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## Platelet Integrins and Signaling

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### INTRODUCTION

The term *integrin* describes an adhesion molecule family and originates from the integrative function of these molecules between extracellular ligands and the intracellular cytoskeleton (1,2). Integrins mediate cell–cell, cell–extracellular matrix, and cell–pathogen interactions. Integrins have two major functions: First, they mechanically couple the cytoskeleton to the extracellular matrix or to surface receptors of other cells. Second, they transmit signals from the inside of the cell to the outside of the cell and vice versa (3). At least 24 different integrins are known in vertebrates (Fig. 1). Resting platelets express integrins  $\alpha_2\beta_1$ ,  $\alpha_5\beta_1$ ,  $\alpha_6\beta_1$ ,  $\alpha_v\beta_3$ , and  $\alpha_{IIb}\beta_3$  (4). In addition to these,  $\alpha_L\beta_2$  and  $\alpha_M\beta_2$  expression on activated platelets has been reported (5).

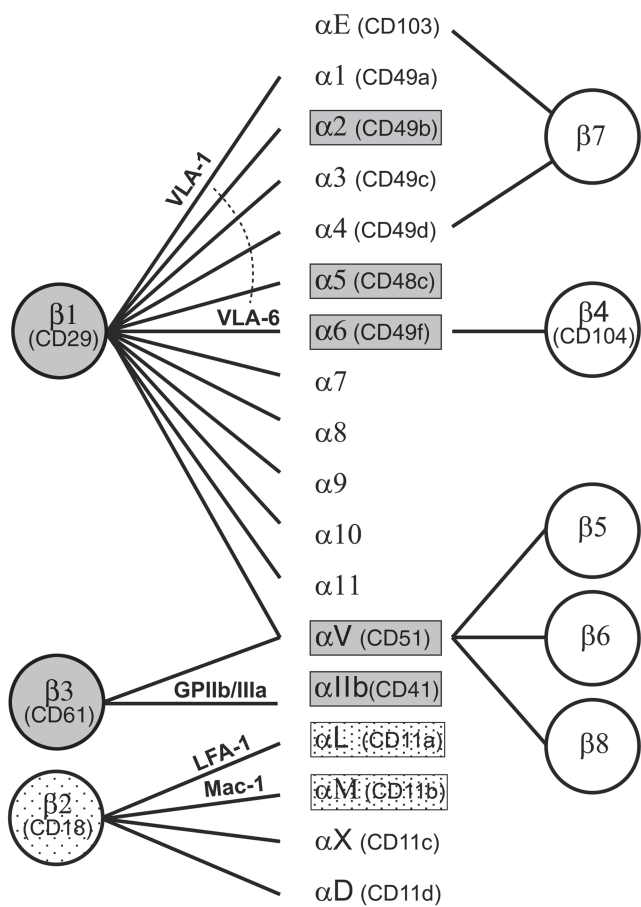
### INTEGRIN STRUCTURE

All integrins consist of two subunits ( $\alpha$  and  $\beta$ ) that are noncovalently linked to each other (Fig. 2). Both subunits are type I membrane glyco-

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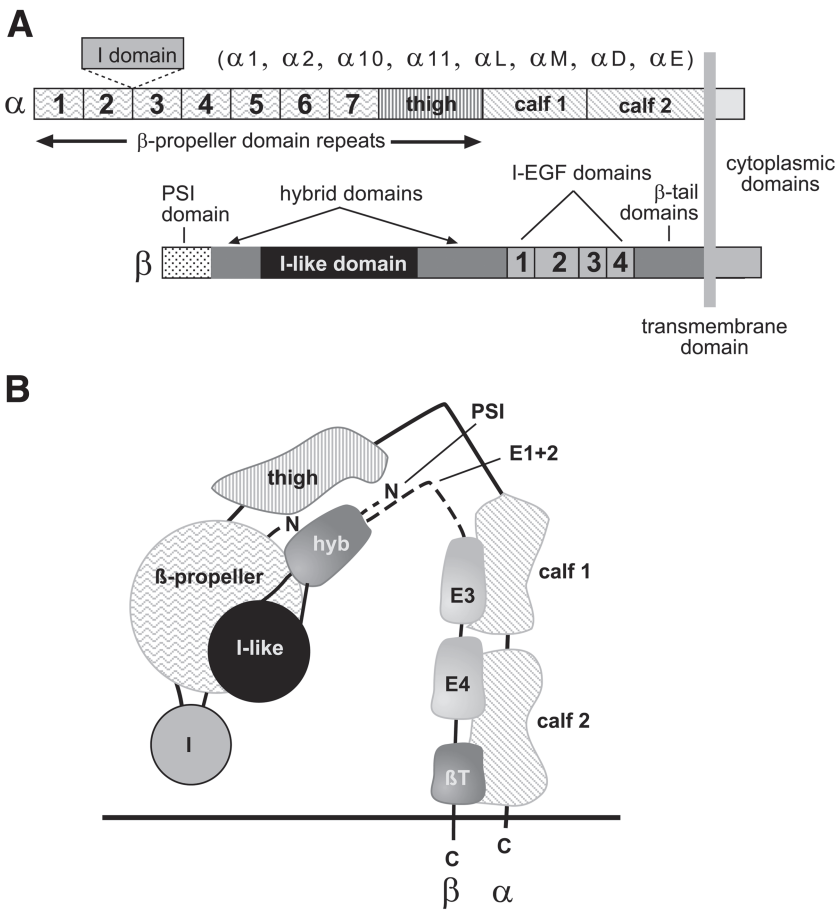
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**Fig. 1.** Integrin  $\alpha/\beta$  heterodimers. Integrin nomenclature and CD numbers are given. Integrins expressed on resting platelets are in dark gray. Integrins reported to be expressed only on activated platelets are in light gray. LFA-1, leukocyte function-associated antigen-1; VLA, very late activation antigen.

