We describe here the alteration of thrombin specificity induced by its interaction with glycocalcin. Glycocalcin is the external part of platelet glycoprotein Ib (GPlb) and contains binding sites for von Willebrand factor and thrombin. Taking advantage of its solubility, we have used glycocalcin in competition assays on various thrombin activities. Glycocalcin did not inhibit chromogenic substrate hydrolysis nor diisopropylfluorophosphate IP2 (PF) incorporation, indicating that thrombin binding to GPlb does not alter access to or the conformation of the thrombin catalytic site. Glycocalcin competitively inhibited thrombin binding to fibrin (Ki = 0.1 μmol/L) and blocked fibrinogen clotting activity of thrombin. Glycocalcin also inhibited thrombin binding to thrombomodulin in a competitive manner (Ki = 3 to 5 μmol/L), but failed to prevent thrombin interaction with protein C in the absence of thrombomodulin. Previous results have indicated that GPlb binds to thrombin within the anion binding exosite masked by the carboxy-terminal hirudin peptide 54-65. The present results confirm the implication of the anion binding exosite in GPlb recognition, and further indicate that the thrombin binding site for GPlb overlaps with the thrombin binding sites for fibrin and thrombomodulin, whereas it is distinct from the thrombin binding site for protein C. Some of the structural requirements for thrombin binding to GPlb appear to be very similar to those reported for binding to its platelet receptor. However, thrombin-GPlb interaction does not appear to compete with receptor hydrolysis but rather increases the sensitivity and the rate of platelet responses elicited by the receptor.

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Glycoprotein Ib (GPlb) is the major sialglycoprotein of the platelet membrane that serves both as a receptor for von Willebrand factor (vWF) and as a high-affinity binding site for α-thrombin.3,4 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,10 several studies using platelets lacking GPlb provide most extensive evidence that GPlb also takes part in the process of thrombin-induced platelet activation. A decrease in the number of GPlb copies, such as in platelets from patients with the Bernard-Soulier syndrome,11,12 or in platelets treated with chymotrypsin,13,14 elastase,15,16 or Serratia marcescens protease,17,18 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 GPlb have been found to inhibit either low-dose thrombin-induced platelet activation only19 or in association with the inhibition of ristocetin-dependent platelet agglutination.20,21 Altogether, these observations suggest that, at low thrombin concentration, binding to GPlb 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 GPlb 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.23 These observations suggest that thrombin-GPlb interaction is mediated by the positively charged surface region remote from the catalytic site, known as the thrombin anion-binding exosite.24 The anion-binding exosite appears as a critical structure of thrombin and is required for the recognition of various substrates or ligands. Therefore, thrombin-GPlb 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 GPlb, glycocalcin, 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), Gla-Pro-Arg-pNA (S2366), and human purified fibrinogen were from Kabi Diagnostics (Uppsala, Sweden); bovine serum albumin (BSA), HEPES, and diisopropylfluorophosphate (IP2-PF) were from Sigma Chemical Co (St Louis, Mo); tetradichlorodiphenylglycouril (Iodogen) was from Pierce Chemical Co (Rockford, IL); and carrier-free Na125I was from CIS Biointerfaces (Saclay, France).

Human α-thrombin was prepared as previously described.25 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.9 Glycocalcin was purified as previously described26 and its purity was assessed by gel electrophoresis (Fig 1). No contaminants are apparent. In some experiments, glycocalcin was reduced and alkylated according to standard procedures.27 Briefly, glycocalcin (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 mol/L mercuric chloride, 8 mol/L iodoacetamide, or 4 mol/L ethylenediamine. Samples were dialyzed in 10 mol/L Tris, 2 mol/L guanidine HCl, pH 8.5, followed by 50 mol/L Tris, pH 8.5.

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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,25 bovine protein C,29 and human antithrombin III (ATIII)25 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 mmol/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 μmol/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 acid solution 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 mmol/L) and variable amounts of glycocalicin. After 2 hours at 37°C, samples were centrifuged (12,000g 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 nmol/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 nmol/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 (Immulon; 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.
Effect of glycocalcin on thrombin catalytic activity. Glycocalcin 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, glycocalcin did not modify the rate of thrombin inhibition by iPr2PF (Fig 2), indicating that the soluble fragment of GPIb does not alter the catalytic site of thrombin.

Effect of glycocalcin on thrombin interaction with fibrin. As previously reported by others, glycocalcin 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 glycocalcin.

When fibrin monomers were allowed to polymerize in the presence of iodinated thrombin, free and fibrin-bound thrombin were conveniently separated by centrifugation. Glycocalcin 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 the concentration of glycocalcin, the inhibition was consistent with those previously reported from kinetic studies.31 It also indicates that the inhibition of thrombin binding to fibrin by glycocalcin is specific.

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.31 It also indicates that the inhibition of thrombin binding to fibrin by glycocalcin is specific.

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

Effect of glycocalcin on thrombin interaction with protein C and thrombomodulin. Glycocalcin, 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 glycocalcin, respectively. In contrast, glycocalcin caused a significant decrease in the rate of protein C activation in the presence of thrombomodulin (Fig 4A), suggesting that glycocalcin inhibited thrombin binding to thrombomodulin. This inhibition was dependent on the concentration of glycocalcin. When the reciprocal of the rate of protein C activation was plotted as a function of the concentration of glycocalcin for two concentrations of thrombin-thrombomodulin complexes (Fig 4B), glycocalcin 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 microtiter plates. Thrombin bound to thrombomodulin with a Ka of 0.4 nmol/L, consistent with published values.28 In addition, compared with 67% inhibition observed with the same amount of alkylated nonreduced glycocalcin and 85% inhibition with control glycocalcin. The decrease in the inhibitory potency of glycocalcin upon reduction suggests that the two disulfide bonds, 209-248 and 211-264, might contribute to maintaining the binding region optimal conformation.
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 pmol/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 pmol/L) activation by (○) 1.25 nmol/L (▲) 2.5 nmol/L of an equimolar complex of α-thrombin and rabbit thrombomodulin was measured in 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.
modulin bind to overlapping sites on thrombin, whereas the
binding sites for GPIb and protein C are clearly distinct.
Glycocalcin competes with both fibrin and thrombomodu-
lin for binding sites, but the inhibition constant (K_i) for
fibrin-thrombin interaction is at least one order of
magnitude lower than the K_i for thrombomodulin-throm-
bin 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 under-
goes a proteolytic cleavage by thrombin unmasking a new
amino-terminal sequence that functions as a tethered
ligand effecting receptor activation.10 The sequence of the
thrombin cleavage site within the receptor resembles that
of bovine protein C, which is a poor substrate for throm-
bin.41 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.34,42 We have previously35
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 ap-
ppears to increase the sensitivity and the rate of the platelet
responses11-18 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 concen-
tration of thrombin or by changing the thrombin specificity.
However, we cannot exclude that receptor-dependent plate-
let 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 phosphatidyl insitol 3
kinase activation, might be provided by receptor proteoly-
sis.43 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 A_2.44 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 polymeriza-
tion.45 Thus, a GPIb-mediated modification of the cytoskel-
eton 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 hirudin-like domain of the
platelet receptor,42 is only a weak inhibitor of platelet
activation by α-thrombin.46 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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