REVIEW

Recent advances in the understanding of the molecular mechanisms regulating platelet integrin $\alpha\text{IIb}\beta 3$ activation

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ABSTRACT

Integrins are allosteric cell adhesion receptors that cycle from a low to a high affinity ligand binding state, a complex process of receptor activation that is of particular importance in blood cells such as platelets or leukocytes. Here we highlight recent progress in the understanding of the molecular pathways that regulate integrin activation in platelets and leukocytes, with a special focus on the structural changes in platelet integrin $\alpha\text{IIb}\beta 3$ brought about by key intracellular proteins, namely talin and kindlins, that are of crucial importance in the regulation of integrin function. Evidence that the small GTPase Rap1 and its guanine exchange factor CalDAG-GEF1, together with RIAM, a Rap1GTP adaptor protein, promote the interaction of talin with the integrin β subunit, has greatly contributed to fill the gap in our understanding of the signaling pathway from G-coupled agonist receptors and their phospholipase C-dependant second messengers, to integrin activation. Studies of patients with the rare blood cell disorder LAD-III have contributed to the identification of kindlins as new co-regulators of the talin-dependent integrin activation process in platelets and leukocytes, underlining the relevance for the in-depth investigation of patients with rare genetic blood cell disorders.

INTRODUCTION

Blood cells in the vasculature are constantly exposed to hemodynamic forces generated by the flow of blood, and rely largely on adhesion receptors such as integrins to counteract

these dispersive forces and to allow anchorage-dependent cell events. Integrins represent a large family of heterodimeric transmembrane adhesion receptors that are formed by the non-covalent association of various α and β subunits. A hallmark of integrins is their ability to promote bidirectional signaling across the cell membrane (Hynes, 2002). For example, endothelial cells of the vasculature use integrins to interact with the extracellular matrix (ECM) of the subendothelium, and engaged integrins transduce signals (outside-in signaling) that are critical for the dynamic regulation of endothelial cell functions such as adhesion, migration and proliferation (Harburger and Calderwood, 2009). Circulating blood cells on the other hand switch from nonadherent to adherent cells as a result of integrin activation (inside-out signaling) leading to platelet aggregation during hemostasis, leukocyte attachment to the vessel wall and extravasation to surrounding tissues during inflammation, or immune B and T cell interactions in immunological synapses (Abram and Lowell, 2009). In platelets, integrin $\alpha\text{IIb}\beta 3$ serves as a receptor for platelet adhesion to the exposed ECM, and additionally functions as a fibrinogen receptor that mediates platelet aggregation through binding of plasma fibrinogen, two platelet functions that are essential for sealing injured blood vessels and preventing blood loss. Indeed, genetic defects in functional integrin $\alpha\text{IIb}\beta 3$ expression in the megakaryocytic/platelet lineage are the cause of a severe bleeding disorder, Glanzmann's thrombasthenia (GT) characterized by defective platelet aggregation (Nurden, 2006). In contrast, excessive platelet aggregation can initiate arterial thrombosis, causing heart attacks and stroke. To ensure that platelets, which are constantly exposed to plasma fibrinogen in the circulating blood, only aggregate at sites of vessel injury,

platelet integrins exist in a low-affinity ligand binding state and shift to a high-affinity state following platelet stimulation by various agonists, such as ADP or thrombin, compounds that are released into the blood stream at sites of vessel injury (Savage et al., 1998). In platelets, this switch in $\alpha\text{IIb}\beta 3$ ligand binding affinity is commonly referred to as $\alpha\text{IIb}\beta 3$ activation or integrin inside-out signaling (Hynes, 2002). Similarly, circulating leukocytes maintain their $\beta 2$ integrins in a nonadhesive state; however, at sites of inflammation or infection, released chemoattractants and cytokines induce $\beta 2$ integrin activation allowing the firm arrest of leukocytes on the inflamed endothelium and transmigration through the subendothelium to allow leukocyte homing to sites of inflammation or infection. The physiologic importance of $\beta 2$ integrin-dependent blood cell adhesion is underlined in patients with genetic defects in leukocyte $\beta 2$ integrin expression (LAD, leukocyte adhesion deficiency), who suffer from recurrent life-threatening bacterial infections (Abram and Lowell, 2009).

