The GPIbα–thrombin interaction: far from crystal clear

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The interaction of thrombin with platelet glycoprotein (GP)-Ibα has been well demonstrated. However, recent data have provided new insights into the GPIb–thrombin interaction. GPIb-clustering, which seems to be required for signal transduction, might be achieved by removal of GPV from the complex. In addition, the GPIbα subunits might need to be relatively mobile, as would occur in rafts or with GPIbα that has dissociated from the cytoskeleton. Finally, by resolving the crystal structures, two groups have identified different interaction sites in both thrombin and GPIbα that could be involved in cross-linking. Our direct comparison of the two structures reveals that, whereas one thrombin molecule binds to exactly the same site in GPIbα in both crystals, the other does not. Nevertheless, present biochemical and structural data complement each other well and help to clarify how GPIb might facilitate platelet activation by thrombin.

The serine-protease thrombin has important roles in haemostasis and thrombosis as a main protease in the coagulation cascade that converts circulating fibrinogen into fibrin. It is also a potent platelet agonist that induces platelet aggregation, and an activator of protein C, the key component of a natural anticoagulant system. Active thrombin, of which the precursor is prothrombin, consists of an A-chain of 36 residues and a B-chain of 259 residues. α-Thrombin contains a typical active-site catalytic triad composed of His43, Asp99 and Ser205 (B-chain numbering). Next to this active site, two recognition domains, designated exosite I (or the fibrinogen binding site) and exosite II (or the heparin binding site), are present as electropositive patches situated at opposite poles of the molecule [1–3].

Three thrombin receptors have been identified on the surface of human platelets: the protease-activated receptors (PAR)-1 and PAR-4 [4,5], and the glycoprotein (GP)-Ib–IX–V complex [6,7].

The PAR receptors belong to the family of G-protein-coupled PARs and are activated by thrombin-induced cleavage at position Arg41-Ser42 for PAR-1, and at position Arg47-Gly48 for PAR-4 [5,8,9]. This receptor proteolysis results in the unmasking of a new N-terminus that serves as a tethered ligand, folding back into the receptor and evoking potent transmembrane signalling involving phosphoinositide hydrolysis, protein phosphorylation, an increase in intracytosolic free calcium levels, and suppression of cAMP synthesis [8] [Figure 1(i)].

The platelet GPIb–IX–V receptor complex consists of four different polypeptide chains: GPIbα, GPIbβ (which are disulfide-bond-linked), GPIX and GPV. GPIb, GPIX and GPV associate on the plasma membrane [10] in a 2:2:1 distribution of which ~25 000 GPIb or GPIX copies are present on the platelet surface [11,12]. The major ligand is von Willebrand factor (VWF), followed by thrombin, Mac-1, P-selectin, high molecular weight kininogen, and coagulation factor XI and XII [10,13]. Virtually the entire ligand-binding capacity of the GPIb–IX–V complex is situated in the N-terminal globular region (~45 kDa) of the GPIbα chain, which can be removed from the GPIbα fragment glycocalicin through treatment with trypsin and mocarhagin [14]. Recently, the crystal structures of an analogous recombinant N-terminal GPIbα fragment, and the fragment in complex with the VWF A1-domain, have been solved [15–17].

By contrast, GPV is a thrombin substrate [18,19], and maximal platelet activation induced by thrombin can be accompanied by hydrolysis of >1% of the GPV [20]. In addition, GPV seems to be a negative modulator of thrombin-induced platelet activation [21,22].

GPIbα as a thrombin receptor

Although GPIbα was the first thrombin receptor to be identified on the platelet surface [7], an understanding of its mechanism-of-action remains elusive.

Thrombin binds to human platelets with either high affinity ($K_d = 0.5$ nmol l^{-1}) or moderate affinity ($K_d = 50$ nmol l^{-1}) [23,24], with the former corresponding to GPIbα binding [25,26]. In addition, GPV modulates the high-affinity thrombin-binding site on GPIbα [27]. However, it remains puzzling why only 105–1050 high-affinity thrombin-binding sites on platelets have been determined [24,25], whereas 25 000 copies of GPIbα are present on the platelet surface [11].