proteins. Eighteen different  $\alpha$ -subunits and 8 different  $\beta$ -subunits have so far been identified (Fig. 1) (1,2). With the exception of  $\alpha_4$ , all integrin subunits have a short cytoplasmic tail, a transmembraneous region, and a large extracellular domain. Both subunits of integrins are rich in cysteine residues; and their pairing in disulfide bonds has been shown to be important in the structure of integrins and may also play a role in the conformational changes during their activation (6,7). In addition, integrins may possess an endogenous thiol isomerase activity that itself may be part of the integrin activation process (8). Their characteristics



**Fig. 2.** Integrin structure. Proposed domain organization within the primary structure (**A**) and within the 3D structure (**B**) of integrins according to Takagi and Springer (9,45). For a detailed description *see* text. hyd, hybrid; I-EGF, integrin epidermal growth factor; PSI, plexin, semaphorin, and integrin domain.

in respect to cell type expression, ligand specificity, cytoplasmic interaction partners, and signaling pathways are often overlapping and are the subject of extensive scientific work in many laboratories. For several of these integrins, different conformational states have been demonstrated (2,9). Upon cell stimulation, integrins can change their conformation rapidly from a low- to a high-affinity state in respect to their ligand binding properties. This is of particular importance for integrins on leukocytes and platelets, since these cells have to be recruited from the blood stream by the rapid activation of their integrins.

Figure 2 shows proposed models for integrin structures. The extracellular part of both integrin subunits seems to form a “globular head” and two long “legs” or “stalks.” In the middle of the stalks a “knee” or “genu” region has been postulated at which the extracellular integrin domains seem to be bend. The globular head contains the ligand binding regions (10,11).

### *Structure of the $\alpha$ -Subunits*

The N termini of the  $\alpha$ -subunits contain seven segments of around 60 amino acids each, which have weak homologies to one another, and these segments are proposed to form a  $\beta$ -propeller with seven four-stranded  $\beta$ -sheets arranged like the blades of a propeller around a pseudosymmetry axis (11,12). Mapping of epitopes, which are far apart in the primary sequence but close in the predicted structure, and X-ray crystallography have confirmed the  $\beta$ -propeller model (13,14). The C-terminal 4-strands of the seven repeats are similar to the so-called EF-hand structure, which is composed of a divalent cation-binding motif (15,16). The problem of the  $\beta$ -propeller model and the finding that EF-hand motifs are based on  $\alpha$ -helical structures was recently elucidated by the finding that a  $\text{Ca}^{2+}$ -binding  $\beta$ -hairpin loop resembles integrin sequence motifs better than the EF hand (16,17). Furthermore, the recently published crystal structure of  $\alpha_V$  (13) indeed revealed a  $\beta$ -propeller structure, as predicted by Springer (11,12). Mutagenesis studies suggest that ligand-binding residues cluster in one portion of the top and side of the  $\beta$ -propeller and that the  $\text{Ca}^{2+}$ -binding domains are in loops on the lower, bottom face of the propeller (18).

Half of the integrin  $\alpha$ -subunits ( $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_{10}$ ,  $\alpha_{11}$ ,  $\alpha_L$ ,  $\alpha_M$ ,  $\alpha_X$ ,  $\alpha_D$ ,  $\alpha_E$ ) contain a domain of around 200 amino acids that is inserted between  $\beta$ -sheets 2 and 3 of the  $\beta$ -propeller structure. This domain is called the I- or A-domain because of its homology to the von Willebrand factor A1-, A2-, and A3-domains and it is the major integrin binding site in the integrins containing an I-domain (19–21). The 3D structure of the dinucleotide-binding or Rossmann fold, with  $\alpha$ -helices surrounding a central  $\beta$ -sheet, is adopted by the I-domain (22). A metal ion-dependent adhesion site (MIDAS) within the I-domain binds negatively charged residues in ligands. The I-domain of  $\alpha_L$ , expressed solely on the cell surface, is able to mediate cell adhesion, providing strong evidence for the central role of the I-domain in ligand binding (23).

Corresponding to the stalk or leg region is a large portion of around 500 residues that is predicted to consist of three two-layer  $\beta$ -sandwich domains, designated the thigh, calf-1, and calf-2 domains (13,24).

