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Margadant, C.

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Mechanisms of integrin activation and trafficking

Coert Margadant¹, Hanneke N. Monsuur¹, Jim C. Norman², and Arnoud Sonnenberg¹

¹Division of Cell Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands. ²Beatson Institute for Cancer Research, Cancer Research UK, Glasgow, G61 3BX, Scotland.

ABSTRACT

Integrin adhesion receptors are essential for the normal function of most multicellular organisms, and defective integrin activation or -signaling is associated with an array of pathological conditions. Integrins are regulated by conformational changes, clustering, and trafficking, and regulatory mechanisms differ strongly between individual integrins and between cell types. Whereas integrins in circulating blood cells are activated by an inside-out-induced conformational change which favors high-affinity ligand binding, β1-integrins in adherent cells can be activated by force or clustering. In addition, endocytosis and recycling play an important role in the regulation of integrin-turnover and -redistribution in adherent cells, especially during dynamic processes such as cell migration and invasion. Integrin trafficking is strongly regulated by their cytoplasmic tails, and the mechanisms are now being identified.

INTRODUCTION

Integrins are heterodimeric αβ transmembrane receptors that connect the extracellular matrix (ECM) to the cytoskeleton. In mammals, 18 α- and eight β-subunits assemble into 24 different integrins, which bind collagens, laminins, or proteins containing an Arg-Gly-Asp (RGD) sequence. In addition, several integrins bind soluble ligands or cellular receptors (Figure 1). Many integrins are known to adopt low-, intermediate-, and high-affinity conformations, and these exist in a dynamic equilibrium with one another. An increase in the proportion of heterodimers adopting high-affinity conformations is termed integrin activation, and can be induced either by cytoplasmic events (‘inside-out’ activation; Figure 2A), or by extracellular factors (‘outside-in’ activation). Ligand-binding triggers integrin clustering (avidity), integrin connection to the cytoskeleton, and the formation of macromolecular adhesion complexes (Figure 2A). Moreover, integrin-ligand interactions induce a plethora of ‘outside-in’ events such as cell spreading and migration, ECM assembly, and the activation of several signal transduction pathways that regulate cell proliferation, survival, and gene expression (1).