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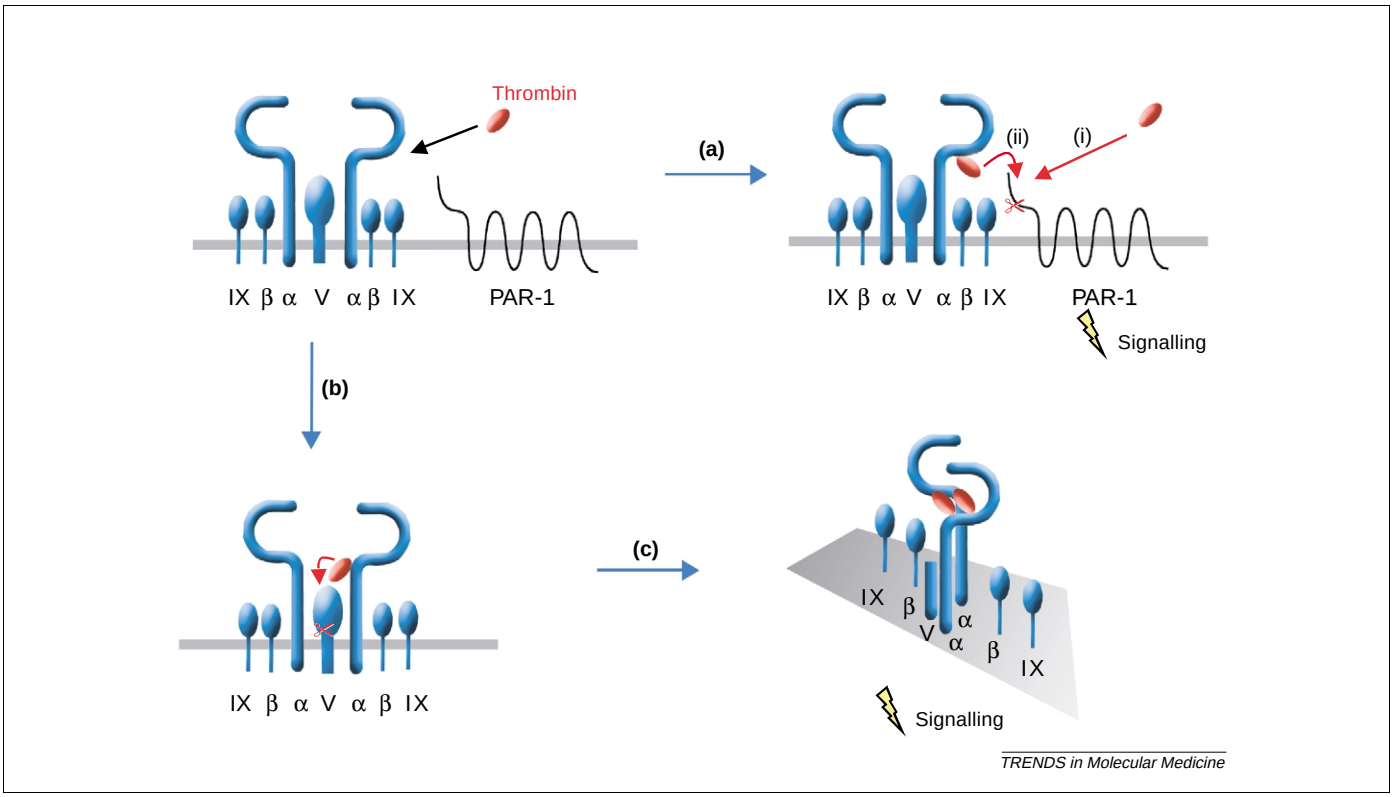