The transmembrane domains of the  $\alpha$ -subunit as well as the  $\beta$ -subunit are predicted to form  $\alpha$ -helical coiled coils; the hand of the coiled coil seems to be uncertain (25). 3D models suggest a close juxtaposition of the transmembrane domains of both subunits (25–27).

Cytoplasmic domains of the  $\alpha$ -subunits are generally between 20 and 40 amino acids in length. There are several sequence similarities between the different  $\alpha$ -subunits including completely conserved motifs. The GFFKR region, which is directly adjacent to the transmembrane domain, is highly conserved, and its direct role in platelet activation has been demonstrated (28–31). A number of signaling, adaptor, and cytoskeletal proteins have been demonstrated to interact with integrin cytoplasmic domains, some of which are able to regulate the integrin activation state. Recent nuclear magnetic resonance (NMR) studies suggest that the N-terminal part of the  $\alpha_{IIb}$ -subunit is an extension of the helical structure of the transmembrane region up to the middle of the cytoplasmic domain (P998), followed by a turn that allows the acidic C-terminal portion to fold back and interact with the positively charged N-terminal region (32). Interestingly, the cytoplasmic domains of both integrin subunits seem to interact directly with each other, and the disturbance of this interaction seems to be one of the central mechanisms of inside-out signaling in integrins (28,29,33–35).

### *Structure of the $\beta$ -Subunits*

The N-terminal residues 1–50 of all  $\beta$ -subunits share sequence homology with other membrane proteins, and therefore the region has been termed the PSI domain for plexins, semaphorins, and integrins (Fig. 2) (36). The PSI domain is predicted to form two  $\alpha$ -helices. It is a cysteine-rich region with seven cysteines in integrins, with the first forming a long-range disulfide bond to the cysteine-rich C-terminal region of the  $\beta$ -subunit extracellular domains (37). The region of approximately residues 100–340 is termed the *I-like domain*, since this evolutionarily conserved region contains a putative metal-binding sequence similar to the MIDAS domain in the  $\alpha$ -subunit I-domain, its secondary structure is similar, and its sequence is homologous to the I-domain. In the  $\alpha_V$  crystal structure a large interface between the  $\beta$ -propeller and the I-like domain could be seen (13). In integrins lacking the I-domain, the I-like domain seems to regulate ligand binding directly, whereas in integrins containing the I-domain, the I-like domain seems to be indirectly involved in ligand binding. On either side of the I-like domain, there is a so-called hybrid domain, which forms  $\beta$ -sandwich domains. Without constituting the N terminus in the primary structure, this insertion between the hybrid

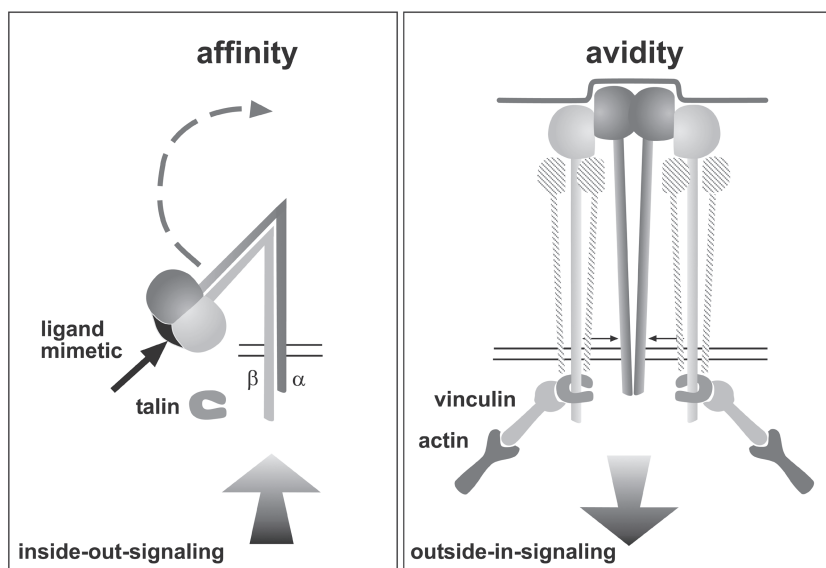
domains positions the I-like domain in a loop at the end of the extracellular domain in the integrin tertiary structure. From around residue 435–600, four cysteine-rich repeats were termed integrin-epidermal growth factor domains (I-EGF) because of their homology to EGF domains. The C-terminal part of the extracellular domain of integrin subunits, termed the  $\beta$ -tail domain, is cysteine-rich and contains an  $\alpha$ -helix and a  $\beta$ -sheet.

Cytoplasmic domains of the  $\beta$ -subunits generally contain 45–60 amino acids. Recent NMR studies suggest that the N-terminal part of the  $\beta_3$ -subunit forms a helical structure as an extension of the transmembrane region up to the middle of the cytoplasmic domain (K735) (32). As in the  $\alpha$ -subunits, in the cytoplasmic domains of  $\beta$ -subunits there are sequence similarities and completely conserved motifs between the various integrins. One of these motifs, the NPXY/F motif, is found twice in most  $\beta$ -subunit cytoplasmic domains. Indeed, very recently several phosphotyrosine-binding (PTB) domain-containing proteins have been shown to bind to  $\beta$ -subunit cytoplasmic domains, revealing a common regulatory mechanism for integrin activation (38). In addition, several other intracellular molecules have been shown to interact with the  $\beta$ -subunit cytoplasmic domains, including calcium- and integrin-binding protein (CIB),  $\beta_3$ -endonexin, cytohesin-1 and talin (39).