CHO CELLS AS A MODEL TO INVESTIGATE RECOMBINANT INTEGRIN $\alpha\text{IIb}\beta 3$ FUNCTION

Much of the progress achieved in integrin $\alpha\text{IIb}\beta 3$ research has been obtained following investigation of wild type or mutant integrin $\alpha\text{IIb}\beta 3$ receptors expressed in Chinese hamster ovary (CHO) cells, used as putative surrogates for anucleated platelets that cannot be genetically manipulated. It has to be mentioned, however, that in these cells, integrin $\alpha\text{IIb}\beta 3$ is in a constitutive low affinity ligand binding state, unable to interact with soluble fibrinogen, but can be artificially activated to bind this ligand either by activating monoclonal antibodies, or by Mn^{2+} that binds to the integrin head A domain and induces the high affinity ligand binding conformation. Over the years, monitoring of integrin $\alpha\text{IIb}\beta 3$ activation has been performed with a monoclonal antibody PAC-1, which does not bind to $\alpha\text{IIb}\beta 3$ in resting platelets, but interacts with $\alpha\text{IIb}\beta 3$ following thrombin stimulation of platelets, and is therefore largely used as a ligand mimetic (Shattil et al., 1987). Although this multivalent IgM antibody has recently been shown to monitor integrin $\alpha\text{IIb}\beta 3$ affinity/clustering, i.e., integrin avidity rather than affinity (Bunch, 2010), for convenience, we will continue to refer to this antibody as a marker of integrin $\alpha\text{IIb}\beta 3$ activation.

STRUCTURAL CHANGES IN INTEGRIN $\alpha\text{IIb}\beta 3$ DURING ACTIVATION

A key contribution to the understanding of integrin $\alpha\text{IIb}\beta 3$ activation was the initial observation that mutation of the highly conserved D⁷²³ residue in the membrane proximal part of the $\beta 3$ subunit cytoplasmic tail or the equivalent R⁹⁹⁵ residue in the α subunit membrane proximal part was sufficient to activate integrin $\alpha\text{IIb}\beta 3$ (Hughes et al., 1996), suggesting that a salt bridge between $\beta 3\text{D}^{723}$ and αIIbR^{995}

keeps the two cytoplasmic tails in close contact and stabilizes integrins in their low affinity state.

The $\beta 3\text{D}^{723}$ and αIIbR^{995} residues are located in two highly conserved sequences in the membrane proximal part of the α (GFFKR) and the β subunits (HDR(R/K)E), reported to form α -helical coiled-coiled interactions (Vinogradova et al., 2002). Deletion or mutations of these sequences leads to integrin activation both *in vitro* and *in vivo* (Luo et al., 2007). However, the notion of helical coiled-coiled interactions between the α and β subunits in the transmembrane domain and the membrane-proximal cytoplasmic part has recently been challenged by two independent reports (Lau et al., 2009; Zhu et al., 2009), which revealed an unusual asymmetric structure of the transmembrane α and β subunit helices that span the membrane at an angle of 25° due to their unequal length. While the $\beta 3$ transmembrane helix extends into the cytoplasm, the α helix is interrupted at the cytoplasmic face of the membrane by a G cap structure of the GFFKR sequence, with the two FF residues partially embedded into the hydrophobic portion of the membrane bilayer into the interface with the $\beta 3$ subunit. This unusual non-helical structure of the αIIb subunit does however not preclude the formation of the ionic salt bridge between residue R⁹⁹⁵ of αIIb and residue D⁷²³ of $\beta 3$ cytoplasmic tails, known to be important to keep integrins in the low affinity state (Kim et al., 2009).

On the basis of structural studies, it has been suggested that integrins in their low affinity state have a bent, v-shape conformation with the head piece oriented toward the cell membrane, while the ligand binding high affinity receptor adopts an extended upright conformation, that would roughly double its molecular height to about 200 Å (Xiong et al., 2001). Also, *in vitro* and *in vivo* data suggest that in the bent conformation, the subunit transmembrane and cytoplasmic domains are in close contact, while they are separated in the high affinity conformation (Kim et al., 2009).

