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Conformational Equilibrium of Human Platelet Integrin Investigated by Three-Dimensional Electron Cryo-Microscopy

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Abstract

Integrins are bidirectional transmembrane receptors that play central roles in hemostasis and arterial thrombosis. They have been subject to structural studies for many years, in particular using X-ray crystallography, nuclear magnetic resonance spectroscopy, and two-dimensional negative stain electron microscopy. Despite considerable progress, a full consensus on the molecular mechanism of integrin activation is still lacking. Three-dimensional reconstructions of full-length human platelet integrin $\alpha_{IIb}\beta_3$ in lipid-bilayer nanodiscs obtained by electron cryo-microscopy and single-particle reconstruction have shed new light on the activation process. These studies show that integrin $\alpha_{IIb}\beta_3$ exists in a continuous conformational equilibrium ranging from a compact nodular conformation similar to that obtained in crystal structures to a fully extended state with the leg domains separated. This equilibrium is shifted towards the extended conformation when extracellular ligands, cytosolic activators and lipid-bilayer nanodiscs are added. Addition of cytosolic activators and extracellular ligands in the absence of nanodiscs produces significantly less dramatic shifts, emphasizing the importance of the membrane bilayer in the activation process.

Keywords

Integrin; Electron cryo-microscopy; Image reconstruction; Single-spanning transmembrane receptors; Activation; Conformation; Three-dimensional structure; Talin head domain; RGD peptides; Membrane bilayer; Nanodiscs

12.1 Introduction

Integrins constitute the principal family of extracellular-matrix receptor that transmit bidirectional signals across the plasma membrane. Their binding to the extracellular matrix enables cells to respond to a wide variety of physical and chemical cues (Hynes and Naba 2012) that regulate many biological processes such as hemostasis, differentiation, migration, proliferation, and cell death. Integrins are heterodimeric transmembrane receptors composed of α - and β - subunits, each with a single transmembrane helix, an extracellular ligand-binding domain and a short cytoplasmic tail (Hynes 2002). Integrin receptors are expressed on the cell surface in either an ‘on’ or an ‘off’ state with respect to ligand binding. The equilibrium between the two states can be modulated by intra or extracellular cues (Campbell and Humphries 2011) The ‘on’ state is thought to be stabilized by extracellular ligand binding and binding to scaffolding proteins such as vinculin or talin, ultimately

linking integrins to the actin cytoskeleton and thereby mediating mechanotransduction (Sun et al. 2016).

The differences between the two affinity states are essential for regulating cell adhesion, particularly in the case of platelets. Platelets need to be able to circulate freely in a non-adherent state to avoid blood clots. Only stimulation by agonists at the sites of wounds turns on the fibrinogen binding function of $\alpha_{IIb}\beta_3$ integrins, which then leads to aggregation of platelets and the formation of a thrombus, halting the loss of blood. The transition between the 'on' and 'off' affinity states is referred to as activation (for reviews see Banno and Ginsberg 2008; Luo et al. 2007; Bouaouina et al. 2012; Ye et al. 2012; Collier 2015). Many of the current models of integrin's molecular activation mechanism have been primarily inferred from crystal structures of β_3 integrin extracellular domains (Xiong et al. 2001, 2002, 2009; Zhu et al. 2008, 2009, 2013; Dong et al. 2012) as well as two-dimensional negativestain electron microscopy (Luo et al. 2007; Nishida et al. 2006; Takagi et al. 2002; Xie et al. 2010; Ye et al. 2010; Zhu et al. 2008; Dai et al. 2015; Su et al. 2016; Eng et al. 2011).

12.2 Conformational States of Integrin Receptors

The crystal structures show the receptor in a 'bent' conformation, where the headpiece containing the ligand-binding site is pointed in the same direction as the cytoplasmic tail. The electron microscopy studies indicate that integrins can also adopt upright conformations, which are generally assumed to correspond to high-affinity states (Liddington 2014). Some of the two-dimensional projection images of upright integrins in negative stain show the α - and β -legs separated, other images show the legs close together even though the β -subunit legs tend to be poorly resolved in either case. In the crystal structures as well as three-dimensional reconstructions of negatively stained bent integrins (Choi et al. 2013; Adair et al. 2005), the legs are always close together.