### ***Conformational Changes of Integrins in Inside-Out and Outside-In Signaling***

Control of the activation state of integrins is vital for cells and needs to be tightly regulated in a coordinated and fast manner (Figs. 2 and 3). This is especially true for cells that circulate in blood such as leukocytes and platelets. GPIIb-IIIa on circulating platelets needs to be maintained in a conformation that does not allow binding of natural ligands, e.g., fibrinogen. However, upon vessel injury, platelets are activated, and they have to change the activation status of GPIIb-IIIa within the shortest possible time to allow fibrinogen binding, platelet aggregation, and thus vessel sealing. Similarly, integrins expressed on the surface of leukocytes are maintained in a low-affinity state for their ligands until they are activated by the immune response. Inappropriate ligand binding of leukocyte integrins would cause chronic inflammation (40).

In integrin activation induced by cell activation, an activating stimulus is transferred from the inside of the cell to the outside and is therefore termed inside-out signaling (Fig. 3). Cell activation results in the binding of signaling molecules to the cytoplasmic domains of integrins. Thus the association between the transmembrane and cytoplasmic domains of the  $\alpha$ - and  $\beta$ -subunits is disturbed and both are separated, which finally results in integrin activation (32,35,41). The cytoskeletal protein talin



**Fig. 3.** Affinity and avidity regulation in inside-out and outside-in signaling in integrins. **(Left)** Inside-out signaling. The transmembrane and cytoplasmic domains of an  $\alpha$ - and  $\beta$ -subunit are associated with each other. The binding of a signaling, adaptor, or cytoskeletal molecule results in separation of the cytoplasmic and transmembrane domains and causes a conformational change from the bend to the extended structure of the extracellular integrin “legs.” Thus the ligand-binding sites within the integrin head pieces are fully exposed and bind native ligands. Integrin clustering with cytoskeletal anchorage is induced by homodimerization of the  $\alpha$ -subunit and a homotrimerization of the  $\beta$ -subunit. High-affinity binding and avidity modulation (cytoskeletal crosslinking) in combination result in a high adhesive strength. **(Right)** Outside-in signaling. Ligand-mimetic peptides can bind to integrins in the bended form. Thus the integrin extracellular domain undergoes a conformational change into the extended form, which enables the head piece to bind native ligands and which can be accompanied by a separation of the transmembrane and cytoplasmic domains and cytoskeletal rearrangements.

may be considered a prototype for regulatory function in integrin activation via its binding to the cytoplasmic integrin domains (2). The head domain of talin is able to bind to the cytoplasmic domain of the  $\beta_3$ -subunit. For exposure of the head domain, talin can be cleaved either by calpain or by phosphatidyl inositols, linking platelet activation directly to the activation of GPIIb-IIIa (38,42,43). The binding of the head of talin results in the conformational change of GPIIb-IIIa from a low-affinity to a high-affinity receptor (43). Furthermore, via the adaptor molecule vinculin, talin mediates the cytoskeletal anchorage of GPIIb-

IIIa to the actin cytoskeleton. This cytoskeletal anchorage is the basis for integrin clustering, whereby integrin avidity is greatly increased. In very elegant recent work, it could be demonstrated that separation of the integrin  $\alpha$ - and  $\beta$ -subunits results in homodimerization of  $\alpha$ -subunits and homotrimerization of  $\beta$ -subunits and that this process runs parallel with the upregulation of ligand affinity and the initiation of receptor clustering (41). This important work thus links affinity and avidity regulation of integrins.

One of the most fascinating areas in integrin research is the question of how the conformational change of the cytoplasmic domains of integrins is transferred to the ligand binding site in the headpiece of the integrins. Over a decade ago it was already realized that platelet activation or ligand binding causes the exposure of so-called ligand-induced binding sites (LIBS; epitopes) at the C-terminal end of the extracellular domains of GPIIb-IIIa, especially on the  $\beta$ -subunit (44). Thus, it was recognized early that there are long-range conformational changes within integrin molecules. However, only very recently, based on studies with NMR, crystallography, and electron microscopy, was a generally accepted model for this conformational change in integrins developed. In this model, the low-affinity integrin is bent at the so-called genu region ( $\alpha$ -subunit: between the tight and the calf-1 domains;  $\beta$ -subunit: between I-EGF1 and I-EGF2), bringing the headpiece in direct contact with the tailpiece. In this bent conformation, the headpiece is accessible for small-ligand mimetics but not for the multivalent macromolecules. Inside-out signaling then results in the transition of the bent form into an extended form, in which the stalk regions of integrins parallel each other.