Most integrins engage the actin cytoskeleton, and a range of integrin-containing actin-associated adhesive structures has been described, including focal complexes, focal adhesions (FAs), fibrillar adhesions (FBs), podosomes, and invadopodia (2) (Figure 2B). In contrast, integrin α6β4 connects to the intermediate filament system, and localizes to hemidesmosomes (3). The relatively short α- and β-cytoplasmic tails (13-70 amino acids, except for β4) contain docking sites for a variety of proteins that control integrin activation, recruitment to adhesion sites, and trafficking. Here, we discuss recent advances in our understanding of how these processes are regulated by integrin cytoplasmic tails, with emphasis on differences between adherent and non-adherent cells, and between individual integrins.
Integrin activation in non-adherent cells such as leukocytes and platelets is rapid, reversible, and tightly-controlled, and this process is best-exemplified by the rapid enhancement of ligand-binding capacity of integrin αIIbβ3 following platelet activation with agonists such as thrombin. In resting platelets, the bent, low-affinity conformation of αIIbβ3 is stabilized by a ‘clasp’ formed between the GFFKR sequence in αIIb and the HDRxE motif in β3, most importantly a salt bridge between R995 and D723 (Figure 3) (4), and this mechanism is thought to help prevent inappropriate platelet aggregation which could lead to thrombosis. Activation of cytoplasmic signaling downstream of G-protein coupled receptors (such as the thrombin receptor) leads to disruption of the salt bridge, and the subsequent separation (‘unclasping’) of the cytoplasmic tails triggers an allosteric change to favor the extended, high-affinity integrin conformation. The last step of this inside-out mechanism is the binding of the four-point-one/ezrin/radixin/moesin (FERM)-containing head-domain of talin to the membrane-proximal (MP)-NPxY motif and an additional MP region of the β3 cytotail (Figures 2A, 3) (4,5). Inside-out activation also requires kindlin-3, a member of the kindlin family of proteins whose FERM domains bind the membrane-distal (MD)-NxxY motif in β-tails (6). Loss of talin or kindlin-3 from platelets prevents platelet adhesion and aggre-
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Figure 2 Integrin activation and adhesion assembly. (A) In leukocytes and platelets, ‘inside-out activation’ is triggered by a cytoplasmic chain-of-events that terminates in the binding of talin and kindlin-3 to the β-subunit, triggering a shift from the low-affinity conformation (bent with a closed head) toward the intermediate- (extended with closed head) and high- (extended with open head) affinity conformation. Ligand-binding induces ‘outside-in’ events including talin-mediated connection to the actin cytoskeleton and cell spreading, integrin clustering, and activation of various signal transduction cascades. (B) Types of integrin-containing adhesion complexes: (1) Focal complexes (red arrows) are Rac-induced peripheral adhesions (≤1 µm) that stabilize membrane protrusions such as lamellipodia, whereas focal adhesions (yellow arrows) are Rho-induced tensioned structures (1-5 µm) that represent major sites of signaling. Red, F-actin; green, phosphotyrosines; blue, nuclei. (2) Fibrillar adhesions (arrows) are central adhesions of 1-10 µm in length, contain integrin α5β1 and tensin but few tyrosine-phosphorylated proteins, and align with FN fibrils. Red, F-actin; green, tensin; blue, FN. (3) Podosomes are punctate structures with an actin core (indicated by white arrows), whereas hemidesmosomes are huge assemblies formed by integrin α6β4. Red, F-actin; green, β4.
The motifs are phosphorylated in β3 after ligand-binding, and prevention of this event by Y>F substitutions causes defects in platelet aggregation and a bleeding defect in mice (13). However, Y>F mutations in the NPxY motifs of β1 in mice do not cause any abnormalities whereas Y>A mutations lead to a complete loss-of-function, suggesting that in β1-integrins, structural integrity of the NPxY motifs is required but tyrosine-phosphorylation is dispensable. Furthermore, mice carrying a D759A substitution in β1 (which prevents formation of a salt bridge) are normal, suggesting that also the salt bridge is not essential for β1-integrin function in vivo (14,15).

As integrins in adherent cells need generally to be in an ‘on’- rather than ‘off’-state, the bent conformation is less likely to predominate in these cells. Nevertheless, the turnover of adhesion complexes that occurs for example during cell migration, requires dynamic regulation of integrin-ligand binding. Conformational changes in α5β1 from the bent to the extended conformation have been observed in FAs, but the mechanism that triggers the unbending remains to be elucidated (16). Probably, integrin activation in adherent cells occurs by an ‘outside-in’ mechanism (e.g. the high concentration of available ligand in the ECM), by regulation of avidity (integrin clustering), or by force. For example, α5β1 can be activated by cytoskeletal tension generated by myosin-II, in response to a stiff ECM. Under low tension, α5β1 forms ‘relaxed’ bonds to the RGD-site of fibronectin (FN), whereas under high tension, α5β1 binds both the RGD and the synergy site, leading to stronger, ‘tensioned’ bonds (17). The lifetime of α5β1-FN bonds is thus increased by tensile force (18). Interestingly, neither force nor high avidity seems to influence talin-induced molecular extension of αIIbβ3 (19). This indicates that talin-regulated αIIbβ3 activation may solely occur through conformational changes, whereas integrin-mediated adhesion in adherent cells may depend principally on talin-dependent increases in integrin avidity through the formation of antiparallel talin homodimers, and/or connection to the actin cytoskeleton via the talin rod-domain (Figure 2A). The latter is strongly suggested by intriguing observations in Drosophila; demonstrating that both talin- and integrin-null mutations cause wing blistering, however in the absence of talin, integrin-ligand binding is not impaired but integrins fail to connect to the cytoskeleton (20). Talin may also indirectly promote integrin function, by preventing the binding of the negative regulator filamin (21). Filamin competes with talin for binding of the β-tail, and its affinity is decreased by phosphorylation of the threonine/serine (T/S)-rich region that separates the two NPxY motifs (Figure 3) (22). T/S phosphorylation is thus thought to act as a molecular switch that increases talin-binding and integrin activation, and this is supported by observations that 1) T/S-phosphorylation occurs in β1, β2, β3, and β7 after stimulation with integrin-activating agonists, and 2) conservative and non-conservative mutations of the T/S residues in β3 or β1A decrease integrin activation and cell adhesion (4). The importance of T/S-phosphorylation in vivo remains to be determined.

