HHS Public Access

Author manuscript

Biorheology. Author manuscript; available in PMC 2016 May 17.

Published in final edited form as:

Biorheology. 2015 December 16; 52(5-6): 353–377. doi:10.3233/BIR-15085.

Leukocyte arrest: Biomechanics and molecular mechanisms of β_2 integrin activation

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Abstract

Integrins are a group of heterodimeric transmembrane receptors that play essential roles in cell–cell and cell–matrix interaction. Integrins are important in many physiological processes and diseases. Integrins acquire affinity to their ligand by undergoing molecular conformational changes called activation. Here we review the molecular biomechanics during conformational changes of integrins, integrin functions in leukocyte biorheology (adhesive functions during rolling and arrest) and molecules involved in integrin activation.

Keywords

Talin; RIAM; kindlin-3; PSGL-1; GPCR; Rap-1

1. Integrins

Integrins are $\alpha\beta$ heterodimers that function as activation-dependent adhesion molecules at the interface between cells and immobilized ligands in the extracellular matrix or other cell surfaces [1–3]. The interactions of integrins with their ligands are broadly relevant to a multitude of physiological and disease situations, such as inflammation [4–7], immune responses [8–11], thrombosis and hemostasis [12–15], extracellular matrix assembly [1–3,16–18], tumor metastasis [15,19–22] and other cellular processes. This review is focused on the four β_2 integrins (Table 1) among the known 24 integrins [9,23]: $\alpha_L\beta_2$ (LFA-1, lymphocyte function-associated antigen 1), $\alpha_M\beta_2$ (Mac-1, macrophage-1 antigen), $\alpha_X\beta_2$ (p150,95), $\alpha_D\beta_2$.

Excellent reviews cover the structures of integrins [9,78–81]. Both α and β subunits of integrins have large ectodomains, a single membrane-spanning helix (transmembrane, TM) and, usually, a short unstructured cytoplasmic tail (Fig. 1). Typically the α and β subunits contain around 1000 and 750 amino acids, respectively [78]. Specifically, human α_L has 1170 amino acids, α_M has 1152 amino acids, α_X has 1163 amino acids, α_D has 1161 amino acids and β_2 has 769 amino acids. All α chains of the β_2 integrins contain an I domain with

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homology to von Willebrand factor (VWF) A domain. The ectodomains are composed of several domains with flexible linkers between them. Each α ectodomain has (from C to N terminus) calf-1 and 2 domains, a thigh domain, a β propeller domain, and an α I domain. The β_2 ectodomains have a β-tail domain, integrin epidermal growth factor like repeat domains (I-EGF-1 to 4), a plexin-semaphorin-integrin (PSI) domain, a hybrid domain, and a β I-like domain. The ectodomains can be divided into headpiece and tailpiece as shown in Fig. 1. The α and β cytoplasmic tails of integrins are extended and flexible and can directly bind several adapter proteins with different functional effects [82–88] (Table 2).

β_2 integrins, also known as leukocyte integrins [4,27], are the most important molecules in recruiting leukocytes, especially neutrophils and naïve lymphocytes, from the blood stream to sites of immune responses and inflammation. β_2 integrins are involved in slowing down rolling [24,100,124–128], promoting arrest [100,129–137], supporting spreading and migration [128,136–143], homotypic and heterotypic cell–cell interactions [8,10,11,144–148] and phagocytosis [6,137,149–153]. Unlike other integrins, leukocyte integrins have few extracellular matrix ligands.

2. Conformational activation

Integrins regulate their adhesiveness through changes in the conformation of their ectodomain [154], which can increase ligand affinity by ~10,000 fold [155]. The first integrin structure was solved for $\alpha_V\beta_3$ integrin [156,157] starting a rich stream of publications with information about integrin ectodomain structures. It is widely accepted that β_2 integrins have at least three conformations with different ligand binding affinities [8,9,88,158–160] (Fig. 2(A)–(C)): bent ectodomain with closed headpiece (E^-H^-, resting state, low affinity), extended ectodomain with closed headpiece (E^+H^-, intermediate [88,100,135,161–163] or low [159] affinity), and extended ectodomain with open headpiece (E^+H^+, high affinity). Furthermore, a structure of bent ectodomain with open headpiece (E^-H^+, Fig. 2(D)), which can bind small soluble ligands [164,165], was found in structures of $\alpha_v\beta_3$ [164] (Electron Microscopy, EM) and $\alpha_x\beta_2$ [67] (X-ray crystallography), but it is not clear whether this structure exists on the cell surface and whether it has any function.

2.1. Models of integrin activation

Most studies on conformational integrin activation are limited to the truncated ectodomains, often modified by site-directed mutagenesis to stabilize conformations. In these studies, amino acids thought to be in close proximity in one of the conformations are replaced by cysteines to form disulfide bonds. By necessity, such stabilized integrins must be studied outside their natural cellular context. These experiments suggested two different models of integrin conformational activation. The “switchblade” model proposes that integrin extension is required for headpiece opening and the bent integrin first undergoes extension followed by rearrangements in the metal ion-dependent adhesion site (MIDAS) motif leading to headpiece-opening and high affinity ligand binding [8,9,154,161,162,166,167] (Fig. 2, (A) to (B) to (C)). An alternative model is the “deadbolt” model, where interactions between the headpiece and the legs keep the integrin in the closed, bent conformation and extension can only occur after the “deadbolt” is released. Two studies have shown that E^-H^+

integrin can exist, suggesting that integrins can open their headpiece prior to extending their ectodomain (Fig. 2, (A) to (D) to (C)). It is not known whether this conformation exists on living cells. A third model is extension first, then force-facilitated headpiece opening [10,11,135].