This extended form has been seen earlier in electron micrographs (44). Initially, it was postulated that the headpieces of the  $\alpha$ - and  $\beta$ -subunits are then separated to allow ligand binding. However, very recent data suggest that the headpieces stay together but the shift from the bent to the extended conformation causes a change in the angle between the hybrid domains of the  $\alpha$ -subunit and the I-like domain of the  $\beta$ -subunit (45,46). The I-domain is the major integrin binding site in those integrins that contain this domain. Crystallographic studies suggest two conformations of the isolated I-domain, an open and a closed conformation. Indeed, the finding of two conformational states in two I-domains ( $\alpha_2$  and  $\alpha_M$ ) that are most distantly related regarding their sequence homologies within the group of integrins implies a general principle of regulation of ligand binding to I-domains (9). Elegant expression studies of the I-domain on the cell membrane alone proved the central role of this domain, since it is sufficient to mediate ligand binding (23). An initial model of I-domain activation from the closed to the open state proposes



a movement of the C-terminal  $\alpha$ -helix within the I-domain, resulting in the exposure of a high-affinity MIDAS (9).

GPIIb-IIIa is best studied example of the integrins that do not contain an I-domain. Both subunits seem to demonstrate ligand binding sites located in the  $\beta$ -propeller in the  $\alpha$ -subunit and the I-like domain in the  $\beta$ -subunit. Epitope mapping suggests that putative ligand binding sites on the top surface of the I-like domain interact with the  $\beta$ -propeller near  $\beta$ -sheets 2 and 3, thus creating a tertiary ligand binding pocket (47). Indeed, in the crystal structure of  $\alpha_V\beta_3$  together with an RGD peptide, the model of a tertiary ligand binding pocket consisting of both subunits was confirmed. The Asp of RGD coordinated to the MIDAS bound metal in the I-like domain of  $\beta_3$ , and the Arg coordinated to the  $\beta$ -propeller within the  $\alpha_V$ -subunit (48).

## THE ROLE OF AFFINITY AND AVIDITY REGULATION IN INTEGRIN FUNCTION

Avidity modulation describes changes in lateral mobility and integrin clustering that strengthen the binding of cells to multivalent ligands and matrices (Fig. 3). The sole expression of integrin binding domains (the I-domain of leukocyte function-associated antigen-1 [LFA-1]) and the monovalent binding of ligands are sufficient to mediate cell adhesion (23). However, the intracellular crosslinking and cytoskeletal anchorage of integrins provide a means to regulate cell adhesion further. The integrin/cytoskeleton association can modulate cell adhesion by coupling of the adhesive forces of the individual integrin/ligand pairs. To break adhesion, all the cytoskeleton-coupled integrin receptors have to be separated from their ligands simultaneously. This would require more physical force than disengagement of individual integrin/ligand bonds. Indeed, for LFA-1, a central role of avidity modulation in the regulation of cell adhesion independent on the affinity regulation could be demonstrated (28,49). Furthermore, in a recent report it was elegantly demonstrated that integrin clustering and conformational changes to high-affinity ligand binding, e.g., avidity and affinity changes, are simultaneously induced by homodimerization and homotrimerization of GPIIb-IIIa subunits (41). For the interaction of integrins with fibronectin, it was proposed that integrins may be regulated in two distinct manners. Affinity changes may be used for the binding of soluble fibronectin and avidity changes for the binding of immobilized, multimeric fibronectin (50). In conclusion, integrin-mediated cell adhesion can be mediated by both affinity change and avidity modulation. Most probably, both together are regulated simultaneously and only the combination of both provides the basis for strong cell adhesion.

## SPECIFIC PLATELET INTEGRINS

### $\alpha_2\beta_1$ (*GPIa-IIa, VLA-2, CD49b/CD29*)

$\alpha_2\beta_1$  is the major collagen receptor on platelets that mediates stable adhesion on collagen, and 2000–4000  $\alpha_2\beta_1$  molecules are found per platelet. Expression of  $\alpha_2\beta_1$  seems to be dependent on  $\alpha_2$ -alleles, and, interestingly, atherothrombotic risk is influenced by  $\alpha_2$  polymorphisms, arguing for an important role of  $\alpha_2\beta_1$  (51) in thrombosis. As in other integrins, it is postulated that  $\alpha_2\beta_1$  is expressed in a low-affinity default state for collagen binding. Upon platelet activation, outside-in signaling at the receptor is supposed to result in a conformational change of the I-domain of  $\alpha_2$ , which was clearly shown to constitute the collagen binding site (52,53). Indeed, in recent X-ray crystallographic studies, the  $\alpha_2$  I-domain revealed an open and closed conformation depending on the presence of ligands (54,55). Similar to other integrins (LFA-1, Mac-1, and GPIIb-IIIa), the highly conserved GFFKR region within the  $\alpha$ -subunit cytoplasmic domain seems to play a crucial role in the activation of integrin  $\alpha_2\beta_1$  (28,29,31,56).