The kindlins are also important for integrin function in adherent cells. In humans,
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Kindlin-1 mutations cause Kindler syndrome, a skin-blistering disorder that resembles loss-of-function of $\alpha_3\beta_1$ in the epidermis, and gene targeting of kindlin-1 and -2 compromises integrin-mediated adhesion in mice (23-25). However, the molecular mechanism remains elusive and requires further investigation. First, the effects of kindlins are cell-type-specific, as kindlin-3 overexpression enhances integrin activation in macrophages but not in CHO cells (9). Second, kindlins exert integrin-specific effects, because co-expression of kindlin-1 or kindlin-2 with talin results in activation of $\alpha_{IIb}\beta_3$ but inhibition of $\alpha_5\beta_1$ in CHO cells, and kindlin-2 regulates adhesion to vitronectin whereas kindlin-3 regulates adhesion to FN in endothelial cells (26,27). Third, kindlins can regulate integrins independent of activation, as cell adhesion and cell spreading were compromised in kindlin-1-deficient-keratinocytes, whereas integrin activation was not impaired (28).

In summary, it is clear that mechanisms of activation are not generic for all

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### Figure 3 Sequences of integrin cytoplasmic tails

Depicted are the sequences of the human $\beta_1A$, $\beta_1D$, $\beta_2$, $\beta_3$, $\beta_5$, $\beta_6$, and $\beta_7$ subunits ($\beta_4$ and $\beta_8$ have been omitted because of poor sequence homology), and the human $\alpha_{IIb}$, $\alpha_2$, and $\alpha_5$ subunits. Numbers indicate the first and last residues. The green box represents the ‘clasp’ that maintains the low-affinity conformation, formed between the HDRxE region (with the D-residue essential for the salt bridge in red), and the GFFKR motif in the $\alpha$-subunit. Yellow boxes indicate the membrane-proximal (MP)-NPxY and membrane-distal (MD)-NxxY motifs, and the red box indicates the intervening T/S region. Shown are the binding sites for key regulators of integrin activation and trafficking including Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein 1 (ACAP1), $\beta_3$-endonexin, Disabled (Dab)-1/-2, Dok-1, filamin, G-protein-signaling GAPIP-interacting protein COOH terminus (GIPC), integrin cytoplasmic domain associated protein-1$\alpha$ (ICAP-$\alpha$), kindlins, Numb, p120RasGAP, protein kinase C (PKC), protein kinase D1 (PKD1), Rab21, Rab25, Src, talin, and tensin. The subunits to which binding has been demonstrated are indicated in brackets, and dashed lines indicate that the exact binding sites have not been mapped.