The bent conformation is generally assumed to correspond to the low-affinity state, in part because the ligand-binding pocket is presumed to be close to the membrane, possibly blocking access to the binding site for extracellular ligands. However, three-dimensional negative-stain reconstructions of $\alpha_V\beta_3$ integrins in the absence of membrane show that they can bind relatively bulky fibronectin fragments in the bent conformation (Adair et al. 2005). It is not clear whether the membrane would prevent ligand binding for this integrin type, but a three-dimensional reconstruction of negatively stained $\alpha_{IIb}\beta_3$ integrins in a nanodisc membrane environment (Choi et al. 2013) shows a bent conformation with the ligand-binding site pointing away from the membrane.

12.3 Models for Integrin Activation

Attempts to reconcile the bent conformation with the existence of upright conformations have led to the idea that the transition between these two conformations could equate to a biochemical transition in ligand binding affinity. This line of thought suggests that the bent integrins represent the 'off' state, and that an upward switchblade-like movement of the integrin headpiece turns the integrins to an 'on' state. While this 'switchblade' model of

activation is widely discussed (Askari et al. 2009; Takagi et al. 2002; Kinashi 2006; Zhu et al. 2007), it is not the only hypothesis on activation. One alternative, the ‘deadbolt’ model (Xiong et al. 2003, 2009; Arnaout et al. 2005), proposes that ligand binding induces integrin extension and thus the bent state must already be capable of ligand binding. Others have proposed that activation is controlled by more subtle structural rearrangements involving receptor clustering (Bunch 2010), binding to cytoplasmic proteins (Moser et al. 2009), application of force (Li and Springer 2017) and even the rigidity of the extracellular matrix (Wei et al. 2008).

To transition into the upright conformation with separated legs from the bent crystal structure conformation, the hybrid domain, which connects the β -head with the β -leg, has to swing out. Indeed, different degrees of hybrid domain opening were observed within a crystal lattice, when ligand was soaked into crystals of integrin $\alpha_{IIb}\beta_3$ headpiece (Zhu et al. 2013). Talin binds to the cytoplasmic tails of β integrin subunits (Calderwood et al. 1999). Binding of talin domains to cytoplasmic fragments causes dissociation of integrin $\alpha_{IIb}\beta_3$ transmembrane helices (Wegener et al. 2007; Kim et al. 2009). Association of talin binding with the separation of the α - and β -legs was also shown in living cells for $\alpha_L\beta_2$ integrin (Kim et al. 2003). However, a direct dissociation effect of talin on the transmembrane helices has not conclusively been shown in the context of the full-length molecule.

Because negative staining approaches involve embedding in a heavy metal stain, absorbing on carbon film support, and dehydration, there is a danger of introducing artifacts into the system (Ye et al. 2010). In addition, the degeneracy of two-dimensional projection images does not allow to distinguish between upright integrins with closed legs and upright integrins with open legs in an orientation that makes the leg appear closed in the projection (Xu et al. 2016). The absence of various domains, especially in the crystal structures, is also a possible source of distortions and artifacts.

12.4 Electron Cryo-Microscopy of Full-Length Integrins in Nanodiscs

To overcome these shortcomings, we investigated full-length $\alpha_{IIb}\beta_3$ integrin in a fully hydrated environment using electron cryo-microscopy (Xu et al. 2016). To provide a membrane environment for the receptors, we utilized nanodiscs, which are nanometer-scale phospholipid bilayer membrane islands (Denisov et al. 2004; Xu et al. 2013; Ye et al. 2010; Choi et al. 2013). Nanodiscs match the properties of biological membranes more closely than liposomes (Shaw et al. 2004) and allow access to the receptor for both extracellular ligands and cytosolic binding partners. Sample preparation protocols were adjusted so that only single integrin molecules were incorporated into the nanodiscs to avoid clustering effects.

