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# Integrin-mediated signal transduction

**C. M. Longhurst[a] and L. K. Jennings[a,b,*]**

[a]University of Tennessee, Memphis, Department of Medicine, 956 Court Avenue, Rm. H335, Memphis (Tennessee 38163, USA), Fax +1 901 448 7181, e-mail: ljennings@utmem1.utmem.edu
[b]University of Tennessee, Memphis, Department of Biochemistry, Memphis (Tennessee 38163, USA)

**Abstract.** Integrins, expressed on virtually every cell type, are proteins that mediate cellular interactions with components of the extracellular matrix (ECM) and cell surface integral plasma membrane proteins. In addition, integrins interact with the cytoskeleton and through this process participate in cell migration, tissue organization, cell growth, haemostasis, inflammation, target recognition of lymphocytes and the differentiation of many cell types. Signals generated from ligand-integrin interactions are propagated via the integrin cytoplasmic tails to signal transduction pathways within the cell (outside-in signalling). Information from within the cell can also be transmitted to the outside via integrin affinity modulation (inside-out signalling). Protein tyrosine phosphorylation has a central role in integrin-initiated cell signalling, leading to cytoskeletal organization and focal adhesion formation. This review will examine the current understanding of integrin function, focusing on the intracellular consequences of integrin-ligand interaction.

**Key words.** Integrin; cell adhesion; extracellular matrix; signalling; focal adhesion kinase.

## Introduction

Integrins bind to a variety of ligands including extracellular matrix (ECM) proteins [1], cell surface immunoglobulin (Ig) superfamily receptors [2] and certain plasma proteins [3]. Integrin-mediated signal transduction contributes to the coordination of the actin cytoskeleton organization and cellular responses to ECM proteins and growth factors. The adaptability and rapid modulation of integrin-ligand interactions make integrins ideal coordinators of dynamic cellular processes. As integrin binding to extracellular ligands precedes diverse cell functions, the accompanying signalling pathways must be tightly regulated and coordinated.
Inactivated integrins usually have a low affinity for ligand; however, on activation via appropriate signals, integrins can participate in high-affinity binding. This unique function allows modulation of cell adhesive properties without changes in integrin gene expression.

* Corresponding author.

The understanding of signal transduction pathways involved in integrin-mediated cellular events has been the focus of intense interest for several years. The manipulation of the integrin $\alpha$IIb$\beta$3 expressed on human platelets by mimetic agents has already demonstrated that integrins provide potential sites for therapeutic intervention in thrombotic states [4]. In this review, we shall discuss the ongoing dissection of integrin-signalling pathways and their potential role in cell function.

## Structure of integrins

Sixteen $\alpha$ and eight $\beta$ integrin subunits have been cloned. These structures assemble to form the 22 described integrin heterodimers. These subunits are the products of two different genes, and the expression of both subunits is required for cell surface expression [5]. The $\alpha$ subunits comprise a short cytoplasmic tail, a transmembrane domain and a large extracellular domain of 1000 amino acids (aa) approximately. The $\alpha$ extracellular domain consists of seven tandem repeats

of 60 aa containing three or four EF-like divalent cation-binding sites. The EF-hand domains have homology with a helical-loop $Ca^{2+}$-binding structure found in proteins such as calmodulin, trophin C and parvalbumin [6]. The non-I domain $\alpha$ subunits ($\alpha$3, $\alpha$5, $\alpha$6, $\alpha$7, $\alpha$IIb, $\alpha$v) contain four EF-like motifs and a disulphide cleavage site near the transmembrane region. $\alpha$IIb$\beta$3 was the first integrin to be purified to homogeneity and demonstrated to be a calcium dependent hetrodimeric complex [7].

