

Theme Issue Article

Talin-dependent integrin signalling *in vivo*

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Summary

Integrins are heterodimeric adhesion receptors essential for metazoan life. In addition to mediating cell-extracellular matrix and cell-cell interactions, integrins are bona fide signalling receptors in that they transmit information in both directions across the plasma membrane. The affinity of integrins for extracellular ligands is regulated through a process termed integrin activation or "inside-out signalling". On the other hand, ligand binding to integrins can induce the recruitment and activation of a number of enzymes and adaptors such as pp125^{FAK} and Src family kinases, to initiate "outside-in signalling". Intensive investigation into the mechanisms of integrin signalling has revealed many of the key players; amongst these, one of the most important is talin. Our understanding of how many of these molecules interact is now understood at the atomic level thanks to detailed structural

studies. Indeed structural information and model cell systems have provided unique opportunities to dissect the molecular mechanisms of many aspects of integrin signalling. Recent studies have begun testing the biological significance of these mechanisms using in-vivo models, particular genetically modified mice. The generation and characterisation of in-vivo models to study integrin signalling has provided valuable information into the functional significance of integrin signalling in fundamental physiological processes as well as within the context of human disease. Here, I will review recent insights that have been gained into integrin signalling through the use of genetically modified mice focusing on integrin α IIb β 3 (GPIIb-IIIa) and the regulation of its function in haemostasis and thrombosis.

Keywords

Adhesion receptors, integrins, thrombosis

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The integrin family of adhesion receptors

Integrins are adhesion receptors that play essential roles in all multicellular organisms by mediating cell-extracellular matrix interactions and cell-cell interactions (1). Integrins are heterodimers comprised of an α and a β subunit each containing a large extracellular domain (>700 amino acids), a single transmembrane domain, and typically a short (13–70 amino acids) cytoplasmic domain (tail) (1). In mammals, 18 α and eight β subunits combine to form at least 24 integrin receptors, many of which exhibit considerable differences in ligand specificity and tissue expression (1, 2). The biological significance of integrins has been demonstrated by genetic deletion of individual integrin subunits in mice that results in a spectrum of developmental and physiological defects (3, 4).

The importance of integrins is particularly appreciated in the field of haemostasis and thrombosis where several integrins play

important, well-defined roles in platelet function. Platelet aggregation occurs through the binding of soluble ligands such as fibrinogen, von Willebrand factor, and fibronectin which depend on integrin α IIb β 3 (GPIIb-IIIa) (5). Defects in either α IIb or β 3 integrins cause the severe bleeding disorder Glanzmann's thrombasthenia, highlighting the importance of α IIb β 3 function in haemostasis (6). Furthermore, this essential role of integrin α IIb β 3 in platelet aggregation has been targeted therapeutically in the form of several α IIb β 3 antagonists that are used very effectively to prevent thrombosis during acute vascular procedures such as angioplasty (7, 8).

In-vitro studies have provided important information about integrin structure and function. However, it has become increasingly clear that a better understanding of integrin biology requires study in living animals. For example, Lammermann et al. found that dendritic cells do not require integrins in order to migrate through the interstitium into lymph nodes of mice (9). Thus,

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studies in the intact organism are essential to establishing the biological functions of integrins. In addition, assessing the role of integrins in complex physiological and pathological processes necessitates *in-vivo* models. Here I will review some of the recent progress in understanding how integrin affinity is regulated with a particular focus on studies utilising *in-vivo* approaches to study the role of talin in integrin activation.

Integrin affinity modulation

The affinity of many integrins for ligand is regulated through intracellular signalling mechanisms. For example, integrins on the surface of circulating platelets and leukocytes are predominately in a low-affinity state. In response to extracellular cues that initiate intracellular signalling, integrins undergo a conformational change that underlies a significant increase in ligand affinity (10, 11). This tight regulatory control over integrin affinity insures that rapid adhesion and aggregation of circulating platelets in the event of vascular injury and adhesion and transmigration of leukocytes in the event of an acute inflammatory stimulus. Furthermore, tight regulation of integrin activity prevents adhesion of platelets and leukocytes under normal circumstances thereby avoiding thrombosis and chronic inflammation. The molecular mechanisms that link extracellular stimuli to the shift in integrin affinity have been intensively studied and many of the key molecules involved have been identified (reviewed in [11, 12]).

