JULS REVIEWS

Integrin $\alpha_{IIb}\beta_3$ in Cardiovascular Thrombotic Events

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Abstract

Integrin signalling mediates several intercellular events in order to maintain hemostasis, including platelet adhesion and aggregation during the process of coagulation. Evolutionary pressures have directed the mechanisms which underpin coagulation to develop in a highly-regulated way, as both hypo- and hyper-coagulable states are incompatible with life. This review focuses on our current understanding of the "inside-out" and "outside-in" signalling events that mediate integrin activation and suppression in order to allow for appropriate aggregation of platelets following vascular trauma. In addition, it characterizes the most abundant integrin receptor, $\alpha_{IIb}\beta_3$, and the important biological roles this receptor plays in the context of cardiovascular thrombotic events.

Hemostatic Mechanisms

The process of hemostasis is carefully regulated by a variety of factors in the blood which become active following vascular trauma1. Exposed extracellular matrix molecules beneath vessel walls, in addition to factors released directly from the damaged endothelial cells, initiate a coagulation cascade that results in the activation of the protease thrombin. Activated thrombin acts enzymatically on circulating fibrinogen to produce polymerized fibrin [2, 3]. This fibrous protein forms crosslinks by transglutaminase factor XIII to provide a scaffold for blood clotting [4, 5]. Simultaneously, circulating platelets produced from megakaryocytes in bone marrow become chemically activated through exposure to markers of tissue damage not encountered in healthy vessels, e.g. collagen proteins, and both adhere to the site of damage and aggregate with each other [6, 7]. The interaction of activated platelets and fibrin initiates a further cascade of signalling events within platelets, encouraging further hemostatic function, and a blood clot or thrombus is formed to repair the damaged vessel wall [8, 9].

While platelet adhesion and aggregation are essential for hemostatic damage repair, excessive aggregation is considered pathological when a 'mural' thrombus binds to a vessel and decreases blood flow or an 'occlusive' thrombus entirely occludes a vessel [10, 11]. A thrombus can remain wedged in the vessel in which it formed, or it can detach from the vessel wall to become a free-flowing embolus. Emboli travel within the blood stream until they lodge within narrower vessels where they can cause ischemia, similar to mural or occlusive thrombi [12]. This can result in tissue damage and can lead to serious cardiovascular complications such as myocardial infarction or stroke [13, 14].

Platelet adhesion and aggregation events are mediated by integrins, heterodimeric adhesion receptors composed of one alpha (α) and one beta (β) subunit which each have unique cellular roles [15]. Platelets first form weak interactions with a damaged site via glycoprotein surface receptors, and then integrin $\alpha_{II}\beta_1$ binds collagen (largely type I and type III collagen) and other molecules in the vessel wall to mediate more robust adhesion [6]. Next, integrin $\alpha_{IIb}\beta_3$ makes connections with fibrinogen to initiate platelet aggregation [2]. Integrins are therefore promising targets for the pharmacological regulation of coagulation in clinical circumstances where it may be beneficial to up- or down-regulate platelet adhesion. This is particularly relevant in the context of patients suffering from trauma requiring massive blood transfusion, or those in genetic or iatrogenic hypercoagulable states.

The process of hemostasis must be tightly-controlled to allow for blood clotting in response to tissue damage while simultaneously preventing thrombus formation in healthy vessels. Integrin activity is therefore carefully regulated by a series of complex signalling events. Integrins act as bi-directional signalling molecules, first becoming activated by "inside-out" signalling events where intracellular signalling cascades initiated by other cellular receptors evoke changes in integrin structure, and then mediating "outside-in" signalling where the binding of extracellular matrix factors to activated integrin receptors stimulates intracellular signal transduction pathways that can regulate intracellular activity [16, 17]. Integrin receptor binding partners include a wide variety of both cytosolic and membrane-bound proteins with several structural and cytoskeletal roles [18]. $\alpha_{IIb}\beta_3$ is abundant in the platelet membrane and crucial in the aggregation events that govern both vascular damage repair and thrombus formation [19]. For this reason, an understanding of the precise activities of $\alpha_{IIb}\beta_3$ in activated platelets is critical in the study and treatment of cardiovascular thrombotic events.