Electron microscopy studies [8] have shown that integrins are asymmetric structures consisting of a mushroom-like extracellular region and two flexible tails. The N-terminal regions of the $\alpha$ and $\beta$ subunits form the extracellular domains which span the membrane ending in cytoplasmic C-terminals of both subunits. The eight described $\beta$ subunits of approximately 750 aa have an overall homology of approximately 40%. Each $\beta$ subunit has a short cytoplasmic tail with the exception of $\beta$4, which has an extended cytoplasmic region containing four fibronectin (FN) type III repeats and two alternatively spliced sites. The $\beta$ extracellular domain has a highly conserved region of approximately 200 aa which has similarities to the I domain (A-like domain) found in some $\alpha$ subunits and may contain a divalent cation-binding site [9]. The $\beta$ subunit contains four cysteine-rich motifs within the C-terminal proximal to the transmembrane region. Additional variability is provided by alternative splicing of the cytoplasmic tails of the $\beta$1 and $\beta$3 subunits [10]. Disulphide bond arrangements and intersubunit contacts of proteolytic fragments of $\alpha$IIb$\beta$3 have been proposed [11]. These models relate to biophysical and electron microscopy data [12]. Integrins are subject to disulphide bond rearrangements and alternative splicing [10].

## Binding of integrins to their ligands

Integrins generally recognize ligand amino acid sequences which contain a key acidic residue that is essential for receptor binding. An example is the Arg-Gly-Asp (RGD) peptide motif which is present in a number of integrin ligands. Studies of the three-dimensional structure of several unrelated RGD-containing ligands showed structural and topological similarities in the RGD motif [13]. The RGD-binding motif of the type III FN repeat is presented in a flexible structure between two $\beta$ loops. Additional discontinuous regions of this protein may provide secondary binding sites [14, 15].

Divalent cations play a role in regulating integrin binding to the majority of ligands [7, 16]. Integrin-mediated cell adhesion as well as ligand binding to purified receptor is dependent on millimolar concentrations of calcium [17–19]. A relationship between cation, ligand and integrin was demonstrated in studies with $\alpha$IIb$\beta$3 utilizing the mAb PMI-I [19, 20]. PMI-I binds minimally to an epitope on the $\alpha$IIb subunit in the presence of physiological levels of cations and binds maximally upon chelation of cations with EDTA. Direct evidence for integrin-cation interactions comes from affinity-labelling experiments using cobalt ions and the integrin $\alpha$v$\beta$3 [21]. An irreversible linkage between $Co^{3+}$ and $\alpha$v$\beta$3 was demonstrated. The $Co^{2+}$ ion also supported ligand binding to $\alpha$v$\beta$3 in a similar manner to other divalent cations. When cobalt ions were irreversibly bound to the receptor by oxidation ($Co^{3+}$), $\alpha$v$\beta$3 was no longer able to interact with vitronectin. A recombinant fragment of $\alpha$IIb containing the four putative cation-binding sites described earlier bound fibrinogen optimally when all four sites had bound cation [22]. In general, the divalent cation $Ca^{2+}$ plays either a facilitating or inhibitory function on integrin binding depending on the integrin-ligand interaction in question.

Ligand-binding domains in the $\alpha$ subunit have been found in or around cation-binding sites [23]. Ligand mimetic peptides can be cross-linked to the N-terminal of the $\alpha$IIb [24] and $\alpha$v [25] subunits. A critical site for divalent cation binding at aa 296–306 in the $\alpha$IIb subunit has been identified [24], and peptides from this region inhibit fibrinogen binding to $\alpha$IIb$\beta$3 [26]. A highly conserved 72-aa sequence in the $\beta$3 chain (D109-E171) has been implicated in $\beta$3 chain ligand recognition and can be chemically cross-linked to bound peptide ligand [27]. Point mutations and monoclonal antibody (mAb) binding within D109-E171 can inhibit ligand binding [27–29]. Divalent metal ions are involved in ligand binding mediated by D109-E171. The naturally occurring mutation $\beta$3 (D119Y) altered the conformation of $\alpha$IIb$\beta$3 in a way that suggests loss of bound divalent cation [19]. Binding of a luminescent calcium analogue to a synthetic peptide corresponding to $\beta$3 118–131 was reduced by the substitution of D119 by alanine [30]. Alanine substitutions at D119 also reduced macromolecular ligand binding [31]. The cluster of D119, S121 and S123 is highly conserved among the $\beta$ integrin subunits. A peptide representative of $\beta$3 (211–222) bound competitively with $\alpha$IIb$\beta$3 to fibrinogen [32]. In addition, $\beta$3 (211–222)-specific mAbs inhibited fibrinogen binding to purified $\alpha$IIb$\beta$3 [32], and mutations in this region ($\beta$3 R214) blocked ligand binding [33] and caused instability of the $\alpha$IIb$\beta$3 heterodimer [34].