Reconstructions of platelet integrin $\alpha_{IIb}\beta_3$ were determined in the presence or absence of RGD peptide extracellular ligand, the talin head domain, and nanodiscs. Because the talin head domain was reported to activate integrin $\alpha_{IIb}\beta_3$ for RGD peptide binding, this represents a minimal system for reconstituting potential activation of un-clustered integrin. In all preparations, we found that integrin $\alpha_{IIb}\beta_3$ exists in a continuous conformational equilibrium centered around four main conformational states (Fig. 12.1). These four

conformations range from (i) a compact state similar to the bent conformer observed in crystal structures; through (ii, iii) two upright conformers with different degrees of hybrid domain opening and the lower legs close together; to (iv) an upright conformation with the lower legs clearly separated by 8 nm. Like in the three-dimensional negative stain reconstruction in the nanodisc (Choi et al. 2013), the ligand-binding site points away from the membrane and is accessible even to bulky ligands in the bent conformation.

The conformations of integrin were analyzed using unbiased iterative multi-reference single-particle reconstruction techniques (Spahn and Penczek 2009; Scheres 2012). The distribution of the four conformers in the population of integrin samples was compared by tallying the fraction of particles observed in each class of conformers. Integrin $\alpha_{\text{IIb}}\beta_3$ by itself is distributed with 12.9% in the bent conformation, 21.1% in the first intermediate, 37.5% in the second intermediate, and 28.5% in the fully upright state. Addition of the RGD peptide ligand or the talin head domain, had a relatively small effect on the distribution of conformers (Fig. 12.2). However, addition of the nanodisc led to a significant shift in the population toward the upright conformation (43.3%).

12.5 Are Integrin Conformations Tightly Linked to Specific Activation State?

These findings indicate that the activation state of the integrin may not be correlated strongly with any particular global conformation of integrin. Many other lines of evidence support the idea that extension of the integrin to an upright stance is not identical to activation of the ligand binding function. Studies on the hydrodynamics of $\alpha_{\text{IIb}}\beta_3$ in the resting state show that it resembles an extended structure rather than a bent conformation (Rocco et al. 2008). Electron cryotomographic studies of $\alpha_{\text{IIb}}\beta_3$ in liposomes fail to detect changes in height or orientation with respect to the membrane in response to activating agents such as Mn^{2+} (Ye et al. 2008) or talin head domain (Ye et al. 2010). Studies on the $\alpha_{\text{V}}\beta_3$ integrin, which shares the same β subunit with $\alpha_{\text{IIb}}\beta_3$, show that it can bind an RGD peptide while in the bent conformation (Xiong et al. 2001) and that it remains bent while in complex with a macromolecular ligand (a fragment of fibronectin) (Adair et al. 2005). Results on other integrins are similar. Studies in which FRET was used to study conformational shifts upon activation of integrin $\alpha_4\beta_1$ (Chigaev et al. 2003) and $\alpha_{\text{IIb}}\beta_3$ (Coutinho et al. 2007) on the cell surface argue against a mechanistic connection between extension and activation. This conclusion is also supported by studies in which the conformation of $\alpha_4\beta_1$ was probed with antibodies (Chigaev et al. 2009). Finally, rotary shadowed images of constitutively inactive integrin $\alpha_5\beta_1$ reveal extended conformers (Takagi et al. 2003), a finding that also dissociates the connection between activation and extension.

The signal for integrin activation has to be transmitted through the membrane using the transmembrane helices in one way or another. It is well established that talin can disrupt the salt-bridge that holds the transmembrane helices together and simultaneously reorients the transmembrane helix of the β subunit in respect to the membrane (Anthis et al. 2009). Our data indicates that the membrane insertion of the full-length integrin shifts the equilibrium towards the conformation with separated legs with much higher probability than talin alone.

