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Review

The Anti-platelet Approach Targeting the Fibrinogen Ligand of the GPIIb/IIIa Receptor[‡]

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Abstract: Activation of the platelet surface receptor GPIIb/IIIa is the final pathway of platelet aggregation, regardless of the initiating stimulus. RGD analogues, peptidomimetics and monoclonal antibodies to GPIIb/IIIa have been developed targeting the blockage of the receptor and inhibition of the fibrinogen binding. However, the intrinsic activating effect of GPIIb/IIIa blockers is widely discussed as one potential contributing factor for the disappointing outcome of trials with GPIIb/IIIa inhibitors. An alternative method for thrombus prevention could be the use of specific fibrinogen blockers since they will act at the final step of the platelet aggregation and are expected to leave the receptor unaffected. To achieve this target the design of the fibrinogen ligands could be based on (i) sequences derived from GPIIb/IIIa ligand binding sites, and (ii) sequences complementary to RGD and/or to fibrinogen γ-chain. The available information, which could be used as a starting point for developing potent fibrinogen ligands, is reviewed. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: $\alpha_{IIb}\beta_3$ receptor; fibrinogen blockers; GPIIb/IIIa binding domains; integrin inhibitors; platelet aggregation inhibitors

GPIIB/IIIA FUNCTION AND DRAWBACKS OF USING ITS BLOCKERS AS ANTI-THROMBOTIC AGENTS

Integrins are non-covalently associated heterodimers of α and β subunits. They belong to a superfamily of cell surface receptors that mediate adhesive processes in many biological functions [1–3]. Integrin mediated cell adhesions have important roles in cell anchorage, migration, proliferation, differentiation, lymphocyte homing and blood clotting [4,5]. The GPIIb/IIIa receptor, specific to platelets and

megakaryocytes, belongs to the β_3 family and is primarily responsible for platelet aggregation [6,7]. GPIIb/IIIa like other integrins, is a non-covalently associated heterodimeric complex composed of the subunits GPIIb (αIIb) and GPIIIa (β_3). Under resting conditions, it has low affinity for its ligands (Figure 1A) and is activated either when platelets adhere to subendothelium matrix (Figure 1B) or are stimulated by agonists such as ADP, thrombin and epinephrine, etc. (Figure 2A) [8]. Activation induces conformational changes and the receptor acquires a high affinity binding for its ligands, principally fibrinogen [9,10]. The binding of fibrinogen leads to platelet aggregation, an early step in the generation of a thrombus (Figure 2B). Anti-thrombotic strategies are based on the inhibition of platelet adhesion and aggregation. One approach to antiplatelet therapy is to block the primary stimulus by any of several

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[‡] Dedicated to my teachers Professor Constantinos Sakarellos and Professor Maria Sakarellos-Daitsiotis on the occasion of Professor Constantinos Sakarellos's 60th birthday.

BIOGRAPHY

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different agonists, e.g thrombin, epinephrine, etc. [11–13]. Another approach involves the interrup-tion of the signal transduction mechanism, which follows the agonist binding to the platelet surface. Intracellular signaling in platelets leads to the acti-vation of arachidonic acid metabolism and platelet secretion, with the release of thromboxane A_2 and

ADP, which contribute largely to platelet recruitment by the growing thrombus. Specific inhibitors of the enzyme thromboxane synthase and the thrombox-ane A_2 receptor have been developed to intercept the arachidonic acid metabolic pathway [14]. However, the specific inhibition of a particular agonist leaves several alternative routes open to platelet activa-tion (Figure 2A). To overcome this problem, specific inhibition of the interaction between GPIIb/IIIa and fibrinogen has been an attractive target of exten-sive research (Figure 3A). This interest arises from the fact that the intervention occurs at the final common step of the platelet aggregation pathway.

Integrins, including GPIIb/IIIa, can recognize a common tripeptide motif, the Arg-Gly-Asp (RGD) sequence. This motif, originally identified as the cell attachment domain on fibronectin, is present in a surprisingly large number of adhesive glyco-proteins including fibrinogen, von Willebrand fac-tor, fibronectin etc [5,7,15]. Integrin GPIIb/IIIa binds fibrinogen via recognition sequences RGDS (α572–575), RGDF (α95–98) and HHLGGAKQAGDV (γ400–411) [16–19]. Small RGD and γ-chain based peptides inhibit the glycoprotein adhesion and the platelet aggregation as well. Thousands of RGD ana-logues, peptidomimetics and monoclonal antibodies to GPIIb/IIIa have been developed targeting the blockage of the receptor ligand interaction [20–33]. Some of them are currently used in clinical trials. In addition, a family of small proteins obtained from
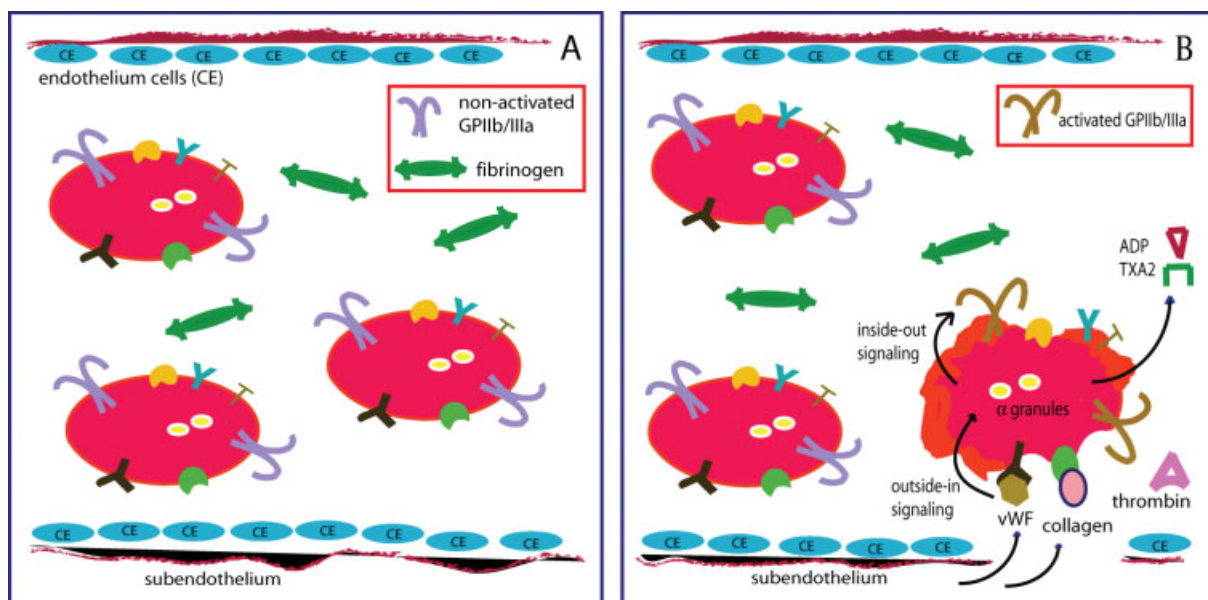