## Affinity modulation of integrins

Cells can rapidly change integrin function by altering the binding affinity of integrin for ligand [35, 36]. Inte-

grins normally exist in a low-affinity state [37, 38] and cannot bind to ligand without an energy-dependent activation process termed 'inside-out' signalling. Integrins of the $\beta 1$, $\beta 2$ and $\beta 3$ integrin families have all been shown to undergo activation in order to bind ligand [39]. It has been proposed that inside-out signalling is mediated by the cytoplasmic domains in response to intracellular signalling events [39, 40]. Recent work has implicated intracellular proteins such as calreticulin, serine/threonine kinases and members of the small GTPase family such as R-Ras [41] and RhoA [42]. The $\alpha$IIb$\beta 3$ receptor expressed on human platelets is constitutively in a low-affinity state and cannot bind soluble fibrinogen or von Willebrand factor (vWF) until it is activated via signal transduction mechanisms into a high-affinity ligand binding conformation. $\alpha$IIb$\beta 3$ activation occurs through the generation or release of soluble agonists such as thrombin, thromboxane A2, adenosine diphosphate (ADP) or serotonin [43]. These agents do not act directly on $\alpha$IIb$\beta 3$ but bind to platelet receptors which lead to the engagement of classical signal transduction pathways. G proteins and tyrosine kinases usually transduce these signals which result in the activation of phospholipase C (PLC), changes in cytosolic calcium and activation of cellular protein kinases. Inhibitors of these signal transduction pathways can inhibit agonist-induced ligand affinity of integrins.

In response to vWF or fibrinogen binding to $\alpha$IIb$\beta 3$, additional conformational changes occur in the activated integrin that can be measured by a specialized class of mAbs called anti-LIBS (ligand-induced binding site). Anti-LIBS such as D3 [44] and others [20] have been used to measure ligand binding to $\alpha$IIb$\beta 3$. In addition, these mAbs have been used to activate $\alpha$IIb$\beta 3$ directly on platelets and in transfected cells as well as to alter $\alpha$IIb$\beta 3$-mediated functions [45]. Certain anti-LIBS inhibit clot retraction, full-scale platelet aggregation or reversible aggregation presumably by interrupting outside-in signalling events that occur upon receptor occupancy [46]. The exact function that LIBS have in regulation of integrin high-affinity states is still under investigation. It has been proposed that integrin affinity states may be ultimately regulated by the cytoplasmic regions of the integrin. Two key regions have been identified. The first is a highly conserved NPXY motif located in the cytoplasmic domains of $\beta 1$–$\beta 7$, excluding $\beta 4$ [47]. Point mutations in this motif abolished $\beta 1$ and $\beta 3$ integrin activation [47]. The second region consists of a highly conserved membrane proximal sequence, KLLxxxxD, where x represents any amino acid. Deletion of (Leu717-Asp723) within this region in $\beta 3$ integrins results in constitutive activation [40, 47]. In addition, the truncation of a conserved membrane proximal motif (KXGFFKR) in the $\alpha$IIb cytoplasmic domain resulted in constitutive integrin activation [39, 40]. Point mutations within this region have revealed that two phenylalaline residues and the terminal arginine residue are mandatory in integrin affinity regulation [47]. The terminal aspartic acid residue in $\beta$ cytoplasmic tails is also necessary for regulation of integrin activation [47]. The terminal residues of the $\alpha$ and $\beta$ cytoplasmic domains may form a salt bridge which stabilizes an inactive conformation, as point mutations at these sites result in constitutive integrin activation [47].

