Characteristics of the Interaction between Thrombin Exosite 1 and the Sequence 269-297 of Platelet Glycoprotein Ibα*

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Summary

The interaction between GPIb and thrombin promotes platelet activation elicited via the hydrolysis of the thrombin receptor and involves structures located on the segment 238-290 within the N-terminal domain of GPIbα and the positively charged exosite 1 on thrombin. We have investigated the ability of peptides derived from the 269-287 sequence of GPIbα to interact with thrombin. Three peptides were synthesized, including Ibα 269-287 and two scrambled peptides R1 and R2 which are comparable to Ibα 269-287 with regards to their content and distribution of anionic residues. However, R2 differs from both Ibα 269-287 and R1 by the shifting of one proline from a central position to the N-terminus. By chemical cross-linking, we observed the formation of a complex between 125I-Ibα 269-287 and α-thrombin that was inhibited by hirudin, the C-terminal peptide of hirudin, sodium pyrophosphate but not by heparin. The complex did not form when γ-thrombin was substituted for α-thrombin. Ibα 269-287 produced only slight changes in thrombin amidolytic activity and inhibited thrombin binding to fibrin. R1 and R2 also formed complexes with α-thrombin, modified slightly its catalytic activity and inhibited its binding to fibrin. Peptides Ibα 269-287 and R1 inhibited platelet aggregation and secretion induced by low thrombin concentrations whereas R2 was without effect. Our results indicate that Ibα 269-287 interacts with thrombin exosite 1 via mainly electrostatic interactions, which explains why the scrambled peptides also interact with exosite 1. Nevertheless, the lack of effect of R2 on thrombin-induced platelet activation suggests that proline 280 is important for thrombin interaction with GPIbα.

Introduction

Glycoprotein (GP) Ib, a major component of the platelet membrane, consists of two disulfide-linked polypeptide chains (α and β), associated with GPIX and GPV to form a non-covalent hetero-oligomeric complex [for a review see (1)]. GPIb has a dual function: first, as a receptor for von Willebrand factor (vWF), GPIb is essential to mediate the initial attachment of platelets to the blood vessel wall at sites of injury; second, GPIb plays a role in thrombin-induced platelet activation, although this role is poorly understood. Platelet responses to thrombin are mainly elicited via the cleavage of a specific receptor (TR) which belongs to the family of the seven transmembrane domain receptors (2). However, it is well established that GPIbα also acts as a binding site for thrombin. Thrombin interaction with GPIbα promotes TR-coupled platelet activation by increasing the rate of platelet responses as well as platelet sensitivity to thrombin (3-6). The mechanism by which GPIbα promotes TR-coupled responses is not clearly understood but appears to require the anchorage of the proteins to the platelet membrane (7). Nevertheless, there are growing evidences indicating that GPIb and the thrombin receptor activate distinct pathways (4, 8-10). The observation that thrombin-induced [Ca²⁺]-mobilization is decreased by 50% in the presence of anti-GPIbα monoclonal antibodies suggests that GPIbα is coupled to a specific activation pathway (8, 9). In addition, the thrombin receptor agonist peptide appears to be a partial agonist when compared to thrombin and in particular, it induces only partial phosphorylation and activation of cytosolic PLA2 and, in contrast to thrombin, it fails to induce a prominent activation of the mitogen-activated protein kinases ERK1 and ERK2 (10).

The interaction between GPIbα and thrombin involves structures located on a segment corresponding to residues 238 to 290 within the N-terminal globular domain of GPIbα (1), and a positively charged patch at the surface of the thrombin molecule, called exosite 1 (11). Thrombin exosite 1 also binds the anionic C-terminal segment of hirudin (12) and the hirudin-like region of the thrombin receptor (13). We have thus hypothesized that a cluster of negatively charged amino acids present within the sequence extending from Asp 269 to Asp 287 of the GPIbα chain, which presents similarities with the COOH-terminal peptide of hirudin, could act as a counterpart of thrombin exosite 1 (11, 14). In accordance with this hypothesis, it has been shown that synthetic peptides based on the sequence Pro 265-Asp 287 of the GPIbα chain inhibited thrombin-induced platelet activation as well as thrombin binding to its high-affinity sites on platelets (15, 16). However, these studies did not examine if the inhibitory effect of the peptides resulted from a direct interaction with thrombin or from their interaction with a complementary sequence on GPIbα itself. The latter mechanism has been recognized in several proteins in which amphiphilic peptides approximately fifteen amino acids in length have been shown to interact with complementary antisense peptides within the same molecule (17, 18). For instance, a peptide derived from a sequence within GPIIIa has been
shown to interact specifically with the ligand-binding domain of GPIb-IIIa, the integrin from which it was derived, thus preventing fibrinogen binding (19). The aim of the present study was to determine if a synthetic peptide corresponding to the sequence 269-287 of GPIbα binds thrombin and modulates some of its activities.