Figure 1. The interaction of thrombin with its platelet receptors. Thrombin binds to the glycoprotein (GP)-Ib–IX–V complex and protease-activated receptor (PAR-1) on human platelets. Both PAR-1 and GPV are known to be thrombin substrates on platelets. **(a)** At high thrombin concentrations, thrombin hydrolyses PAR-1 directly, resulting in potent platelet activation **(i)**. At low thrombin concentrations, thrombin bound to GPIb is able to prime the hydrolyses of PAR-1 **(ii)** and/or induces platelet activation through GPIb **(b,c)**. The latter is only observed when GPIbα receptors are cross-linked, as, for example, in the study using knockout GPV murine platelets [21], where removal of GPV by thrombin enables GPIb-receptor cross-linking and subsequent platelet activation (b,c).

A physiological role for GPIbα as a thrombin receptor was difficult to assign because the GPIbα dependency of thrombin-induced platelet activation is only detectable at low concentrations of thrombin, whereas high thrombin concentrations resulted in a strong activation of platelets in a GPIbα-independent way. Furthermore, studies to further identify GPIbα as a true thrombin receptor were overruled by the identification of PAR receptors on the platelet surface. It has been suggested that binding of thrombin to GPIbα primes the hydrolysis of PAR-1 by acting as a cofactor for PAR-1 activation [Figure 1(ii)], but no direct intracellular signals were initially observed to result from binding of thrombin to GPIbα [28]. However, identification of the PAR receptors could not account for all the phenomena observed when thrombin interacts with platelets. For example, platelets with proteolytically removed GPIbα, or platelets from Bernard-Soulier patients, which lack or have dysfunctional GPIb–IX–V complexes but are also giant, fail to respond to low doses of thrombin [29–31]. Thus, the question as to whether GPIbα is a thrombin receptor – that is, whether this receptor generates activation signals – remained unanswered.

In the first part of this review, we discuss recent data that could provide new insights into activation signals triggered by the GPIb–thrombin interaction. In addition, we highlight why only 105–1050 GPIbα receptors have a high-affinity interaction with thrombin. In the second part of the review, we discuss the molecular interactions between thrombin and GPIbα.

Platelet signalling induced by thrombin through GPIbα

Study of the platelet activation pathway triggered by the GPIbα–thrombin interaction, without interference of the much stronger PAR-coupled signals, was set up using either catalytically inactive thrombin or by de-sensitizing the PAR-1 and PAR-4 receptors. Ramakrishnan *et al.* demonstrated that catalytically inactive thrombin could induce platelet signalling events if GPV was no longer present in the GPIb–IX–V complex [21]. They used either murine-GPV-knockout platelets or human platelets from which GPV had been proteolytically removed. Addition of inactive thrombin resulted in platelet aggregation (Figure 1b,c). This activation pathway was dependent on ADP secretion.

Soslau *et al.* de-sensitized only the PAR-1 receptor, and demonstrated that, under these conditions, both active and inactive thrombin could induce platelet activation via a pathway that requires polymerizing fibrin [32]. However, this observation was disputed by Jarvis *et al.* who suggested that the platelets merely became trapped in the fibrin network because they were still aggregating when GPIbα was removed by proteolytic cleavage [33]. Dubois *et al.* desensitized both PAR-1 and PAR-4 and showed that thrombin binding to GPIbα also resulted in fibrin-dependent platelet aggregation, involving the activation of Rho kinase p160ROCK (independent of calcium mobilization), MEK-1 phosphorylation, and cleavage of talin through a calcium-independent mechanism [34].

Adam *et al.* also used catalytically inactivated thrombin to identify signalling events dependent on GPIb and

independent of PAR, although, in this case, thrombin needed to be immobilized [35]. Under these circumstances, platelet adhesion and spreading, dense-granule secretion and $\alpha IIb\beta_3$-dependent platelet interactions did occur, with ADP as an important secondary agonist. Molecules involved in this pathway were phosphatidylinositol-3-kinases, Src family kinases and protein kinase C.

