding to GpIbα and the catalytic interaction of this enzyme com-
plex with PAR-1. It was previously shown that GpIbα in solution does not alter the kinetic constants of hydrolysis of the PAR-1-(38–60) peptide (7, 26). This implies that membrane phenomena are responsible for the effect observed using intact platelets.

One possible mechanism of action of GpIb could be that this transmembrane glycoprotein mediates a signal transduction event that could modify the membrane and/or PAR-1-folding, so that PAR-1 would be more activatable by thrombin. However, experiments with PPACK-thrombin did not confirm this hypothesis. In fact, intraplatelet Ca2+ flux was not observed using PPACK-thrombin that binds to GpIb but does not cleave PAR-1 (data not shown). Moreover, when PPACK-thrombin was used along with α-thrombin, it caused, as shown in Fig. 4, a 3-fold reduction of the apparent $k_{cat}/K_m$ value pertaining to PAR-1 cleavage, in agreement with the effect of the other inhibitors of thrombin-GpIb binding. PPACK-thrombin would have caused an enhancement of PAR-1 cleavage, if a positive intracellular signal were generated. It is postulated that von Willebrand factor binding to GpIb induces signal transduction (27), whereas this phenomenon has not yet been demonstrated for thrombin binding to GpIb. Accordingly, very recent findings have indicated that thrombin-GpIb interaction is a necessary but not sufficient condition to induce a procoagulant capacity in the procoagulant platelet membrane (28). In addition to thrombin binding to GpIb, the procoagulant platelet activity has been demonstrated to arise from both the involvement of the GpIIb-IIIa complex and the platelet-platelet interaction as well (28).

It may be hypothesized that GpIb would enhance the productive collisions between the enzyme and the substrate, PAR-1, on the membrane surface, essentially acting as a catalyst for the reaction. This hypothesis hinges on the structural properties of the components involved in the interaction. The formation of a ternary thrombin-GpIb-PAR-1 complex can be hypothesized, as GpIb binds to the thrombin HBS, whereas PAR-1 interacts with both the exosite referred to as fibrinogen recognition site and the catalytic pocket (6, 9, 23). The positive effect of GpIb interaction with thrombin might be considered as a template function, which could enhance the number of productive collisions between the enzyme and the PAR-1 substrate. The mechanisms through which this effect takes place is not in the realm of this study. Further studies are needed to demonstrate experimentally the formation of the ternary thrombin-GpIb-PAR-1 complex, as well as to unravel some structural issues pertaining to the components of this complex. GpIb is in fact an elongated molecule with a longitudinal axis of ~550 Å (29). Because the thrombin binding site on GpIbα and the NH2-terminal PAR-1 domain are expected to be located at roughly 300 and 65–70 Å from the membrane surface, respectively, it has to be demonstrated how a ternary thrombin-GpIb-PAR-1 adduct could form on the platelet membrane.

From a physical standpoint, one can reasonably assume that the kinetics of thrombin binding to GpIb is faster than kinetics of formation of the thrombin-PAR-1 Michaelis adduct. Thus, although the association rate constants of thrombin-GpIb and thrombin-PAR-1 may be similar, the concentration of GpIb is at least 1–2 orders of magnitude higher than that of PAR-1 on the platelet membrane (19–20), and thus the bimolecular interaction of thrombin with GpIb is faster than the formation of the thrombin-PAR-1 adduct. Hence GpIb may function as a cofactor for PAR-1 activation by thrombin in situ. It is not clear from this investigation whether this cofactor activity is exclusively mediated by GpIb alone, or through more complex interactions involving the entire glycoprotein adduct, GpIb-IX-V. GpV in fact is known to be cleaved by thrombin, but its role in platelet activation remains unknown (30).

The cofactorial function of GpIb would resemble the one recently identified for thrombin interaction with PAR-3 and PAR-4 on mouse platelets (31). Similarly to GpIb for human platelets, PAR-3 in mouse platelets does not mediate transmembrane signaling, but its loss inhibits the mouse platelet activation by low thrombin concentrations. The model for PAR-3 function predicts that this receptor binds thrombin that remains available on platelet membrane to cleave and activate nearby PAR-4 molecules. Although PAR-3 and GpIb are completely different molecules, they might share a cofactorial function for PARs activation. This model may be of interest for human platelets, because it introduces the concept that cofactors for PARs activation might regulate the specificity of response to proteases of target cells. Regulation of cofactor function, rather than that of the receptor itself, might thus become crucial in modulating the effects of proteases on cells. This concept seems particularly to fit for proteins and enzymes that do not bear phospholipid binding sites. Membrane surface is one of the most relevant cofactors usually involved in strongly accelerating coagulation reactions (32). Many coagulation factors, such as Factor V, and Factor VIII, operating on a membrane surface, bear specific phospholipid binding sites. The interaction of thrombin with its PAR-1 substrate, inserted in the platelet membrane, could be hampered by the lack in the thrombin molecule of a domain capable of binding to membrane phospholipids. The high affinity interaction of thrombin with GpIb could overcome this limitation. The thrombin-GpIb interaction could pay the energetic cost to favor an otherwise hampered interaction between the enzyme and its macromolecular substrate. Likewise, an interesting model for this kind of cofactorial function of protease receptors is that recently described for the endothelial Protein C receptor involved in activating Protein C along with the thrombin-thrombomodulin complex on the surface of endothelial cells (33).

At this point one might question whether the 5–6-fold increase of the $k_{cat}/K_m$ value of PAR-1 hydrolysis caused by thrombin interaction with GpIb could be of physiological relevance. The present data cannot indicate whether the increase of the $k_{cat}/K_m$ value arises from either an increase of $k_{cat}$, or a decrease of $K_m$ value, or else a combination of both phenomena. The present study shows that, under physiological conditions, thrombin cleaves PAR-1 according to a pseudo first-order kinetics. Under this condition, whatever the mechanism, an increase of 5–6-fold of the $k_{cat}/K_m$ value of PAR-1 hydrolysis leads to a 5–6-fold increase of the net velocity of its cleavage, according to Eq. 2. Although the GpIb effect on PAR-1 activation is not dramatic, if compared with other similar phenomena of the blood coagulation system (33), it could likely be physiologically relevant. In fact, the PAR-1 shut-off is a very rapid process after cell activation by thrombin (34), and thus a very efficient enzyme interaction with PAR-1 molecules is needed to generate an optimal amount of activated receptors required for a full cell activation.

The results from the present and previous studies show that α-thrombin binding to GpIbα has a net prohemostatic effect, as not only is this interaction able to enhance the hydrolysis of PAR-1 on the platelet membrane, but also protects the enzyme from the heparin-catalyzed inhibition by anti-thrombin III (9). The demonstration that the specific inhibition of thrombin-GpIb interaction leads to reduced thrombin cleavage of PAR-1 on intact platelets, might open the way to new strategies for specific modulation of platelet responses to thrombin stimulation in different clinical settings.

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