Figure 1   Schematic representation of the platelets under physiological conditions (A) and their activation induced by adhesion to subendothelium substrates (von Willebrand factor, collagen) (B).
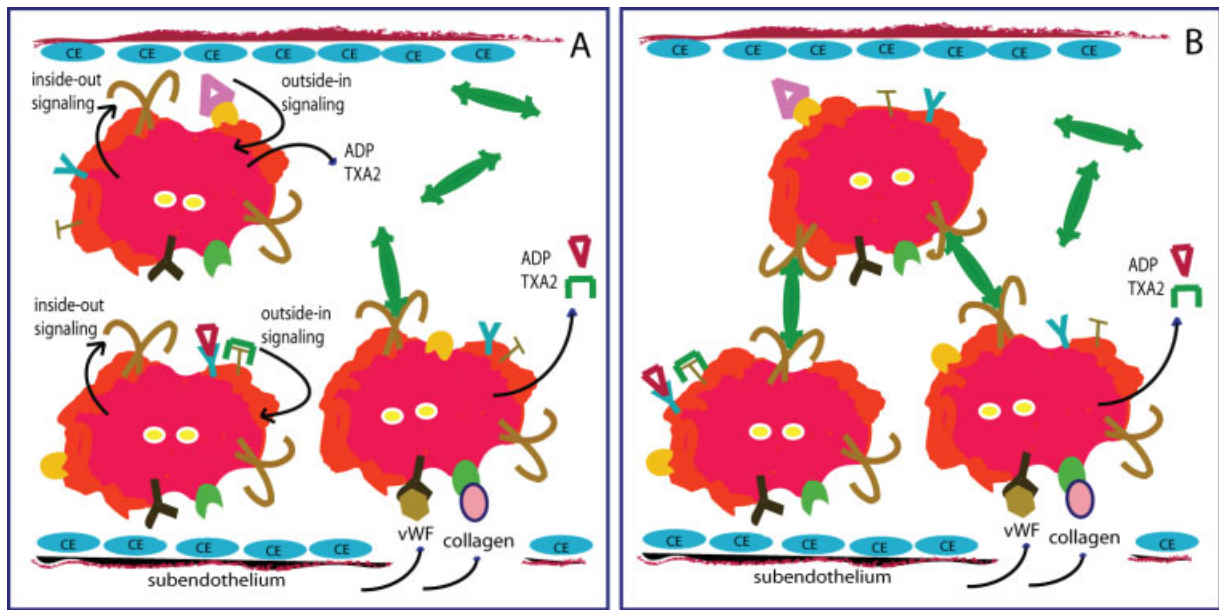
Figure 2   Binding of various agonists to their receptors induces platelet activation and intracellular signaling (A). GPIIb/IIIa acquire the high affinity binding for fibrinogen leading to thrombus formation (B). The symbols are the same as those in Figure 1.
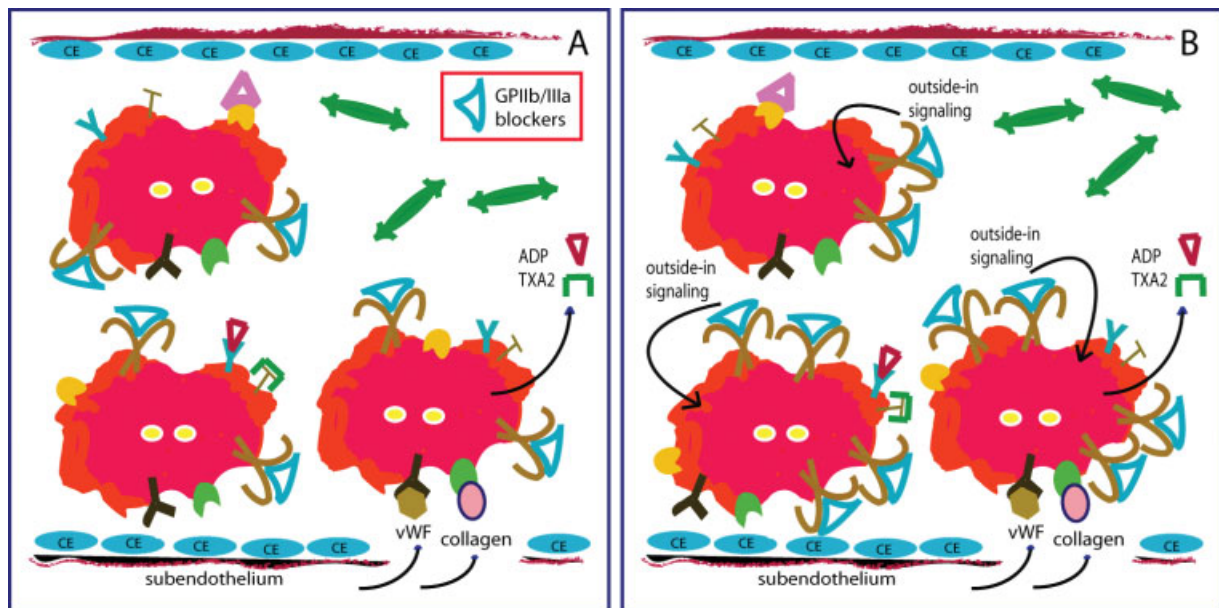

Figure 3   Inhibition of platelet aggregation by GPIIb/IIIa blockers (A) and clustering of the activated GPIIb/IIIa induced by GPIIb/IIIa blockers leading to outside-in signaling (B). The symbols are the same as those in Figure 1.

snake venoms, called disintegrins, which possess the RGD or the KGD sequence are among the most potent inhibitors of GPIIb/IIIa [34].

Despite the very different nature of the GPIIb/IIIa inhibitors, their mechanism of action is similar.

Their competition with fibrinogen for the target receptor GPIIb/IIIa results in the inhibition of platelet aggregation. All the RGD- or fibrinogen γ-chain dodecapeptide-based inhibitors possessing specificity and high affinity for the receptor bind to

resting and activated forms of GPIIb/IIIa [35,36]. It is well documented that besides the receptor conformational alteration upon agonist-induced platelet activation, ligand binding to GPIIb/IIIa induces further conformational changes of the receptor extracellular domain, resulting in clustering (Figure 3B) and the exposure of neoantigenic sites termed ligand-induced binding sites (LIBS) [37–39]. LIBS modulate secondary function by shifting the conformational equilibrium in the presence of ligand [40,41]. Some of the affected functions include the secondary wave of platelet aggregation, platelet adhesion to collagen, clot retraction and platelet secretion [42,43]. The intrinsic activating effect of GPIIb/IIIa blockers is widely discussed as one potential contribution factor for the disappointing outcome of trials with GPIIb/IIIa inhibitors [25,26,44,45]. Although the existence of an activating property of GPIIb/IIIa blockers leading to platelet aggregation under physiological conditions is questioned [46,47], the ligand-induced conformational alteration, activation, clustering and signaling of the receptor have been extensively documented [39,40,48–61]. The finding that RGD- and fibrinogen γ-chain based-ligands and other ligand mimetic peptide sequences act directly or indirectly as partial agonists of integrin function raises some key questions. Is the development of specific GPIIb/IIIa blockers a suitable approach in anti-platelet therapy? Could fibrinogen ligands be used? The latter could act at the final common step of the platelet aggregation pathway without affecting the state of the receptor.

The aim of this review article is to focus on the accumulated knowledge of GPIIb/IIIa ligand binding sites, which could be the starting point for the development of specific fibrinogen ligands as potential candidates for the anti-platelet therapy (Figure 4). Inhibition of the fibrinogen binding to GPIIb/IIIa using fibrinogen blockers requires agents that could specifically recognize the RGD and/or the fibrinogen γ-chain sequences, other fragments or the intact fibrinogen. To achieve this target the design of the fibrinogen ligands could be based on: (i) sequences derived from GPIIb/IIIa ligand binding sites and (ii) sequences complementary to RGD and/or to fibrinogen γ-chain.

GPIIB/IIIA LIGAND BINDING SITES

The studies for determining the binding sites of the GPIIb/IIIa receptor started three decades ago.
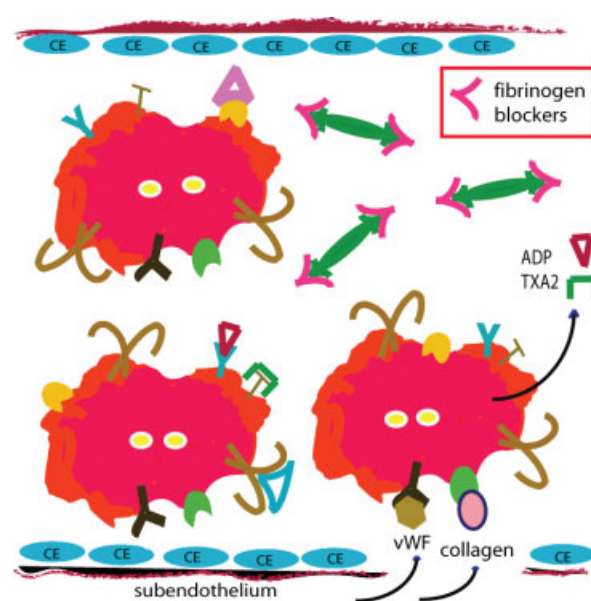

Figure 4 Schematic representation of the inhibition of platelets aggregation by fibrinogen blockers. The receptor remains unaffected overcoming the clustering and the additional outside-in signaling. The symbols are the same as those in Figure 1.

Substantial data have been accumulated regarding the location of potential ligand contact sites within GPIIb/IIIa. Combinations of immunological, biochemical, mutational approaches and peptide studies have been applied for mapping epitopes and identifying residues of GPIIb/IIIa implicated in ligand binding function.

Studies using anti-peptide antibodies and recombinant mutant fibrinogen suggest that platelet GPIIb/IIIa interacts primarily with the γ-chain sequence rather than with RGD sequences in fibrinogen [62–65]. Both the GPIIIa and the GPIIb subunits of integrin participate to the ligand-binding capability of the receptor involving their amino-terminal portions [35,66–70]. Experiments with radiolabeled, photoactivatable aryl azide RGDS and HHLGGAKQAGDV containing derivatives showed that the binding sites on the receptor for the two peptides do not appear to be entirely identical [70]. Little labeling of the GPIIb/IIIa complex with either the GRGDSC or the HHLGGAKQAGDV derivative was observed in the absence of platelet activation. The extent of labeling was markedly enhanced when the platelets were activated with thrombin. From this study it was concluded that the RGDS containing derivative binds on both

subunits, whereas the HHLGGAKQAGDV containing derivative specifically binds only on subunit GPIIb.

