We describe here the alteration of thrombin specificity induced by its interaction with glycocalicin. Although a newly described thrombin receptor that belongs to the family of seven-transmembrane domain receptors has recently been shown to play a major role in mediating platelet activation, several studies using platelets lacking GPIb provide most extensive evidence that GPIb also takes part in the process of thrombin-induced platelet activation. A decrease in the number of GPIb copies, such as in platelets from patients with the Bernard-Soulier syndrome, or in platelets treated with chymotrypsin, elastase, or Serratia marcescens protease, induces a decrease in platelet sensitivity to thrombin together with an increase in the lag phase preceding the platelets responses. In addition, some monoclonal antibodies directed against the 45-Kd region of the α-chain of GPIb have been found to inhibit either low-dose thrombin-induced platelet activation only or in association with the inhibition of ristocetin-dependent platelet agglutination. Altogether, these observations suggest that, at low thrombin concentrations, binding to GPIb could serve either to accelerate reactions occurring through the newly described thrombin receptor or to trigger an alternative platelet activation pathway.

We have previously shown that thrombin binding to GPIb occurs through lysine residues located within the segment 18-73 of the human thrombin B chain and is abolished by a peptide mimicking the carboxy-terminal tail of hirudin. These observations suggest that thrombin-GPIb interaction is mediated by the positively charged surface region remote from the catalytic site, known as the thrombin anion-binding exosite. The anion-binding exosite appears as a critical structure of thrombin and is required for the recognition of various substrates or ligands. Therefore, thrombin-GPIb interaction, regardless of its participation in mediating platelet activation, could modulate the various activities of thrombin. In this study, we show that the soluble proteolytic fragment derived from GPIb, glycocalicin, binds thrombin without affecting its affinity for small substrates or protein C, but competes with fibrinogen and thrombomodulin for thrombin binding.

**MATERIALS AND METHODS**

**Materials.** H-D-Phe-Pip-Arg-pNA (S2238), Glu-Pro-Arg-pNA (S2366), and human purified fibrinogen were from Kabi Diagnostics (Uppsala, Sweden); bovine serum albumin (BSA), HEPES, and diisopropylfluorophosphate (IPr2PF) were from Sigma Chemical Co (St Louis, Mo); tetradichlorodiphenylglycouril (Iodogen) was from Pierce Chemical Co (Rockford, IL); and carrier-free Na125I was from CIS Bioindustries (Saclay, France).

Human α-thrombin was prepared as previously described. All the preparations used throughout this study were more than 97% α-thrombin, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). α-Thrombin was 125I-labeled using the solid-phase reactant Iodogen. Glycocalicin was purified as previously described and its purity was assessed by gel electrophoresis (Fig 1). No contaminants are apparent. In some experiments, glycocalicin was reduced and alkylated according to standard procedures. Briefly, glycocalicin (10 mg/mL in 0.5 mol/L Tris, 6 mol/L guanidine-HCl, pH 8.5) was incubated with 10 mmol/L dithiothreithol for 2 hours at 37°C and then for 2 hours at 0°C with 20 mmol/L dithiothreithol. Samples were dialyzed in 10 mmol/L Tris, 2 mol/L guanidine HCl, pH 8.5, followed by 50 mmol/L Tris,

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0.3 mol/L NaCl, pH 7.4. Nonreduced glycocalicin was alkylated using the same procedure.

Fibrin monomers were prepared by incubating fibrinogen (30 μmol/L in 50 mmol/L HEPES, 125 mmol/L NaCl, pH 7.4) with 1 nmol/L α-thrombin. After washing with 500 mmol/L NaCl, 50 mmol/L HEPES, pH 7.4, the clot was dissolved in 20 mmol/L acetic acid.

Rabbit lung thrombomodulin,28 bovine protein C,29 and human antithrombin III (ATIII)28 were isolated according to published procedures.

Amidolytic activity assays. The hydrolysis of S2238 by thrombin was measured at room temperature in 10 mmol/L HEPES, 10 mmol/L Tris, 100 mmol/L NaCl, 0.1% polyethylene glycol (PEG), pH 7.8.27 The release of p-nitroaniline was followed by monitoring absorbance at 405 nm in a Beckman (DU 70) spectrophotometer (Fullerton, CA).

Kinetics of thrombin inhibition by iPr2PF. The rate of inactivation of thrombin by iPr2PF was determined at room temperature as previously described.30 Briefly, thrombin (1 nmol/L in 10 mmol/L Tris, 100 mmol/L NaCl, 0.1% PEG, pH 7.8) was incubated with iPr2PF (0.15 mmol/L) in the absence or presence of glycocalicin (5 μmol/L). At timed intervals, aliquots were assayed for residual thrombin activity on S2238 (0.125 mmol/L).