A probable hypothesis clearly defined by Adam *et al.* [35] to link these various studies is that, to have a thrombin-induced GPIbα-dependent signal, GPIbα needs to become cross-linked, as might occur when several thrombin molecules are bound to the same surface (i.e. polymerizing fibrin [32,34] or plastic [35]). The removal of GPV might facilitate the dimerization of GPIbα and thus amplify the signals generated by the thrombin–GPIbα interaction [21]. The same reasoning could hold true for GPIbα-dependent signals induced by: (i) cross-linking GPIb molecules, (ii) multimeric VWF or (iii) bivalent anti-GPIb antibodies.

Recent studies therefore clearly demonstrate that thrombin-induced platelet signalling through GPIbα can be observed if PAR receptors are blocked and GPIbα receptors are cross-linked. Not only do the signalling pathways evoked by GPIbα–thrombin and PAR–thrombin differ [35], but it is also clear that GPIbα-dependent platelet activation can occur with inactive thrombin in which the active site is blocked, whereas for PAR activation, active thrombin is a prerequisite. However, active thrombin is needed to convert fibrinogen to polymerizing fibrin [32,34] or to remove GPV from the platelet surface before platelet signalling through the GPIbα–thrombin interaction can be shown (Figure 1b,c) [21]. This is probably owing to the requirement for cross-linking of the GPIbα receptors.

Nevertheless, it is clear that GPIbα can act as a receptor for platelet activation by low-dose thrombin either by inducing direct signalling events by receptor cross-linking, or by modulating other thrombin-dependent events at the platelet surface. Therefore, from a physiological point of view, the GPIbα–thrombin interaction enhances the sensitivity of platelets to thrombin, enabling them to respond to lower amounts of this agonist.

Less than 5% of the GPIbα receptors bind thrombin

One mystery that remains largely unsolved is why only a small fraction of the GPIb-complexes present on the platelet membrane function as high-affinity receptors for thrombin.

In recent years, the role of lipid rafts as an area where signalling complexes are built in cellular membranes has become increasingly evident; indeed, a minor fraction of the GPIb–IX–V complex is situated in lipid rafts [36]. Disruption of lipid rafts resulted in a dramatic decrease in the GPIb-dependent adhesion of platelets to immobilized catalytically inactive thrombin [35]. The number of high-affinity binding sites for thrombin on platelets is increased by enrichment in cholesterol and decreased by cholesterol depletion. The presence of cholesterol is known to be a main characteristic of lipid rafts [37].

It is tempting to speculate that the small fraction of GPIb–IX–V complexes associated with lipid rafts might account for the unexpectedly low number of high-affinity binding sites for thrombin on platelets. Moreover, because receptor clustering is an essential feature of signalling through rafts, localization of the GPIb–IX–V complex could be necessary for cross-linking and, hence, signalling by thrombin. However, at present, it is not clear how the localization of GPIbα in rafts might facilitate this.

Alternatively, linkage of the cytosolic tail of GPIbα to the membrane skeleton via filamin has been shown to have a strong negative-regulatory effect on the GPIb–VWF interaction [38]. It is not unreasonable to suggest that the percentage of GPIb not linked to the cytoskeleton might represent the high-affinity thrombin binding sites, and that this free-GPIb is more readily cross-linked and activated.

Interaction sites in GPIbα and thrombin

Many studies have identified sites in thrombin and GPIbα that could account for their interaction. However, several studies have identified different sites in both proteins. Furthermore, two groups recently resolved the crystal structure of the N-terminal fragment of GPIbα in complex with thrombin, each showing different interaction sites. A direct comparison of both crystal structures reveals a striking similarity (see later).