Platelet Activation

Vascular injury exposures molecules in the subendothelium, such as von Willebrand factor (vWF) and collagen fibres, allowing platelets travelling in the bloodstream to bind to these proteins with membrane surface receptors GPIb and GPVI, respectively [10]. In addition to these two binding events, platelet adhesion is also regulated by the collagen-binding integrin $\alpha_{II}\beta_I$ [6, 20]. These adhesion events initiate platelet activation, during which intracellular molecular switches including small GTPases, regulate a variety of cellular activities [2]. Specifically, the GTP-binding proteins RhoA and Rac1 regulate cytoskeletal reorganization leading to platelet shape change, Rab27 and Ral control the secretion of platelet granules, and Rap1B and RhoA regulate platelet aggregation [2, 21]. Atomic force microscopy has allowed for detailed illustration of these morphological changes accompanying platelet activation, primarily characterized by the surrogate measure of filopodial growth which represents remodelling of the actin cytoskeleton [3, 22].

Adherent platelets undergo a process of exocytosis wherein dense intracellular granules merge with the cell membrane in order to expose transmembrane P-Selectin proteins to the extracellular space and release several cell products, including adenosine disphosphate (ADP), α-granules containing factor V, fibrinogen, vWF, thrombospondin, and α_2-antiplasmin, into the extracellular matrix [2, 4, 23]. These soluble factors, in concert with thrombin and thromboxane A2 produced by activated platelets, act through their receptors to increase platelet activity through a positive feedback loop and initiate an intracellular signalling cascade leading to the activation of integrin $\alpha_{IIb}\beta_3$ [24]. The precise characterization of activated platelets has traditionally been ambiguous, but attempts have been made to divide them into more well-defined subpopulations. Recently, a novel transglutaminase peptide substrate was used to identify and characterize such a subpopulation of transglutaminase-active platelets [4, 25]. While the precise roles of each subpopulation of platelets have yet to be elucidated, activated platelets as a whole are credited with integrin $\alpha_{IIb}\beta_3$-mediated aggregation in vascular repair and thrombosis. Future work in this area can be expected to elucidate specific hemostatic activities that subpopulations of platelets are responsible for, creating novel treatments of coagulopathic diseases.

Integrin αIIbβ3

The integrin $\alpha_{IIb}\beta_3$ is a heterodimer composed of transmembrane subunits α_{IIb} and β_3 subunit, which each traverse the cell membrane once. It is the most abundant receptor on the platelet surface at 50,000-80,000 copies per cell19,24. The literature has been controversial on the structure of $\alpha_{IIb}\beta_3$ in its inactive state, but many discussions of structure focus on a "switchblade hypothesis" with three core tenants: the inactive receptor is bent and extends with activation, the head region points towards to platelet membrane, and extension is achieved when the α_{IIb} and β_3 subunits' cytosolic domains separate and destabilize the interfaces of the extracellular domains in order to expose a ligand binding site [19, 26]. Recent three-dimensional reconstructions of $\alpha_{IIb}\beta_3$ using synthetic nanodisc lipid bilayers have refined this model through observation of the head region of inactive $\alpha_{IIb}\beta_3$ pointing outwards from the platelet surface [27]. The cytosolic legs of α_{IIb} are bent, while those of β_3 are freely coiled [27].

$\alpha_{IIb}\beta_3$ has high affinity for fibrinogen, as well as Arg-Gly-Asp (RGD) peptide sequences [3]. Fibrinogen has binding sites for $\alpha_{IIb}\beta_3$ in both its α and β chains, and so it is expected to link aggregating platelets via their $\alpha_{IIb}\beta_3$ integrins, which can each bind only one fibrinogen molecule [24]. Giant Unilamellar Vesicles (GUVs) are cell-sized lipid vesicles that are a useful model for the study of reconstituted integrin because, unlike comparable methods, they do not leave a space between the substrate and the lower bilayer leaflet that could impact protein diffusion rates. GUVs were used to determine that the diffusion of active $\alpha_{IIb}\beta_3$ bound by fibrinogen is slowed [24]. Based on the magnitude of diffusion diminution, active $\alpha_{IIb}\beta_3$ likely undergoes intracellular clustering induced by ligand binding24. This report of $\alpha_{IIb}\beta_3$ clustering is supported by new studies exploring integrin activity using immunohistochemical staining techniques [17, 28]. The aggregation of platelets in the blood stream in the absence of injury would threaten hemostasis, and for that reason integrins exist in a low-affinity basal state and shift into a high-affinity active state (called integrin activation, priming, or "inside-out" signalling) when contact with a wounded vessel is made [10, 17].