Integrins can adopt different stages of ligand binding affinity and to date, it is still unclear whether ligand binding only occurs to the extended conformation. Indeed, two models have been proposed, (a) the switchblade model which predicts that only extended integrins will bind ligand (Luo et al., 2007), whereas (b) the deadbolt model (Xiong et al., 2003) suggests that ligand binding occurs to the bent integrin and that extension occurs only following ligand binding. *In vitro* integrin activation by Mn^{2+} only alters the cation coordination in the integrin A domain (Shimaoka et al., 2002) and does not promote separation of the cytoplasmic tails (Kim et al., 2009), a mechanism thought to be indispensable for integrin extension.

ROLE OF CYTOPLASMIC PROTEIN TALIN IN INTEGRIN $\alpha\text{IIb}\beta 3$ ACTIVATION

For many years, the biochemical pathways that take place in platelets and link agonist receptor signaling to integrin $\alpha\text{IIb}\beta 3$

Figure 1. Structural models of integrin α IIb β 3. (A) (a) Amino acid sequence and structure of the transmembrane (red), juxtamembrane (blue) and cytoplasmic segments (green) of integrin α IIb β 3 in its inactive conformation. (b) Enlarged view of the juxtamembrane domain showing the inter-subunit amino acid contacts: hydrogen bonds are dashed green lines; nitrogens, oxygens, and sulfurs are blue, red and yellow, respectively. (from Zhu et al., 2009). (B) Structural model illustrating integrin α IIb β 3 inside-out activation by the talin head F3 domain according to Lau et al. (2009). Prior to talin binding, integrin α IIb β 3 adopts a bent, v-shaped conformation stabilized in the juxtamembrane domain by inter-subunit ionic bonds. The talin F3 domain interaction with the integrin β 3 subunit cytoplasmic tail disrupts the ionic bond α IIbR⁹⁹⁵- β 3D⁷²³ and promotes separation of the 2 subunits with long range conformational changes in the extracellular domain necessary for the induction of the extended conformation.

activation have remained elusive. It was therefore quite surprising when the cytoskeletal protein talin, known to link integrin β subunit cytoplasmic tails to the actin cytoskeleton,

was identified as the final effector of integrin activation (Tadokoro et al., 2003). Talin is a 270-kDa cytoplasmic protein, which can be cleaved by calpain into a globular head

domain and a rod like tail domain. Although both the talin head and the talin rod domain contain an integrin binding site, it is the talin head interaction with the integrin β subunit cytoplasmic tail that activates integrins (Calderwood et al., 1999). The talin head contains a FERM (band 4.1/ezrin/radixin/moesin) homology domain, composed of 3 subdomains F1–F3, with a phosphotyrosine binding (PTB)-like fold in the F3 subdomain. By solely overexpressing the talin head F3 subdomain in the CHO cell model, Calderwood et al. were able to induce mAb PAC-1 binding to integrin α IIb β 3.

The talin head-integrin contact site was mapped to the β 3 cytoplasmic tail NPXY⁷⁴⁷ sequence, a typical binding motif for proteins with a PTB domain and highly conserved in integrin β subunits. Also, mutation of the Y⁷⁴⁷ residue or the upstream W⁷³⁹ residue was sufficient to disrupt this interaction (Calderwood et al., 2002; Calderwood and Ginsberg, 2003; García-Alvarez et al., 2003). Interestingly however, other PTB-domain containing proteins known to interact with the NPXY

motif of integrins, such as Dok1, tensin or Numb, were unable to induce integrin activation. Recent structural data of the F3 domain of talin1 in complex with the β 3 cytoplasmic tail (Wegener et al., 2007) or the F2–F3 subdomains of talin2 in complex with the integrin β 1D cytoplasmic tail have provided a possible explanation for this observation by revealing additional talin head contact sites in the β subunit, upstream of the NPXY⁷⁴⁷ motif, and located in the β subunit membrane proximal α -helix. In particular, a loop in the talin F3 domain that does not exist in Dok1, tensin or Numb, establishes contacts with 2 F residues, F⁷²⁷ and F⁷³⁰ (Wegener et al., 2007), while a K residue in this loop engages with the β 1D subunit D residue, known to form a salt bridge with the α subunit, thus providing the clue on how talin head, by binding to the integrin β subunit, can break this salt bridge and activate integrins (Anthis et al., 2009).