The binding of the cytoskeletal protein talin to the integrin β subunit is a key final step in activating integrins (13). Talin consists of a 47 kD globular head which contains a FERM domain and a 190 kD flexible rod region (14, 15). Studies using Chinese hamster ovary (CHO) cells engineered to express integrin α IIb β 3 showed that expression of the talin head region or FERM domain is sufficient to activate integrins and this activation required the binding of talin to the β integrin subunit (13, 16). Furthermore, shRNA knock-down of talin in embryonic cell-derived megakaryocytes markedly impaired agonist-induced integrin activation (13). In addition, point mutations within either the β integrin tail or in the talin FERM domain that disrupt integrin-talin interactions also abolish talin-mediated integrin activation (13, 17). Together these *in-vitro* studies showed that talin binding to the β integrin cytoplasmic domain was both sufficient and necessary for integrin activation.

How does binding of talin to the β integrin cytoplasmic domain activate integrins? Recent structural studies have provided insights into this mechanism by identifying key interactions between the talin FERM domain and the integrin tail that are important for talin binding to and activating integrins. The F3 subdomain of talin resembles a phosphotyrosine-binding domain and interacts with an NPxY motif in a membrane distal portion of the integrin tail, an interaction critical for integrin activation (18). In addition, Ulmer et al. reported that the talin F3 subdomain also interacts with a membrane proximal region of the β integrin subunit (19). The functional significance of this membrane proximal interaction with respect to talin-dependent integrin activation was demonstrated in experiments in which mutations in either talin or β integrin that disrupted this membrane proximal interaction also abolished talin-mediated integrin activation (20). These results suggested a model in which talin acti-

vates integrins in a two-step manner. Talin binds to the β integrin membrane distal region subsequently facilitating the relatively lower affinity membrane proximal interaction (20, 21). This talin-membrane proximal integrin interaction might then separate the integrin α/β tails and activate the integrin (20).

Regulation of talin-integrin interactions

The intracellular signalling events that link extracellular stimuli to talin-dependent integrin activation have begun to be defined and this topic has been recently reviewed (12). Han et al. ordered the sequence of molecular events that regulates α IIb β 3 integrin activation using a CHO cell model system (22). Their results suggest a model whereby PKC or a Rap guanine nucleotide exchanger (such as CalDAG-GEFI [23]) activate the GTPase Rap1 and promotes the assembly of an "activation complex" including RIAM (Rap1 interacting adaptor molecule) and talin. This model is in line with the defects reported in α IIb β 3 integrin activation in platelets from Rap1b and CalDAG-GEFI knock-out mice (24, 25). It appears that Rap1 activity leads to RIAM-dependent recruitment of talin to the membrane and thereby promotes talin-dependent integrin activation (22, 26, 27). How talin activation and recruitment are regulated and the ordering of the sequence of these events will provide a better understanding of integrin activation and may identify new therapeutic targets for thrombotic and inflammatory diseases.

Additional mechanisms regulating integrin activation

In addition to talin, several proteins have been identified that bind to β integrin tails, some of which modulate integrin activation. Filamin, which like talin is a cytoskeletal protein involved in linking integrins to the actin cytoskeleton, binds to β integrin tails and may modulate talin-dependent integrin activation by competing with talin for overlapping binding sites on the integrin (17, 28). Like talin, many proteins that interact with β integrin cytoplasmic domains contain PTB-like domains (29). Some of these proteins, such as Numb, DOK and ICAP1 appear to have little direct effect on integrin activation but may instead modulate integrin activation by competing with talin for integrin binding (20, 30, 31).