2.2. Extension

The resting state of β_2 integrins shows a bent structure shaped like an inverted "V" with the low affinity headpiece closely approaching the plasma membrane [154,168], experimentally verified in live leukocytes by Förster resonance energy transfer (FRET) [169,170], in which FRET from α I domain (FITC-conjugated antibodies) to plasma membrane (Octadecyl rhodamine B, ORB) was observed in resting leukocytes and disappeared when the cells were activated. The bent ectodomain of β_2 integrins is about 11 nm above the plasma membrane, whereas the extended ectodomain is about 23 nm (with α I domain) [78]. To allow the headpiece to bind ligands on other cells or surfaces in trans, the ectodomain needs to be extended. Integrin extension is initiated by inside-out signaling [9]. EM and FRET studies show that the α and β feet of extended integrins are more separated than those of bent integrins [154,160]. This could be achieved by lateral displacement of the cytoplasmic tails or by a change of the angle between the α and β transmembrane domains, or both. Such molecular rearrangements could conceivably provide the force necessary to extend the ectodomain. There is good evidence that β_2 integrin extension is mediated by talin binding in the β cytoplasmic tail of integrin [88,100], thus causing the conformational changes of cytoplasmic tail and transmembrane domain [171].

2.3. Headpiece opening

The integrin headpiece includes the α I domain, the β propeller domain and the thigh domain of the α subunit and the β I-like domain, the hybrid domain, the PSI domain and the I-EGF-1 domain of the β subunit [9]. In β_2 integrins, the ligand-binding pocket is located in the α I domain. During integrin activation, the headpiece undergoes conformational changes allowing two ligand binding sites to be exposed, one for the external ligand like ICAM-1 and one for an internal ligand formed by the α I domain, binding to the β I-like domain. On α_M or α_L, the MIDAS is formed by the metal ion (such as Mg^{2+}) and the residues T209, D242 and the D140XSXS motif of the α_M I domain [9,172], or the residues T206, D239 and the D137XSXS motif of α_L I domain [155], respectively. It has been demonstrated by introducing disulfide bonds that wild-type isolated α_L I-domain has low affinity for ICAM-1, whereas pulling down the α7 helix of the I-domain partially or completely will include the stabilized intermediate or high affinity α_L I-domain [155,173]. The α I-domain sits on top of the β propeller domain, in close proximity to the β I-like domain. In natural integrin without disulfide bonds, it is thought that upon integrin activation, the β I-like domain binds an internal ligand (amino acid residue G310) of the α_L I domain. This binding pulls down the α7 helix and stabilizes the high affinity conformation of α I [9,67,154,174]. The internal ligand binding requires that the MIDAS in the β I-like domain is open, which is thought to be induced by hybrid domain swing-out [175,176]. In the "switchblade" model, it is suggested that integrin extension enables hybrid domain swing-out [175,176], thus inducing further conformational changes of the α and β I and I-like domains and acquiring high affinity for ligand [9,174]. However, in cell-free systems it has also been observed that

bent integrin can have swung-out hybrid domain and open headpiece [154,174]. This bent conformation with open headpiece [67,164,177] ($E^{-}H^{+}$) can bind (small) soluble ligands [164,165,177] prior to extension, suggesting that integrin extension is not necessary for headpiece-opening. These observations are difficult to reconcile with the switchblade model. Kindlin-3 (another important adapter protein) deficient murine neutrophils or kindlin-3 knock down HL-60 cells show a defect in headpiece-opening as reported by conformation-specific antibodies [100]. A mutant talin-1 (L325R) [178] was also demonstrated to prevent headpiece-opening of β_2 integrin on neutrophils, which exhibit a similar phenotype as kindlin-3 knock out neutrophils [100]: both show normal slow rolling but deficient arrest.

2.4. Cytoplasmic tail separation

In live cells, the cytoplasmic domains of the integrin α and β subunits are close to one another [171] in the resting (bent) state, close enough so FRET occurs between fluorescent proteins fused to the α and β cytoplasmic domains [171]. Replacement of the α and β cytoplasmic domains with acidic and basic amino acids that form a heterodimeric α-helical coiled-coil forces the two cytoplasmic domain together and stabilizes integrins in their inactive state [179]. The natural integrin cytoplasmic tails have flexible structures and several binding sites for different regulatory adaptor proteins (Table 2). Important regions of the cytoplasmic tails are the NPxY motifs in the β_2 tail, which bind talin (membrane-proximal NPxY) [83,101] and kindlin (membrane-distal NPxY) [109,110], respectively. The integrin “off” state is stabilized by binding of filamin or other phosphotyrosine-binding (PTB) domain-containing proteins. It is thought that filamin competes with talin. Tyrosine phosphorylation of the β-tail by some Src family kinases (SFK) has also been suggested to be involved in stabilizing the bent conformation through inhibiting talin binding [180]. β tail phosphorylation promotes the binding of PTB-containing proteins such as Dok1 (docking protein 1), thus preventing the binding of talin. The binding of other PTB-containing talin competitors, such as ICAP1 (integrin cytoplasmic domain associated protein 1), is not dependent on integrin tyrosine phosphorylation [84]. Upon talin binding, cytoplasmic tails separation occurred, resulting in diminished FRET between fluorescent proteins fused to the α and β cytoplasmic domains [171]. This separation of α and β cytoplasmic tails is thought to be critical for integrin extension and, in the switchblade model, also for headpiece opening [179].