Additional basic residues in the F2 and F3 domains engage with acidic phospholipids of the cell membrane (Anthis et al.,

Figure 2. Sequence alignment of integrin β subunits and binding model of talin head-integrin tail. The highly conserved PTB protein binding motifs NPXY/F and NXXY/F are shown in blue and yellow, respectively. The Src 'RGT' binding site in β 3 is shown in purple. Residues of the membrane proximal α helix involved in talin head binding are shown in pink. The D residue that establishes an ionic salt bridge with integrin α subunits is marked with an asterisk. The overlapping talin head and talin rod binding domains are indicated. (B) Crystal structure of talin2 F2–F3 domain bound to the β 1D integrin tail (in green). Residues in the integrin β 1D subunit shown to be important for talin binding are highlighted (β 3 residue numbering is indicated in parenthesis). Acidic residues in talin are shown in red and basic residues in blue. (from Anthis et al., 2009).

2009; Saltel et al., 2009) and reorient the talin head together with the integrin β subunit, promoting the separation of the transmembrane helices of the integrin α and β subunits. It has to be mentioned that only the non-phosphorylated NPXY⁷⁴⁷ motif in the integrin β subunit can associate with the PTB-like F3 domain of the talin head, suggesting that phosphorylation of Y⁷⁴⁷ in $\beta 3$ negatively regulates talin binding and integrin activation (Anthis et al., 2009). And finally, recent data have shown that talin head binding to a single integrin $\alpha\text{IIb}\beta 3$ molecule inserted into a phospholipid layer is sufficient to induce the long-range conformational change that converts $\alpha\text{IIb}\beta 3$ from a bent to an extended receptor (Ye et al., 2010).

How then do agonists, which stimulate platelets and promote integrin activation, induce the translocation of talin from the cytosol to the platelet membrane? Agonist regulated redistribution of platelet talin from the cytosol to the inner face of the platelet membrane was initially reported in thrombin- and glass-activated, substratum-adherent platelets (Beckerle et al., 1989). Also, agonist-induced activation of $\alpha\text{IIb}\beta 3$ in platelets has been known for years to be dependent on the generation of Ca^{2+} and diacylglycerol (DAG). These second messengers are generated by phospholipase C downstream of platelet agonist receptors such as the thrombin receptor (PAR) or the ADP receptor (P2Y₁), two members of the G protein coupled receptor family (GPCRs) (Rivera et al., 2009). Research in the leukocyte field had already identified the guanosine triphosphatases (GTPases) Rap1 and Rap2 as important effectors downstream of PKC in integrin activation in thymocytes and neutrophils. Also, through genetic ablation in mice of the guanine exchange factor CalDAG-GEF1, that, similar to PKC, binds Ca^{2+} and DAG and regulates the GDP-GTP cycle of Rap1, it was shown that CalDAG-GEF1 is involved in an additional signaling pathway that links platelet agonist receptors to integrin activation, as platelets from these mice failed to aggregate (Crittenden et al., 2004; Bergmeier et al., 2007). The final missing link between activated Rap1 and talin-dependent integrin activation was however established by Ginsberg's group who used the CHO cell model to reconstitute the entire signaling pathway mimicking integrin $\alpha\text{IIb}\beta 3$ activation in human platelets. They first demonstrated that the low expression levels of talin and PKC α in CHO cells were responsible for the inability of integrin $\alpha\text{IIb}\beta 3$ to become activated in these cells (Han et al., 2006). Next, by stimulating CHO cells (over-expressing talin and PKC α) with phorbol 12-myristate 13-acetate (PMA), which triggers PLC activation and the formation of IP₃ and DAG, they showed that Rap1 activates integrins by forming a complex with talin and RIAM, a Rap1-GTP-interacting adaptor molecule. A similar integrin $\alpha\text{IIb}\beta 3$ activation in CHO cells was also achieved following over-expression of the thrombin receptor (Watanabe et al., 2008). More recently, it has been shown that activated Rap1 targets to the lipid membrane following farnesylation of its CAAX motif, while RIAM functions as a linker and binds to both talin

and Rap1, thereby recruiting talin to the plasma membrane for its interaction with integrins. Interestingly, overexpression of an artificial fusion protein composed of the CAAX motif of Rap1 (10 residues) and the talin binding motif of RIAM (30 residues) is sufficient to promote integrin activation (Lee et al., 2009).