Several recent reports have described a role for kindlin proteins in regulating integrin activation (32–35). The kindlin family of proteins is comprised of three members (kindlin-1, kindlin-2, and kindlin-3) each encoded by a separate gene (36). Kindlin-1 and kindlin-2 are widely expressed and kindlin-3 expression is restricted primarily to haematopoietic cells (36). Structurally, kindlins are characterised by a central FERM domain bisected by a pleckstrin homology domain (37). It is notable that the kindlin F3 domain is intact and highly similar to the F3 subdomain of talin (37). Kindlins bind to β 1 and β 3 integrins (35, 37) and the binding site within the β integrin cytoplasmic domain has been mapped to the membrane distal NPxY motif, distinct from the talin binding site (32, 34). In CHO cells, expression of kindlin-2 augmented talin-induced integrin activation (32, 33). Furthermore, depleting kindlin-2 significantly

blunted talin-induced integrin activation (32, 33). Analysis of kindlin-3-deficient murine platelets suggests that kindlin-3 is required for activation of α IIB β 3 and β 1 integrins (34). Interestingly, over-expression of kindlin-3 in a mouse macrophage cell line activated integrins, an effect not observed when kindlin-3 was expressed in CHO cells (32, 34). Thus, the role of kindlins in the activation of integrins appears to be cell-type-specific and perhaps kindlin isoform-specific.

Genetic manipulation of talin in invertebrates

Important insights have been gained into mammalian integrin biology and talin-dependent integrin function through genetic studies in invertebrates. Deletion of talin in both *Caenorhabditis elegans* and *Drosophila* results in failure to form the linkage between the extracellular matrix (ECM) and cytoskeleton and is manifest in the detachment of contractile muscles (38, 39). It is noteworthy that talin-deficiency phenocopies integrin loss-of-function mutations in both of these invertebrates suggesting that talin and integrins may function together to link the ECM to the cytoskeleton (40–43). Tanentzapf et al. examined this hypothesis by analysing a talin mutant that does not bind to integrins, talin^{R367A}, in *Drosophila* (44). This talin mutant was recruited to sites of integrin adhesions but could not rescue the muscle detachment and embryonic lethality that occurs in the absence of talin indicating that interactions between talin and the integrin are essential to link the ECM to the cytoskeleton. In addition, there was a dissociation between integrins and the ECM in talin^{R367A} embryos, an observation interpreted as impaired integrin activation (44). However, it is important to bear in mind that the apparent reduction in integrin affinity for ligand in this study was inferred from indirect assays. Indeed, direct measurements of integrin activation in a heterologous system have shown that talin (be it mammalian talin or *Drosophila* talin) does not activate *Drosophila* integrin α PS2 β PS (45). At any rate, it seems likely that talin-integrin interactions play an important role in *Drosophila* development independent of integrin affinity modulation, perhaps by promoting integrin clustering (44) and/or facilitating interactions of additional proteins with the integrin cytoplasmic domain (26).

In addition to providing new information regarding the biological roles of talin-integrin interactions, these studies raise important questions. If *Drosophila* α PS2 β PS integrin affinity is regulated in the intact fly what molecular interaction(s) underlie this regulation? How do these regulatory mechanisms differ in mammalian systems and do these differences provide clues about the regulation of mammalian integrins? Future studies utilising genetic manipulation of invertebrates should continue to provide new information about talin-dependent integrin activation and guide the experimental approaches used to dissect mammalian integrin signalling *in vivo*.

Deletion of talin in mouse platelets

The biological importance of talin is highlighted by the striking phenotypes of talin-deficient *Drosophila* and *C. elegans* described above and by the observation that global genetic deletion of talin1 in mice results in defects in gastrulation and lethality

around embryonic day 9 (46). To examine the effects of genetically deleting talin in murine platelets and circumvent the lethality associated with global deletion of talin1, we crossed a conditional talin1 knock-out (KO) mouse with a mouse expressing Cre recombinase under the control of the platelet factor 4 promoter (47) to selectively delete talin1 in platelets and megakaryocytes (48). Platelet talin1 KO animals were viable but showed reduced survival associated with spontaneous haemorrhage, chronic gastrointestinal bleeding and anaemia. Despite deletion of 95% of talin, platelet talin KO mice showed a very modest reduction in circulating platelet counts and talin-deficient platelets were morphologically similar to control platelets suggesting that talin may not be required for megakaryopoiesis (48). It is noteworthy that while talin was deleted in mature megakaryocytes of platelet talin1 KO mice, the precise stage during megakaryopoiesis at which talin was deleted is unknown. Indeed, previous studies have identified important roles for integrin-dependent adhesion during megakaryopoiesis (49, 50) and it would be interesting to more closely examine the requirement of talin-dependent integrin signalling in megakaryocyte function and proplatelet formation.