Fibrinogen clotting assay. Clotting was determined at 37°C using thrombin (8 nmol/L in 20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1% BSA) and purified human fibrinogen (8 μmol/L) in the presence of variable amounts of glycocalicin.

Thrombin interaction with fibrin. Reaggregation of fibrin monomers from acetic acid solutions was initiated by diluting an aliquot of solubilized fibrin into 9 vol of 50 mmol/L Tris, 50 mmol/L NaCl, pH 7.5, 1% BSA in the presence of iodinated thrombin (2 nmol/L) and variable amounts of glycocalicin. After 2 hours at 37°C, samples were centrifuged (12,000 g for 10 minutes). Free thrombin in the supernatant and thrombin associated to the fibrin polymer in the pellet were quantified by gamma counting. Control experiments were performed using purified human ATIII in place of glycocalicin.

Thrombin interaction with protein C. The rate of protein C activation was measured in the absence or presence of thrombomodulin as previously described.30 In the absence of thrombomodulin, 12.5 μmol/L thrombin was incubated at 37°C with 0.12 μmol/L protein C in 20 mmol/L Tris, 0.15 mol/L NaCl, pH 7.5, 1% BSA, in the presence of buffer or glycocalicin (6.5 μmol/L). At timed intervals, the activation was quenched by adding ATIII (3.8 μmol/L) and heparin (0.5 U/mL), and activated protein C was measured using S2366. Protein C activation by thrombin-thrombomodulin was measured using thrombin (1.25 and 2.5 mmol/L in 20 mmol/L Tris, 0.15 mol/L NaCl, 10 mmol/L CaCl2, pH 7.5, 1% BSA) and thrombomodulin in a 1:1 molar ratio, in the presence of various amounts of glycocalicin. At timed intervals, the reaction was quenched and activated protein C was measured as described above.

Thrombin binding to insolubilized thrombomodulin. Thrombin binding to thrombomodulin was measured using a solid-phase assay. Microwell plates (Immuno; Dynatech, Saint-Cloud, France) were coated with 100 μL rabbit thrombomodulin (0.25 μg/mL in 15 mmol/L Na2CO3, 35 mmol/L NaHCO3, pH 9.4) for 18 hours at 4°C. Unbound material was removed by washing with 50 mmol/L Tris, 50 mmol/L NaCl, pH 7.4. The wells were blocked with 150 μL 1% BSA in the same buffer for 2 hours and then washed with Tris-NaCl buffer. Then, 100 μL thrombin at various concentrations in 50 mmol/L Tris, 50 mmol/L NaCl, pH 7.4, containing 0.5% BSA and 0.05% Tween 20, was added to the wells in the presence of various amounts of glycocalicin. Thrombin bound to thrombomodulin was detected using S2238 (0.2 mmol/L in 50 mmol/L Tris, 0.15 mmol/L NaCl, pH 8.3). Paranitrophenol was detected at 405 nm on a microplate reader (Dynatech) at various times between 30 and 120 minutes. Over this period, the rate of S2238 hydrolysis was linear with time. Blanks were obtained by omitting the coating with thrombomodulin or by replacing thrombin by buffer.
RESULTS

Effect of glycocalicin on thrombin catalytic activity. Glycocalicin up to 4 μmol/L did not modify the initial rate of hydrolysis of S2238 (2 to 16 μmol/L) by 0.5 nmol/L α-thrombin (data not shown). Moreover, glycocalicin did not modify the rate of thrombin inhibition by iPrzPF (Fig 2), indicating that the soluble fragment of GPIb does not alter the catalytic site of thrombin.

Effect of glycocalicin on thrombin interaction with fibrin (ogen). As previously reported by others, glycocalicin prolonged the thrombin clotting time of purified fibrinogen in a dose-dependent manner. Using 8 nmol/L α-thrombin and 8 μmol/L fibrinogen, 50% thrombin inhibition was achieved with 6 μmol/L glycocalicin.

When fibrin monomers were allowed to polymerize in the presence of iodinated thrombin, free and fibrin-bound thrombin were conveniently separated by centrifugation. Glycocalicin was shown to inhibit the binding of thrombin to fibrin in a dose-dependent manner. When the reciprocal of fibrin-bound thrombin was plotted as a function of glycocalicin concentration at several concentrations of fibrin monomers (Fig 3), glycocalicin appeared to behave as a competitive inhibitor with a Ki of 0.1 μmol/L. An identical value was obtained using a double reciprocal plot of fibrin-bound thrombin versus fibrin monomers concentrations, with the slopes obtained being secondarily plotted versus the glycocalicin concentration.