ROLE OF TALIN IN LINKING INTEGRINS TO THE CYTOSKELETON

Although talin functions as the final effector in integrin activation, it was initially identified as a cytoskeletal protein that links integrins to the actin cytoskeleton (Horwitz et al., 1986). Interaction of homo-dimerized talin with integrins is thought to promote the lateral mobility and clustering of integrins within the plane of the plasma membrane, and to recruit additional cytoplasmic proteins, such as vinculin and actin, to form transient self-assembling complexes, called focal contacts (FC), that eventually mature into focal adhesions (FA) (Critchley, 2009). FAs function as integrin-rich signaling hubs, which promote strong contacts with the surrounding matrix, link integrins to the actin cytoskeleton and orchestrate outside-in signaling pathways by recruiting a large number of cytoskeletal, adaptor and signaling proteins (Geiger et al., 2009). Some of these signaling proteins, namely p125FAK (focal adhesion kinase), Src and ILK (integrin linked kinase), have been reported to directly associate with integrin β subunit cytoplasmic tails. Src binding however only occurs to $\beta 3$ among integrin β subunits and targets its last three C-terminal residues, RGT (Arias-Salgado et al., 2003). Interestingly, a membrane permeable RGT peptide has recently been shown to inhibit integrin $\alpha\text{IIb}\beta 3$ outside-in signaling in platelets without affecting integrin activation (Su et al., 2008).

Using the drosophila model, Tanentzapf et al. were the first to show that the talin head and talin rod serve different functions, as interaction between integrin and the talin FERM domain mediates integrin activation but does not promote linkage to the cytoskeleton, thus underlining the importance of the additional integrin binding site in the talin rod domain (Tanentzapf and Brown, 2006). The talin rod is composed of 62 α -helices organized into a series of four or five-helix bundles followed by a single C-terminal helix that allows talin dimerization. A number of these helical bundles are involved in protein-protein interactions and function as binding sites for vinculin (VBS1, 2, 3), actin (ABS2) and integrins (IBS2) (Critchley, 2009). The second integrin binding site (IBS2) in the talin rod domain appears to play a major role in integrin outside-in signaling, as expression in mouse talin1(−/−) cells of a full-length mouse talin with the IBS2 binding site inactivated by a LI/AA mutation, was unable to rescue the inability of these cells to assemble focal adhesions (Tremuth et al., 2004; Moes et al., 2007). Within IBS2, talin rod helix 50 has been shown in vitro to establish direct contacts with the

membrane proximal α helix of $\beta 3$ and $\beta 1$ integrins (Rodius et al., 2008; Gingras et al., 2009), based on charge complementary ionic bonds. As the talin head and talin rod contact sites in the integrin membrane proximal domain are in close proximity and partially overlapping, it remains to be determined whether talin head binding to the integrin β subunits is simultaneous or sequential.

FUNCTIONAL ROLE OF KINDLINS IN INTEGRIN ACTIVATION AND OUTSIDE-IN SIGNALING

Over the recent years, talin has evolved as a major activator of integrin ligand binding function. However, experimental data have suggested the existence of additional co-activators, as talin head binding to the integrin cytoplasmic tail in the CHO cell model was unable to achieve the same level of integrin activation as that measured following Mn^{2+} binding or antibody activation (Ma et al., 2007; Bouaouina et al., 2008). Among the numerous proteins known to interact with integrin cytoplasmic tails, kindlins have recently emerged as new co-regulators of integrin activation.