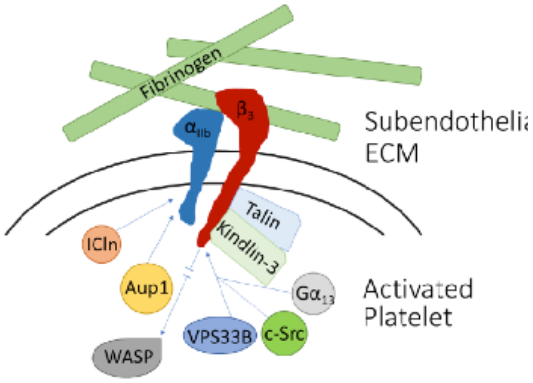

Figure 1. Summary of αIIbβ3 interactions. A representation of the binding interactions between platelet integrin αIIbβ3 and fibrinogen, ICln, Aup1, WASP, VPS33B, c-Src, Gα13, Talin, and Kindlin-3. ICln, Aup1, Talin, and Kindlin-3 have demonstrable roles in the inside-out signalling activation of αIIbβ3. Talin, Kindlin-3, Gα13, c-Src, and VPS33B have demonstrable roles in outside-in signalling initiated by fibrinogen binding to αIIbβ3. Together, this complicated set of interactions contributes to the ability of integrin αIIbβ3 in mediating the activation of platelets and their subsequent adhesion to sites of vascular tissue damage in the endogenous coagulation response.

Integrin αIIbβ3 Inside-Out Signalling

Integrin $\alpha_{IIb}\beta_3$ activation, via inside-out signalling, begins with the actions of molecules like thrombin, ADP, collagen, and thromboxane A_2 on their platelet surface receptors [6, 24]. These agonists increase intracellular levels of cytosolic calcium, activate kinases like PKC and PI3K, and result in the direct engagement of molecules with the cytosolic tail sequences of $\alpha_{IIb}\beta_3$ [24]. A vast array of intermediate signalling molecules exist within these signalling cascades, several of which are shown in Figure 1. Partial deletions of the αIIb cytoplasmic tail, mutations in a conserved N-terminal sequence (KVGFFKR) of this subunit, or mutations in the cytoplasmic tail of β_3 enhance the affinity of $\alpha_{IIb}\beta_3$ for its ligands [29]. Thus, these membrane proximal regions seem to have a negative regulatory function, locking $\alpha_{IIb}\beta_3$ in its low-affinity basal state. Transmembrane helix-helix interactions are critical in maintaining $\alpha_{IIb}\beta_3$ in an inactive state, and resting αIIbβ3 is poised to undergo conformational changes to expose its ligand-binding site [28]. In fact, synthetic peptides that bind this transmembrane domain and

interrupt these interactions have comparable effects to thrombin treatment [28, 30].

Inside-out signalling reaches its conclusion in a similar fashion when molecules bind the integrin's β subunit cytoplasmic tail, disrupting the helical interactions of inactive $\alpha_{IIb}\beta_3$. One such molecule is Talin, which has a head domain containing many integrin binding sites and a tail domain with binding sites for a host of other molecules such as vinculin and F-actin [6, 10]. Talin binds the integrin subunit β_3 between residues 722 and 738 [31]. Talin-null megakaryocytes were demonstrated to produce platelets with normal morphology, but impaired hemostatic function [6]. Another molecule that binds and activates the integrin β_3 subunit cytoplasmic tail, though at a site distinct (slightly more membrane-distal) from Talin, is Kindlin-3 [10]. The Kindlin family has three members, which all localize to integrin adhesion sites [10]. Kindlin-3 homozygous null mice had no change in the number of platelets, but developed hemorrhages within one week from birth [10]. In addition to their role as the final regulatory molecules of $\alpha_{IIb}\beta_3$ inside-out signalling, Talin and Kindlin-3 are also players in the subsequent outside-in integrin signalling events [10, 32].