The calcium-binding protein calreticulin interacts with a KXGFFKR motif in the cytoplasmic tails of $\alpha$ subunits [48]. $\alpha 2\beta 1$/calreticulin complexes were only detectable during integrin activation, suggesting a role for this protein in affinity modulation [49]. Anti-calreticulin antibodies specifically inhibited the activation of $\alpha 2\beta 1$ in Jurkat cells [49]. Calreticulin binding to the KXGFFKR motif could sterically disrupt the salt bridge between the integrin cytoplasmic termini, thus stabilizing the active conformation state. Regulation mechanisms mediating calreticulin binding could involve either the phosphorylation state of calreticulin or the integrins themselves [50]. Some studies have shown evidence of phosphorylation of integrin cytoplasmic domains. There are reports that the $\beta 3$ cytoplasmic tail of $\alpha$IIb$\beta 3$ is phosphorylated predominantly on serine and threonine residues on platelet activation [51], and phosphorylation of these sites may regulate exposure of LIBS [52]. However, other groups have found an $\alpha$v-dependent phosphorylation of the $\beta 3$ cytoplasmic tail at Tyr747 in vivo after stimulation by specific ligand, antibody or manganese [53]. This phosphorylation was found to induce a specific association between $\alpha$v$\beta 3$ and Grb-2 [53]. In addition, phosphorylation at Tyr747 appears to be required for affinity modulation of $\alpha$v$\beta 3$ [54].

Other intracellular elements bind to the cytoplasmic domains of integrins to create an integrin-activating complex including talin [55] and $\alpha$-actinin [56]. These proteins are abundant in all cells, and integrin affinity modulation is often cell specific event dependent on the widely divergent cytoplasmic domain of a specific integrin [39, 40]. Competition studies performed in whole cells have demonstrated the dependence of integrin interaction with cytoplasmic elements. Introduction of isolated $\beta$-subunit cytoplasmic domains into cells induced a dose-dependent decrease in ligand binding affinity of integrins, suggesting that limiting amounts of an intracellular protein are required for integrin high-affinity states [57]. Constitutively expressed R-Ras conferred an ECM-adherent phenotype in suspension cells by affinity modulation of expressed integrins which was reduced by the expression of dominant negative R-Ras [41].

Ligand binding could also alter the conformation of the cytoplasmic domains to induce a number of the effects discussed. Displacement of divalent cations during ligand binding may trigger conformational changes across

the membrane [30]. $\beta 3$ endonexin is a novel $\beta 3$ specific intracellular protein [58] which has no known homology with any as yet identified proteins. The binding specificity of $\beta 3$-endonexin was demonstrated by point mutation of the $\beta 3$ cytoplasmic region leading to inhibition of $\beta 3$-endonexin binding. A NIKY amino acid motif at position 756–759 is critical for $\beta 3$-endonexin binding to the cytoplasmic tail of $\alpha$IIb$\beta 3$ [59]. Exchange of the NPKY motif at $\beta 1$ 772–775 for the NIKY motif endowed $\beta 1$ with the ability to bind to $\beta 3$ endonexin. A core 91 residue sequence of $\beta 3$ endonexin is sufficient for specific binding to the $\beta 3$ cytoplasmic domain [59]. In a transfected cell system, $\beta 3$ endonexin binding induced activation of $\alpha$IIb$\beta 3$ in an energy-dependent fashion, resulting in fibrinogen-dependent aggregation. The affinity modulation of $\alpha$IIb$\beta 3$ by $\beta 3$ endonexin was inhibited by either coexpression of monomeric $\beta 3$ cytoplasmic tails or by an activated form of H-Ras [60]. Recently, a novel calcium binding protein (CIB) has been found specifically associated with the cytoplasmic domain of $\alpha$IIb in platelets. CIB has significant homology with calmodulin and calcineurin B and may be a regulatory molecule for $\alpha$IIb$\beta 3$ [61]. Integrin-linked kinase (p59ILK) is a novel serine/threonine kinase which interacts with the cytoplasmic tail of $\beta 1$ integrins [62]. The gene encoding p59ILK has been mapped to human chromosome 11p15.5–p15.4. This chromosomal region is associated with both genomic imprinting and loss of heterozygosity in certain tumours [63]. Overexpression of p59ILK in epithelial cells dramatically stimulated FN matrix assembly [64]. p59ILK overexpressing epithelial cells readily formed tumours in nude mice despite forming an extensive FN matrix. Overexpression of p59ILK in rat epithelial cells promoted anchorage-independent cell cycle progression [65]. Further investigation of the nature and regulation of p59ILK will be a key to the clarification of the role of integrins in tumerogenicity. Cytohesin-1 is a novel $\beta 2$ cytoplasmic binding protein [66]. Cytohesin-1 contains a pleckstrin homology domain and shows homology to the yeast SEC7 gene product which is involved in protein transport. The overexpression of cytohesin-1 induced $\alpha$L$\beta 2$-mediated adhesion of Jurkat cells to ICAM-1. This effect appeared to be mediated by the SEC7 domain. The expression of the isolated cytohesin-1 pleckstrin homology domain inhibited the adhesion of Jurkat cells to ICAM-1 [66]. It is evident that many proteins have been recently identified that interact with integrin cytoplasmic domains. Further studies will identify which of these proteins modulates integrin ligation in vivo.