Thrombin interaction sites in GPIbα

Several studies using synthetic peptides [25,39], proteolytic fragments [14], or site-directed mutants of GPIbα [40] identified the thrombin binding site as a negatively-charged region (271–284) containing three sulfated-tyrosine residues (Tyr276, Tyr278, Tyr279 [14,41]). Other thrombin-binding proteins, such as hirudin [42], the PAR receptor [5], fibrinogen, thrombomodulin, FV and FVIII [43], also have negatively charged regions, but no homology or consensus sequences are observed between these regions [44]. Sulfation of all three Tyr residues in the negatively charged region of GPIbα seems to be a prerequisite for thrombin interaction [40,45]. Tyrosine sulfation within hirudin, FV and fibrin also contributes to their binding to thrombin [44,46,47], but these proteins interact with different regions in thrombin.

Peptides corresponding to the negatively charged region of GPIbα not only inhibited thrombin binding but also inhibited thrombin-induced platelet aggregation [25]. Another peptide corresponding to a region immediately upstream of the negatively charged region in GPIbα (Phe247–Thr265) also inhibited binding of thrombin to GPIbα [39], although to a lesser degree. However, this peptide had no effect on thrombin-induced platelet aggregation. By contrast, a peptide comprising residues Phe216–Thr240 of GPIbα inhibited thrombin-induced platelet aggregation [39,48] but not thrombin binding to GPIbα [25,39].

Although the role of the negatively charged region appears to be well-established, the function of other regions of GPIbα is less clear and might be restricted either to thrombin binding or to thrombin-induced platelet aggregation.

GPIbα interaction sites in thrombin

Whereas exosite I alone is crucial for the interaction of thrombin with the PARs, both exosite I and exosite II have been identified as interaction sites in thrombin for GPIbα.

Chemical modification or blockage of exosite I by hirudin affected thrombin-induced serotonin release or aggregation [24,49]. The involvement of exosite I was also demonstrated by the ability of hirudin to inhibit thrombin binding to GPIb or GPIbα peptide (269–287) by competing for exosite I [50–52] and by the ability of glycocalicin to inhibit the interaction of exosite I with fibrin or thrombomodulin [53]. Limited mutagenesis data are available where residues in exosite I have been altered. These mutations did not influence thrombin binding to GPIbα [54–56] (Table 1).

The involvement of exosite II has also been demonstrated. Heparin and the prothrombin fragment 2, which bind to exosite II, did compete with glycocalicin (a GPIbα fragment) for the interaction with thrombin [57,58]. Here, more mutagenesis data are available to demonstrate a decreased binding of these thrombin mutants to GPIb [54–56] (Table 1).

Two crystal structures of the GPIbα–thrombin complex

Crystal structures of a complex between thrombin and a GPIbα fragment were published in the same issue of *Science* by Celikel *et al.* [59] and Dumas *et al.* [60]. These groups of investigators used N-terminal GPIbα fragments of comparable length that included the anionic region, with sulfated tyrosine residues at their C terminus. These GPIbα fragments were mixed with thrombin and a 1:1 complex was isolated by gel filtration and crystallized.

Large differences

The two groups obtained different crystal forms. In both crystal forms, GPIbα and thrombin were present in a 1:1 ratio. Both groups of investigators observed extensive contacts between GPIbα and exosite I of thrombin, and between GPIbα and exosite II of a symmetry-related thrombin molecule, suggesting the existence of 1:2 GPIbα–thrombin complexes. The precise interactions between the thrombin exosites and GPIbα are very different in the two crystal forms [61] [Figure 2(i)]. In the structure of Dumas *et al.*, interactions involving thrombin exosite I are mainly polar [60]. In the structure of Celikel *et al.*, exosite I interacts with approximately the same surface-patch of GPIbα, but the thrombin molecule is rotated by 180° and the interaction involves more hydrophobic contacts [59]. Moreover, in the Celikel structure, the exosite I interaction includes the sulfated tyrosines of GPIbα, whereas in the Dumas structure it does not. The interactions involving exosite II both involve the anionic region of GPIbα, but the position of the anionic region with respect to the main body of GPIbα is entirely different; in the Dumas structure, the interaction involves an additional region of the convex face of GPIbα.