A membrane-proximal KVGFFKR sequence on the α_{IIb} subunit is also involved in the regulation of integrin $\alpha_{IIb}\beta_3$ activity [18]. ICln, a chloride channel regulatory protein, is highly expressed in platelets and has been shown to bind α_{IIb} at this sequence and inhibit its activity [18]. Similarly, the binding of Ancient Ubiquitous Protein 1 (Aup1) to this sequence in α_{IIb} negatively modulates $\alpha_{IIb}\beta_3$ signalling in platelets [29]. Previous reports suggest that as much as 40% of α_{IIb} in cultured megakaryocytes is complexed with Aup1 [29]. Thus, numerous binding sites within the cytoplasmic domains of both α_{IIb} and β_3 are implicated in the regulation and activation of $\alpha_{IIb}\beta_3$ via inside-out signalling.

Integrin $\alpha_{IIb}\beta_3$ Outside-In Signalling

The binding of active $\alpha_{IIb}\beta_3$ to fibrinogen leads to platelet shape change, aggregation, and release of α-granules, mediated by calcium mobilization, an increase in cytosolic pH, generation of thromboxane A_2, and the tyrosine phosphorylation of many intracellular proteins like Focal Adhesion Kinase (FAK) and Src family members which complex with the actin cytoskeleton and are recruited to focal contacts [29, 33]. It is possible that initial binding of fibrinogen to activated $\alpha_{IIb}\beta_3$ is followed by secondary binding of the growing fibrin fiber to other platelet surface proteins by means of transglutaminase activity [4]. Integrin-mediated cytoskeletal reorganization causes platelet cell spreading, stabilization of cell adhesions and aggregation, secretion, and clot retraction [31]. The aggregatory effects of activated $\alpha_{IIb}\beta_3$ are due to its affinity for multivalent fibrinogen, but its many intracellular signalling outputs are mediated by a wide variety of signalling molecules (Figure 1) [34].

c-Src binds the β_3 subunit of active integrins via an SH3 domain, and itself becomes active [35]. c-Src binding appears to involve an "unlatching" of its own structure via the dephosphorylation of pTyr530, enabling Tyr419 autophosphorylation and c-Src activation [35]. The signal transduction of c-Src culminates in cytoskeletal reorganization and platelet spreading via a clustering of the $\alpha_{IIb}\beta_3$ integrin receptors [28]. Clustering is an essential step because it brings c-Src molecules together, required for Tyr419 autophosphorylation [28].

The protein WASP is mobilized in $\alpha_{IIb}\beta_3$ outside-in signalling, and localizes to the membrane skeleton of platelets [36]. WASP is a scaffolding protein that integrates cellular activation and cytoskeletal rearrangements by binding actin and actin-related protein complex 2/3 (Arp2/3) in order to bring about the polymerization and cross-linking of the actin cytoskeleton [36]. WASP knockout mice have no change in platelet size, integrin quantity, or fibrinogen binding, but fewer platelets and a marked reduction in the spreading of platelets on immobilized fibrinogen [36]. While WASP impairs the retraction of fibrin clots and the stabilization of the primary platelet plug, it does not effect inside-out signalling [36].

Ligand binding to $\alpha_{IIb}\beta_3$ also promotes the binding of the heterotrimeric G protein $G\alpha_{13}$ to integrin subunit β337. Ordinarily, $G\alpha_{13}$ is activated by G Protein-Coupled Receptors (GPCRs) and then employs RhoGEF to activate RhoA and cause morphological changes [37,38]. In contrast, the interaction of Gα13 with $\alpha_{IIb}\beta_3$appears to inhibit RhoA, and so $\alpha_{IIb}\beta_3$ has been established as a non-canonical $G\alpha_{13}$-coupled receptor that dynamically regulates RhoA [37]. This was demonstrated through the interference of $G\alpha_{13}$'s interaction with $\alpha_{IIb}\beta_3$ in mice, resulting in diminished c-Src activity and a stimulation of RhoA [37].