Isolated transmembrane helices and the heterodimeric complex of the helices have essentially the same configuration in respect to the membrane and there is apparently no reorientation induced by separation (Lau et al. 2009). Also, the relatively slow transmembrane helix dissociation does not interfere with the much faster activation and there is a fair amount of separated transmembrane helices in membrane environments even if the experimental conditions were tweaked to favor heterodimeric transmembrane helices (Lau et al. 2009).

12.6 Conclusions

Together, these findings raise the possibility that integrin leg separation is primarily driven by the membrane insertion while talin's main role would be to reorient the transmembrane helix of the β subunit in the membrane. In this scenario, the reorientation of the transmembrane helix triggers the signal transmission to the ligand-binding site that leads to activation of the ligand-binding capability, not the leg separation (Fig. 12.3). In this model, neither full leg separation nor extension is necessary for activation. All that would be required is breaking the salt bridge that locks the transmembrane helices and a subsequent reorientation of the β subunit helix in the membrane. Because this can be achieved without completely separating the legs and extending the extracellular domain, it would allow activation of non-extended integrin conformations as well as extended conformations with closed legs.

Switching between signaling modes should carry a reasonable energy expense. The energy expense for separating the legs and to go from bent to extended conformation would be much higher than merely breaking the salt bridge and reorienting the transmembrane helix. The talin F2F3 head fragment is a much stronger activator than talin F3 domain alone. Within the framework of the model, this is consistent with the F2 domain being the main factor responsible for reorienting the transmembrane helix (Anthis et al. 2009). The model also explains why other molecules that do not affect the salt-bridge (such as kindlin) can activate integrin. They would induce a reorientation in the transmembrane helix rather than triggering leg separation.

As a consequence of recent hardware and software developments, electron cryo-microscopy has been able to reach near-atomic resolution more frequently than ever before (reviewed in Subramaniam et al. 2016; Fernandez-Leiro and Scheres 2016), including for low-molecular weight assemblies embedded in nanodiscs (Liang et al. 2017). Furthermore, detailed analyses of cellular systems using electron cryo-tomography are becoming more and more feasible (reviewed in Oikonomou and Jensen 2017; Beck and Baumeister 2016). With these technologies in hand, high-resolution electron cryo-microscopy studies of integrin receptors in nanodiscs and detailed analyses of integrins in their cellular context should become possible in the near future.