Methods

Reagents

Bovine serum albumin (BSA), SDS, poly(ethylene glycol) 8000 and recombinant hirudin were from Sigma Chemical Co. (St. Louis, MO, USA). Iodogen and bis(sulfosuccinimidyl)suberate (BS3) were from Pierce (Rockford, IL, USA). 5-[14C]-hydroxytryptamine and 125I[Na were from Amersham (Les Ulis, France) and sulfated hirudin 54-65 was from Bachem Feinchemikalien AG (Bubendorf, Switzerland). The chromogenic substrates S-2238 (H-D-Phe-Pip-Arg-NH-nitroanilide), S-2765 (N-α-benzyloxy carbonyl-D-Arg-Gly-Arg-NH-nitroanilide) and S-2288 (D-Ile-Pro-Arg-NH-nitroanilide) were from Chromogenix (Stockholm, Sweden), CBS 65-25 (D-Lys-benzylcarbonyl-Pro-Arg-NH-nitroanilide), CBS 46-25 (ethoxycarbonyl-L-Lys-Gly-Arg-NH-nitroanilide), and chromothrombin (p-toluene sulfonyl-Gly-Pro-Arg-NH-nitroanilide) were from Diagnostica Stago (Asnières, France). Human fibrinogen was from Kabi Diagnostic (Stockholm, Sweden).

Human α-thrombin (EC.3.4.21.5) was purified as previously described (20). The preparations used throughout this study were more than 97% α-thrombin, as shown by SDS-PAGE. α-Thrombin was 125I-labeled using the solid-phase reactant Iodogen (18). γ-Thrombin was prepared according to a published procedure (20).

Three different peptides of the same amino acid composition (Table 1) were synthesized. The peptide Ibα 269-287 is a 19-residue peptide corresponding to the sequence extending from Asp 269 to Asp 287 within the GPIbα chain. The two other peptides have the same composition but amino acids were randomly ordered. Peptide R1 is characterized by the presence of a proline residue in a central position as in the native peptide. Peptide R2 is characterized by the location of the proline residue at the amino-terminal extremity. The three peptides were synthesized by the solid-phase method using FMI chemistry and a model 9050 peptide synthesizer (Millipore, USA). The peptides were purified by reverse phase h.p.l.c. (Depta prep 300 Waters, USA) on a Vydac C18 (50 x 300 mm) column. The authenticity of the 19-residue peptide was confirmed by amino acid analysis and FAB mass spectrometry on a Normag R10-10C apparatus. The lyophilized peptides were reconstituted in distilled water/2 M Tris, pH 11, to adjust the peptide solutions to pH 7.5 ± 0.5. Peptides were iodinated using the solid-phase reagent Iodogen. The specific radioactivity was similar for the three peptides and was of 22 ± 3 x 10^6 Bq/mg.

Two unrelated peptides differing from the GPIbα-derived peptides by their composition and their content in negatively charged residues were also used as controls in some experiments: neurotensin (PyrELYGNKPRRPYIL) and mastoparan (INLKALAALAKK) from Bachem Feinchemikalien AG (Bubendorf, Switzerland).

Cross-linking Experiments

α-Thrombin, γ-thrombin or bovine serum albumin, at the same concentration (420 nM), were incubated with various amounts of 125I-peptide Ibα for 45 min at 37°C. BS3 (0.5 mM) was then added and the incubation was continued for one hour at room temperature. The experiments were performed in 20 mM phosphate, 150 mM NaCl, pH 7.5 (PBS buffer). Some experiments were conducted in the presence or absence of 5-65 sulfated on Tyr 63 (H 54-65), or heparin. Other experiments were performed in the presence of the unlabeled peptides in up to a 500-fold excess. Cross-linking was stopped by the addition of 2 M Tris (0.1 vol.) and 2% SDS and boiling for 5 min. Radiolabeled bands were detected after separation of the proteins by 10% SDS-PAGE and autoradiography.