Ligand Binding Sites on the GPIIIa Subunit

The chemical cross-linking approach using peptide inhibitors derived from adhesion proteins was initially applied. A radioiodinated fibronectin derived RGD peptide (KYGRGDS) was cross-linked to thrombin-stimulated washed platelets [35]. It was found that the RGD peptide preferentially binds to GPIIIa and much more efficiently on stimulated compared with unstimulated platelets. Thrombin was shown to be more effective than ADP in enhancing cross-linking of the peptide. The ligand recognition site was estimated to include GPIIIa residues 109–171 [71]. This 63-amino acid stretch of GPIIIa is highly conserved among integrin β-subunits and has been postulated to participate in the recognition of RGD sequences within adhesive proteins [72]. A monoclonal antibody produced against the synthetic peptide GPIIIa 109–128 has been shown to interact with thrombin or ADP stimulated washed platelets and to inhibit fibrinogen binding and platelet aggregation [73]. There were no reported data to provide evidence that this peptide (GPIIa 109–128) inhibits either platelet aggregation or fibrinogen binding. However, the GPIIIa 118–131 synthetic peptide was shown to function both as a ligand and as a cation-binding site [74,75]. This peptide blocks platelet aggregation and platelet adhesion to fibrinogen and inhibits the binding of fibrinogen to purified GPIIb/IIIa. The formation of GPIIIa 118–131/RGDF and GPIIIa 118–131/GRGDSP stoichiometric complexes evidenced by electrospray ionization mass spectrometry suggests that this region is a putative RGD ligand binding site on the GPIIIa subunit. These results were supported by the observation that the peptide GPIIIa 118–131 contains the sequence DDLW (residues 126–129) which was identified by the phage display libraries technology to be an RGD binding motif [76]. The overlapping domain GPIIIa 100–348 was also suggested to play a prominent role in the binding of fibrinogen to its platelet receptor [77]. This conclusion was based on a combination of chemical and enzymic cleavage procedures of GPIIIa in whole platelets, synthetic peptides and enzyme immunoassay studies. The importance of the experimental approach used for determining the ligand binding sites on GPIIb/IIIa receptor was proved by chemical cross-linking studies. Significant differences of the peptide cross-linking to GPIIb/IIIa were observed in solution and in activated platelets [78]. Four peptides CKRKRKRKRRGDV, (α1), GGRGDF (α2), CVHHLGGAKQAGDV (γ1) and CGAKQAGDV (γ2) incorporating a photoactivable cross-linker, a fluorescent reporter group, an amino acid sequence as spacer and fibrinogen α- and γ- chain derived sequences were used to study the ligand binding sites of GPIIb/IIIa in solution. By comparing the results obtained in solution with those in activated platelets, it was concluded that, while in activated platelets the RGD and KQAGDV peptides bind mainly to GPIIIa and GPIIb subunits, respectively, in solution both types of peptides bind indiscriminately to GPIIb and GPIIIa, regardless of the peptide length [66,71,78]. The identified sequence stretches corresponding to the tryptic peptides of GPIIIa within which the inhibitory peptides (γ1, γ2, α1 and α2) cross-linked to purified GPIIb/IIIa in solution were 9–37 for γ1, 63–83 and 303–350 for α1, 303–350 for γ2 and 151–199 for α2.

Aimed at determining the binding sites on GPIIa, synthetic peptides were also used. Peptides derived from residues 1–288 of the amino-terminal sequence of GPIIIa were tested for their abilities to block the binding of fibrinogen and other adhesive proteins to GPIIb/IIIa [79]. It was found that a specific sequence of 12 amino acids within GPIIa (SVSRNRDAPEGG, residues 211–222) blocked the binding of at least four adhesive proteins (fibrinogen, fibronectin, von Willebrand factor and vitronectin) to purified GPIIb/IIIa. This sequence is highly conserved between the β-subunits of the other two families of the integrin superfamily, the fibronectin receptor family (β1) and the leukocyte receptor family (β2) [80,81]. In addition to the GPIIIa 211–222 sequence, peptides GPIIIa 204–222 and 211–229 inhibited fibrinogen binding to immobilized GPIIb/IIIa, while peptides GPIIIa 204–216 and 217–229 did not have any significant effect. From dissociation experiments of GPIIb/IIIa and the fact that the peptide 211–221 does not induce GPIIb to become a substrate for thrombin it was concluded that the GPIIb/IIIa complex does not undergo major conformational changes when incubated with the GPIIIa peptide. Therefore, this peptide binds directly to fibrinogen and fibronectin [79]. However, studies with synthetic peptides derived from the GPIIIa sequence 211–221 have shown that these GPIIIa peptides bind specifically to GPIIb/IIIa and not to fibrinogen [82]. Evidence from various sources suggest that peptides 211–221 (SVSRNRDAPEG), 214–221 (RNRDAPEG) and 214–218

(RNRDA) of GPIIIa, like RGDS, bind at or near the fibrinogen binding site. This conclusion was supported by the following data: (i) the binding of RGDV and GPIIIa 214–221 was mutually exclusive. RGDV eluted GPIIb/IIIa bound to a GPIIIa 214–221 affinity column and, similarly, GPIIIa 214–221 and 211–221 eluted GPIIb/IIIa bound to an RGDS column; (ii) GPIIIa 214–218 was capable of inducing a conformational change in GPIIb/IIIa similar to RGDS and (iii) RGDV and GPIIIa 214–221 peptides inhibited the binding of pl-55 and PAC-1, two monoclonal antibodies that recognize only the activated complex and that are thought to bind at or near the fibrinogen binding site [82,83]. Among the above mentioned peptides, the GPIIIa sequence 214–218 was proposed as the most active in inhibiting fibrinogen binding to purified GPIIb/IIIa.

The high homology between the platelet GPIIIa 217–231 sequence and similar regions of other β subunits of integrin receptors leads to the hypothesis that this region may participate in ligand binding [84]. Enzyme-linked immunosorbent assays evidenced that the synthetic peptide DAPEGGF-DAIMQATVY (217–231 (Y)) binds to fibrinogen, von Willebrand factor and fibronectin and inhibits binding of GPIIb/IIIa receptor to immobilized fibrinogen. Interestingly, an analogous peptide in which Pro 219 was substituted with alanine did not bind to fibrinogen. The inhibitory effect of the GPIIIa 217–230 peptide on ADP-induced platelet aggregation and ^{125}I-fibrinogen binding to ADP-stimulated platelets was also confirmed [85]. Cross-linking experiments of ^{125}I-peptide 217–230 (Y) with fibrinogen showed that the interaction of the GPIIIa 217–230 sequence with fibrinogen occurs through an RGD dependent mechanism [85]. This peptide blocks platelet aggregation by interacting with the fibrinogen Aa chain.

Comparing the results obtained for the amino terminal region 211–231 of GPIIIa it can be concluded that this region certainly plays a significant and peculiar role in the fibrinogen recognition process by GPIIIa [79,82,84,85]. Some of the reported disagreements may arise from the different experimental approaches and conditions used in each particular case. In this connection, an interesting contribution was reported [86]. The GPIIIa derived peptides 214–218 and 217–231 were used in a comparative study for evaluating the activity of both peptides in inhibition of binding of biotin-labeled fibrinogen to ADP stimulated platelets, binding of GPIIb/IIIa receptor to immobilized fibrinogen and binding of biotin-labeled fibrinogen to immobilized GPIIb/IIIa receptor. It was found that in all three systems the inhibitory effect of peptide GPIIIa 214–218 was higher compared with that of peptide GPIIIa 217–231. Cross-linking experiments with ^{125}I-labeled peptides verified the specific interaction of peptide GPIIIa 214–218 with GPIIb/IIIa receptor on ADP-stimulated platelets, while peptide GPIIIa 217–231 does not cross-link to the receptor. In contrast, the latter cross-links to the Aa chain of native fibrinogen and its recombinant wild type and γ variant. From these results it was concluded that the mechanism of inhibition by each peptide is different. Peptide GPIIIa 217–231 interacts directly with fibrinogen, while peptide GPIIIa 214–218 interacts directly with the receptor [86].

The results obtained by using bacterial-expressed fragments, spanning defined regions of the extracellular domain of the GPIIIa subunit, are in good agreement with these data while an additional ligand binding site on GPIIIa was identified [87]. Four recombinant fragments, GPIIIa 56–231, 188–368, 274–403 and 274–368, were prepared and tested for their ability to bind both soluble and immobilized fibrinogen and to inhibit platelet aggregation. It was found that the recombinant fragments GPIIIa 56–231 and 188–368, which contain both the GPIIIa 214–218 and 217–231 sequences, bind soluble and immobilized fibrinogen. Moreover, the GPIIIa 274–368 sequence was proposed as a ligand recognition and binding domain for the γ-chain of fibrinogen that is independent of platelet activation. Recently, two recombinant fragments, GPIIIa 95–373 and 95–301 were expressed and used in fibrinogen binding assays. It was shown that the central segment 95–373 of the GPIIIa subunit binds Fg in a cation-dependent manner [88]. In agreement with these results, the participation of the GPIIIa 179–183 region in a direct contact with ligand-mimetic antibodies and native ligands was suggested from studies with human-to-mouse chimeras, which are expected to maintain the functional integrity of GPIIb/IIIa and ligand-mimetic antibodies [89].

Ligand Binding Sites Supported by GPIIIa Mutants

The exchange of the individual amino acids in the receptor protein sequence affords valuable information for the elucidation of their role in the ligand binding. One has to keep in mind, however, that this exchange cannot prove the direct participation of the respective amino acid in the binding process. Conformational changes, as a

consequence of alteration of amino acids, have to be taken into consideration as well.