$\alpha_2\beta_1$  has long been considered *the* major collagen receptor of platelets. However, the most recent model of platelet adhesion to collagen proposes that the initial contact with collagen under high shear stress is made by GPVI (57–59). The fast off-rate of the interaction between GPVI and collagen allows tethering of platelets but no firm adhesion. However, collagen binding to GPVI causes platelet activation, which by inside-out signaling results in a conformational change of  $\alpha_2\beta_1$  from a low- to a high-affinity receptor and which by receptor clustering (avidity modulation) finally allows stable platelet adhesion on collagen (59). Indeed, in mice with  $\beta_1$ -deficient platelets, clot stability on collagen was decreased, resulting in frequent emboli originating from those unstable thrombi (60). Recent data also describe outside-in signaling in  $\alpha_2\beta_1$  similar to that of GPIIb-IIIa (61).  $\alpha_2\beta_1$ -mediated spreading of platelets on collagen results in tyrosine phosphorylation of proteins such as Src, Syk, SLP-76, and focal adhesion kinase (61). Interestingly as in the interaction between fibrinogen and GPIIb-IIIa, outside-in signaling does not occur with the binding of monovalent collagen or collagen peptide but does occur on immobilized, multivalent collagen, arguing for the central role of integrin clustering (61).

### $\alpha_v\beta_3$ (*CD51/CD61*)

Integrin  $\alpha_v\beta_3$  is expressed at a low copy number of only a few hundred molecules per platelet. Its role, especially relative to the GPIIb-IIIa receptor, which is expressed 50–500-fold as often compared with  $\alpha_v\beta_3$ ,

has not yet been defined. Osteopontin, a glycoprotein expressed in atherosclerotic plaques and in injured arteries but not in normal arteries, is a ligand for  $\alpha_v\beta_3$ . Activated platelets, but not nonactivated platelets, adhere to immobilized osteopontin, mediated solely by the  $\alpha_v\beta_3$  receptor (62). These findings argue that the default state of  $\alpha_v\beta_3$  expressed on platelets is also a low-affinity state comparable to the low-affinity state of GPIIb-IIIa on unstimulated platelets. Interestingly, in other cell types the default state of  $\alpha_v\beta_3$  seems to be a high-affinity state, indicating a cell type-specific regulation of the  $\alpha_v\beta_3$  affinity states, as was described for other integrins (33,63). Indeed, it seems to be reasonable that a cell circulating in the blood has a low-affinity default state of its integrins and a cell embedded in tissue has a high-affinity default state of its integrins.

#### $\alpha_5\beta_1$ (VLA-5, CD49c/CD29) and $\alpha_6\beta_1$ (VLA-6, CD49f/CD29)

$\alpha_5\beta_1$  and  $\alpha_6\beta_1$  mediate platelet adhesion to fibronectin and laminin, respectively. Both are thought to have supplementary roles in platelet adhesion at injured vessel areas (4,64). However, no information on the relative roles of  $\alpha_5\beta_1$  and  $\alpha_6\beta_1$ , compared with other platelet adhesion receptors, is available.

#### $\alpha_L\beta_2$ (LFA-1, CD11a/CD18) and $\alpha_M\beta_2$ (Mac-1, CD11b/CD18)

Activated but not resting platelets have been reported to express  $\beta_2$ -integrins (LFA-1 and Mac-1). The role of these  $\beta_2$ -integrins in platelet adhesion or aggregation is not known. However, they seem to modulate caspase activation and consequently the life span of mouse platelets (5,65).

#### GPIIb-IIIa ( $\alpha_2\beta_1$ , CD41/CD61)

The term GPIIb-IIIa originates from platelet protein gel electrophoresis describing band numbers IIb and IIIa (66). GPIIb consists of two chains (a heavy chain of 105 kDa and a light chain of 25 kDa) linked by a disulfide bond (67,68). GPIIIa is a single-chain protein of 95 kDa (67,68). Cloning of the genes was achieved in the late 1980s, and GPIIb and GPIIIa are both located in close physical proximity on chromosome 17 (69,70). GPIIb-IIIa is the most abundant platelet membrane receptor, with 50,000–80,000 molecules per platelet, thus constituting up to 2% of the amount of total platelet protein and 15% of total surface protein (71,72). The genetic defect of Glanzmann's disease helped to identify the role of this glycoprotein for fibrinogen binding and for platelet aggregation (73,74). Direct binding experiments with fibrinogen (75,76), blocking experiments with monoclonal antibodies (77), and reconstitution of GPIIb-IIIa binding function either after purification of the protein

(78) or by expression of the transfected cDNA on model cell lines (79) provided definite proof of the binding of fibrinogen and the consequent role of GPIIb-IIIa in platelet aggregation. In addition to fibrinogen, several other ligands that bind to GPIIb-IIIa (von Willebrand factor, fibronectin, vitronectin, thrombospondin, CD40 ligand) have been identified (80,81). The two glycoproteins GPIIb and GPIIIa were later shown to be identical to the antibody epitopes CD41 and CD61. At the end of the 1980s, it was recognized that GPIIb-IIIa is a member of the adhesion molecule family of integrins, and their integrative function between extracellular ligands and the intracellular cytoskeleton was described (82). According to the original term, GPIIb-IIIa,  $\alpha_{IIb}\beta_3$  was chosen as the term within the integrin nomenclature (82).