Thrombin Amidolytic Activity

The influence of GPIbα 269-287 on thrombin catalytic activity was examined using several chromogenic substrates (S-2238, S-2765, S-2288, CBS 65-25, CBS 46-25 and chromothrombin). These peptides were chosen because of their differences in residues corresponding to positions P1 to P3 that are known to play an important role in the docking of the substrates into the catalytic site of thrombin (21). Hydrolysis of the substrates (10 µM) by α-thrombin (0.4 µM) was measured in 10 mM Heps, 10 mM Tris, 100 mM NaCl, pH 7.8, 0.1% poly(ethylene glycol) 8000 at 37°C in the absence or presence of the peptide. The rate of paranitroanilide release was followed at 405 nm in a Beckmann DU70 spectrophotometer. The influence of the random 1 and 2 peptides on thrombin catalytic activity was examined using S-2238. Kinetic parameters of S-2238 hydrolysis were derived from the determination of initial velocities for eight substrate concentrations ranging over 2-20 µM.

Thrombin Binding to Fibrin

Thrombin interaction with fibrin was measured as previously described (3) by measuring at equilibrium the association of 125I-α-thrombin with repolymerizing purified fibrin monomers.

Thrombin-induced Platelet Aggregation and Secretion

Platelets were collected from fresh human blood according to published procedures (22). Platelet-rich plasma was incubated for 25 min at 37°C with 0.6 µM S-2238 or 5-[14C]-hydroxytryptamine before isolation of washed platelets. Platelet aggregation was induced by the addition of α-thrombin, and was followed at 37°C with stirring at 1100 rpm. Platelet secretion was measured after 3 min incubation. The reaction was stopped by adding 100 mM ice-cold EDTA (0.2 vol.). After centrifugation at 11,000 x g, supernatants were assayed for 5-[14C]-hydroxytryptamine by scintillation counting.

Results

We have previously shown that thrombin binding to platelet GPIbα may be visualized by chemical cross-linking (22). We have used this approach to examine whether a synthetic peptide corresponding to residues 269-287 in GPIbα (Ibo 269-287) interacts with α-thrombin or not. Incubation of 125I-labeled Ibo 269-287 with α-thrombin in the presence of the chemical cross-linking agent BS3 resulted in the formation of a 39 kDa radiolabeled band detected by autoradiography after migration on 10% acrylamide SDS gels (Fig. 1A). The observed molecular mass of this band corresponded to combined mass of thrombin (36.5 kDa) and peptide Ibo 269-287 (2.5 kDa). A molar ratio of labeled peptide to thrombin of 20:1 was optimal in visualizing the cross-linked complex. In these conditions, the recovery of the cross-linking calculated by counting the thrombin-associated radioactivity was of about 0.6% for the peptide and 12% for thrombin. When bovine serum albumin replaced α-thrombin, no association with Ibo 269-287 was observed (not shown). The intensity of the 39 kDa band was clearly decreased in the presence of a 500-fold molar excess of cold peptide indicating that the binding of the labeled peptide to thrombin was specific.

<table>
<thead>
<tr>
<th>Table 1 Peptide sequences</th>
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<tbody>
<tr>
<td>Ibo 269-287</td>
</tr>
<tr>
<td>Random 1</td>
</tr>
<tr>
<td>Random 2</td>
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The amino acid sequence between Asp 269 and Asp 287 in GPIbα is particularly rich in negatively charged residues, suggesting that electrostatic interactions could be critical for Ibα 269-287 binding to α-thrombin. This was supported by the observation that sodium pyrophosphate, a highly negatively charged compound, prevented the cross-linking of Ibα 269-287 to α-thrombin (Fig. 1B). An important structural feature in the thrombin molecule is the existence of two basic patches, exosites 1 and 2, which interact with the C-terminal tail of hirudin (23) and heparin (24) respectively and play an important role in thrombin interaction with its physiological substrates or ligands. Hirudin and the acidic hirudin-derived peptide H 54-65 reduced the interaction between Ibα 269-287 and α-thrombin (Fig. 1B), suggesting an interaction between Ibα 269-287 and thrombin exosite 1. We then examined the capacity of the γ derivative of thrombin, in which exosite 1 is disrupted, to bind Ibα 269-287. Under the same conditions of cross-linking as above, no labeled complex was observed between γ-thrombin and Ibα 269-287 (not shown). Since the thrombin exosite 2 can also make ionic interactions, cross-linking experiments were performed in the presence of the polyanion heparin. The intensity of the band corresponding to α-thrombin cross-linked to Ibα 269-287 was not modified by heparin at concentrations up to 100 IU/ml (~60 µM) (Fig. 1C).