The kindlin family consists of three members (kindlin-1, -2, -3) that are encoded by 3 separate genes and are expressed in a tissue- or cell type-specific manner. Kindlin-1 is ubiquitously expressed in murine and human tissues, including skin, heart, lung, liver, kidney, colon, prostate, ovary and pancreas (Siegel et al., 2003). Kindlin-2 is also expressed ubiquitously with the exception of hematopoietic cells and it is the only kindlin expressed in embryonic stem (ES) cells (Ussar et al., 2006; Dowling et al., 2008). In contrast, kindlin-3 is restricted to hematopoietic cells where it is particularly abundant in megakaryocytes and platelets (Ussar et al., 2006). Within the cells, all 3 kindlins have a similar subcellular distribution, as they colocalize with integrins in adhesion structures, suggesting that they have very similar functions (Tu et al., 2003; Weinstein et al., 2003). However, certain kindlins are also found to localize to unique structures, e.g., kindlin-2 is present in cell-cell contacts or in the nucleus, indicating exclusive functions of some of the kindlin family members (Ussar et al., 2006). The three mammalian kindlins, kindlin-1 (also known as kindlerin and FERMT1), kindlin-2 (MIG-2) and kindlin-3 (URP2) exhibit high sequence similarities with identical domain architecture (Larjava et al., 2008). Kindlins contain a C-terminally located FERM domain which is divided into three subdomains F1, F2, F3. A structural hallmark of kindlins is the interruption of the F2 subdomain by a pleckstrin homology (PH) domain, suggesting that these proteins can be recruited to the cell membrane by binding to phosphoinositides (Weinstein et al., 2003; Larjava et al., 2008). In comparison to other FERM-domain containing proteins, the F3 subdomain of kindlins shares the highest homology with the talin F3 domain (Kloeker et al., 2004). Also, *in vitro* binding studies using recombinant proteins have shown that the F3 subdomain of

Figure 3. Schematic drawing of the signaling cascade that leads to integrin activation in platelets. Agonist stimulation of G protein coupled receptors (GPCR) triggers PLC activation and the formation of IP3 and DAG. DAG and Ca^{2+} , the latter being released by IP3, activate CalDAG-GEF1 and PKC. Activated CalDAG-GEF1 along with PKC leads to the shift of Rap-1GDP to Rap-1GTP. Rap-1GTP targets to the lipid membrane following farnesylation of its CAAX motif. RIAM, a RAP-1GTP interacting adaptor protein, functions as a linker and binds to both the talin head and Rap-1 to form a complex, thereby recruiting talin to the plasma membrane for its interaction with the integrin β subunit. Talin head binding to the conserved NPLY motif disrupts the salt bridge between the α and β subunits and promotes integrin activation with a shift from a bent to an extended, ligand binding conformation. Kindlin binding to the NXXY motif and Src binding to the C-terminal RGT motif is shown.

kindlins can interact directly with integrin β subunit cytoplasmic tails. All three kindlins interact with $\beta 1$ and $\beta 3$ integrin tails, while kindlin-3 additionally binds to $\beta 2$ (Larjava et al., 2008; Montanez et al., 2008; Harburger et al., 2009; Moser et al., 2009).

An initial hint on the functional role of kindlins came from studies in the nematode *Caenorhabditis elegans* in which the absence of the unique kindlin gene product, UNC-112, induced the PAT phenotype, characterized by defective worm motility (Rogalski et al., 2000). In addition, in wild type animals, UNC-112 was shown to co-localize with PAT-3, the *C.elegans* unique integrin β subunit, and to be essential for the assembly of proper cell-matrix adhesion structures. As kindlins do not contain catalytic domains, it was suggested that they might function as adaptor proteins promoting protein-protein interactions. And indeed, in addition to the integrin cytoplasmic tails, kindlins have also been shown to interact with ILK (Mackinnon et al., 2002; Tu et al., 2003;

Legate et al., 2006; Montanez et al., 2008) and migfilin (Tu et al., 2003; Wu, 2005), two FA proteins that directly or indirectly regulate actin dynamics and integrin-dependent intracellular signaling pathways.

Further studies using CHO cells stably expressing integrin $\alpha\text{IIb}\beta 3$ revealed that co-expression of the talin head together with kindlin-1 or kindlin-2 resulted in enhanced $\alpha\text{IIb}\beta 3$ activation, as monitored by mAb PAC-1 binding (Ma et al., 2007; Bouaouina et al., 2008; Harburger et al., 2009). This effect was dependent on a direct interaction of the kindlin FERM domain with $\alpha\text{IIb}\beta 3$. Mutational analysis showed that the second conserved NXXY⁷⁵⁹ motif of the integrin $\beta 3$ tail, and a conserved tryptophane in the F3 subdomain of the kindlin FERM domain, were directly involved in kindlin-integrin interactions (Shi et al., 2007; Moser et al., 2008; Harburger et al., 2009). Thus, in contrast to the talin head domain, which interacts with the membrane proximal NPXY motif of integrin β subunits, kindlins target the membrane-distal NXXY motif of integrin β subunits.