Significant similarity

Although it might appear that the crystal structures are surprisingly different, significant similarity exists in the interaction between the anionic region of GPIbα and thrombin exosite II, which, to the best of our knowledge, has not been described in any detail previously. When the complexes are overlaid by superimposing thrombin molecules, the bulk of the GPIbα molecules that interact with exosite II finish-up in different positions; however, residues Leu275–Tyr279 of the anionic region superimpose very well [Figure 2(ii)]. In both crystal forms, residues 276YDYY279 have extensive and almost identical interactions with thrombin exosite II [Figure 2(iii)], whereas residue Leu275 might play a role in positioning the side-chain of sulfated residue Tyr276. As can be seen in Figure 2b, the structurally conserved interaction provided by the 275LYDYY279 sequence accounts for most of the GPIbα–exosite II interactions observed in the structure of Celikel *et al.* [59], whereas additional interactions

Table 1. Comparison of the effect of thrombin mutations on GPIbα binding and the involvement of these thrombin residues in GPIbα interactions as observed from crystal structures

Mutation[b]	Exosite	Involvement in interactions with GPIbα[a]		Fold decrease of binding affinity	Refs
		Dumas *et al.* [60]	Celikel *et al.* [59][c]		
R62A	I	+	+ (67)	No effect	[54]
R68E	I	−	+ (73)	No effect	[55,56]
R70E	I	−	− (75)	No effect	[55,56]
R71A	I	+	+ (76)	No effect	[56]
R73A	I	+	+ (77A)	No effect	[56]
R89A	II	+	+ (93)	22	[54]
R89E	II	+	+ (93)	10	[55]
R93A	II	−	− (97)	8	[54]
R98A	II	+	+ (101)	13	[54,56]
R245A	II	+	+ (233)	29	[54,56]
K248A	II	+	+ (236)	21	[54,56]
K248E	II	+	+ (236)	25	[55]
R245A/	II	+	+ (233)	31	[54]
K248A/	II	+	+ (236)		
Q251A	II	−	− (239)		
Q251A	II	−	− (239)	No effect	[54]
K252A	II	+	+ (240)	5	[54]

[a]Distance cut-off of 4 Å used.
[b]Amino acid residues are numbered from the first residue of the human thrombin B-chain. This residue numbering scheme is also used by Dumas *et al.* [60].
[c]Listed in parentheses are residue numbers according to the thrombin–chymotrypsin numbering scheme [2] used in the publication by Celikel *et al.* [59].