Despite normal numbers of circulating platelets and normal surface expression of integrin α IIB β 3, platelet talin KO mice showed profound defects in haemostasis including spontaneous haemorrhage, chronic bleeding, and markedly prolonged tail bleeding times (48). Functional characterisation of talin-deficient platelets revealed defects in agonist-induced aggregation and agonist-induced soluble fibrinogen binding. Importantly, talin-deficient and control platelets bound similar amounts of fibrinogen in the presence of Mn²⁺, an agent known to stabilise a high-affinity conformation by binding to the extracellular domain of the integrin (51, 52), indicating that the α IIB β 3 integrins expressed on talin-deficient platelets are capable of binding fibrinogen if extrinsically activated.

The same talin1 conditional mouse discussed above was also crossed with an Mx-Cre mouse resulting in the deletion of talin1 in all haematopoietic cells (53). Functional analysis of Mx-Cre mediated talin1 KO mice showed defects in agonist-induced α IIB β 3 activation, platelet aggregation, haemostasis and thrombosis equivalent to those observed in PF4-Cre mediated talin1 KO mice (48, 53). Thus, the results from these studies conducted in separate laboratories using different Cre recombinase mouse lines to induce talin1 gene disruption offer compelling evidence that talin is required for α IIB β 3 activation *in vivo*.

The cytoplasmic domains of many of the integrin β subunits are highly conserved and, in addition to β 3 integrins, talin has been shown to activate β 1 and β 2 integrins *in vitro* (13, 54, 55). Talin-deficient platelets were unable to adhere to a collagen coated surface in flowing blood, a process dependent on α 2 β 1 integrins (56). Furthermore, direct measurement of β 1 integrin activation with an antibody that recognises the high-affinity conformation of the receptor indicated that agonist-induced activation of β 1 integrins in platelets requires talin (48). Since α 2 β 1 integrin serves as an important collagen adhesion receptor in platelets, the defective β 1 integrin activation observed in platelet talin1 KO mice could be one factor that contributed to the more severe bleeding diathesis observed in platelet talin1 KO mice relative to β 3 integrin KO mice (48, 57).

Disruption of $\beta 3$ integrin-talin interactions

The observation that agonist-induced fibrinogen binding is markedly reduced in talin-deficient platelets provided strong evidence that talin is required for integrin activation. The results obtained from the platelet talin KO did not, however, indicate whether binding of talin to the integrin tail was the key molecular event essential for activation. For instance, it is possible that loss of talin could result in impaired integrin activation due to failure to recruit a cytoplasmic factor(s) necessary for activation. Indeed, talin has been shown to directly recruit PIPKI γ to the cell membrane (58, 59). To address this issue, genetically engineered mice expressing mutant $\beta 3$ integrins that disrupt the binding of talin were generated (60).

Structural studies of talin- $\beta 3$ integrin interactions have provided important insights into the mechanisms that regulate talin-dependent integrin activation. X-ray crystal structure of the talin F3 subdomain complexed with a portion of the $\beta 3$ integrin tail identified amino acids that mediate talin- $\beta 3$ integrin interactions (17). In $\beta 3$ integrin, Tyr⁷⁴⁷ sits in a hydrophobic and acidic pocket formed by two β strands of talin and the $\beta 3$ integrin Leu⁷⁴⁶ residue. Based on this structure, mutating either the Tyr⁷⁴⁷ or Leu⁷⁴⁶ was predicted to disrupt binding of talin to $\beta 3$ integrin. Keima et al. reported the structural details of how the cytoskeletal protein filamin interacts with the $\beta 7$ integrin cytoplasmic domain (28). Modeling a portion of $\beta 3$ integrin, which is highly similar to $\beta 7$, based on the structure of Kamei et al. indicated that the $\beta 3$ integrin Tyr⁷⁴⁷ made key contacts with filamin. In contrast, $\beta 3$ integrin Leu⁷⁴⁶ did not significantly contribute to $\beta 3$ integrin-filamin interactions. Together, these studies predicted that mutation of $\beta 3$ integrin Leu⁷⁴⁶ would disrupt $\beta 3$ integrin-talin binding without affecting $\beta 3$ -filamin interactions. This concept was validated by measuring the binding of talin, filamin and other known $\beta 3$ integrin cytoplasmic interacting proteins from platelet lysates to recombinant $\beta 3$ integrin tail proteins (13, 60).