2.5. Transmembrane domain (TMD) structure changes

The TMD of α and β are both α helices. The α and β helices cross at specific angles relative to the plane of the plasma membrane. This is stabilized by in-register alignment of side-chain arrays (Fig. 3) [181]. Nuclear magnetic resonance (NMR) spectroscopy showed the structures of the $\alpha_{IIb}\beta_3$ TMD complex – the α_{IIb} TMD helix is roughly perpendicular to the membrane while the β_3 TMD helix is tilted [182,183]. A specific α-helical interface (salt-bridge) between the α and β transmembrane domains stabilizes integrin in the resting state [181,184,185]. Three models have been proposed for the movement of the transmembrane domains during integrin activation: moving apart [184], pistoning up and down [181], or an angle change [181] between α and β. The pistoning model is related to the angle change model, because it suggests that intracellular activating signals could shorten the portion of helix which is buried within the lipid bilayer, thereby changing membrane tilt angle and

register with the neighboring helix to avoid hydrophobic mismatch with the fixed width of the membrane bilayer. This change in tilt angle could be the critical event in disrupting transmembrane interactions that stabilize the low-affinity conformation, leading to integrin activation [181,186]. Structural studies of a complex between talin and the integrin β_3 cytoplasmic domain [102] suggested two talin binding sites on the β_3 cytoplasmic domain – membrane-proximal (MP) and membrane-distal (MD) sites. Talin binding to the MD part of the β cytoplasmic domain subsequently engages the MP binding site, resulting in reorientation of the TMD (Fig. 3(A)). The interaction of the MP part with talin results in the position and angle change of MP part, thus leading to the pistoning [102] and moving apart [187] of the transmembrane helices. A recent study showed that phosphatidylinositol 4,5-bisphosphate (PIP2) can interact with residue Arg995 within the TMD and break the salt-bridge when talin is bound, thus helping integrin activation [188].

3. Adaptor proteins and integrin activation

The main adaptor molecules regulating integrin activation include talin, Rap1-GTP interacting adapter molecule (RIAM) and kindlin (kindlin-3 in leukocytes). Talin and kindlin are reported to bind the integrin β cytoplasmic tail directly, whereas RIAM is thought to bind and recruit talin to integrin.

3.1. Talin

Talin is a high-molecular-weight cytoskeletal protein concentrated at regions of cell-substratum contact [189]. There are two genes, talin-1 and talin-2. Talin-1 contains an N-terminal 47-kD head domain and a ~220-kD C-terminal flexible rod domain [190]. Talin-1 interacts with both actin and integrin [103]. The head domain consists of an N-terminal FERM domain (protein 4.1, ezrin, radixin and moesin) with three subdomains (F1, F2, F3) and an F0 subdomain with no homology to known domains [191]. It is suggested that the talin head domain has a unique extended structure different from the typical cloverleaf structure seen in other FERM domains [192]. The F3 subdomain contains a PTB domain that binds the integrin β subunit tail at the membrane-proximal NxxY site and promotes integrin activation [101–104]. The talin rod domain is composed of a series of helical bundles (R1 to 13) and a C-terminal single helix dimerization domain (DD). The rod domain contains multiple binding sites for RIAM [193–195], vinculin [195,196], deleted in liver cancer 1 (DLC1) [197], synemin [198], a second binding site for integrin [199,200], binding sites for actin [87,201,202] and internal binding sites for the talin F3 subdomain [203]. It is not clear whether and how all these binding sites are occupied when the attendant integrins are resting or activated.

Talin binding to integrin β cytoplasmic tail leads to spatial changes of the tail and TMD. Two models have been proposed for these changes: the “moving apart” model, in which talin binding leads to the moving apart of the α/β “legs” as demonstrated by FRET assay [171]; and the “piston and angle change” model, in which talin binding leads to angle change, pistoning up and down along with moving apart of the α and β TMDs (Fig. 3), supported by NMR structure studies using short peptides including the TMDs [102,181,187,204,205]. It is thought that the change of the position of the α and β chains relative to each other induces

the conformation changes in the ectodomain, but it is not known how the forces are transduced.

The interaction of the talin F3 subdomain with the β_2 cytoplasmic tail is necessary but not sufficient for integrin activation. The interaction of talin and the membrane is also important [87]. Some basic residues in the F2 subdomain (Fig. 3), which is located close to the plasma membrane during talin-integrin binding, can bind acidic phospholipids in the plasma membrane [187]. This interaction is also thought to be necessary for integrin activation [204], because mutations of the membrane binding residues on F2 domain significantly decrease the binding of PAC-1 antibody, which is specific for activated $\alpha_{IIb}\beta_3$ integrin. A recent molecular dynamics study using atomistic molecular stimulation demonstrated that PIP2 and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) are important for talin-mediated salt bridge breaking, which leads to spatial changes of the β TMD and leads to integrin activation [188].

3.2. RIAM

RIAM is a broadly expressed adapter protein that contains an RA (Ras association)-like domain, a pleckstrin homology (PH) domain, and several proline-rich sequences. RIAM is important for talin recruitment and integrin activation in leukocytes [206,207], but not platelets [208]. A short amphipathic helix (residues 6–30) in the N-terminal region of RIAM binds talin. A fusion protein containing this helix fused to the membrane-targeting sequence of Rap1A mimics recruitment of talin to the plasma membrane by the RIAM-Rap1 complex and supports integrin activation [193]. This result shows that RIAM is sufficient to recruit talin, but does not address the question whether it is required. RIAM and vinculin compete for the same binding site on talin-1 and their binding to talin is mutually exclusive [194]. RIAM is located in the newly formed protrusions of adherent cells, whereas vinculin is located in mature focal adhesions [194]. This suggests a model where RIAM binding to talin initializes the talin recruitment and integrin activation, then RIAM is replaced by vinculin to further stabilize the activated state of integrin. Interestingly, RIAM was also shown to immunoprecipitate with kindlin-3, even before it immunoprecipitates with talin [209]. However, whether RIAM directly interact with kindlin-3 is unknown.