Identification of natural GPIIb/IIIa mutations occurring in Glanzmann's thrombasthenic patients and mutants of GPIIb/IIIa expressed in chinese hamster ovary (CHO) cells have contributed greatly to understanding the function of the receptors and to evaluating the critical role of various amino acids within the GPIIb/IIIa sequence.

Various amino acid substitutions in mutants of the GPIIIa subunit that affect the receptor function have been reported [90–98]. Interestingly, naturally occurring point mutations have been identified within the regions 109–171 and 211–222 of GPIIIa that have been proposed as ligand binding domains. Thus, the GPIIIa Asp119 → Tyr mutation in a receptor variant is characterized by a complete loss of ligand binding function of GPIIb/IIIa [90]. The ligand binding function of recombinant GPIIb/IIIa expressed in CHO cells following scanning Ala mutagenesis of residues Asp119, Ser121, Ser123, Asp126, Asp127 and Ser130 in GPIIIa has also been examined [91]. It was found that Ala substitution at positions Asp119 or Ser121 produces a complete loss of receptor function. Substitution at position Ser123 produces defects in both ligand binding and conformational changes in the receptor induced by ligand binding. In contrast, substitution at positions Asp126, Asp127 or Ser130 does not affect ligand binding function.

In the second potential ligand-interactive site of GPIIIa two natural receptor variants, characterized by loss of ligand binding function, contain substitutions at Arg214, (Arg214 → Gln) or (Arg214 → Trp) [92,93]. It is interesting to note that substitution of R214 by Gln in the synthetic peptide containing the sequence GPIIIa 211–222 resulted in a decreased ability of this peptide to block fibrinogen binding to purified GPIIbIIIa. Studies using mutagenesis of various amino acid residues within the GPIIIa subunit concluded that Asp217 and Glu220 are essential residues for the ligand binding function of GPIIb/IIIa [94]. Ala substitution of these residues does not affect receptor expression but abolishes the binding of activation-dependent PAC1 and -independent OPG2 ligand mimetic antibodies.

Ligand Binding Sites on the GPIIb Subunit

Various studies using the chemical cross-linking approach concluded that the RGD ligands bind mainly on GPIIIa while the fibrinogen γ-chain derived peptide γ400–411 binds specifically on GPIIb subunit [66,70]. The first ligand binding site on GPIIb was reported to comprise residues 294–314 (AVTDVNGDGRHDLLVGAPLYM) [66]. It was identified by cross-linking experiments using a suitably modified 16-amino acid fibrinogen γ-chain peptide (KYGGHHLGGAKQAGDV). The interaction of this peptide with the receptor was greatly increased in thrombin stimulated compared with non-stimulated platelets. On the other hand, the cross-linking reaction was markedly inhibited by fibrinogen and the GRGDSP peptide. The synthetic peptides GPIIb 296–306 and GPIIb 300–312 derived from the identified binding site inhibit platelet aggregation and binding of fibrinogen to stimulated platelets and interact directly with fibrinogen [99,100]. Cross-linking studies using peptide derivatives incorporating the fibrinogen RGD (α-chain) and KQAGDV (C-terminal γ-chain) sequences have pointed to binding domains that vary considerably with the peptide length and are very different in solution from those observed in activated platelets [78]. The small peptides cross-link to the N-terminal of both the heavy (GPIIbH 42–73) and the light (GPIIbL$_2$ 30–75) chains of GPIIb, while the longer peptides are cross-linked to the C-terminal of GPIIbH within the 696–724 and 752–768 sequences. The different pattern of peptide cross-linking to GPIIb/IIIa observed in solution and in activated platelets was attributed to the molecular flexibility of the GPIIb subunit. In another approach, the combination of the information derived from studies aimed at determining the epitopes of anti-GPIIb/IIIa monoclonal antibodies and the hydropathic complementarity to the fibrinogen γ402–411 sequence led to the hypothesis that the GPIIb 656–667 (GAHYMRALSNVE) sequence could be a fibrinogen γ-chain putative binding site on the GPIIb/IIIa receptor [101,102]. It was shown that the synthetic peptide GPIIb 656–667 binds to soluble human fibrinogen and inhibits the fibrinogen-mediated aggregation of washed platelets activated with ADP [102]. It is worth noting that the interaction of fibrinogen with the GPIIb 296–306 and 300–312 peptides can be inhibited by both the RGD and γ-chain peptides [93,94], while that of GPIIb 656–667 is inhibited by the synthetic fibrinogen γ-chain peptide 400–411 but not by GRGDS [102]. In a very recent study, 82 synthetic 20-peptides (overlapping by eight residues), covering the extracellular region (1–992) of the GPIIb subunit, were tested for their ability to inhibit the ADP induced human platelet aggregation [103]. It was found that the peptides corresponding to regions GPIIb 57–64 (PWRAEGGQ), GPIIb

265–284 (GAVEILDSYYQRLHRLRAEQ) and GPIIb 313–332 (YMESRADRKLAEVGRVYLFL) inhibited platelet aggregation and antagonized fibrinogen association. In addition, the above peptides did not interfere with the binding of PAC-1 to the activated form of GPIIb/IIIa. Solid-phase binding assays on fibrinogen-coated plates showed that the peptide GPIIb 313–332 bound to fibrinogen in a concentration-dependent manner [103]. In the same study the peptide GPIIb 294–314, which has been proposed as a putative fibrinogen binding domain [66], was found to be 8- and 13-times less active than the GPIIb 313–332 peptide in inhibiting platelet aggregation and fibrinogen binding, respectively [103]. Studies focusing on determining the minimum length of the GPIIb 313–332 site, which is required for the maintenance of the inhibitory effect, concluded that the GPIIb 313–320 sequence (YMES-RADR) inhibited human platelet aggregation, bound to immobilized fibrinogen and did not affect the binding of PAC-1 to the activated form of GPIIb/IIIa [104]. Moreover, the GPIIb 313–320 octapeptide seemed to inhibit platelet activation through the GPIIb/IIIa-dependent outside-in signal transduction pathway as evidenced by inhibition of ATP secretion. In support of these results the peptide complementary to region GPIIb 315–321 (LSARLAF) bound to the receptor and induced conformational changes and platelet aggregation [105,106]. Binding of the LSAR-LAF peptide to GPIIb also induced platelet secretion and further activation through a GPIIb/IIIa mediated outside-in signal transduction [107].

Ligand Binding Sites Supported by GPIIb Mutants

Residues of GPIIb implicated in ligand binding function have been identified by characterization of mutations present in patients with Glanzmann's thrombasthenia and by site-directed mutagenesis studies [108,109]. Among the most frequently naturally occurring mutations within GPIIb found in patients with Glanzmann's thrombasthenia are E324K and R327H [110–115]. Both mutations are correlated with the rate of maturation and/or intracellular transport of the complex to the cell surface probably due to the induced conformational changes. Interestingly, these two naturally occurring mutations (E324K and R327H) are located within the sequence GPIIb 313–332 proposed recently as a putative ligand binding site on GPIIb [103]. Both amino acids are not probably directly involved in the ligand–receptor interaction since the sequence that maintains almost the same amount of inhibitory

activity as the parent peptide was restricted to GPIIb 313–320 [104]. Moreover, two additional naturally occurring mutations have been identified (L55P and V298F) [116,117] within the GPIIb domains, which have been proposed as putative ligand binding sites [78,99,100,103]. The GPIIb L214P, D224V and P145A naturally occurring mutations which were found to impair both GPIIb/IIIa expression and its ligand binding activity [118–120] can not be correlated directly with any of the proposed GPIIb binding sites. Loop swapping and site directed mutagenesis studies concluded that more than 30 discontinuous residues in the *N*-terminal portion of GPIIb are critical for ligand binding [121]. Interestingly, in the same study it was shown that fibrinogen binding to mutants of residues 283–285 is completely abolished, in agreement with the proposed binding site of GPIIb 265–284 [103].

COMPLEMENTARY PEPTIDES TO FIBRINOGEN SEQUENCES

The anticomplementary method provides an alternative approach for predicting amino acid sequences of peptide ligand and receptor binding domain mimics [122]. The sequence GAPLRV was predicted as a putative fibrinogen binding site on GPIIb/IIIa [123]. This peptide has been shown to bind fibrinogen and to inhibit platelet aggregation and clot retraction. Interestingly, the sequence GAPL is present as residues 309–312 in glycoprotein GPIIb and is within or adjacent to the proposed binding sites [99,100,103,104]. Moreover, it has been found that three of the four complementary peptides (EHIPA, GAPL and APLHL) predicted by the m-RNA nucleotides coding for the RGD sequence of Vn and vWf have characteristics expected for a mimic of a glycoprotein GPIIb/IIIa ligand binding site [124–126]. These peptides are inhibitors of platelet functions, which are dependent on Fg binding to platelets.