## The role of focal adhesion kinase in integrin signalling

The involvement of focal adhesion kinase (FAK) in integrin signalling cascades is supported by elevated FAK phosphotyrosine levels and enzymatic activity upon ligand-integrin interactions. Integrin-mediated adhesion to ECM proteins regulates FAK phosphorylation, leading to the formation of the FAK signalling complex. In fibroblasts, tyrosine phosphorylation of FAK requires cells to adhere to ECM and is reduced in cell suspension [67]. FAK is a highly conserved protein which can be detected in the majority of cell types. The major structural features of FAK include a central catalytic domain flanked by large NH2 and C-terminals of about 400 amino acids each which are both involved in integrin signalling (fig. 1). FAK does not contain SH2 or SH3 domains which mediate protein interactions or a myristylation site that anchors proteins to membranes. The NH2 domain may interact with integrin cytoplasmic domains, providing a means for FAK activation upon integrin clustering [68]. The C-terminal flanking region contains two proline-rich segments (PR1 and PR2) involved in protein interactions. A portion of the C-terminal of FAK (150 residues) is required for localization to focal adhesions, suggesting that this sequence interacts with one or more focal adhesion proteins [69]. This region is known as the focal adhesion targeting sequence (FAT). The C-terminal domain also binds the focal adhesion protein, paxillin [70].

Alternative splicing of the *fak* gene leads to the autonomous expression of the C-terminal portion of FAK [71]. This molecule is called FAK-related non-kinase (FRNK). As FRNK lacks the central region of the whole FAK structure, it possesses no catalytic activity. FRNK does have the FAT sequence and can localize to focal adhesion sites. FRNK may act as a negative regulator of FAK activity [72], as overexpression of FRNK inhibited tyrosine phosphorylation of FAK and delayed cell spreading on FN [72]. Serine phosphoryla-

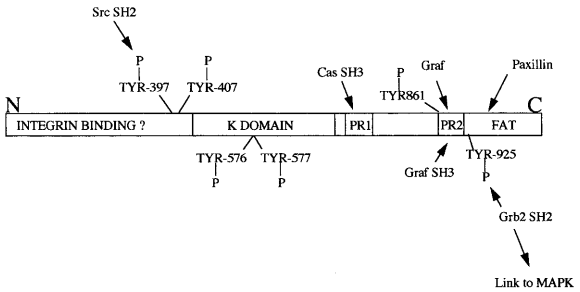


Figure 1. FAK consists of a kinase (K) domain surrounded by N- and C-terminal regions. There are six tyrosine phosphorylation sites as indicated. Tyr-397 is the autophosphorylation site and the binding site of Src. The C-terminal domain contains the FAT sequence involved in targeting FAK to focal adhesion sites. Two proline-rich domains (PR1, PR2) facilitate the binding of SH3 domain-containing proteins such as Cas and/or Graf.