3.3. Kindlin-3

Kindlin is a family of focal adhesion proteins including three subtypes – kindlin-1, 2, and 3, which are also known as fermitin family homolog 1, 2 and 3 (FERMT1, FERMT2, FERMT3) respectively [210]. Kindlin-3 is expressed in leukocytes and platelets and is another essential player that binds to the integrin β_2 subunit tail at the membrane-distal NxxY site [109,110]. Kindlin binding to β_2 does not compete with talin binding. Kindlin shows high levels of sequence similarity to talin FERM domain [211]. The main difference is that kindlin contains a PH domain that may be involved in membrane recruitment by phosphoinositide binding [190]. The subdomain 3 in kindlin binds the distal NPxY motif of integrin β tails [109,212,213]. Kindlin-3 is expressed exclusively in cells of haematopoietic origin. Mutations in kindlin-3 are found in leukocyte adhesion deficiency III (LAD-III) patients, which have severe bleeding and immune deficiency caused by defective integrin activation in leukocytes and platelets [109,214– 217]. These findings demonstrate a critical

role for kindlin-3 in leukocyte integrin activation, but the specific mechanisms remain controversial. Kindlin-3 knockout mice show a defect in neutrophil arrest but not slow rolling, which suggests that kindlin-3 is required for headpiece-opening but not extension of β_2 integrin [100]. Kindlin-3 knockdown in the HL-60 promyeloid cell line results in a binding defect of mAb24 [218–221], which is specific for open β_2 headpiece, but not KIM127 [222,223], which reports β_2 integrin extension. These results suggest essential roles of kindlin-3 in headpiece-opening of β_2 integrin [100]. An alternative hypothesis suggests that kindlin can promote talin binding to integrin cytoplasmic tail [224]. However, although kindlin, talin and integrin cytoplasmic tail can form a ternary complex, the binding of kindlin to integrins neither enhances talin–integrin binding nor increases talin targeting to the membrane [225,226], and no direct talin–kindlin interaction has been reported. Another hypothesis suggests that kindlin-binding may induce integrin clustering to enhance binding of multivalent ligands via increased avidity, rather than through conformational changes that lead to increased affinity for monovalent ligand [190,227], which is experimentally supported [228].

4. Leukocyte arrest and signaling of integrin activation

During infection or inflammation, leukocytes in blood, including granulocytes, monocytes and lymphocytes are recruited to the site of immune responses in a cascade-like fashion [17,27,136,229–234] that includes rolling, arrest, crawling, transendothelial migration and migration in tissues. Integrins play vital roles in all these processes. From rolling to arrest, leukocytes need to adhere with sufficient force to balance the force imposed by the drag and torque exerted by the flowing blood. Arrest is thought to be triggered by rapid changes in β_2 integrin conformation through inside-out signaling, which may be triggered from surface receptors P-selectin glycoprotein ligand-1 (PSGL-1) or chemokine receptors. This requires regulation of several kinases and assembly and disassembly of multiprotein complexes that form around the cytoplasmic tails of integrins.

4.1. Rolling

The dominant molecules involved in leukocyte rolling are selectins and their ligands, whose biomechanics has been reviewed before [24,25,125,127,235–238]. The interaction of endothelial selectins with leukocyte PSGL-1 primes integrin activation, specifically promoting extension of β_2 integrins [24,88,125,126,239]. Assays using conformation-specific antibodies have confirmed that rolling on P-or E-selectins can induce integrin extension on leukocytes within human blood [24]. Thus, β_2 integrins also serve as “rolling receptors” [27]: they transiently engage their ligand ICAM-1, thereby reducing rolling velocity (slow rolling). Slow rolling has been proposed to be mediated by extended integrin with intermediate affinity [24,100,125–127,135,161–163,240]. This mechanism of integrin-dependent slow rolling was confirmed by using an allosteric inhibitor [125,163] that allows integrin to assume the extended, but not high affinity conformation, and by using kindlin-3 knocked out mice, in which integrin can be extended but the headpiece cannot be opened [100].

4.2. PSGL-1 signaling (Fig. 4)

During leukocyte rolling, interactions of endothelial E- and P-selectins with PSGL-1 activate a key signaling pathway. PSGL-1 is the major ligand for P-selectin [239,241,242] and also binds E-selectin [236,243] and L-selectin [237] under flow conditions. PSGL-1 is preferentially located on the tips of leukocyte microvilli [25,244], which enables interactions with selectins during rolling. Granulocytes rolling on P- or E-selectin partially activate β_2 integrin through PSGL-1 induced inside-out signaling, which was demonstrated by observation of slow rolling on ICAM-1 [24,125,126] accompanied by increased staining with the β_2 extension reporter antibody KIM127 [24]. A mutational study of PSGL-1 showed that the cytoplasmic tail was crucial for this signal transduction, whereas the extracellular domain is responsible for leukocyte rolling [238].

Under no-flow conditions, soluble P-selectin-Fc chimeric protein was shown to induce SFK-dependent phosphorylation of Naf-1, followed by recruitment of the phosphoinositide-3-OH kinase (PI3K) p85-p110δ (PI3Kδ) heterodimer and priming of integrin activation [245]. This pathway may be important when leukocytes bind platelets, which have much higher density of P-selectin than endothelial cells. This pathway is not operative in rolling leukocytes, because PI3Kδ deficient leukocytes showed normal slow rolling on P-selectin and ICAM-1 under flow conditions [246].

On the neutrophil surface, PSGL-1 is closely associated with L-selectin and this does not require interaction of the L-selectin lectin domain with the known selectin-binding domain of PSGL-1 [127]. Engagement of P- or E-selectin with PSGL-1 triggers signaling through lateral interaction with L-selectin [127], followed by phosphorylation of the SFKs Fgr, Hck and Lyn [126,127,246,247]. Further studies indicated that Fgr is the main SFK required for slow rolling [126]. The phosphorylation of Fgr induces phosphorylation of the ITAM-containing adaptor proteins DAP12 or FcRγ [126], which subsequently interact with and phosphorylate the tyrosine kinase Syk [126,246]. Bruton tyrosine kinase (Btk) [246,248], which is one of Tec family kinase, acts downstream from Syk, where the signaling appears to divide into two pathways: one through phospholipase C-γ2 (PLCγ2); the other through PI3Kγ [248]. The downstream molecules of PLCγ2 are CalDAG-GEFI and p38 MAPK, which are known to activate the small GTPase Rap1a [240]. Rap-1a activation is associated with LFA-1 activation, but the details are unknown. Rap-1 has been proposed to bring talin-1 to the β_2 subunit, or it may be involved in kindlin-3 recruitment. In the PI3Kγ pathway, Akt is phosphorylated [127,248], which was reported to inhibit the integrin negative regulator GSK3α/β in platelets [249]. Again, the details are unknown. The PI3Kγ pathway could also be involved in integrin clustering.