Another breakthrough on the functional role of kindlins came from studies of kindlin-knockout mice. Mice with a kindlin-1 gene deletion had skin as well as intestinal defects (Ussar et al., 2008), whereas knockout of the kindlin-2 gene in mice resulted in early embryonic lethality (Dowling et al., 2008; Montanez et al., 2008). Mice with the kindlin-3 gene knockout only survived for about 1 week, due to severe leukocyte and platelet defects (Krüger et al., 2008; Moser et al., 2008). Kindlin-3 ($-/-$) mice showed normal platelet counts but severe platelet dysfunction, such as defective platelet aggregation *in vitro* or thrombus formation *in vivo*, that led to severe hemorrhages and resistance to arterial thrombosis. Although platelets from kindlin-3 ($-/-$) mice were able to establish initial contacts with von Willebrand factor (vWF)/collagen via the vWF receptor GPIb and the collagen receptor GPVI, they were unable to spread on these ligands as a result of defective $\alpha\text{IIb}\beta 3$ and $\alpha 2\beta 1$ integrin activation (Moser et al., 2008). Interestingly, Mn^{2+} treatment of kindlin-3 deficient platelets allowing artificial activation of the platelet integrins, did not restore platelet spreading, suggesting an additional functional role of kindlins in integrin outside-in signaling. On the other hand, studies using kindlin-3 ($-/-$) polymorphonuclear granulocytes isolated from kindlin-3 ($-/-$) chimeric mice showed that kindlin-3 is not required for leukocyte rolling on inflamed endothelium but is essential for their firm adhesion (Moser et al., 2009). Indeed, kindlins have been shown to cooperate with ILK by interacting with each other and to orchestrate the recruitment of other proteins, such as migfilin to FAs. However, how kindlins regulate integrin-mediated outside-in signaling is still unclear.

Kindlin deficiency in humans has further contributed to the understanding of the functional role of these proteins. The kindlin name refers to the Kindler syndrome first described by Dr. Kindler in 1954, of patients with congenital skin blistering, skin fragility and sun sensitivity, with often oral and colonic

involvement (Kindler, 1954). Histological analysis of the skin blisters revealed epithelial detachment from the basal membrane, pointing to a defect in integrin function. Kindlin-2 deficiency in patients has not been reported, and is predicted to be embryonically lethal. Finally, the real breakthrough in identifying the functional role of kindlins in integrin activation came from the investigation of patients with a rare genetic blood cell disorder, called LAD-III. Patients with LAD-III suffer from Glanzmann's thrombasthenia-like bleeding problems in addition to life-threatening infections, typical of the LAD disorder which is caused by a genetic defect in $\beta 2$ integrin expression (Kuijpers et al., 2009; Svensson et al., 2009). However, LAD-III patients have normal integrin expression in their platelets and leukocytes, and their defect relates to an inability of integrins $\beta 1$, $\beta 2$ and $\beta 3$ to become activated in platelets, neutrophils and lymphocytes. Initially, evidence was provided that LAD-III results from a homozygous single nucleotide C→A splice-junction mutation in the CalDAG-GEF1 gene (Kinashi et al., 2004; Pasvolsky et al., 2007) whose product is known to regulate Rap1. However, more recently, 2 independent groups have shown that mutations in the gene encoding kindlin-3 constitute the primary molecular defect in LAD-III, with all of the LAD-III patients described so far having undetectable kindlin-3 expression (Mory et al., 2008; Kuijpers et al., 2009). This observation was further confirmed by complementation studies showing that expression of kindlin-3, but not CalDAG-GEF1 in LAD-III patient-derived cell lines restored their adhesive phenotype, thus underlining the role of kindlins in integrin activation. Interestingly however, some but not all of the patients also presented the C→A splice-junction mutation in the CalDAG-GEF1 gene. Whether this CalDAG-GEF1 mutation is an innocuous single nucleotide polymorphism (SNP), or whether it also contributes to the LAD-III disorder is still unclear, as CalDAG-GEF1 acts upstream of Rap1 in the talin-dependent integrin activation pathway.