tion of FRNK decreases the electrophoretic mobility of the protein from 41 kilodaltons (kDa) to 43 kDa [71]. Two major sites of serine phosphorylation in FRNK are responsible for the formation of the 43-kDa isoform [73]. The phosphorylation of these sites does not regulate the inhibitory action of FRNK. FRNK cannot be the only regulator of FAK localization, as FRNK has not been found in all cells that express FAK [74]. FAK has six phosphorylation sites (Tyr 397, 407, 576, 577, 861, 925) [75, 76]. Autophosphorylation at site Tyr397 generates a high-affinty site for the binding of the Src family of nonreceptor kinases via an SH2 domain [77]. Both c-Src and Fyn have been detected in FAK immunoprecipitates [78]. The interaction of c-Src with FAK is modulated by integrin-mediated cell adhesion [75]. The events that link integrin-ligand clustering with the autophosphorylation of FAK are not clear. Src interaction with FAK causes the phosphorylation of Tyr 407, 576, 577 and 861, causing the maximal activation of FAK [76]. A conformational change in FAK may play a major role in its activation, as autophosphorylated FAK has been detected in quiescent cells. The interaction of Src with FAK Tyr397 may upregulate the activity of Src kinase [79] by exposure of the Src C terminal tail, mediating its dephosphorylation [80]. Csk inactivates Src by phosphorylating its C-terminal tyrosine [81], thus leading indirectly to a reduction in FAK activity by downregulation of FAK tyrosine phosphorylation. In addition, overexpressed Csk has been found in focal adhesion sites, suggesting that Csk may regulate the FAK signalling complex [82]. Src also contains an SH3 domain which could interact with the FAK-associated proteins paxillin or Cas or both. Cas has been immunoprecipitated with FAK and associates with FAK via the proline-rich domain flanking the C-terminal of FAK [83].

Cas is a proposed docking protein that coordinates the binding activities of multiple signalling proteins and associates with the SH2 domain of Crk [84]. Cas has been detected at focal adhesions and becomes phosphorylated in a manner very similar to FAK in response to integrin-mediated cell adhesion [85]. Gene knockout experiments have shown that adhesion-dependent phosphorylation of Cas is more likely to be mediated by Src than by FAK [86, 87]. The phosphorylation of Cas potentiates SH2-mediated interactions with other proteins. The SH2 domain of the focal adhesion site associated protein, tensin, binds to Cas in vitro [88]. Recent studies suggest that the FAK-Cas complex activates the extracellular signal-related kinases (Erks) [89]. Mutational studies have shown that the FAK-Cas complex is a mediator of FAK-promoted cell migration in an Erks-independent manner [90].

A number of targets for FAK have been proposed. Paxillin and tensin are potential FAK substrates [72]. FAK may not be the only mediator of paxillin and tensin phosphorylation, as these proteins have been found to be equally phosphorylated in FAK-deficient fibroblasts [74]. Recent characterization of integrin-mediated phosphorylation of paxillin showed that 96% of phosphorylation of paxillin occurred on serine residues [91]. It is interesting that studies have demonstrated that serine phosphorylation as well as tyrosine can mediate protein-protein interactions [92]. Paxillin, able to bind both Src and Csk, could also act as a regulator of the FAK complex [93]. The formation of the FAK complex in platelets could be regulated by the cleavage of FAK by calpain, causing downregulation of autokinase activity [94]. Graf (GTPase regulator associated with FAK) binds to the second proline-rich domain of FAK [95]. Graf is thought to stimulate the GTPase activity of cdc42 and Rho, which are proposed regulators of filopodial extension and stress fibre/focal adhesion assembly [96].