4.3. Chemokine receptor signaling (Fig. 4)

Although PSGL-1 signaling can prime integrin activation by promoting integrin extension and cause slow rolling of leukocytes [24,100,125,126,250], chemokine receptor signaling is thought to be required for full integrin activation (probably associated with headpiece opening) and leukocyte arrest [125,130,135,251]. Chemokine receptors are G-protein coupled receptors (GPCRs), which are seven-transmembrane proteins that are identified by the coupling with the heterotrimeric G protein containing a particular α-subunit ($G_{\alpha i}$) paired

with a $\beta\gamma$-complex ($G_{\beta\gamma}$) [252]. Several chemokine receptors are expressed on leukocytes, and many of them can trigger arrest [253]. The best-studied chemokine receptor in this context is the C-X-C chemokine receptor type 2 (CXCR2), which binds the chemokine (C-X-C motif) ligands (CXCL) 1, 2, 3, 5, 6 and 7.

The chemokine receptor signaling pathway triggering integrin activation is incompletely understood [88,234]. In neutrophils, the most relevant chemokine receptor responsible for arrest is CXCR2 [254]. In vitro flow chamber assays show that immobilized CXCL1 stimulating CXCR2 leads to LFA-1 activation and neutrophil arrest on an E-selectin/intercellular adhesion molecule 1 (ICAM-1) substrate through $G_{\alpha i}$ depended signaling [125]. Specifically, $G_{\alpha i2}$ but not $G_{\alpha i3}$ is required. $G_{\alpha i}$-dependent Ras activation leads to the activation of phosphoinositide 3-kinase (PI3K) by binding to its catalytic subunit [255]. The dissociated $G_{\beta\gamma}$ subunit has been shown to interact with a number of molecules, including PI3K isoforms [256,257], PDZ proteins [258], guanine nucleotide exchange factors (GEFs) such as PIP3-dependent Rac exchanger (P-Rex) [259] and protein kinase D [260]. A recent study showed that the β subunits 1, 2, 4, and 5 in $G_{\beta\gamma}$ are indispensable for GPCR-mediated integrin activation and leukocyte recruitment, in which Ras-related C3 botulinum toxin substrate 1 (Rac-1) and phosphoinositide phospholipase C (PLC) β_2 and β_3 are involved in downstream signaling pathway [261]. In vivo, PI3Kγ [262,263] and protein kinase C (PKC) θ [264] deficient neutrophils show no defect in arrest, but they revert to rolling again, suggesting that there is a defect in integrin bond maturation. Blockade of p44/42 and p38 mitogen-activated protein kinases (MAPK), PI3K, or PKC signaling does not affect chemokine triggered integrin activation on monocytes. The role of inositol triphosphate (IP3) and intracellular free calcium (Ca^{2+}), the calcium-binding messenger protein calmodulin, and inositol-1,4,5-triphosphate receptors as downstream events of PLC activation is controversial [265,266]. Ca^{2+}/diacylglycerol (DAG)-regulated guanine nucleotide exchange factor I (CalDAG-GEFI) is required for activation of Ras-related protein 1 (Rap1) and integrin in neutrophils [267]. In contrast, when the neutrophils were stimulated by fMLP, integrins were activated by activation of Rac through proto-oncogene vav (Vav1) and P-Rex 1 [268]. Two (incomplete) chemokine receptor pathways leading to activation of different integrins were demonstrated in T-lymphocytes stimulated by CXCL12 or PMA: PLC$\gamma \rightarrow Ca^{2+}$/DAG $\rightarrow$ CalDAG-GEFI $\rightarrow$ Rap1 $\rightarrow$ LFA-1 and PLC$\gamma \rightarrow Ca^{2+}$/diacylglycerol $\rightarrow$ PKC $\rightarrow$ very late antigen-4 (VLA-4) [269] respectively. Another GTPase, cell division control protein 42 (Cdc42), may be a negative regulator of integrin activation upon chemokine stimulation [270]. Phospholipase D1 (PLD-1) was demonstrated as the downstream of Rac1 and RhoA [270]. Phosphatidylinositol-4-phosphate 5-kinase type I gamma (PIP5KC) is downstream of PLD-1 and found to specifically control the transition of LFA-1 from an extended low-intermediate state to a high-affinity state [270]. In a recent study [271] CXCL12 was shown to induce Janus kinase (JAK) 2 and 3 activation in a $G_{\alpha i}$-independent manner. JAK2 and 3 were upstream of Vav1, which then activates Ras homolog gene family member A (RhoA) and Rac-1. Blockade of RhoA and PLD-1 inhibits the activation of Rap1a. Another study on $\alpha_{IIb}\beta_3$ suggests that Rap1 is downstream of PKC [272].

In conclusion, the proximal (GPCR to $G_{\alpha i2}$ to PLCβ) and distal (GALDAG-GEFI to Rap1 to talin-1 to integrin) parts of the pathway are clear, but the middle parts are not clear. It is also

unknown whether integrin activation is local, i.e. in the microvillus where the leukocyte is in touch with chemokine, or global (whole cell).

4.4. Rap-1

As reviewed, the pathways of PSGL-1 signaling and chemokine receptor signaling may converge on Rap1. However, PSGL-1 ligation triggers only extension and chemokine receptor ligation triggers high affinity (open headpiece). This discrepancy requires that molecules other than Rap-1 are involved. During integrin activation, activated Rap1 needs to be recruited to the plasma membrane by a signaling module comprising the cytosolic adapter proteins ADAP (adhesion and degranulation promoting adapter protein) and SKAP55 (src kinase-associated protein of 55 kDa) [273]. RapL is one of the Rap1 downstream effectors, which was identified by a yeast two-hybrid screen [89,274]. RapL binds to a site consisting of two lysine residues (K1097/K1099) following the GFFKR motif, which is found only in the α_L [90] subunit. RapL forms a complex with the serine/threonine kinase Mst1. Rap1 activation recruits both RapL and Mst1 to LFA-1 and actives Mst1 kinase activity. RapL-deficiency impairs Mst1 activation in cells. Knocking down Mst1 expression reduces integrin activation in response to chemokine stimulation [91], but it is not known how Mst1 is involved in extension or high affinity.