GPIIB/IIIA DERIVED SEQUENCES AS CANDIDATES FOR DEVELOPING FIBRINOGEN BLOCKERS

Table 1 summarizes the proposed contact sites of GPIIIa and GPIIb subunits with fibrinogen. It is evident from the presented data that there is a very good agreement about the location of the binding

Table 1 Proposed Ligand Binding Sites on GPIIb/IIIa and Functional Properties of the Derived Synthetic Peptides

Subunit	Proposed ligand binding site	Approach[a] used for determining the binding site	Inhibition of platelet aggregation	Binding to fibrinogen	Reference
GPIIIa	109–171	CL	—	—	71
	118–131	SP	Yes	Yes	74
	63–83	CL	—	—	78
	303–350	CL	—	—	78
	151–199	CL	—	—	78
	211–222	SP	Yes	Yes(no)	79(82,86)
	217–231	SP	Yes	Yes	84,85,86
	274–368	RF	Yes	Yes	87
GPIIb	57–64	SP	Yes	Yes	103
	265–284	SP	Yes	Yes	103
	294–314	CL	Yes	Yes	66,99,100
	300–312	SP	Yes	Yes	100
	313–320	SP	Yes	Yes	103,104
	656–667	HC	Yes	Yes	102

[a] Cross-linking (CL), synthetic peptides (SP), recombinant fragments (RF) and hydropathic complementarity (HC).

sites at the *N*-terminal part of both subunits, regardless of the approach which was used. The most precise information for the ligand binding sites was furnished from studies with peptide analogues derived from GPIIIa and GPIIb sequences. The performed groundwork reveals that at least the GPIIIa 211–222, 217–231 and 118–131 and the GPIIb 265–284, 300–312, 313–320 and 656–667 regions, respectively, can constitute a suitable starting point for designing and developing potent and specific fibrinogen ligands. To this end, the fact that continuous sequences were found to represent functional contact sites is very encouraging, since in the case of discontinuous sites, the design and the development of synthetic analogues would be very difficult.

CONCLUDING REMARKS AND OUTLOOK

Peptide sequences derived from GPIIb/IIIa binding sites have proved their ability to inhibit platelet aggregation via a mechanism that involves fibrinogen blocking. Although more studies are needed, the mode of action of these peptide sequences seems to prevent platelet activation, which was a key point for the drawbacks observed in the case of the GPIIb/IIIa blockers.

Despite the extensive studies performed for determining the binding sites of the fibrinogen on the GPIIb/IIIa receptor and the fact that well defined sequences have been found that inhibit platelet aggregation and bind to fibrinogen, these data were not suitably exploited toward developing potent fibrinogen ligands. As a result, there is actually a deficit of valuable data, which could advance this approach. Studies aimed at evaluating (a) the role of each amino acid within sequences derived from the binding sites, (b) the conformational determinants of the fibrinogen ligands required for high binding affinity and (c) the specificity of the GPIIb/IIIa derived peptides for the RGD or the fibrinogen γ-chain sequences are expected to contribute greatly to this field.

Acknowledgements

REFERENCES

1. Hynes RO. Integrins: a family of cell surface receptors. *Cell* 1987; **48**: 549–554.
2. Loftus JC, Smith JW, Ginsberg MH. Integrin-mediated cell adhesion: The extracellular face. *J. Biol. Chem.* 1994; **269**: 25 235–25 238.

3. Marguerie GA, Plow EF, Edgington TS. Human platelets possess an inducible and saturable receptor specific for fibrinogen. *J. Biol. Chem.* 1979; **254**: 5357–5363.

4. Ruoslahti E, Pierschbacher MD. New perspectives in cell adhesion: RGD and integrins. *Science* 1987; **238**: 491–497.

5. Plow EF, Haas TA, Zhang L, Loftus J, Smith JW. Ligand binding to integrins. *J. Biol. Chem.* 2000; **275**: 21 785–21 788.

6. Ginsberg MH, Loftus JC, Plow EF. Cytoadhesins, integrins and platelets. *Thromb. Haemost.* 1988; **59**: 1–6.

7. Pytela R, Pierschbacher MD, Ginsberg MH, Plow EF, Ruoslahti E. Platelet membrane glycoprotein IIb/IIIa: member of a family of Arg-Gly-Asp specific adhesion receptors. *Science* 1986; **231**: 1559–1562.

8. Bennett JS, Vilaire G. Exposure of platelet fibrinogen receptors by ADP and epinephrine. *J. Clin. Invest.* 1979; **64**: 1393–1401.

9. Müller B, Zerwes H-G, Tangemann K, Peter J, Engel J. Two-step binding mechanism of fibrinogen to $\alpha_{\text{IIb}}\beta_3$ integrin reconstituted into planar lipid bilayers. *J. Biol. Chem.* 1993; **268**: 6800–6808.

10. Fox JEB, Shattil SJ, Kinlough-Rathbone RL, Richardson M, Packhamm MA, Sanan DA. The platelet cytoskeleton stabilizes the interaction between $\alpha_{\text{IIb}}\beta_3$ and its ligand and induces selective movements of ligand-occupied integrin. *J. Biol. Chem.* 1996; **271**: 7004–7011.

11. Coller BS. Platelets and thrombolytic therapy. *N. Engl. J. Med.* 1990; **322**: 33–42.

12. Gresele P, Agnelli G. Novel approaches to the treatment of thrombosis. *Trends Pharmacol. Sci.* 2002; **23**: 25–32.

13. Weitz JI, Crowther M. Direct thrombin inhibitors. *Thromb. Res.* 2002; **106**: V275–V284.

14. Geiger J. Inhibitors of platelet signal transduction as anti-aggregatory drugs. *Exp. Opin. Invest. Drugs* 2001; **10**: 865–890.

15. Pierschbacher MD, Ruoslahti E, Sundelin J, Lind P, Peterson PA. The cell attachment domain of fibronectin. Determination of the primary structure. *J. Biol. Chem.* 1982; **257**: 9593–9597.

16. Lam SCT, Plow EF, Ginsberg MH. Evidence that arginyl-glycyl-aspartate peptides and fibrinogen γ chain peptides share a common binding site on platelets. *J. Biol. Chem.* 1987; **262**: 947–950.

17. Gartner TK, Bennett JS. The tetrapeptide analogue of the cell attachment site of fibronectin inhibits platelet aggregation and fibrinogen binding in activated platelets. *J. Biol. Chem.* 1985; **260**: 11 891–11 894.

18. Plow EF, Srouji AH, Meyer D, Marguerie G, Ginsberg MH. Evidence that three adhesive proteins interact with a common recognition site on activated platelets. *J. Biol. Chem.* 1984; **259**: 5388–5391.

19. Andrieux A, Hudry-Glergeon G, Ryckewaert J-J, Chapel A, Ginsberg MH, Plow EF, Marguerie G. Amino acid sequences in fibrinogen mediating its interaction with its platelet receptor, GPIIbIIIa. *J. Biol. Chem.* 1989; **264**: 9258–9265.

20. Ojima I, Chakravarty S, Dong Q. Antithrombotic agents: From RGD to peptide mimetics. *Bioorg. Med. Chem.* 1995; **3**: 337–360.

21. Craig WS, Cheng S, Mullen DG, Blevitt J, Pierschbacher MD. Concept and progress in the development of RGD-containing peptide pharmaceuticals. *Biopolymers* 1995; **37**: 157–175.

22. Stavrakoudis A, Bizos G, Eleftheriadis D, Kouki A, Panou-Pomonis E, Sakarellos-Daitsiotis M, Sakarellos C, Tsoukatos D, Tsikaris V. A three-residue cyclic scaffold of non-RGD containing peptide analogues as platelet aggregation inhibitors: design, synthesis and structure–function relationships. *Biopolymers* 2001; **56**: 20–26.

23. Casserly IP, Topol EJ. Glycoprotein IIb/IIIa antagonists — from bench to practice. *Cell. Mol. Life Sci.* 2002; **59**: 478–500.

24. Thibault G, Tardif P, Lapalme G. Comparative specificity of platelet $\alpha_{\text{IIb}}\beta_3$ integrin antagonists. *J. Pharmacol. Exp. Ther.* 2001; **296**: 690–696.

25. Topol EJ, Byzova TV, Plow EF. Platelet GPIIb-IIIa blockers. *Lancet* 1999; **353**: 227–231.

26. Scarborough RM, Kleiman NS, Philips DR. Platelet glycoprotein IIb/IIIa antagonists. What are the relevant issues concerning their pharmacology and clinical use? *Circulation* 1999; **100**: 437–444.

27. Verstraete M. Synthetic inhibitors of platelet glycoprotein IIb/IIIa in clinical development. *Circulation* 2000; **102**: e76–e80.

28. Pfaff M, Tangemann K, Muller B, Gurrath M, Muller G, Kessler H, Timpl R, Engel J. Selective recognition of cyclic RGD peptides of NMR defined conformation by $\alpha_{\text{IIb}}\beta_3$, $\alpha_{\text{v}}\beta_3$, and $\alpha_5\beta_1$ integrins. *J. Biol. Chem.* 1994; **269**: 20 233–20 238.

29. Samanen J, Ali F, Romoff T, Calvo R, Sorenson E, Vasco J, Storer B, Berry D, Bennett D, Strohsacker M, Powers D, Stadel J, Nichols A. Development of a small RGD peptide fibrinogen receptor antagonist with potent antiaggregatory activity *in vitro*. *J. Med. Chem.* 1991; **34**: 3114–3125.