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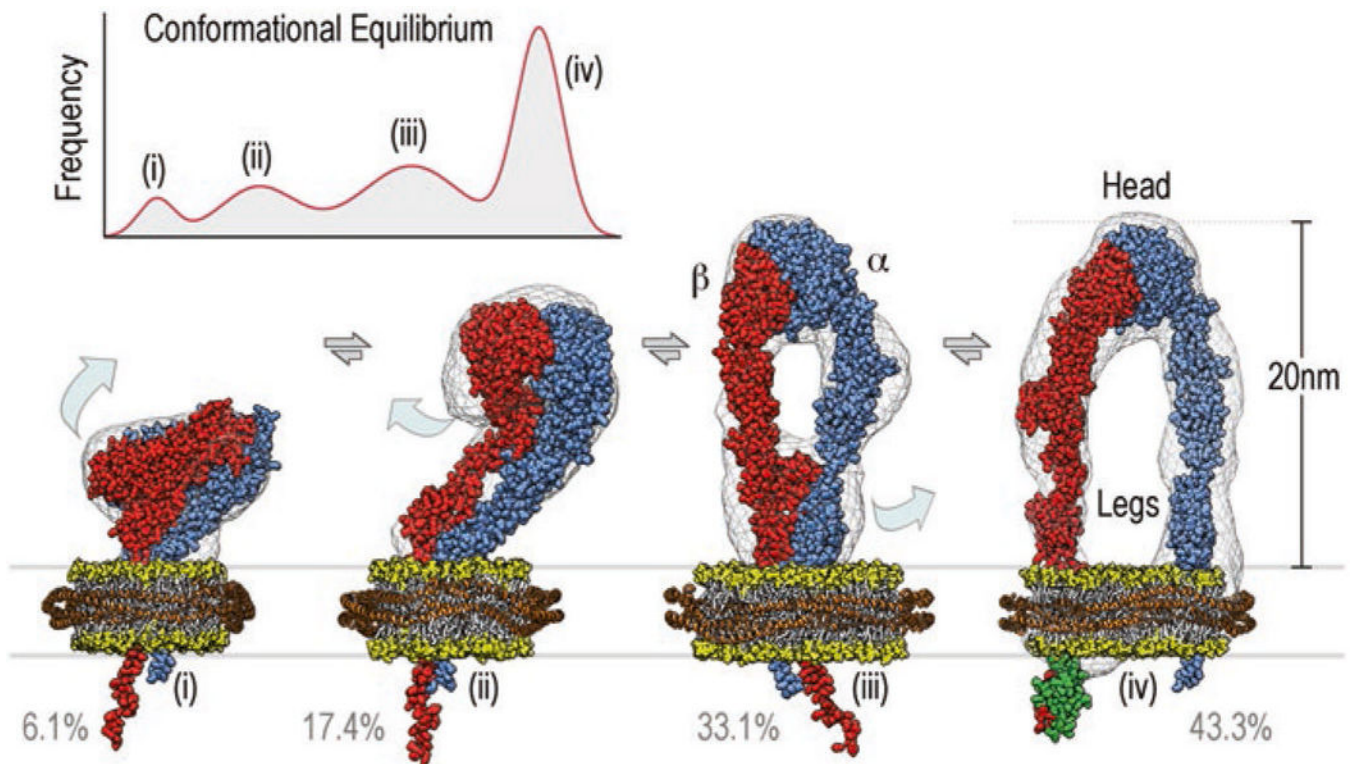


Fig. 12.1.

Integrin $\alpha_{IIb}\beta_3$ exists in a continuous conformational equilibrium centered around four main conformational states. The height of the peaks is given by the percentage of particles assigned to a given conformation, the width was estimated from the structural variability within each group. The equilibrium in the presence of the extracellular ligand RGD, cytosolic binding partner talin head, and lipid bilayers is depicted in the top left corner and centers around the four conformations shown in the lower part of the figure. Space-filling atomic models of $\alpha_{IIb}\beta_3$ integrin (α -subunit in blue, β -subunit in red) embedded in nanodiscs (lipid head groups in yellow, belt protein in orange) are shown fitted into their respective three-dimensional reconstructions (grey wire representation). Required structural transitions are depicted as light blue arrows. In the three-dimensional reconstruction of the upright state (*iv*) density for bound talin head (F3 domain shown in green) was evident