RIAM is another important Rap1 binding protein, which is very important in integrin activation [275]. RIAM has been reported to interact with SKAP-55 and play key roles in the recruitment of Rap1/RIAM complex to the plasma membrane [276]. The ability of Rap1 to activate integrins depends on talin binding to integrin cytoplasmic tail [272]. Rap1 induces the formation of an integrin-activation complex containing talin in combination with RIAM [272,277]. RIAM deficient mice have no defect in platelet arrest [206,208], but a severe defect in neutrophil arrest [207], macrophage adhesion [207], and lymphocyte adhesion [206,207]. A recent study shows that SLAT (SWAP-70-like adaptor of T cells) can interact with Rap-1 through its PH domain and promotes TCR-mediated, Rap1-dependent LFA-1 activation and adhesion [278]. Since SLAT is activated by T cell receptor engagement, this mechanism is unlikely to be involved in triggering arrest.

5. Conclusions and open questions

β_2 integrins are fascinating molecular machines that translate intracellular adaptor binding (kindlin-3, talin-1, RIAM) into conformation changes of the ectodomains. This may be transmitted through moving the cytoplasmic and transmembrane domains apart, or changing their crossing angle. The molecular mechanics by which these movements are translated to ectodomain changes are unknown. β_2 integrin activation is triggered by PSGL-1 ligation or chemokine receptor ligation or both. Under physiologic conditions, PSGL-1 ligation alone reduces the rolling velocity in a β_2 integrin-dependent fashion. When (soluble or immobilized) chemokines are available, full integrin activation (E^+H^+) ensues and leads to arrest of the rolling cell. Both the PSGL-1and the chemokine receptor signaling pathways to β_2 integrins are only partially understood. The initial hypothesis that all integrins are activated in the same way was refuted: not only are different integrins activated in different ways, but activation mechanisms even appear to be cell type-specific. The methods used to

study integrin activation include crystallography of conformationally stabilized integrins, rotary shadowing EM, NMR, reporter antibodies, and functional studies of rolling and arrest. It is very challenging to integrate findings obtained with these fundamentally different and mutually exclusive methods.

Acknowledgments

This research was supported by funding from National Institutes of Health, USA (NIH, HL078784).

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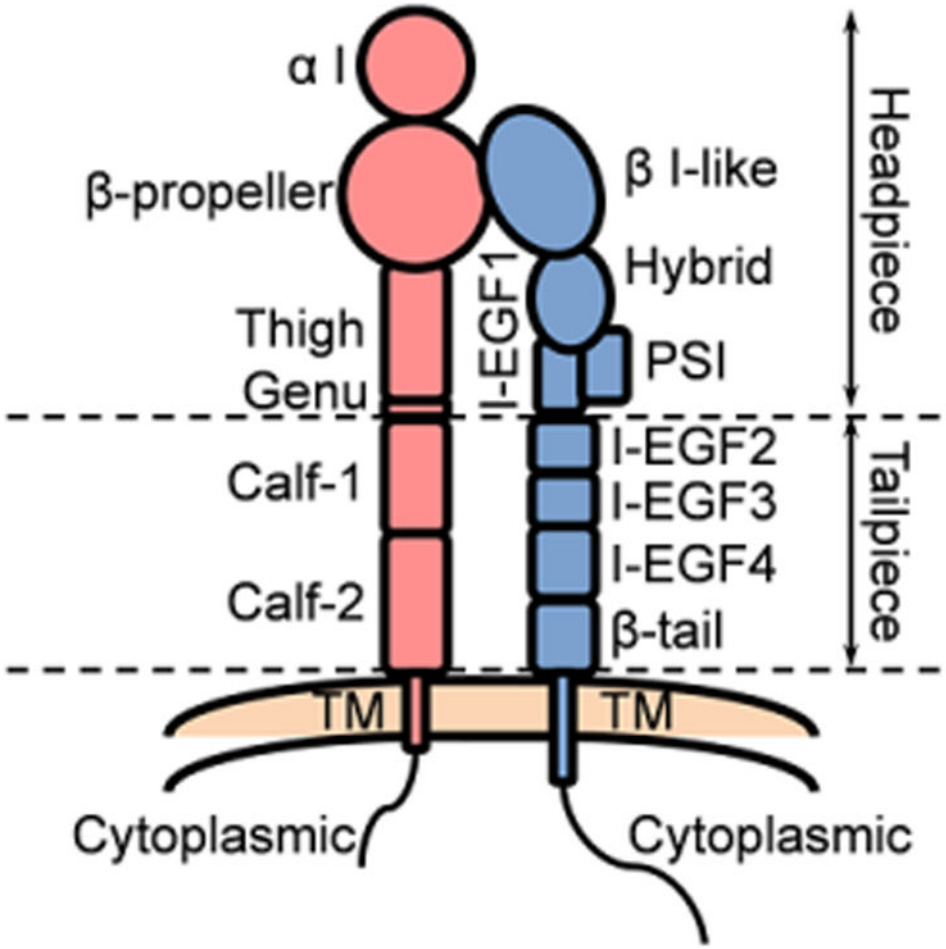

Fig. 1.

Structural schematic of the extended β_2 integrin. α chain red, β chain blue. Subdomains and headpiece/tailpiece portions labeled.