### **INSIDE-OUT SIGNALING OF GPIIb-IIIa**

The signaling pathways from platelet membrane receptors to the cytoplasmic domains of GPIIb-IIIa are far from being completely understood, but it is well accepted that activation of GPIIb-IIIa is the common final pathway of platelet activation. Small G protein GTPases such as Rac and Rho may regulate GPIIb-IIIa activation (83). Tyrosine kinases such as Src and Syk, phospholipases, and protein kinase C (PKC) may be involved in this signaling. For two proteins that bind directly to the  $\beta_3$ -cytoplasmic domain, a direct effect on affinity regulation could be demonstrated. The binding of the N-terminal head of talin to the  $\beta_3$ -cytoplasmic domain causes a direct upregulation of fibrinogen binding to GPIIb-IIIa (43). Also, the binding of  $\beta_3$ -endonexin to the  $\beta_3$ -cytoplasmic domain causes an increase in GPIIb-IIIa affinity for fibrinogen (84).

### **OUTSIDE-IN SIGNALING OF GPIIb-IIIa**

Ligand binding to GPIIb-IIIa results in outside-in signaling in platelets. Platelets can adhere to immobilized fibrinogen without being activated and thus without the precondition of a high-affinity GPIIb-IIIa receptor. In contrast to soluble fibrinogen, which only binds to the activated, high-affinity GPIIb-IIIa, immobilized fibrinogen exposes so-called receptor-induced binding site (RIBS) epitopes that allow binding to the nonactivated, low-affinity GPIIb-IIIa receptor (85). Ligand binding causes conformational changes of the cytoplasmic domains and integrin clustering (30,86–88). However, only the combination of both results in full outside-in signaling (89). At least three different signaling pathways have been identified:

1. Activation of the tyrosine kinase Syk is triggered by integrin clustering within seconds after cell attachment (90). One of the targets of Syk is the multifunctional adaptor molecule SLP-76, which directly relays signals from GPIIb-IIIa to the actin cytoskeleton (91).

2. Activation of pp125<sup>FAK</sup> requires integrin clustering and platelet activation. pp125<sup>FAK</sup> seems to play a regulatory role in dynamic changes of focal contacts and actin stress fibers (92).
3. Platelet aggregation causes tyrosine phosphorylation of two NXXY motifs within the  $\beta_3$ -cytoplasmic domain (93). Indeed, the importance of this pathway for outside-in signaling was demonstrated in a knockout mouse model in which both tyrosines of the  $\beta_3$ -cytoplasmic domain were substituted by phenylalanines (94). A diminished stability of platelet aggregates and a reduced clot retraction was reflected in a pronounced tendency to rebleed (94). Furthermore, the protein Shc has been identified as a direct downstream signaling partner of GPIIb-IIIa in phosphorylation-dependent signaling (95).

### THE GPIIb-IIIa LIGAND FIBRINOGEN

Fibrinogen is a 340,000-Dalton dimeric macromolecule consisting of three pairs of disulfide-bonded polypeptide chains, designated A $\alpha$ -, B $\beta$ -, and  $\gamma$ -chain. Fibrinogen is transformed to fibrin monomers by the cleavage of fibrinopeptides A and B. The symmetric bivalent structure of fibrinogen provides the basis for its bridging function between two GPIIb-IIIa receptors and thus for platelet aggregation. Three sites on fibrinogen that are potentially involved in binding to GPIIb-IIIa have been identified: two RGD sequences within the  $\alpha$ -chain and a sequence of 12 amino acids (HHLGGAKQAGDV) close to the carboxy terminal end of the  $\gamma$ -chain (96). The  $\gamma$ -chain sequence seems to be the primary interaction site. Fibrinogen molecules without this sequence did not mediate binding to GPIIb-IIIa and platelet aggregation (97,98), whereas recombinant fibrinogen molecules designed to contain RGE sequences instead of the native RGD sequences still support fibrinogen binding and platelet aggregation (97). In addition, blocking experiments with peptide-specific antibodies provided further evidence for the primary role of the  $\gamma$ -chain sequence but not the RGD sequences (99). Nevertheless, peptides based on both sequences block fibrinogen binding to GPIIb-IIIa. Interestingly, naturally occurring GPIIb-IIIa inhibitors, e.g., in snake venoms and pharmaceutically designed drugs, are imitating the RGD sequence, as discussed in the next section.

### GLANZMANN'S THROMBASTHENIA: GPIIb-IIIa BLOCKADE BY NATURE

In 1918, Glanzmann first described patients with a hereditary form of hemorrhagic thrombasthenia. Abnormalities in GPIIb-IIIa have been defined beginning in the 1970s as the basis of the autosomal recessive inheritance of Glanzmann's thrombasthenia (73). Meanwhile, several mutations have been identified on GPIIb or GPIIIa resulting either in total loss or considerable reduction in platelet surface expression of GPIIb-IIIa or in the expression of nonfunctional GPIIb-IIIa (74,100,101).