The analysis of the effect of Ibα 269-287 on thrombin-catalyzed cleavage of different tripeptide substrates indicated that the peptide did not block the catalytic site of thrombin. Furthermore, in the presence of Ibα 269-287 the rate of hydrolysis of S-2238, S-2288, chromothrombin and CBS 65-25 by α-thrombin was increased (10 to 30%), while the rate of hydrolysis of S-2765 was not modified (Fig. 2). The kinetic constants of S-2238 hydrolysis were determined (Table 2), and indicate that Ibα 269-287 increased the catalytic efficiency of α-thrombin by increasing the turn-over number (kcat) by 11% without affecting the enzyme’s affinity for its substrate (Km). The unrelated peptides neurotensin and mastoparan had no effect on thrombin catalytic activity (not shown).

The peptide Ibα 269-287 exhibited concentration-dependent inhibition of α-thrombin binding to fibrin (Fig. 3) while polymerization of fibrin monomers was not modified by the presence of the peptide (not shown). The plot of fibrin-bound α-thrombin versus Ibα 269-287 concentrations in the presence of different fixed concentrations of fibrin indicated that Ibα 269-287 behaved as a competitive inhibitor with a Ki of 53.3 ± 6.0 µM. The unrelated peptides mastoparan and neurotensin had no effect on thrombin binding to fibrin (not shown).

The peptide Ibα 269-287 inhibited platelet aggregation and secretion induced by low concentrations (<1 nM) of α-thrombin (Fig. 4A). Inhibition was concentration-dependent. Half-maximal inhibition for serotonin release was obtained for 50 µM Ibα 269-287 when α-thrombin concentration was 0.25 nM. Increasing α-thrombin concentration overcame the inhibitory effect of the peptide: when platelets were stimulated with 2 nM α-thrombin, 200 µM Ibα 269-287 failed to inhibit platelet aggregation (not shown).

In order to examine whether the interaction of Ibα 269-287 with α-thrombin was sequence-specific or related to the amino acid composition of the peptide, we used two different peptides (R1 and R2) in which the amino acids were randomly ordered (Tab. 1). In presence of the cross-linking agent BS3, the two radiolabeled peptides, R1 and R2, formed a complex with α-thrombin, with a molecular mass (39 kDa) similar to that of the complex formed between Ibα 269-287 and α-

![Fig. 1](image-url)  
**Fig. 1** Cross-linking of peptide 125I-Ibα 269-285 to α-thrombin. 125I-labeled peptide Ibα 269-287 (8.4 µM) was incubated with 420 nM α-thrombin for 45 min at 37°C in the presence of buffer or inhibitors. Cross-linking was performed by the addition of 0.5 mM BS3, for one hour at room temperature. Samples were analyzed by 10% SDS-PAGE and autoradiography. The heavy arrow indicates the position of the complex formed between α-thrombin and peptide Ibα 269-287, whereas the light arrow indicates the position at which free α-thrombin migrates. Results A to C are from three independent experiments. Cross-linking was performed in the presence of: buffer (lanes A1, B1, C1); 4.2 mM cold peptide Ibα 269-287 (lane A2); 20 mM sodium pyrophosphate (lane B2); 420 U/ml (4.2 µM) hirudin (lane B3); 21 µM sulfated hirudin 54-65 (lane B4); 100 U/ml heparin (lane C2)