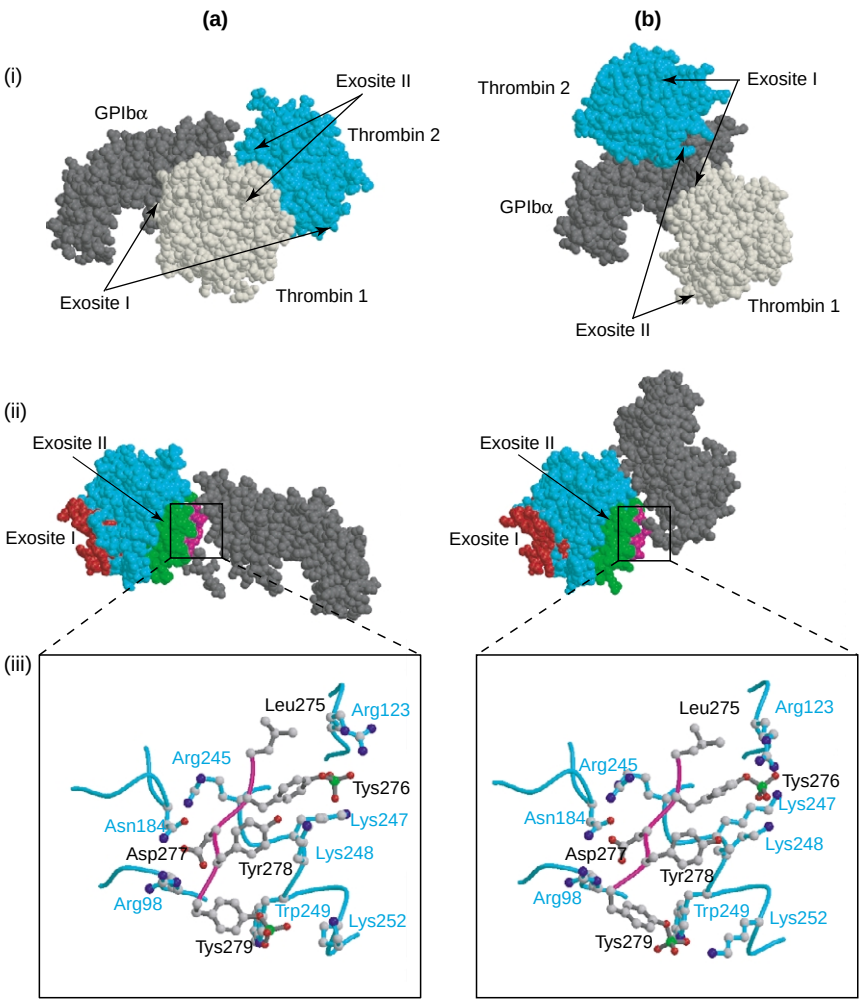

Figure 2. Comparison of the crystal structures of the glycoprotein (GP)-Ibα–thrombin complexes solved by **(a)** Celikel *et al.* and **(b)** Dumas *et al.* Almost identical contacts between thrombin exosite II and the GPIbα anionic region observed in highly different crystal forms might identify a dominant thrombin–GPIbα interaction. **(i)** Different overall arrangements of GPIbα and thrombin are apparent from space-filling models of the crystal structures published by Celikel *et al.* [59] and Dumas *et al.* [60]. GPIbα–thrombin complexes are depicted with their respective GPIbα molecules (dark grey) in the same orientation. Two copies of thrombin (light grey and cyan) are shown to illustrate the existence of two types of GPIbα–thrombin contact sites in both crystal lattices. One type of contact site involves thrombin exosite I, the other involves exosite II from a symmetry-related thrombin molecule. The majority of GPIbα residues involved in these contact sites are different between the two crystal forms. **(ii)** GPIbα–thrombin pairs interacting through exosite II and depicted with thrombin in the same orientation reveal almost identical interactions between thrombin exosite II (green) and part of the anionic region of GPIbα (magenta). Flexibility of the residues that connect the C-terminal anionic region of GPIbα to its main body enables GPIbα to adopt different orientations in the two crystal forms. **(iii)** Magnifications of the boxed areas of (ii) show almost identical interactions between thrombin exosite II and the 275LYDYY279 sequence of the anionic region of GPIbα in both crystal forms. Labelled residues are involved in intermolecular contacts (see also Table 1). The backbone trace of GPIbα is shown in magenta, and the trace and chemical bonds of thrombin are shown in cyan. Note that, in both crystal forms, residues Tyr276 and Tyr279 are sulfated, whereas Tyr278 is not. Thrombin residues are numbered from the first residue of the human thrombin B-chain. Figures generated from the atomic coordinates of protein data bank entries 1OOK [59] and 1P8V [60] using the computer programs Molscript [62] and Raster3D [63].

involving the convex face of GPIbα are present in the structure of Dumas *et al.* [60]. Interestingly, the short 275LYDYY279 sequence of GPIbα interacts with five out of six exosite II residues that were shown by site-directed mutagenesis to be involved in GPIbα binding (Table 1), thus explaining much of the available mutagenesis data.

Entirely different packing arrangements and structurally conserved interactions of GPIbα peptide 275LYDYY279 with thrombin exosite II are made possible by the flexibility of GPIbα residues 267–274, which adopt very different conformations in the two crystal forms. The flexibility of this region of GPIbα is also evident in three out of four molecules in crystal structures of free GPIbα [15,16], and in the structure of GPIbα bound to the VWF-A1 domain [16], where C-terminal residues beyond residue 267 are not visible. This apparent flexibility would enable the relatively independent movement of thrombin bound to GPIbα residues Leu275–Tyr279 with respect to the rest of GPIbα. This flexible complex apparently crystallizes with GPIbα and thrombin in totally different overall orientations, possibly owing to different crystallization conditions.