30. Haubner R, Gratias R, Diefenbach B, Goodman SL, Jonczyk A, Kessler H. Structural and functional aspects of RGD-containing cyclic pentapeptides as highly potent and selective integrin $\alpha_{\text{v}}\beta_3$ antagonists. *J. Am. Chem. Soc.* 1996; **118**: 7461–7472.

31. Ali F, Bennett D, Calvo R, Elliott J, Hwang S-M, Ku T, Lago M, Nichols A, Romoff T, Shah D, Vasko J, Wong A, Yellin T, Yuan C-K, Samanen J. Conformationally constrained peptides and semipeptides derived from RGD as potent inhibitors of platelet fibrinogen receptor and platelet aggregation. *J. Med. Chem.* 1994; **37**: 769–780.

32. Bach A, Eyermann C, Gross J, Bower M, Harlow R, Weber P, DeGrado W. Structural studies of a family

of high affinity ligands for GPIIb/IIIa. *J. Am. Chem. Soc.* 1994; **116**: 3207–3219.

33. Schabbert S, Pierschbacher M, Mattern R-H, Goodman M. Incorporation of (2S,3S) and (2S,3R) β-methyl aspartic acid into RGD-containing peptides. *Bioorg. Med. Chem.* 2002; **10**: 3331–3337.

34. McLance MA, Marcinkiewcz C, Vijay-Kumar S, Wierzbicka-Patynowski I, Niewiarowski S. Viper venom disintegrins and related molecules. *Proc. Soc. Exp. Biol. Med.* 1998; **219**: 109–119.

35. D'Souza SE, Ginsberg MH, Lam SC-T, Plow EF. Chemical cross-linking of arginyl-glycyl-aspartic acid peptides to an adhesion receptor on platelets. *J. Biol. Chem.* 1988; **263**: 3943–3951.

36. Bednar RA, Gaul SL, Hamill TG, Egbertson MS, Shafer JA, Hartman GD, Gould RJ, Bednar B. Identification of low molecular weight GPIIb/IIIa antagonists that bind preferentially to activated platelets. *J. Pharmacol. Exp. Ther.* 1998; **285**: 1317–1326.

37. Frelinger III AL, Cohen I, Plow EF, Smith MA, Roberts J, Lam SC-T, Ginsberg MH. Selective inhibition of integrin function by antibodies specific for ligand-occupied receptor conformers. *J. Biol. Chem.* 1990; **265**: 6346–6352.

38. Kouns WC, Wall CD, White MM, Fox CF, Jennings LK. A conformation-dependent epitope of human platelet glycoprotein IIIa. *J. Biol. Chem.* 1990; **265**: 20 594–20 601.

39. Leisner TM, Wencel-Drake JD, Wang W, Lam Sc-T. Bidirectional transmembrane modulation of integrin $\alpha_{IIb}\beta_3$ conformations. *J. Biol. Chem.* 1999; **274**: 12 945–12 949.

40. Cierniewski CS, Byzova T, Papierak M, Haas TA, Niewiarowska J, Zhang L, Cieslak M, Plow EF. Peptide ligands can bind to distinct sites in integrin $\alpha_{IIb}\beta_3$ and elicit different functional responses. *J. Biol. Chem.* 1999; **274**: 16 923–16 932.

41. Mondoro TH, White MM, Jennings LK. Active GPIIb-IIIa conformations that link ligand interaction with cytoskeletal reorganization. *Blood* 2000; **96**: 2487–2495.

42. Peter K, Stranb A, Kohler B, Volkmann M, Schwarz M, Kubler W, Bode C. Platelet activation as a potential mechanism of GPIIb/IIIa inhibitor-induced thrombocytopenia. *Am. J. Cardiol.* 1999; **84**: 519–524.

43. Peter K, Schwarz M, Nordt T, Bode C. Intrinsic activating properties of GP IIb/IIIa blockers. *Thromb. Res.* 2001; **103**: S21–S27.

44. Chew DP, Bhatt DL, Sapp S, Topol EJ. Increased mortality with oral platelet glycoprotein IIb/IIIa antagonists. A metaanalysis of phase III multicenter randomized trials. *Circulation* 2001; **103**: 201–206.

45. Peter K, Schwarz M, Ylänne J, Kohler B, Moser M, Nordt T, Salbach P, Kübler W, Bode C. Induction of fibrinogen binding and platelet aggregation as a potential intrinsic property of various

glycoprotein IIb/IIIa ($\alpha_{IIb}\beta_3$) inhibitors. *Blood* 1998; **92**: 3240–3249.

46. Peter K, Schwarz M, Bode C. Activating effects of GPIIb/IIIa blockers: An intrinsic consequence of ligand mimetic properties. *Circulation* 2002; **105**: E180–E181.

47. Frelinger III AL, Furman MI, Krueger LA, Barnard MR, Michelson AD. Dissociation of glycoprotein IIb/IIIa antagonists from platelets does not result in fibrinogen binding or platelet aggregation. *Circulation* 2001; **104**: 1374–1379.

48. Zolotarjova NI, Hollis GF, Wynn R. Unusually stable and long-lived ligand-induced conformations of integrins. *J. Biol. Chem.* 2001; **276**: 17 063–17 068.

49. Hantgan RR, Paumi C, Rocco M, Weisel JW. Effects of ligand-mimetic peptides Arg-Gly-Asp-X (X = Phe, Trp, Ser) on $\alpha_{IIb}\beta_3$ integrin conformation and oligomerization. *Biochemistry* 1999; **38**: 14 461–14 474.

50. Hu DD, White CA, Panzer-Knodle S, Page JD, Nicholson N, Smith JW. A new model of dual interacting ligand binding sites on integrin $\alpha_{IIb}\beta_3$. *J. Biol. Chem.* 1999; **274**: 4633–4639.

51. Calzada MJ, Alvarez MV, González-Rodríguez J. Agonist-specific structural rearrangement of integrin $\alpha_{IIb}\beta_3$. *J. Biol. Chem.* 2002; **277**: 39 899–39 908.

52. Li R, Babu CR, Lear JD, Wand AJ, Bennett JS, DeGrado WF. Oligomerization of the integrin $\alpha_{IIb}\beta_3$. Roles of the transmembrane and cytoplasmic domains. *Proc. Natl Acad. Sci. USA* 2001; **98**: 12 462–12 467.

53. Du X, Gu M, Weisel JW, Nagaswami G, Bennett JS, Bowditch R, Ginsberg MH. Long range propagation of conformational changes in integrin $\alpha_{IIb}\beta_3$. *J. Biol. Chem.* 1993; **268**: 23 087–23 092.

54. Du X, Plow EF, Frelinger III AL, O'Toole TE, Loftus JC, Ginsberg MH. Ligands 'activate' integrin $\alpha_{IIb}\beta_3$ (platelet GPIIb-IIIa). *Cell* 1991; **65**: 409–416.

55. Hantgan RR, Rocco M, Nagaswami C, Weisel JW. Binding of a fibrinogen mimetic stabilizes integrin $\alpha_{IIb}\beta_3$'s open conformation. *Protein Sci.* 2001; **10**: 1614–1626.

56. Hantgan RR, Lyles DS, Mallett TC, Rocco M, Nagaswami C, Weisel JW. Ligand binding promotes the entropy-driven oligomerization of integrin $\alpha_{IIb}\beta_3$. *J. Biol. Chem.* 2003; **278**: 3417–3426.

57. Frelinger III AL, Du X, Plow EF, Ginsberg MH. Monoclonal antibodies to ligand-occupied conformers of integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa) alter receptor affinity, specificity, and function. *J. Biol. Chem.* 1991; **266**: 17 106–17 111.

58. Basani RB, D'Andrea G, Mitra N, Vilaire G, Richberg M, Kowalska MA, Bennett JS, Poncz M. RGD-containing peptides inhibit fibrinogen binding to platelet $\alpha_{IIb}\beta_3$ by inducing an allosteric change in the amino-terminal portion of αIIb. *J. Biol. Chem.* 2001; **276**: 13 975–13 981.

59. Buensuceso C, de Virgilio M, Shattil SJ. Detection of integrin $\alpha_{IIb}\beta_3$ clustering in living cells. *J. Biol. Chem.* 2003; **278**: 15 217–15 224.

60. Mayo KH, Fan F, Beavers MP, Eckardt A, Keane P, Hoekstra WJ, Andrade-Gordon P. RGD induces conformational transition in purified platelet integrin GPIIb/IIIa-SDS system yielding multiple binding states for fibrinogen γ-chain *C*-terminal peptide. *FEBS Lett.* 1996; **378**: 79–82.

61. Schneider DJ, Taatjes DJ, Sobel BE. Paradoxical inhibition of fibrinogen binding and potentiation of α-granule release by specific types of inhibitors of glycoprotein IIb-IIIa. *Cardiovasc. Res.* 2000; **45**: 437–446.

62. Smith JW, Ruggeri ZM, Kunicki TJ, Cheresh DA. Interaction of integrins $\alpha_v\beta_3$ and glycoprotein IIb-IIIa with fibrinogen. Differential peptide recognition accounts for distinct binding sites. *J. Biol. Chem.* 1990; **265**: 12 267–12 271.