| $\beta_1A$ | 751 | WKLLMII | HDRREFAKFEKEKMNACKWDQGE | NPIY | KSAATTLY | NPKY | EGK | 799 |
| $\beta_1D$ | 751 | WKLLMII | HDRREFAKFEKEKMNACKWDQGE | NPIY | KSPNPICK | NPNY | GRKAGL | 802 |
| $\beta_2$ | 723 | WAKLHL | SLREYRRFEKEKLKSLQWNNCD | NPLF | KSAATTVM | NPKE | AES | 770 |
| $\beta_3$ | 716 | WKLLITI | HDRREFAKFEKEEERARKNACKWDAN | NPLY | KEAFFPT | NITY | RGFT | 762 |
| $\beta_5$ | 742 | WKLLVTI | HDRREFAKFEKQGERSRARYEMAS | NPLY | RKPSWSTH | VDFTFNKF | NIKY | NGTVD | 800 |
| $\beta_6$ | 730 | WKLVSF | HDRKEVAFKEAERSKACKWQGTG | NPLY | RGSTSF | NVTY | KHERKQVDSLTD | 789 |
| $\beta_7$ | 746 | YRLSVEI | YDRREYSPFESEQQQQLNWKGD | NPLY | KSAATTI | NPFM | GEAAPTL | 799 |

**Kindlins**

| $\alpha_{IIb}$ | KVGFFKRNPPPLEDEEDEGE |
| $\alpha_5$ | KLGGFKIRSLP YGTAEMKAQL KPPATEDA |
| $\alpha_2$ | KGLFFKRYE KMTKNPDEID ETTELSS |

| talin | MP-NPxY | T/S region | MD-NxxY |
| talin | MP-NPxY | T/S region | MD-NxxY |

| kindlins | (p1,3) |
| kindlins | (p1,3) |

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integrins. Instead, different integrins are subject to different regulatory mechanisms, which have evolved to meet varying biological requirements. Such diversity is at least in part accomplished through sequence differences among β-tails, which can strongly influence the affinities for binding proteins (29). This is illustrated by the recently resolved structures of various β-tails in complex with talin-1 or talin-2; the tightest link is formed between β1D and talin-2, which are predominant in the myotendinous junctions of striated muscle, and has to withstand the forces of muscle contraction (30).

INTEGRIN CYTOPLASMIC MOTIFS AND THE REGULATION OF INTEGRIN TRAFFICKING

Over the past years, it has been firmly established that integrin trafficking in adherent cells is important for integrin-dependent cell adhesion, spreading and migration, as well as cancer cell invasion. Integrin trafficking regulates FA disassembly, matrix turnover, and localized integrin redistribution to new adhesion sites, for example at the leading edge in migrating cells (31). Trafficking mechanisms include the delivery of newly synthesized integrins via the biosynthetic-secretory pathway, integrin internalization, and recycling of internalized integrins.

Early reports have suggested that talin-binding at the endoplasmic reticulum (ER) may regulate delivery of newly synthesized integrins to the membrane via myosin-mediated vesicular transport along actin filaments (32). Talin-binding exposes the GFFKR sequence which is a suggested ER-export signal, and mutations in this sequence cause reduced surface expression, as observed in some GT patients. It remains unclear how talin-integrin binding at the ER is regulated, and how this affects integrin conformation. Using conformation-specific antibodies, it was recently suggested that β1-integrins are in the bent conformation after association with α-subunits at the ER, and that they stay like this as they journey through the Golgi to the plasma membrane (33).