GPIbα cross-linking by thrombin

Thus, the two crystal structures reveal several interaction sites, one of which is common to both structures. Celikel *et al.* propose that alternating GPIbα–exosite I and GPIbα–exosite II interactions create the possibility of

GPIbα-cross-linking on the platelet membrane, and suggest that, in this process, the GPIbα–exosite I interaction should be stronger because it involves a larger area [59]. Dumas *et al.* suggest that alternating GPIbα–thrombin interactions could serve as a driving force for strong platelet adhesion by cross-linking of GPIbα located on different platelets, but do not suggest which is the stronger interaction [60]. From structural data alone, it is difficult to assess the relative contributions of the different interactions to the free energy of binding. We postulate that the interaction between GPIbα residues Leu275–Tyr279 and exosite II could be the dominant driving force for the formation of the 1:1 complex that exists in solution because it is observed in both crystal structures and explains much of the available mutagenesis data. The physiological role in platelet activation of this interaction and additional promiscuous interactions observed in the two crystal forms remains to be established.

In summary, a prominent interaction between thrombin and GPIbα might involve exosite II of thrombin and the 275LYDYY279 sequence of GPIbα. This conclusion is supported by a large amount of data from binding studies using protein fragments, peptides and mutants. In addition, this is the only contact site between thrombin and GPIbα where we could demonstrate identical interactions in both published crystal structures (Figure 2). A weaker interaction site might involve exosite I of thrombin. Elucidation of the thrombin–GPIbα crystal structures reveals possible modes of interaction between thrombin exosite I and GPIbα. Because no identical interaction sites have been identified between the two structures, and as only few mutations in exosite I have been investigated, further studies are needed to identify which residues in exosite I interact with GPIbα, and which residues in GPIbα are involved. The experimental design of these studies needs to account for the difficulty in studying exosite I interactions in the presence of strong exosite II interactions.

As more evidence accumulates to suggest that signal transduction through GPIbα–thrombin interaction occurs through receptor cross-linking, it is tempting to speculate that the alternating exosite I and exosite II interactions with GPIbα enable this receptor cross-linking. This alternating exosite I and exosite II interaction might also explain why GPIbα peptides outside the 276YDYY279 region cannot inhibit thrombin binding but can inhibit platelet aggregation. These peptides would therefore not inhibit the interaction through the thrombin exosite II site but might prevent receptor cross-linking.

Concluding remarks

Although the thrombin receptor function of GPIbα has long been recognized, the precise physiological role and the mechanism and contribution of GPIbα in thrombin-mediated platelet activation remained unclear. Recently, new data became available that shed light on both the signal transduction mechanisms and the molecular interactions involved.

The emerging picture is that GPIbα-clustering seems to be required to achieve signal transduction. This cross-linking can be achieved by removal of GPV from the complex. Thrombin can then cross-link the GPIbα-subunits if they are relatively mobile, as would occur in rafts or with GPIbα that has dissociated from the cytoskeleton. As the number of mobile GPIbα-receptors is expected to be low, this could explain the discrepancy between the total number of GPIbα molecules on the platelet surface and the estimate for the number of high-affinity copies. This higher affinity might, in turn, result from binding of thrombin to two GPIbα molecules. Indeed, resolution of the crystal structure by two independent groups demonstrates that two thrombin molecules bind, albeit differently, to a single GPIbα, thus enabling cross-linking. However, our direct comparison of the two structures reveals that one thrombin molecule binds to exactly the same site in GPIbα in both crystals, whereas the other does not. It is clear that additional biochemical experiments are required to resolve this discrepancy. Nevertheless, present biochemical and structural data complement each other well and further clarify how GPIbα might facilitate platelet activation by thrombin.

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