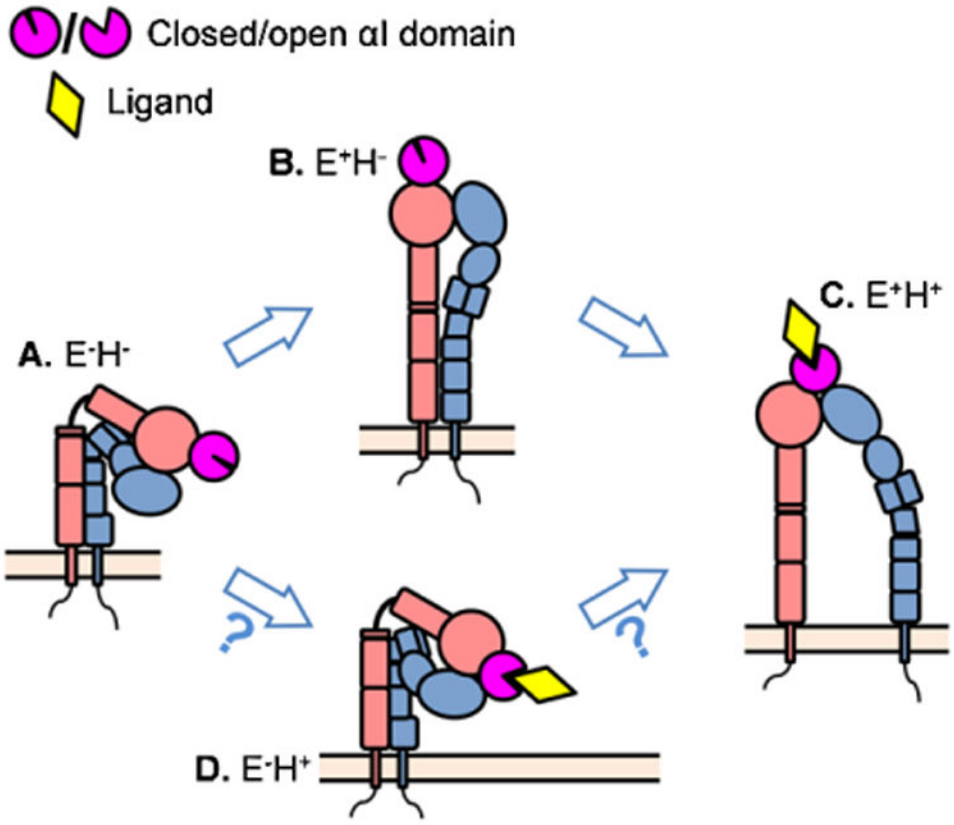

Fig. 2.

The existing conformations of the β_2 integrins and the proposed consequences of conformational changes. (A) The resting state of β_2 integrin has bent ectodomain with closed headpiece. (B) The resting β_2 integrin can extend its ectodomain. This conformation may have intermediate affinity to the ligand and mediate leukocyte slow rolling. (C) Further conformational changes can induce headpiece-opening and acquire fully activated β_2 integrin. This conformation has high affinity to the ligands and is thought to support arrest and leukocyte adhesion. (D) The structure of bent ectodomain with open headpiece was also crystallized in β_2 integrin.

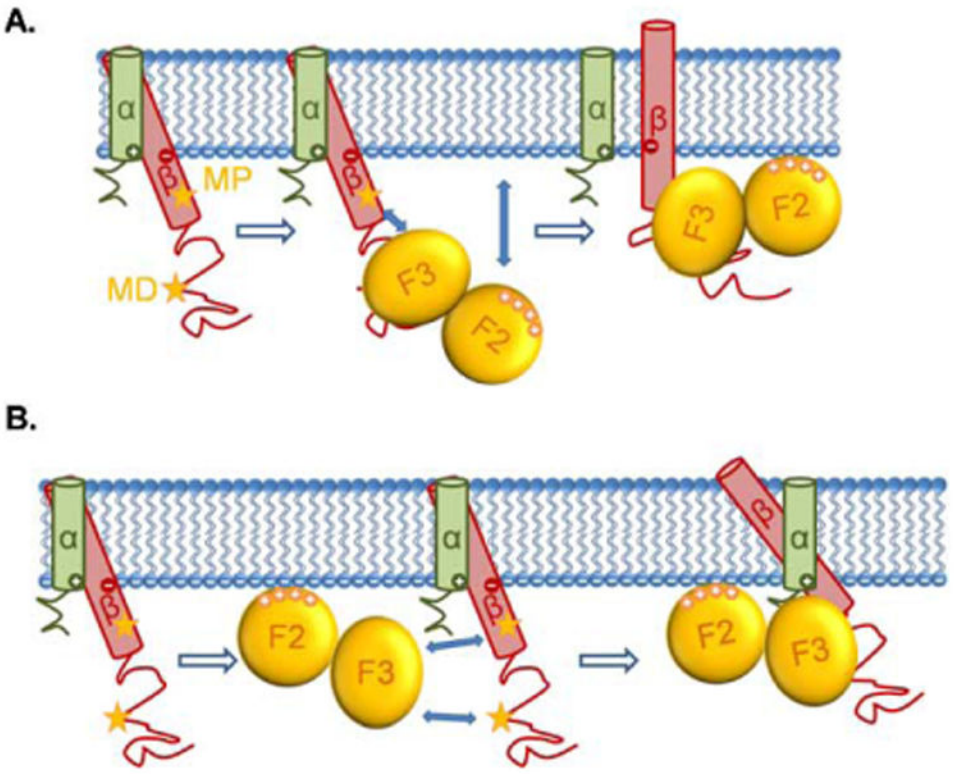

Fig. 3.

The movements of β_2 integrins' TMD induced by talin binding. The α and β TMDs of β_2 integrins cross at an angle at rest. The two stars indicate the talin binding sites (MP and MD) of the β cytoplasmic domain. (A) Upon binding to the talin F3 domain through the MD binding site of the β cytoplasmic domain, the attractions between talin F3 domain and the MP binding site of β cytoplasmic domain, as well as the attractions between the talin F2 domain and the plasma membrane, force the β TMD to move, resulting in an angle change between α and β TMDs, along with the pistoning of β TMD and the dissociation of α and β TMDs. (B) A more recent study suggested that talin first interacts with the plasma membrane through its F2 domain, then the attractions between talin F3 domain and the two β cytoplasmic binding sites force the β TMD to move and change their crossing angle, along with the pistoning of β TMD and the dissociation of α and β TMDs.