Under the same conditions, purified human ATIII (0.5 μmol/L) failed to inhibit thrombin binding to fibrin. This result is consistent with those previously reported from kinetic studies. It also indicates that the inhibition of thrombin binding to fibrin by glycocalicin is specific.

The effect of reduction and/or alkylation of glycocalicin on its ability to inhibit thrombin binding to fibrin was investigated. Using 0.5 μmol/L fibrin monomers, 2 μmol/L reduced and alkylated glycocalicin produced 45% inhibition, compared with 67% inhibition observed with the same amount of alkylated nonreduced glycocalicin and 85% inhibition with control glycocalicin. The decrease in the inhibitory potency of glycocalicin upon reduction suggests that the two disulfide bonds, 209-248 and 211-264, might contribute to maintaining the binding region optimal conformation.

Effect of glycocalicin on thrombin interaction with protein C and thrombomodulin. Glycocalicin, up to 6.5 μmol/L, did not modify the rate of protein C activation in the absence of thrombomodulin. After 1 hour of incubation of 0.12 μmol/L protein C with 12.5 nmol/L thrombin, the amount of activated protein C generated, expressed as the change in absorbance at 405 nm per hour, was 0.21 and 0.22 in the absence or presence of 6.5 μmol/L glycocalicin, respectively. In contrast, glycocalicin caused a significant decrease in the rate of protein C activation in the presence of thrombomodulin (Fig 4A), suggesting that glycocalicin inhibited thrombin binding to thrombomodulin. This inhibition was dependent on the concentration of glycocalicin. When the reciprocal of the rate of protein C activation was plotted as a function of the concentration of glycocalicin for two concentrations of thrombin-thrombomodulin complexes (Fig 4B), glycocalicin appeared to behave as a competitive inhibitor with a Ki of 3 μmol/L.

Binding of thrombin to thrombomodulin was further studied using thrombomodulin immobilized on microtitration plates. Thrombin bound to thrombomodulin with a Ka of 0.4 nmol/L, consistent with published values. In addi-
Fig 4. Effect of glycocalcin on protein C activation by thrombin. (A) Bovine protein C (0.12 μmol/L) was incubated with 1.25 nmol/L of an equimolar complex of α-thrombin and rabbit thrombomodulin in the absence or presence of 6.5 μmol/L glycocalcin. Activated protein C, measured using S2366 (0.2 mmol/L), was expressed as the 405 nm absorbance change in 10 minutes. (B) Bovine protein C (0.12 μmol/L) activation by 1.25 nmol/L thrombin was measured in the presence of various amounts of glycocalcin. The reciprocal of the rate of protein C activation (v) versus the concentration of glycocalcin is presented.

FIG 5. Effect of glycocalcin on thrombin binding to thrombomodulin. Thrombin ([●] 0.125; [■] 0.25; or [△] 0.5 nmol/L in 50 mmol/L Tris, 50 mmol/L NaCl, pH 7.4, 0.5% BSA, 0.05% Tween 20) was incubated in microplate wells coated with 25 ng thrombomodulin in the presence of various amounts of glycocalcin. Bound thrombin, measured using S2238, was expressed as the 405 nm absorbance change per minute at 37°C. A double reciprocal plot of the amount thrombomodulin bound versus the concentration of glycocalcin is presented.


discussion

This study shows that glycocalcin interacts with thrombin at a site shared by other ligands, leading to modifications of thrombin specificity. Glycocalcin consists of the extracellular part of the α-chain of GPIb, which can be removed from the platelet surface by treatment with various proteases. Glycocalcin contains a hydrophilic region between residues 215-299 involved in vWF receptor function. Although the precise location of the thrombin binding site on GPIb is not yet determined, all the studies performed indicate that it is also found in this hydrophilic portion of glycocalcin. Thus, glycocalcin-thrombin interaction is well representative of GPIb-thrombin interaction.

Previous studies have indicated that thrombin catalytic activity is not required for binding to GPIb. In the present study, we show that glycocalcin-thrombin interaction does not modify the amidolytic activity of the enzyme or the kinetics of its inhibition by iPrzPF, indicating that GPIb does not alter the conformation of the catalytic site of bound thrombin. This feature of GPIb binding is shared with fibrin monomers or thrombomodulin binding, which also preserves the catalytic activity of the enzyme. In contrast, binding of thrombin to its platelet receptor has been shown to inhibit chromogenic substrate hydrolytic activity, which is consistent with the fact that the receptor is a thrombin substrate, whereas GPIb is not.