INTEGRIN AFFINITY REGULATION IN CONSTITUTIVELY ADHERENT CELLS

Most information on the molecular events that lead to integrin activation have been obtained by studying platelets or leukocytes, two blood-derived cell types in which integrin activation is initiated through stimulation of agonist receptors, such as the thrombin receptor or the ADP receptor in platelets, or the chemokine receptors in leukocytes, all of which are G-coupled receptors. In contrast, integrin activation in constitutively adherent cells, such as endothelial cells or fibroblasts, appears to rely on a different pathway, since agonist receptors are not involved in this process, with integrins being the primary sensors that establish the initial cell-extracellular matrix contact. Recently, evidence has been provided that in adherent cells, integrin activation might rely on mechanical signaling initiated by force generated tension.

Figure 4. Schematic drawing of the domain architecture of the FERM domain in kindlins and talin (drawing not to scale).

Talin and kindlins contain a FERM domain that shares high structural similarity and consists of three subdomains (F1, F2, and F3). A significant difference between the 2 FERM domains is that the F2 subdomain in kindlins is split by a Pleckstrin Homology (PH) motif. Arrows indicate the regions in kindlins and talin that interact with integrin β subunits, ILK or migfilin.

For example, integrin $\alpha 5 \beta 1$, which interacts with its ligand fibronectin through an auxiliary binding site in addition to the canonical RGD site, has been shown to cycle between a relaxed (low affinity) and tensioned (high affinity) state in response to force generated by the cytoskeleton (Friedland et al., 2009). Interestingly, the binding interface in the relaxed state only included the RGD site of fibronectin, while in the tension state it also included the synergy site. Application of force thus switches the relaxed state, which relies on RGD recognition, to a new tension state with increased bond strength, involving both the RGD site and the synergy site, thus raising the question on whether synergy sites exist more broadly in integrin ligands. It also remains to be determined whether talin and kindlins are required in this force generated activation process, or whether in adherent cells, they function essentially during the process of integrin outside-in signaling.

PERSPECTIVES

The last two years have witnessed an explosion of new data that have now established a fairly comprehensive picture of the molecular events that lead to integrin $\alpha \text{IIb} \beta 3$ activation in human platelets, or $\beta 2$ integrin activation in leukocytes. Some of these new data have been derived from the investigation of patients with rare genetic blood cell disorders, and underline the relevance for the identification and in-depth investigation of such patients. The recent data inevitably also raise numerous new questions. With the increasing number of proteins that directly interact with the short cytoplasmic tail of integrin β subunits, it remains to be seen whether additional proteins are involved in integrin activation, or whether different activation states are regulated by different proteins. Also, the identified overlapping contact sites, such as those of talin IBS1 and talin IBS2 in the integrin β subunit membrane proximal helix, or the binding sites that are in close proximity in the $\beta 3$ C-terminal part, namely those of kindlin and Src, underline the need for further studies to elucidate whether these protein interactions are cooperative, competitive or sequential. Finally, posttranslational modifications, involving

Y or S/T phosphorylation of the integrin cytoplasmic tail or phosphorylation of the binding partners are most likely of major importance in the fine-tuning of integrin activation and outside-in signaling.

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ABBREVIATIONS

ABS, actin binding site; CHO, Chinese hamster ovary; DAG, diacylglycerol; ECM, extracellular matrix; ESC, embryonic stem cell; FA, focal adhesions; FAK, focal adhesion kinase; FC, focal contacts; GPCR, G protein coupled receptor family; GT, Glanzmann's thrombasthenia; IBS, integrin binding site; ILK, integrin linked kinase; LAD, leukocyte adhesion deficiency; PAR, proteinase activated receptor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear granulocyte; PTB, phosphotyrosine binding; RIAM, Rap1GTP-interacting adaptor molecular; VBS, vinculin binding site; SNP, single nucleotide polymorphism

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