## Stimulators and downstream effectors of the FAK-signalling complex

The FAK-signalling complex and associated signalling pathways are summarized in figure 2. The mechanism by which FAK activation is mediated by integrins is unclear, but disruption of F-actin filaments by cytochalasin D can block FAK activation and phosphorylation induced by integrin binding and other agents [97]. Bioactive peptides such as endothelin and bombesin and the serum factor lysophosphatic acid (LPA) are able to activate FAK by interaction with their specific receptors. [98]. Growth factors such platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) trigger cytoskeletal organization. The action of these factors is thought to be mediated by the small GTP-binding proteins such as Rho. Inhibition of RhoA activation by C3 exoenzyme causes downregulation of both FAK activation and focal adhesion formation in LPA-stimulated cells [99]. This suggests that RhoA activation may be an upstream event of FAK activation and may act as a regulator of FAK activity. As FAK is activated by stimuli apart from integrin-ligand interactions, it is a challenging task to dissect the distinct relationship between integrin function and FAK-mediated events.

The dissection of integrin-mediated FAK complex formation and signalling pathways was addressed by Miyamoto et al. [100, 101]. $\beta 1$ integrins on fibroblasts clustered using beads coated with anti-$\beta 1$ antibodies under conventional conditions did not inhibit integrin function and did not activate FAK. Under these conditions, FAK was found recruited at the bead site, suggesting that FAK activation is not mandatory for focal adhesion recruitment. Src kinases and Ras/mitogen-