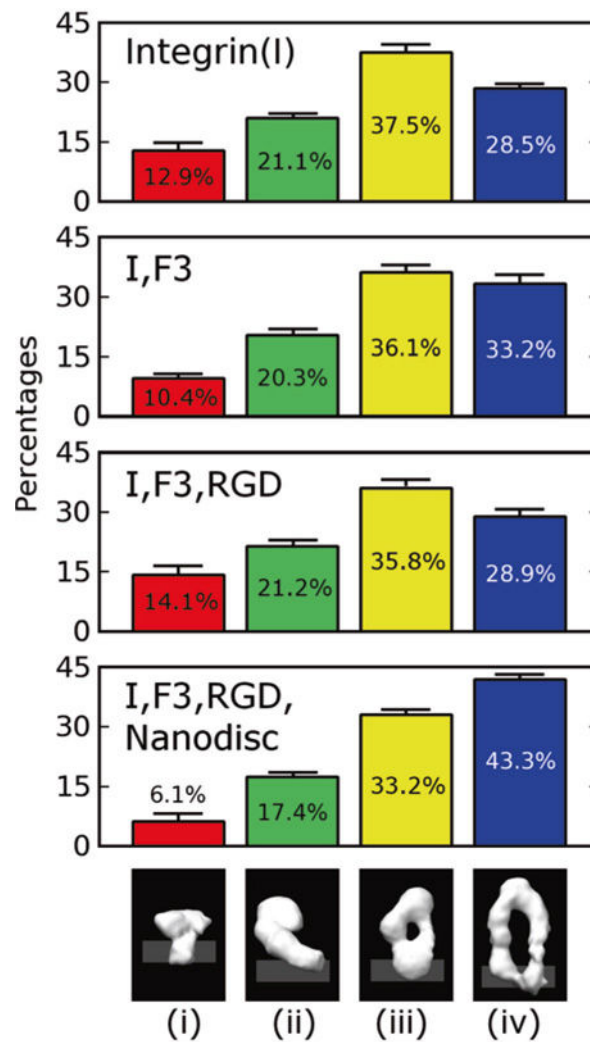


Fig. 12.2. Distributions of integrin $\alpha_{IIb}\beta_3$ conformers in the presence and absence of extracellular ligands (RGD), cytosolic binding partners (talins head domain F3) and lipid bilayer nanodiscs. Between two and four independent data sets were acquired for each condition. The standard deviation for the percentages was below 2 percentage points for all measurements (see error bars). The presence of talin head domain shifts the equilibrium slightly by 4.7% towards the upright conformation with separated legs. The trend is somewhat reversed if RGD is added as well. When nanodiscs are added, there is a major shift of 14.8% towards the upright (*iv*) conformation

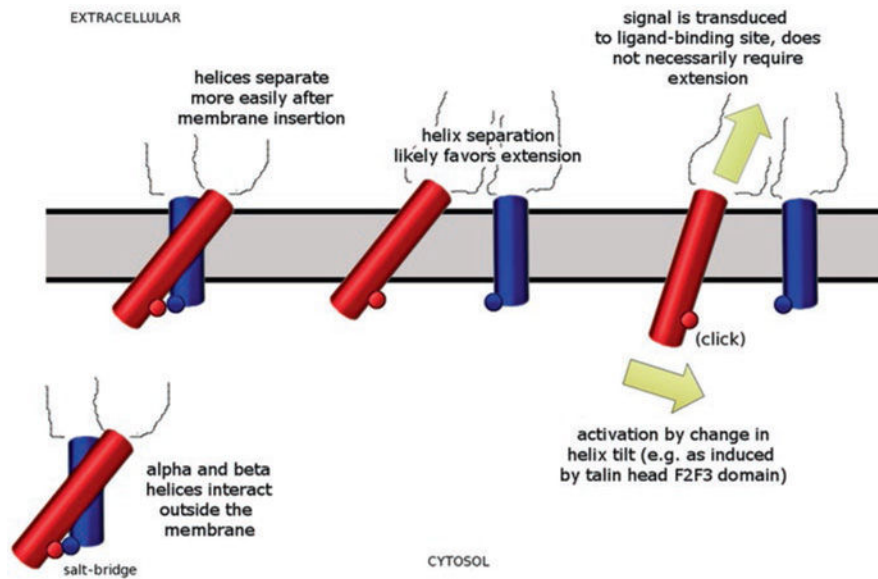


Fig. 12.3.

Possible involvement of helix β -subunit transmembrane helix reorientation in integrin activation. Outside the membrane, the two transmembrane helices (β -subunit red, α -subunit blue) are strongly associated and linked by a salt bridge (lower left corner). Inside the membrane the association is weakened (top left) and the helices can separate (top center) more easily. The separation likely favors extension of the molecule from the bent conformation but does not require or deterministically induce it. Activation could be achieved primarily by a change in helix tilt of the β -subunit transmembrane helix (red) similar to that induced by binding of talin head F2F3 domain. This reorientation triggers the signal transduction to the ligand-binding site (yellow arrows). This action would resemble more a 'light switch' rather than 'switchblade' or a 'deadbolt' mechanism. It would require significantly less energy and could prime the ligand-binding site for binding in all conformational states, including the bent conformation