63. Cheresh DA, Berliner SA, Vicente V, Ruggeri ZM. Recognition of distinct adhesive sites on fibrinogen by related integrins on platelets and endothelial cells. *Cell* 1989; **58**: 945–953.

64. Farell DH, Thiagarajan P. Binding of recombinant fibrinogen mutants to platelets. *J. Biol. Chem.* 1994; **269**: 226–231.

65. Farell DH, Thiagarajan P, Chung DW, Davie EW. Role of fibrinogen α and γ chain sites in platelet aggregation. *Proc. Natl Acad. Sci. USA* 1992; **89**: 10 729–10 732.

66. D'Souza SE, Ginsberg MH, Burke TA, Plow EF. The ligand binding site of the platelet integrin receptor GPIIb-IIIa is proximal to the second calcium binding domain of its α subunit. *J. Biol. Chem.* 1990; **265**: 3440–3446.

67. Kouns WC, Hadvary P, Haering P, Steiner B. Conformational modulation of purified glycoprotein (GP)IIb-IIIa allows proteolytic generation of inactive GPIIb-IIIa. *J. Biol. Chem.* 1992; **267**: 18 844–18 851.

68. Lam SC-T. Isolation and characterization of a chymotryptic fragment of platelet glycoprotein IIb-IIIa retaining Arg-Gly-Asp binding activity. *J. Biol. Chem.* 1992; **267**: 5649–5655.

69. Loftus JC, Halloran CE, Ginsberg MH, Feigens LP, Zablocki JA, Smith JW. The amino-terminal one-third of αIIb defines the ligand recognition specificity of integrin $\alpha_{IIb}\beta$. *J. Biol. Chem.* 1996; **271**: 2033–2039.

70. Santoro SA, Lawing WJ. Competition for related but nonidentical binding sites on the glycoprotein IIb-IIIa complex by peptides derived from platelet adhesive proteins. *Cell* 1987; **48**: 867–873.

71. D'Souza SE, Ginsberg MH, Burke TA, Lam SC-T, Plow EF. Localization of an Arg-Gly-Asp recognition site within an integrin adhesion receptor. *Science* 1988; **242**: 91–93.

72. Smith JW, Cheresh DA. The Arg-Gly-Asp binding domain of the vitronectin receptor. Photoaffinity cross-linking implicates amino acid residues 61–203 of the β subunit. *J. Biol. Chem.* 1988; **263**: 18 726–18 731.

73. Andrieux A, Rabiet M-J, Chapel A, Concord E, Marguerie G. A highly conserved sequence of the Arg-Gly-Asp-binding domain of the integrin β_3 subunit is sensitive to stimulation. *J. Biol. Chem.* 1991; **266**: 14 202–14 207.

74. D'Souza SE, Haas TA, Piotrowicz RS, Byers-Ward V, McGrath DE, Soule HR, Cierniewski C, Ginsberg MH, Plow EF, Smith JW. Ligand and cation binding are dual functions of a discrete segment of the integrin β_3 subunit: Cation displacement is involved in ligand binding. *Cell* 1994; **79**: 659–667.

75. Cierniewski CS, Haas TA, Smith JW, Plow EF. Characterization of cation-binding sequences in the platelet integrin GPIIb-IIIa ($\alpha_{IIb}\beta_3$) by terbium luminescence. *Biochemistry* 1994; **33**: 12 238–12 246.

76. Pasqualini R, Koivunen E, Ruoslahti E. A peptide isolated from phage display libraries is a structural and functional mimic of an RGD-binding site on integrins. *J. Cell Biol.* 1995; **130**: 1189–1196.

77. Calvete JJ, Arias J, Alvarez MV, Lopez MM, Henschen A, Gonzalez-Rodriguez J. Further studies on the topography of the *N*-terminal region of human platelet glycoprotein IIIa. Localization of monoclonal antibody epitopes and the putative fibrinogen-binding sites. *Biochem. J.* 1991; **247**: 457–463.

78. Calvete JJ, Schäfer W, Mann K, Henschen A, González-Rodríguez J. Localization of the cross-linking sites of RGD and KQAGDV peptides on the isolated fibrinogen receptor, the human platelet integrin glycoprotein IIb/IIIa. Influence of peptide length. *Eur. J. Biochem.* 1992; **206**: 759–765.

79. Charo IF, Nannizzi L, Philips DR, Hsu MA, Scarborough RM. Inhibition of fibrinogen binding to GPIIb-IIIa by a GPIIIa peptide. *J. Biol. Chem.* 1991; **266**: 1415–1421.

80. Argraves WS, Suzuki S, Arai H, Thompson K, Pierschbacher MD, Ruoslahti E. Amino acid sequence of the human fibronectin receptor. *J. Cell Biol.* 1987; **105**: 1183–1190.

81. Kishimoto TK, O'Connor K, Lee A, Roberts TM, Srpinger TA. Cloning of the β subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family. *Cell* 1987; **48**: 681–690.

82. Steiner B, Trzeciak A, Pfenninger G, Kouns WC. Peptides derived from a sequence within β_3 integrin bind to platelet $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) and inhibit ligand binding. *J. Biol. Chem.* 1993; **268**: 6870–6873.

83. Shattil SJ, Hoxie JA, Cunningham M, Brass LF. Changes in the platelet membrane glycoprotein IIb-IIIa complex during platelet activation. *J. Biol. Chem.* 1985; **260**: 11 107–11 114.

84. Cook JJ, Trybulec M, Lasz EC, Khan S, Niewiarowski S. Binding of glycoprotein IIIa-derived peptide

217–231 to fibrinogen and von Willebrand factors and its inhibition by platelet glycoprotein IIb/IIIa complex. *Biochim. Biophys. Acta* 1992; **1119**: 312–321.

85. Lasz EC, Mclane M-A, Trybulec M, Kowalska A-M, Khan S, Budzynski AZ, Niewiarowski S. β_3 Integrin derived peptide 217–230 inhibits fibrinogen binding and platelet aggregation: significance of RGD sequences and fibrinogen Aα-chain. *Biochem. Biophys. Res. Commun.* 1993; **190**: 118–124.

86. Wierzbicka I, Kowalska M-A, Lasz EC, Farrell DH, Budzynski AZ, Niewiarowski S. Interaction of β_3 integrin-derived peptides 214–218 and 217–231 with $\alpha_{IIb}\beta_3$ complex and with fibrinogen Aα -chain. *Thromb. Res.* 1997; **85**: 115–126.

87. Alemany M, Concord E, Garin J, Vincon M, Giles A, Marguerie G, Gulino D. Sequence 274–368 in the β_3-subunit of the integrin $\alpha_{IIb}\beta_3$ provides a ligand recognition and binding domain for the γ-chain of fibrinogen that is independent of platelet activation. *Blood* 1996; **87**: 592–601.

88. Cierniewska-Cieslak A, Cierniewski CS, Blecka K, Papierak M, Michalec L, Zhang L, Haas TA, Plow EF. Identification and characterization of two cation binding sites in the integrin β_3 subunit. *J. Biol. Chem.* 2002; **277**: 11126–11134.

89. Puzon-McLaughlin W, Kamata T, Takada Y. Multiple discontinuous ligand-mimetic antibody binding sites define a ligand binding pocket in integrin $\alpha_{IIb}\beta_3$. *J. Biol. Chem.* 2000; **275**: 7795–7802.

90. Loftus JC, O'Toole TE, Plow EF, Glass A, Frelinger III AL, Ginsberg MH. A β_3 integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. *Science* 1990; **249**: 915–918.

91. Bajt ML, Loftus JC. Mutation of a ligand binding domain of β_3 integrin. Integral role of oxygenated residues in $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) receptor function. *J. Biol. Chem.* 1994; **269**: 20913–20919.

92. Bajt ML, Ginsberg MH, Frelinger III AL, Berndt MC, Loftus JC. A spontaneous mutation of integrin $\alpha_{IIb}\beta_3$ (platelet glycoprotein IIb-IIIa) helps define a ligand binding site. *J. Biol. Chem.* 1992; **267**: 3789–3794.

93. Lanza F, Stierle A, Fournier D, Morales M, Andre G, Nurden AT, Cazenave JP. A new variant of Glanzmann's thrombasthenia (Strasbourg I). Platelets with functionally defective glycoprotein IIb-IIIa complexes and a glycoprotein IIIa 214Arg → 214Trp mutation. *J. Clin. Invest.* 1992; **89**: 1995–2004.

94. Takagi J, Kamata T, Meredith J, Puzon-McLaughlin W, Takada Y. Changing ligand specificities of $\alpha_V\beta_1$ and $\alpha_V\beta_3$ integrins by swapping a short diverse sequence of the β subunit. *J. Biol. Chem.* 1997; **272**: 19794–19800.

95. Tozer EC, Liddington RC, Sutcliffe MJ, Smeeton AH, Loftus JC. Ligand binding of integrin $\alpha_{IIb}\beta_3$ is dependent on a MIDAS-like domain in the β_3 subunit. *J. Biol. Chem.* 1996; **271**: 21978–21984.