VPS33B has been recently identified as a binding partner of β_3 integrin using receptor pulldown methods [31]. VPS33B is a member of the Sec1/Munc18 (SM) family with a well-characterized involvement in granule biogenesis [31]. VPS33B binds integrin β_3 between residues 716 and 730, which overlaps the Talin binding site [31]. VPS33B knockout mice have normal platelet morphology but reduced platelet activation, longer bleeding times, and impaired fibrinogen spreading and clot retraction [31]. The latter two phenotypes have been explained by VPS33B's actions upstream of the RhoA-ROCK-MLC and Rac1 dependant pathways that lead to clot retraction and cell spreading, respectively [31]. Given the phenotypic associations of VPS33B alterations and the proximity of its binding site in relation to that of Talin, a promising future direction for research in the mechanisms of coagulation will be investigating the possibility of VPS33B binding to the integrin subunit β_3 preceding and if this can potentiate the binding of Kindlin-3 and Talin.

In summary, a wide variety of molecules interact with the β_3 subunit of $\alpha_{IIb}\beta_3$ in order to elicit diverse downstream effects. Outside-in signalling has an involvement in the spreading of activated platelets, the stability of adhesions (both between platelets and to the subendothelium), granule secretion, and clot retraction. The regulation of each of these events is critical to proper hemostatic function, and each step can potentially be error-prone leading to thrombotic events. The complexity of this pathway also illustrates several unique and potentially actionable targets for medical intervention.

Clinical Perspective

Integrin $\alpha_{IIb}\beta_3$ plays a crucial role in the aggregation of platelets during the healing response of damaged vessels. However, dysregulated aggregation can lead to the formation of a pathological thrombus and leading to further cardiovascular diseases [38]. Integrin αIIbβ3 and its numerous regulatory factors are therefore potential therapeutic targets in the treatment of thrombosis or embolisms, in order to prevent further vascular complications. However, over-inhibition of the mechanisms of hemostasis also poses a variety of risks, like intracranial or gastrointestinal bleeding [39]. An obstacle therefore implied in pharmacological targeting of

these molecules is striking the proper balance of $\alpha_{IIb}\beta_3$ activity so as to halt pathological thrombotic events without increasing patient morbidity and mortality related to iatrogenic hypo-coagulation. Several levels of the complex signalling pathway of integrin $\alpha_{IIb}\beta_3$ may hold promising therapeutic targets in the context of clot prevention and dissolution, and future work is expected to explore potential the clinical benefits of antagonizing $\alpha_{IIb}\beta_3$ itself or disrupting its interactions with molecules like fibrin and Kindlin-340-42.

Conclusion

The integrin $\alpha_{IIb}\beta_3$ plays a crucial role in the aggregation of activated platelets during the vascular repair process as well as in thrombosis. $\alpha_{IIb}\beta_3$, like other integrins, is activated through inside-out signalling initiated through binding of platelet agonists during the adhesion of platelets to the subendothelium at sites of vascular damage, as well as the binding of various molecules to the cytosolic α_{IIb} and β_3 subunit sequences which regulates $\alpha_{IIb}\beta_3$'s propensity to become active by modulating the integrin receptor's conformation. Following activation, $\alpha_{IIb}\beta_3$ binds to multivalent fibrinogen resulting in the aggregation of activated platelets; simultaneously, $\alpha_{IIb}\beta_3$ performs outside-in signalling by binding a diverse suite of molecules, and perhaps many more still undiscovered, at the cytosolic face of the membrane.

Some questions remain unanswered regarding $\alpha_{IIb}\beta_3$ signalling, such as the precise mechanism by which receptor clustering is mediated, and how Talin and Kindlin-3, two major players in integrin signalling, might interact with each other to mediate $\alpha_{IIb}\beta_3$ activation. These molecules and their downstream binding partners are known to cause changes in cell conformation via actin cytoskeletal reorganization, as well as granule secretion, further aggregation, and clot retraction. The pathways mediating these processes are complex, and future studies are anticipated to offer a deeper understanding of their control over, and relevance to, the process of hemostasis. The proper regulation of platelet integrin $\alpha_{IIb}\beta_3$ and its many partners is critical to cardiovascular health, and understanding the full mechanisms underpinning the integrin's activity in this domain promises to be an important area of research.

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