Integrin internalization occurs through clathrin-dependent and clathrin-independent (via caveolae or macropinocytosis) mechanisms, and many integrins can follow more than one route into the cell. Caveolar internalization of α5β1 and FN regulates matrix turnover, and early reports have shown that also α2β1 is endocytosed in a caveolin-dependent manner, which may involve a direct association of protein kinase Cα (PKCα) with the β1-tail (34,35). Recently, it has become clear that αvβ3 can be internalized by macropinocytosis from dorsal ruffles that are induced by platelet-derived growth factor (PDGF) stimulation. The internalized integrins then transit through recycling endosomal compartments to repopulate newly formed FAs on the ventral surface (36). Clathrin-mediated integrin endocytosis is emerging as an important mechanism of adhesion disassembly during cell migration, and recent studies have identified many of the molecules involved in this process. Although there is an early study suggesting that αvβ5 binds clathrin directly, it is gener-
ally thought that most integrins require adaptor proteins to recruit them into clathrin-coated pits. Indeed, clathrin adaptors such as Disabled (Dab)-2 and adaptor protein-2 (AP-2) accumulate at or near FAs shortly before their disassembly. Furthermore, dynamin-dependent integrin internalization and transport along microtubules to Rab5-positive endosomes has been reported following manipulations that lead to synchronous disruption of FAs (37,38). Accordingly, depletion of clathrin or any of these adaptors leads to increased integrin surface expression and reduced migration (37-39). In addition to Dab-2, the clathrin adaptor Numb localizes in FAs at the leading edge of migrating cells to mediate integrin endocytosis and migration (40). Studies in Drosophila now indicate that clathrin- and Rab5-dependent integrin turnover is also important in vivo, in particular for the maintenance of the myotendinous junction (41). NPxY motifs are canonical signals for clathrin-mediated endocytosis of plasma membrane receptors, and Numb and Dab-2 bind respectively to the MP-NPxY and the MD-NxxY of β-tails (29). Several observations suggest that mutations in these motifs impair integrin internalization. First, Y>A substitution in the MD-NxxY of β3A impairs the β3-endonexin-mediated internalization of ligand-bound integrins. Second, F>A substitutions in either one of the NPxF motifs of β2-integrins compromises their endocytosis. Third, Y>F mutations in both NPxY motifs of β1 reduces clathrin-dependent integrin endocytosis in fibroblasts (42). The latter result is surprising as β1YY/FF mice are apparently normal, and it suggests that another endocytic mechanism can compensate for loss of clathrin-dependent internalization of β1-integrins in vivo (14,15). Indeed, endocytosis of β1YY/FF can be rescued by overexpression of Rab21 and this is most likely via the upregulation a clathrin-independent pathway (42). Despite these advances, it remains unclear how the individual NPxY motifs regulate integrin trafficking, and through which proteins. Furthermore, the interplay between internalization and activation requires further investigation. It has been suggested that upon microtubule-induced FA disassembly, active integrins endocytose more rapidly than inactive integrins (38). In addition, whereas some reports find Dab-2 enrichment in FAs, others suggest that Dab-2 is required for bulk endocytosis of inactive integrins, since it colocalizes with β1-integrins distributed over the entire cell surface (39). The possibility that internalization of active and inactive integrins is regulated by separate pathways is intriguing, but has so far only been substantiated for α5β1. Indeed, selective internalization of active α5β1 from FBs and vesicular intracellular transport along actin filaments is mediated by Neuropilin1- and GIPC-mediated connection to myosinVI, whereas endocytosis of inactive α5β1 occurs by Neuropilin1-independent mechanisms (43).

Internalized integrins may be either returned to the plasma membrane or routed to lysosomes for degradation, and the decision to recycle or degrade internalized α5β1 is likely influenced by its ubiquitination. In migrating fibroblasts, the α5-tail is ubiquitinated upon FN binding, and this is required to direct FN-α5β1 complexes to lysosomes. It was suggested that ubiquitination functions to prevent
endosomal accumulation of ligated integrins, which may interfere with cell signaling and migration (44). However, most internalized integrins are not degraded, but are returned to the plasma membrane, and a number of growth factors, kinases, and GTPases of the Rab and Arf families are now known to influence this process. For example, PDGF stimulates rapid, short-loop recycling of internalized αvβ3 from early endosomes (EEs), whereas α5β1 travels from EEs to the perinuclear recycling compartment (PNRC) and is then recycled via a longer loop to the plasma membrane (Figure 4) (45). Short-loop recycling depends on Rab4 and protein kinase D1 (PKD1), the latter of which binds directly to the β3-tail (Figure 3). By contrast, long-loop recycling requires Rab11, Arf6, and protein kinase B (PKB)/Akt (46,47). PKB phosphorylates the Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein1 (ACAP1), which then associates directly with the β1-tail and mediates recycling of β1-integrins from recycling endosomes (Figure 4) (47). Different routes of integrin recycling can exert differences in Rho GTPase signaling and directional migration (48).