96. Ward CM, Kestin AS, Newman PJ. A Leu262 Pro mutation in the integrin β_3 subunit results in an $\alpha_{IIb} - \beta_3$ complex that binds fibrin but not fibrinogen. *Blood* 2000; **96**: 161–169.

97. Fullard J, Murphy R, O'Neill S, Moran N, Ottridge B, Fitzgerald DJ. A Val93Met mutation in GPIIa results in a GPIIb/IIIa receptor with a constitutively high affinity for a small ligand. *Br. J. Haematol.* 2001; **115**: 131–139.

98. Ruiz C, Liu C-Y, Sun Q-H, Sigaud-Fiks M, Fressinaud E, Muller, J-Y, Nurden P, Nurden AT, Newman PJ, Valentin N. A point mutation in the cysteine-rich domain of glycoprotein (GP)IIIa results in the expression of a GPIIb-IIIa ($\alpha_{IIb}\beta_3$) integrin receptor locked in a high-affinity state and a Glanzmann thrombasthenia-like phenotype. *Blood* 2001; **98**: 2432–2441.

99. D'Souza SE, Ginsberg MH, Matsueda GR, Plow EF. A discrete sequence in a platelet integrin is involved in ligand recognition. *Nature* 1991; **350**: 66–68.

100. Taylor DB, Gartner TK. A peptide corresponding to GPIIb$_\alpha$ 300–312, a presumptive fibrinogen γ-chain binding site on the platelet integrin GPIIb/IIIa, inhibits the adhesion of platelets to at least four adhesive ligands. *J. Biol. Chem.* 1992; **267**: 11729–11733.

101. Calvete JJ, Arias J, Alvarez MV, Lopez MM, Henschen A, González Rodríguez J. Further studies on the topography of human platelet glycoprotein IIb. Localization of monoclonal antibody epitopes and the putative glycoprotein IIIa and fibrinogen-binding regions. *Biochem. J.* 1991; **273**: 767–775.

102. Calvete JJ, German R, Schäfer W, McLane MA, Niewiarowski S. Glycoprotein IIb peptide 656–667 mimics the fibrinogen γ chain 402–411 binding site on platelet integrin GPIIb/IIIa. *FEBS Lett.* 1993; **335**: 132–135.

103. Biris N, Abatzis M, Mitsios JV, Sakarellos-Daitsiotis M, Sakarellos C, Tsoukatos D, Tselepis AD, Michalis L, Sideris D, Konidou G, Soteriadou K, Tsikaris V. Mapping the binding domains of the α_{IIb} subunit. A study performed on the activated form of the platelet integrin $\alpha_{IIb}\beta_3$. *Eur. J. Biochem.* 2003; **270**: 3760–3767.

104. Mitsios JV, Tambaki AP, Abatzis M, Biris N, Sakarellos-Daitsiotis M, Sakarellos C, Soteriadou K, Goudevenos J, Elisaf M, Tsoukatos D, Tsikaris V, Tselepis AD. Effect of synthetic peptides corresponding to residues 313–332 of α_{IIb} subunit on platelet activation and fibrinogen binding to $\alpha_{IIb}\beta_3$. *Eur. J. Biochem* 2004; **271**: 855–862.

105. Derrick JM, Taylor DB, Loudon RG, Gartner TK. The peptide LSARLAF causes platelet secretion and aggregation by directly activating the integrin $\alpha_{IIb}\beta_3$. *Biochem. J.* 1997; **325**: 309–313.

106. Derrick JM, Loudon RG, Gartner TK. Peptide LSARLAF activates $\alpha_{IIb}\beta_3$ on resting platelets and causes resting platelet aggregate formation without

platelet shape change. *Thromb. Res.* 1998; **89**: 31–40.

107. Derrick JM, Shattil SJ, Poncz M, Gruppo RA, Gartner TK. Distinct domains of $\alpha_{IIb}\beta_3$ support different aspects of outside-in signal transduction and platelet activation induced by LSARLAF an $\alpha_{IIb}\beta_3$ interacting peptide. *Thromb. Haemost.* 2001; **86**: 894–901.

108. French DL, Coller BS. Hematologically important mutations: Glanzmann thrombasthenia. *Blood Cells Mol. Dis.* 1997; **23**: 39–51.

109. French DL. The molecular genetics of Glanzmann's thrombasthenia. *Platelets* 1998; **9**: 5–20.

110. Milet-Marsal S, Breillat C, Peyruchaud O, Nurden P, Combrie R, Nurden AT, Bourre F. Analysis of the amino acid requirement for a normal $\alpha_{IIb}\beta_3$ maturation at α_{IIb}Glu324 commonly mutated in Glanzmann thrombasthenia. *Thromb. Haemost.* 2002; **88**: 655–662.

111. Ambo H, Kamata T, Handa M, Kawai Y, Oda A, Murata M, Takada Y, Ikeda Y. Novel point mutations in the α_{IIb} subunit (Phe^{289} → Ser, Glu^{324} → Lys and Gln^{747} → Pro) causing thrombasthenic phenotypes in four Japanese patients. *Br. J. Haematol.* 1998; **102**: 829–840.

112. Tao J, Arias-Salgado EG, González-Manchón C, Iruin G, Butta N, Ayuso MS, Parrilla R. A 1063G → A mutation in exon 12 of glycoprotein (GP)IIb associated with a thrombasthenic phenotype: mutation analysis of [324E]GPIIb. *Br. J. Haematol.* 2000; **111**: 965–973.

113. Ferrer M, Fernandez-Pinel M, González-Manchón C, González J, Ayuso MS, Parrilla R. A mutant (Arg327 → His) GPIIb associated to thrombasthenia exerts a dominant negative effect in stably transfected CHO cells. *Thromb. Haemost.* 1996; **76**: 292–301.

114. Ruan J, Peyruchaud O, Alberio L, Valles G, Clemetson K, Bourre F, Nurden AT. Double heterozygosity of the GPIIb gene in a Swiss patient with Glanzmann's thrombasthenia. *Br. J. Haematol.* 1998; **102**: 918–925.

115. Wilcox DA, Paddock CM, Lyman S, Gill SC, Newman PJ. Glanzmann thrombasthenia resulting from a single amino acid substitution between the second and third calcium-binding domains of GPIIb. Role of the GPIIb amino terminus in integrin subunit association. *J. Clin. Invest.* 1995; **95**: 1553–1560.

116. Tanaka S, Hayashi T, Hori Y, Terada C, Han KS, Ahn HS, Bourre F, Tani Y. A Leu^{55} to Pro substitution in the integrin α_{IIb} is responsible for a case of Glanzmann's thrombasthenia. *Br. J. Haematol.* 2002; **118**: 833–835.

117. Mitchell BW, Li JH, Singh F, Michelson AD, Bussel J, Coller BS, French DL. Two novel mutations in the α_{IIb} calcium-binding domains identify hydrophobic regions essential for $\alpha_{IIb}\beta_3$ biogenesis. *Blood* 2003; **101**: 2268–2276.

118. Grimaldi CM, Chen F, Wu C, Weiss HJ, Coller BS, French DL. Glycoprotein IIb Leu^{214}Pro mutation produces Glanzmann thrombasthenia with both quantitative and qualitative abnormalities in GPIIb/IIIa. *Blood* 1998; **91**: 1562–1571.

119. Tozer EC, Baker EK, Ginsberg MH, Loftus JC. A mutation in the α subunit of the platelet integrin $\alpha_{IIb}\beta_3$ identifies a novel region important for ligand binding. *Blood* 1999; **93**: 918–924.

120. Basani RB, French DC, Vilaire G, Brown DL, Chen F, Coller BS, Derrick JM, Gartner TK. A naturally occurring mutation near the amino terminus of α_{IIb} defines a new region involved in ligand binding to $\alpha_{IIb}\beta_3$. *Blood* 2000; **95**: 180–188.

121. Kamata T, Tien KK, Irie A, Springer AT, Takada Y. Amino acid residues in the α_{IIb} subunit that are critical for ligand binding to integrin $\alpha_{IIb}\beta_3$ are clustered in the β-propeller model. *J. Biol. Chem.* 2001; **276**: 44 275–44 283.

122. Bost KL, Blalock JE. Preparation and use of complementary peptides. *Methods Enzymol.* 1989; **168**: 16–28.

123. Gartner TK, Taylor DB. The amino acid sequence Gly-Ala-Pro-Leu appears to be a fibrinogen binding site in the platelet integrin glycoprotein IIb. *Thromb. Res.* 1990; **60**: 291–309.

124. Gartner TK, Loudon R, Taylor DB. The peptides APLHK, EHIPA and GAPL are hydropathically equivalent peptide mimics of a fibrinogen binding domain of glycoprotein IIb/IIIa. *Biochem. Biophys. Res. Commun.* 1991; **180**: 1446–1452.

125. Gartner TK, Taylor DB. The peptide Glu-His-Ile-Pro-Ala binds fibrinogen and inhibits platelet aggregation and adhesion to fibrinogen and vitronectin. *Proc. Soc. Exp. Biol. Med.* 1991; **198**: 649–655.

126. Gartner TK, Taylor DB, Derrick J. Complementary peptides that interfere with platelet aggregation and adherence. *Immunomethods* 1994; **5**: 148–157.