<table>
<thead>
<tr>
<th>Control</th>
<th>Km (µM)</th>
<th>kcat (s⁻¹)</th>
<th>kcat/Km (µM⁻¹ s⁻¹)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>8.4 ± 1.6</td>
<td>89 ± 11</td>
<td>10.6</td>
</tr>
<tr>
<td>Ibα 269-287</td>
<td>8.5 ± 1.6</td>
<td>95 ± 12</td>
<td>11.2</td>
</tr>
<tr>
<td>Random 1</td>
<td>9.6 ± 1.7</td>
<td>131 ± 24</td>
<td>13.7</td>
</tr>
<tr>
<td>Random 2</td>
<td>7.2 ± 0.8</td>
<td>98 ± 15</td>
<td>13.6</td>
</tr>
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Hydrolysis of S-2238 by 0.4 nM α-thrombin was measured in 10 mM Tris, 10 mM Hepes, 100 mM NaCl, 0.1% poly(ethylene glycol)8000, pH 7.8 in the absence or presence (50 µM) of different peptides. Data are the means ± SEM of 4 to 9 experiments.
thrombin (Fig. 5). The recovery of the cross-linking was identical to that observed with $^{125}$I-Ib 269-287. Both hirudin and the hirudin C-terminal peptide H 54-65 (Fig. 5A) reduced the intensity of the band corresponding to the complex between either R1 or R2 and $\alpha$-thrombin. Complex formation between $^{125}$I-Ib 269-287 and $\alpha$-thrombin was inhibited by an excess of either R1 or R2 cold peptide (Fig. 5B). Reciprocally, cross-linking of $^{125}$I-R1 and $^{125}$I-R2 to thrombin was inhibited by the cold homologous peptide and by Ib 269-287 in excess (Fig. 6A-B). In addition, sodium pyrophosphate (20 mM) reduced the cross-linking of $^{125}$I-R1 and $^{125}$I-R2 to thrombin (Fig. 6C).

The two scrambled peptides, R1 and R2, also induced changes in the catalytic activity of $\alpha$-thrombin. Both peptides increased the catalytic efficiency of $\alpha$-thrombin on S-2238 (Tab. 2). R1 and R2 also behaved as competitive inhibitors of $\alpha$-thrombin binding to fibrin, with Ki of 28.0 ± 5.2 µM and 36.0 ± 4.1 µM, respectively. R1 inhibited platelet aggregation and secretion (Fig. 7) induced by low concentrations of $\alpha$-thrombin with an inhibitory potency similar to that of Ib 269-287. In contrast, R2, even at concentrations up to 200 µM, had no detectable effect on platelet aggregation and secretion (Fig. 7) induced by low concentrations of $\alpha$-thrombin.

**Discussion**

The present report demonstrates for the first time that a peptide corresponding to sequence 269-287 of GP Ibα directly binds to $\alpha$-thrombin and modulates some of its activities. Inhibition of the Ib 269-287-thrombin complex formation by the COOH-terminal peptide of hirudin, which specifically binds thrombin exosite 1 (23), indicates that thrombin exosite 1 is involved in the interaction with the GP Ibα-derived peptide. This is further supported by the absence of interaction between Ib 269-287 and $\gamma$-thrombin, a proteolytic derivative characterized by disruption of the exosite 1 without major reorganization of the folded structure of thrombin (25). Ib 269-287 also slightly increased the catalytic efficiency of $\alpha$-thrombin towards several tripeptide substrates, in agreement with the general observation that docking of a protein or a peptide within thrombin exosite 1 produces subtle changes in the catalytic center, probably through allosteric interactions (26, 27). Evidence supporting overlapping binding sites for Ib 269-287 and fibrin was obtained from competition experiments. Altogether, these observations are consistent with previous studies which demonstrate that thrombin exosite 1 mediates thrombin interaction with GP Ibα via structures which overlap with the fibrin-binding site (7). Recent studies indicate that the thrombin exosite 2 might also be involved in thrombin interaction with GP Ibα (28). Although we cannot exclude this possibility for GP Ibα, an interaction between Ib 269-287 and the exosite 2 is unlikely since heparin did not inhibit the cross-linking of the peptide to $\alpha$-thrombin.
The present study points out the importance of electrostatic interactions in the binding of peptide Ib to thrombin as indicated by the inhibitory effect of sodium pyrophosphate on the cross-linking. The importance of the charges in the region 269-287 of GPIbα has previously been indicated by the fact that the sulfatation of the three Tyr residues Tyr 276, Tyr 278 and Tyr 279, in the native molecule, not only contributes to the high density of negative charges within this sequence (29), but also increases the avidity of GPIbα for thrombin. The binding of the scrambled peptides to thrombin is a new interesting observation. The results obtained with these peptides confirm the key role of ionic forces in thrombin binding to the GPIb-derived peptide. One characteristic of the 269-287 sequence of GPIbα is its high proportion of negatively charged residues (10 out of 19). In consequence, whichever scrambled sequence is chosen, the distribution of negatively charged residues in the randomly ordered peptides is similar to that found in the peptide Ibα 269-287. Thus, both scrambled peptides and the peptide Ibα 269-287 could all interact with thrombin exosite 1 via ionic interactions. Moreover, the three peptides appear to interact, at least to some extent, with overlapping structures on thrombin because they bind and inhibit thrombin in a comparable manner and they compete with each other for the binding to thrombin.