To selectively disrupt $\beta 3$ integrin-talin interactions *in vivo*, single point mutations ($\beta 3$ (L746A) and $\beta 3$ (Y747A)) were inserted into the mouse $\beta 3$ integrin locus by gene targeting. Analysis of these animals showed both $\beta 3$ (L746A) mice, harboring a mutation predicted to selectively disrupt $\beta 3$ integrin-talin interaction, and $\beta 3$ (Y747A), harboring a mutation predicted to disrupt the interaction of $\beta 3$ integrin with multiple cytoplasmic proteins, exhibited markedly impaired haemostasis (60). Agonist-induced aggregation and soluble fibrinogen binding were markedly impaired in platelets from $\beta 3$ (L746A) and $\beta 3$ (Y747A)

mice and, notably, were similar to results obtained with the platelet talin1 KO mouse. Thus, either deletion of talin or disrupting the ability of talin to interact with the $\beta 3$ integrin tail resulted similarly in markedly reduced α IIb $\beta 3$ integrin activation.

Whereas platelets from either $\beta 3$ (L746A) and $\beta 3$ (Y747A) mice showed similar behavior with respect to aggregation and soluble fibrinogen binding, there were clear phenotypic differences between these two mutant mice. The $\beta 3$ (Y747A) mutants displayed chronic gastrointestinal bleeding, anaemia and reduced survival, similar to that reported for $\beta 3$ integrin KO mice (57). The talin-selective $\beta 3$ (L746A) mutants, on the other hand, showed no such pathological bleeding phenotype and had lifespans similar to wildtype littermates. What accounts for the phenotypic differences observed in $\beta 3$ (L746A) and $\beta 3$ (Y747A)? First, it is important to keep in mind that agonist-induced soluble fibrinogen binding and aggregation are but single functional measurements of a complex process that takes place during thrombus formation *in vivo* (5, 61). Second, it is possible that there are biologically significant differences in platelet fibrinogen binding, adhesion and aggregation that require particular experimental conditions (i.e. choice and dose of agonist, shear rates, etc.) to be observed. Regardless, it is clear that $\beta 3$ (Y747A) and $\beta 3$ (L746A) mice display distinct haemostatic phenotypes. One hypothesis is that certain aspects of $\beta 3$ integrin signalling that are important for haemostatic platelet function are disrupted by the $\beta 3$ (Y747A) mutation but preserved in the $\beta 3$ (L746A) mutation. Analysis of signalling in platelets from these mutant mice may speak to this possibility and will provide new insights into the role of talin- $\beta 3$ integrin interactions in platelet function.

Agents that block ligand binding to α IIb $\beta 3$ are currently used effectively as anti-thrombotics in the setting of percutaneous coronary intervention (8). Unfortunately, chronic inhibition of α IIb $\beta 3$ with oral α IIb $\beta 3$ antagonists are ineffective and even associated with increased mortality (62). One potential reason for failure of clinical trials of oral α IIb $\beta 3$ inhibitors is the difficulty of keeping patients within a therapeutic window such that cardiovascular events were minimised without inducing bleeding. Indeed, $\beta 3$ (L746A) mice were protected from thrombosis in a model of pulmonary thromboembolism and in response to ferric-chloride-induced injury of the carotid artery (60). These results, combined with the observation that selective disruption of talin- $\beta 3$ integrin interactions is associated with minimal pathological bleeding *in vivo*, suggest that modulating talin-integrin α IIb $\beta 3$ interactions may provide useful therapeutic targets.

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