The clinical features of these patients are quite interesting, since their situation resembles a chronic blockade of GPIIb-IIIa. The vast majority of these patients suffer from easy bruising, purpura, epistaxis, and gingival bleeding. In women, menorrhagia is a major problem. Gastrointestinal hemorrhage is seen in only a few patients. Intracranial bleeding is described in three patients. Two of these had a trauma, and the circumstances for the third patient are not reported (100). Many of these patients needed blood transfusions during life. Indeed, some patients, especially at younger age, died of hemorrhagic problems mostly combined with trauma (100). Interestingly, no myocardial infarctions or strokes have been reported (100). Nevertheless, since most of these patients are at younger age, conclusions have to be drawn with caution.

Overall, the clinical sequelae of the loss of the platelets' ability to aggregate via GPIIb-IIIa are astonishingly mild. Indeed, the clinical features of Glanzmann's disease resemble the clinical consequences of pharmaceutical blockade of GPIIb-IIIa. Spontaneous bleeding is of minor concern, whereas bleeding with trauma constitutes a major problem that very often can only be managed with blood transfusions. Spontaneous cerebral bleeding in Glanzmann's thrombasthenia as well as in pharmaceutical blockade of GPIIb-IIIa fortunately is not a concern. Certainly, this advantageous safety profile is one of the major reasons for the broad use of GPIIb-IIIa blockers.

## IMPORTANCE OF STRUCTURAL KNOWLEDGE FOR ANTI-INTEGRIN DRUG DEVELOPMENT

Integrins are attractive targets for therapeutic agents. Anti-GPIIb-IIIa agents have already been used successfully in hundred of thousands of patients during coronary angioplasty. Three different agents are used, all of which can be considered ligand mimetics. Abciximab, a humanized Fab fragment, binds at or near the binding pocket of fibrinogen, and the original antibody 7E3 was described as presenting some degree of specificity for the activated GPIIb-IIIa receptor (102). Eptifibatide, a cyclic KGD peptide mimetic, was designed based on the snake venom disintegrin barbourin, which inhibits GPIIb-IIIa by its ligand-mimetic binding (103). Tirofiban is a chemical modeled after the RGD binding sequence within fibrinogen and therefore by its design is a pure ligand mimetic. The consequence of the strategy to block GPIIb-IIIa with ligand mimetics is the induction of a conformational change of GPIIb-IIIa by these agents, similar to the conformational change induced by RGD peptides (104). Indeed, as a measure of this conformational change, all three clinically used GPIIb-IIIa blockers induce binding of anti-LIBS

antibodies (104,105). Binding of these GPIIb-IIIa blockers is expected to change the GPIIb-IIIa structure from the bent to the extended form.

This conformational change may result in two unwanted and thus paradoxical effects of the ligand-mimetic GPIIb-IIIa blockers. First, after dissociation of the blockers, the GPIIb-IIIa receptor may stay in the extended form and may be able to bind fibrinogen (104). For  $\alpha_v\beta_3$ , induction of ligand binding could be induced by RGD peptides, and the authors proposed a mechanism of conformational memory of the integrin after dissociation of the ligand-mimetic blockers (106). Second, outside-in signal transduction may be induced by the binding of GPIIb-IIIa blockers, which may then result in platelet activation. Indeed, platelet activation has been observed in trials with oral GPIIb-IIIa blockers (107–110).

In conclusion, structural knowledge of the conformational changes within the integrin GPIIb-IIIa gives rise to the question of whether ligand mimetics are optimal candidates as therapeutic GPIIb-IIIa blockers. Indeed, the importance of this question is emphasized since it became clear that the benefits of the intravenous use of GPIIb-IIIa blockers are less than expected and the trials with oral GPIIb-IIIa blockers showed an increase in mortality, resulting in a halt of further development of these agents (110–112). Thus, future strategies for the therapeutic blockade of GPIIb-IIIa should consider the now available structural knowledge of integrin receptors and might thereby avoid the development of agents that cause conformational changes of GPIIb-IIIa.

Interestingly, newly developed anti-integrin agents directed against LFA-1 and Mac-1 already follow a different strategy. Several anti- $\alpha_L$ -subunit agents have been developed that bind between the C-terminal  $\alpha$ -helix and the  $\beta$ -sheet of the I-domain, a site that is distant from the ligand binding site at the MIDAS (9,23,113–115). Thus these agents favor the closed state of the I-domain but do not directly compete with ligand binding. Another class of compounds that inhibit ligand binding to Mac-1 and LFA-1 to the same extent has been demonstrated to bind to the MIDAS domain of the  $\beta_2$ -subunit I-like domain, thereby only indirectly inhibiting ligand binding to the I-domain (9,116,117). A recent report describing an antibody (4F8) that binds to a ligand-attenuated binding site (LABS) on GPIIb-IIIa and thus prevents platelet aggregation may indeed define a potential binding site for anti-GPIIb-IIIa agents that may be able to lock the receptor in a low-affinity state for fibrinogen (118). A similar epitope has also been described for  $\alpha_5\beta_1$  (119). Overall, these anti-integrin agents are not prone to induce conformational changes and potential outside-in signaling, as has been shown for ligand-mimetics drugs.



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