The involvement of the thrombin anion binding exosite in thrombin-GPIb interaction has been previously documented. Thrombin binding to GPIb has been shown to be prevented when the anion binding exosite is disrupted by trypsin-catalyzed β cleavage at Arg 73, altered by chemical modification of lysine residues, or masked by the carboxy-terminal tail of hirudin. The exosite has also been shown to mediate the recognition of fibrinogen, protein C, thrombomodulin, and platelet receptor. However, the study of recombinant mutant thrombins with single amino acid substitutions has indicated that, although fibrinogen, protein C, and thrombomodulin may interact with some common residues within the anion binding exosite, other contacts are unique for each thrombin-ligand pair, suggesting that these three ligands bind to overlapping but not identical sites. This raised the question of the extent to which the binding site for GPIb overlaps with the binding sites for fibrinogen, protein C, and thrombomodulin. We show in the present study that GPIbs, fibrin, and thrombo-
modulin bind to overlapping sites on thrombin, whereas the binding sites for GPIb and protein C are clearly distinct. Glycocalicin competes with both fibrin and thrombomodulin for thrombin binding, but the inhibition constant (Ki) for fibrin-thrombin interaction is at least one order of magnitude lower than the Ki for thrombomodulin-thrombin interaction. This raises the possibility that the binding sites for GPIb and fibrin share larger common structures than the sites for GPIb and thrombomodulin.

The newly described platelet thrombin receptor undergoes a proteolytic cleavage by thrombin unmasking a new amino-terminal sequence that functions as a tethered ligand effecting receptor activation. The sequence of the thrombin cleavage site within the receptor resembles that of bovine protein C, which is a poor substrate for thrombin. However, it has been proposed that the proteolytic attack on the receptor is favored by the binding of thrombin to a hirugen-like sequence on the receptor via the anion-binding exosite of thrombin. We have previously pointed to a highly negatively charged sequence in GPIbα (residues 269-287) that also resembles the C-terminal hirudin sequence and we confirm in the present study that the thrombin anion-binding exosite is involved in the thrombin-GPIb interaction. Thus, some of the structural requirements for thrombin binding to its platelet receptor and to GPIb appear to be very similar. However, there is no evidence of competition between GPIb and the receptor for thrombin binding because thrombin-GPIb interaction appears to increase the sensitivity and the rate of the platelet responses that are now known to be elicited via the thrombin receptor. This raises the possibility that binding of thrombin to the large number of GPIb copies present on the platelet surface might promote the proteolytic attack on the thrombin receptor, either by increasing the local concentration of thrombin or by changing the thrombin specificity. However, we cannot exclude that receptor-dependent platelet activation might be favored by other mechanisms. From recent reports using synthetic peptides mimicking the unmasked N-terminus of thrombin receptor, it appears that all the signal transduction necessary for platelet activation, particularly for phospholipase C and phosphatidylinositol 3 kinase activation, might be provided by receptor proteolysis. However, the activation of a GPIb-coupled alternative pathway priming phospholipase C activation cannot be ruled out. Indeed, such a GPIb-coupled signal elicited by vWF binding to GPIb has been recently shown to induce activation of phospholipase A2. In addition, elements within the cytoplasmic domain of GPIb are connected to actin through actin binding protein and a CAMP-dependent phosphorylated serine may be involved in actin polymerization. Thus, a GPIb-mediated modification of the cytoskeleton upon thrombin binding cannot be ruled out. Studies of the molecular interactions between GPIb and the thrombin receptor should help to clarify their respective roles in the mechanisms of thrombin-induced platelet activation.

A major unsolved problem is why PPACK active site inhibited α-thrombin, despite the fact that it still binds to GPIb and presumably to the hirugin-like domain of the platelet receptor, is only a weak inhibitor of platelet activation by α-thrombin. This might imply that only a few sites need to be occupied by active thrombin and that the apolar domain surrounding the active site of thrombin and occupied by PPACK might contribute to stabilize the binding of thrombin to its receptor.

The observations presented here that GPIb alters the specificity of thrombin open the possibility that GPIb modulates thrombin functions. Although an anticoagulant effect might be expected through trapping of thrombin in platelet aggregates, in vitro the presence of GPIb has always been shown to have an amplifying effect on the platelet responses to thrombin. The mechanism of this effect remains to be further defined.

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REFERENCES

13. Tam SW, Fenton JW, Detwiler TC: Platelet thrombin


Thrombin interaction with platelet glycoprotein Ib: effect of glycocalicin on thrombin specificity

M Jandrot-Perrus, KJ Clemetson, MG Huisse and MC Guillin