The α-tails of integrins are now known to recruit GTPases and other factors that control their trafficking. Rab21 is a Rab5-related GTPase which associates directly with a region of the α2- and α5-tails that is close to the GFFKR motif (49). Very recently, it has become clear that p120RasGAP also binds to this portion of the α-tail, and does so in a way to replace Rab21, which then mediates integrin

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**Figure 4 Integrin trafficking mechanisms regulate adhesion (dis-)assembly and migration.** Integrin endocytosis is mediated by Rab5 and Rab21. From early endosomes, integrins are recycled to the membrane either via a short loop that involves Rab4, or via the perinuclear recycling compartment in a Rab11- or Rab25-dependent manner. Alternatively, they are delivered to late endosomes/lysosomes for degradation, which may require α-chain ubiquitination. Integrins can also be redistributed to dorsal ruffles after PDGF-stimulation, after which uptake occurs by macropinocytosis. They are then delivered to endosomes and exocytosed at the ventral surface. ACAP1, Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein1; EE, early endosome; MPS, macropinosome; PKB, protein kinase B; PKD1, protein kinase D1; RE, recycling endosome; Ub, ubiquitination.
recycling from EEs (Figure 4) (50). These results indicate that as integrins travel from one intracellular compartment to the other they are able to swap proteins that are associated with their tails in a way that supports their recycling. Possibly, other Rabs also bind directly to the α-tail, and it is conceivable that sequence differences in the region adjacent to the GFFKR are responsible for the integrin-specific effects of Rabs on individual heterodimers (31,42,45,48,49). Moreover, this might explain the long-standing observation that various integrins traffic with different kinetics depending on the α-subunit; whereas rapid and constitutive endocytosis occurs for α5β1 and αMβ2, no or slow endocytosis is observed for α3β1, α4β1 and α6β2 (31). Interestingly, the Rabs contain phosphotyrosine-binding domains that recognize NPxY motifs, and can thus potentially also bind to β-tails. Indeed, Rab25 interacts with the β1-tail, and drives recycling of α5β1 to the tips of pseudopods in invading cancer cells (51).

In conclusion, motifs in the cytotails of integrins are key to the regulation of integrin endocytosis and recycling via a number of pathways, and although many players in these processes have now been identified, major issues still need to be resolved. These include the function of the individual NPxY motifs in trafficking of various integrins, the role of the α-subunit, and the interplay between activation and internalization.

CONCLUSIONS

Our knowledge of the diverse mechanisms of integrin regulation has steadily increased over the years, and it is now clear that mechanisms of integrin activation and trafficking differ between different integrins and across cell types. Future work requires more focus on how integrin trafficking is regulated by the α-subunits and associated proteins, and by the two NPxY motifs in the β-subunits. Furthermore, the interplay between trafficking and activation merits further exploration, which will reveal how differential trafficking of active and inactive integrins is regulated, and via which proteins.

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ABBREVIATIONS

ACAP1, Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein1; AP-2, adaptor protein-2; Dab, disabled; ECM, extracellular matrix; EE, early endosome; ER, endoplasmic reticulum; FA, focal adhesion; FB, fibrillar adhesion; FERM, four-point-one/ezrin/radixin/moesin; FN, fibronectin; GIPC, G-protein signaling...
GAIP-interacting protein COOH terminus; GT, Glanzmann’s thrombasthenia; ICAP-1, integrin cytoplasmic domain-associated protein; LAD, leukocyte adhesion deficiency; MD, membrane-distal; MP, membrane-proximal; PDGF, platelet-derived growth factor; PKB, protein kinase B; PKC, protein kinase C; PKD1, protein kinase D1; PNRC, perinuclear recycling compartment; RGD, Arg-Gly-Asp

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