In agreement with previous reports (15, 16), we have observed that the peptide corresponding to the sequence 269-287 of GPIbα inhibited platelet aggregation and secretion induced by low concentrations of thrombin. An inhibition of thrombin-induced platelet activation can result from an impaired thrombin interaction with its seven transmembrane domain receptor (30) or with GPIbα (15). The first hypothesis can be excluded since peptide Ibα 269-287 did not modify the rate of hydrolysis by thrombin of the Arg 41-Ser 42 peptide bond in the recombinant fragment corresponding to sequence 25-97 of the thrombin receptor (data not shown), in agreement with our previous results obtained with glyocalcin (7). Thus the inhibition of thrombin-induced platelet activation by peptide Ibα 269-287 probably results from the inhibition of thrombin binding to high-affinity GPIb sites. Thrombin-induced platelet activation was also inhibited by the scrambled peptide R1 but not by R2. The major difference between R2 and the two other peptides is the position of the proline residue located at the N-terminus instead of a central position. The C-terminal tail of hirudin (sequence 54-65) also possesses a proline (Pro 60) located at a position comparable to that of Pro 280 in Ibα 269-287. In hirudin, Pro 60 is required for optimal interaction between hirudin 54-67 and thrombin (31). Taken together, these observations strongly suggest that in GPIbα Pro 280 could make specific contacts with structures in thrombin exosite 1. These contacts should be critical for the promoting effect of GPIbα on platelet activation. Pro 280 might also play a role by introducing a conformational element within the 265-87 sequence of GPIbα. The importance of GPIbα conformation for its recognition by thrombin has already been suggested by the observation that reduction and alkylation of glyocalcin decrease its affinity for thrombin (3).

Peptide Ibα 269-287 has a low affinity for thrombin compared to glyocalcin as indicated by a 100-fold reduced potency in inhibiting thrombin binding to fibrin (3). Compared to their parent native protein,
small synthetic peptides often have a decreased affinity for their acceptor molecule. This might be due, at least in part, to the lack of an adequate three-dimensional structure compared to the corresponding sequence in the native protein. Nevertheless, it cannot be excluded that additional sites to the 269-287 segment of GPIbα might be involved in the interaction with thrombin, as proposed by Gralnick et al. (16) who have reported that a peptide corresponding to sequence 247-265 of GPIbα also inhibited thrombin binding to platelets. Katagiri et al. (32) have also shown that a synthetic peptide based on the sequence Phe 216-Thr 240 inhibits activation by low concentrations of thrombin. Alternatively, thrombin interaction with GPIbαs may be controlled by intramolecular interactions within GPIbα. The possibility that intramolecular interactions within GPIbα may play a role in the regulation of von Willebrand factor-dependent platelet aggregation has been recently raised by Miller and Lyle (33). The existence of a regulatory mechanism controlling thrombin interaction with GPIbαs is strongly suggested by the puzzling discrepancy between the number of GPIbα copies at the platelet surface (25,000) and the low number (50 to 100) of high-affinity thrombin-binding sites provided by GPIbα (15, 34). Even if the present study suggests that all GPIbα molecules have the potential to interact with thrombin through the 269-297 segment, the question why, at the platelet surface, only a small proportion of GPIbα molecules are able to bind thrombin remains open.

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