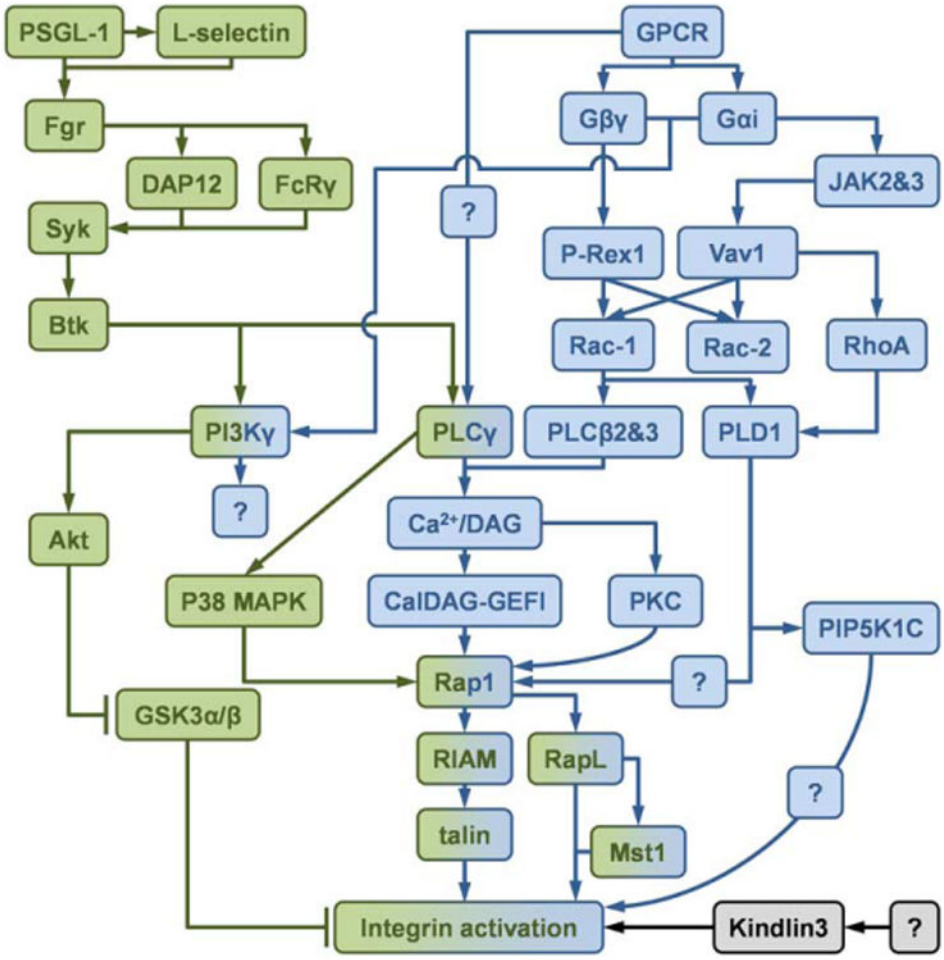

Fig. 4.

Schematics of the inside-out signaling of integrin activation. The integrin activation through inside-out signaling can be divided into PSGL-1 signaling (green) and chemokine receptor (GPCR) signaling (blue). Molecules involved in both two signaling pathway are shown in both colors. The signaling source of kindlin-3 (black) is unknown.

Table 1

β_2 integrins expressed on leukocytes

α subunit	β subunit	Alternative name	Expressed in cells	Main ligands
α_L (CD11a)	β_2 (CD18)	LFA-1	Neutrophils [24–26], T-lymphocytes [11,27,28], B-lymphocytes [28,29], Monocyte [26], Macrophage [30], Dendritic cells [31], Natural killer cells [32]	ICAM-1 [28,33–36], ICAM-2 [25,37], ICAM-3 [38], ICAM-4 [39,40], ICAM-5 [41,42], Collagen [34]
α_M (CD11b)	β_2 (CD18)	Mac-1	Neutrophils [24,26,43], T-lymphocytes [27], B-lymphocytes [44], Monocytes [26,45], Macrophages [46], Dendritic cells [47], Natural killer cells [32]	ICAM-1 [48–50], ICAM-2 [51], ICAM-4 [39], Fibrinogen [43,45,52], Collagen [34], iC3b [34,53], Heparin [54], GPIbα [55], JAM- 3 [56], Thy-1 [57], Plasminogen [58]
α_X (CD11c)	β_2 (CD18)	p150,95	Neutrophils [59,60], T-lymphocytes [27], B-lymphocytes [61,62], Monocytes [59,63], Macrophages [59], Dendritic cells [46], Natural killer cells [32]	ICAM-1 [64,65], ICAM-2 [63], ICAM-4 [66], VCAM-1 [63], Fibrinogen [60,62,65], Collagen [34], iC3b [34,59,65,67], Heparin [68], GPIbα [69], Thy-1 [70], Plasminogen [71]
α_D (CD11d)	β_2 (CD18)		Neutrophils [72,73], T-lymphocytes [27], Monocytes [72,73], Macrophages [72–74], Dendritic cells [73]	ICAM-3 [72], VCAM-1 [75,76], Fibrinogen [77], Vitronectin [77], Cyr61 [77], Plasminogen [77]

Table 2

Cytoplasmic tail binding proteins of integrins*

Tail of subunits	Activating	Inactivating	Signaling	Recycling
α	RapL [89–92]	SHARPIN [21], Nischarin [95,96], MDGI [97], Paxillin [98,99]	PP2A [93]	Rab21 [94], p120RasGAP [94]
β	Talin [100–104], Kindlin [100,109–111], Cytohesin [117,118]	Dok1 [105,106], Filamin [112–114], ICAP1 [119]	α-actinin [103,107], 14-3-3 [115], Arg [120], Src [122,123]	Numb [108], SNX17 [116], DAB2 [121]

*Not necessarily shown for β_2 integrins.