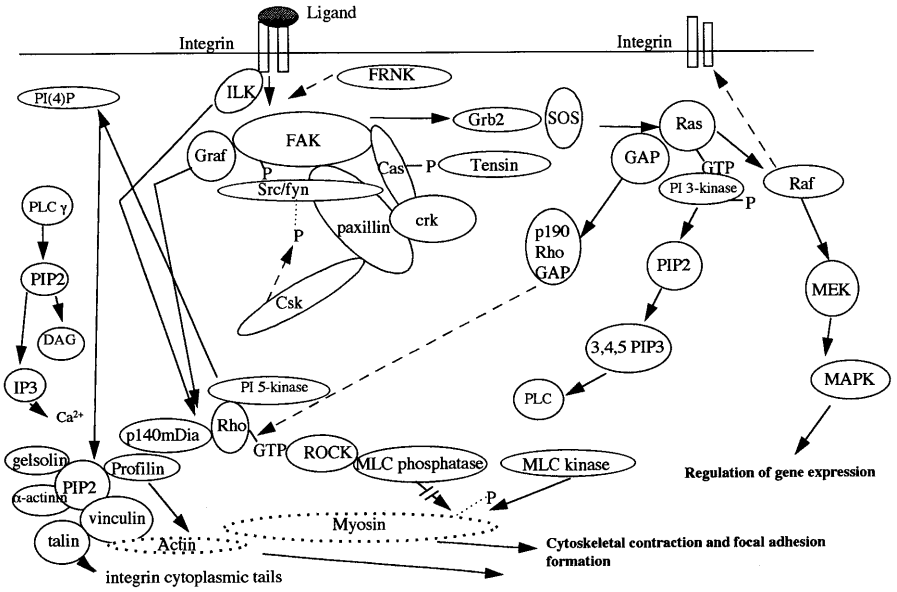


Figure 2. Integrin-ligand interaction promotes the autophosphorylation of FAK at Tyr-397, facilitating Src-Fyn binding, resulting in phosphorylation and activation of FAK. The FAK-Src complex phosphorylates sites on Cas and possibly paxillin and tensin. Other proteins are then recruited to the complex, including the SH2-containing protein Grb2, which links FAK activity to the MAPK pathway. Csk binds to paxillin and can inactivate Src by phosphorylation of the Src C-terminal, thus downregulating the activity of the FAK complex. Negative regulation of FAK may also occur via FRNK. Both the FAK-associated protein Graf and ILK may activate Rho, which in the GTP-bound form is able to interact with ROCK and p140mDia to cause cytoskeletal contraction and focal adhesion formation via the recruitment of p140mDia and the downregulation of MLC phosphatase. $PIP_2$, generated by the action of PI 5-kinase, complexes with $\alpha$-actinin and vinculin, generating binding sites on vinculin for actin and talin. $PIP_2$ also binds to gelsolin, causing uncapping of actin filaments. PLC $\gamma$ hydrolysis of $PIP_2$ leads to the formation of DAG and IP3, which in turn activate PLC subtypes and promote the release of intracellular calcium stores. $PIP_2$ can also undergo phosphorylation by PI 3-kinase to generate 3,4,5,-$PIP_3$. PI 3-kinase is associated with Ras, phosphorylated and associated with FAK. H-Ras and its effector kinase Raf1 are proposed inhibitors of integrin activation. Due to the complexity of the pathways described, not all associations and relationships are illustrated. Solid arrows represent association and/or activation. Broken arrows represent inhibitory effects.

activated protein kinase (MAPK) cascade members were not found at bead sites. When these experiments were repeated using activating $\beta$1 mAbs or FN, many focal adhesion site-associated molecules were found at the bead site. Similar studies reported the localization of Rho and Rho GAP member p190-B to FN-coated bead sites [102]. These studies show first that FAK activation is required for the recruitment of Src kinases and activation of the RAS/MAPK cascade, and second that FAK activation is not mandatory for its localization to focal adhesion sites.

Further links between integrin activation, FAK and the MAPK pathway have been reported. The MAPK pathway can be activated in response to growth factor stimulation and integrin-ligand binding. Studies with NIH 3T3 cells have shown that both PDGF and ECM can induce tyrosine phosphorylation of p44MAPK but by distinct mechanisms [103]. Adhesion-induced p44MAPK activation appears to be associated with cell spreading, and its activation is blocked by cellular treatment with cytochalasin D [103]. This suggests a potential role for FAK in the regulation of MAPK and a requirement for cytoskeletal organization, as MAPK activity may be related to cell shape change [103]. In contrast, PDGF activation of p44MAPK is not related to cell shape change and therefore may not be sensitive to cytochalasin D. Phosphorylation on FAK Tyr925 generates a Grb2-binding site mediating interaction with the Ras/MAPK pathway [104]. Grb2 is able to couple to the guanine nucleotide exchanger SOS for Ras, providing a link with the MAPK pathway. The GTPase cdc42 interacts with an intracellular protein called WASP (the product of Wilcott-Aldrich syndrome locus) [105]. Studies in Jurkat cells have demonstrated that overexpression of WASP leads to large cytoplasmic clusters of polymerized actin that is colocalized with WASP [106]. The coexpression of dominant negative cdc42 demonstrated that WASP is associated with the cytoskeleton and its activity is modulated by cdc42 [106]. WASP, however, is only expressed in haematopoietic cells, suggesting the presence of WASP isoforms in other cell types.

In addition to the promotion of cytoskeletal events, MAPK elements appear to have a moderating effect on

integrin-signalling pathways. For example, H-Ras and its effector kinase Raf-1 act as negative regulators of integrin activation [107]. This protein synthesis-independent event correlated with Erk MAPK pathway activation and was independent of integrin phosphorylation. These findings may explain altered integrin-mediated functions in transformed cells. Integrin-mediated activation of the MAPK pathway in fibroblasts can occur independently of FAK, suggesting the existence of as yet uncharacterized integrin-signalling pathway [108].

## The Rho pathway and regulation of the cytoskeleton

The small GTPase Rho acts by shuttling between its active GTP bound form to an inactive GDP form. The use of inhibitors and activators of Rho has been useful in defining the downstream effectors of Rho and for identifying the biochemical changes induced by Rho activation. The role of Rho in cytoskeletal assembly has been examined by either ADP ribosylation, C3 exoenzyme inhibition or by microinjection of active forms of this protein into cultured cells. The formation of bundles of actin filaments can be inhibited or promoted as described above, and thus Rho has been implicated in integrin-mediated focal adhesion formation [109]. Integrin clustering, however, is not mandatory for Rho-mediated cell adhesion [96]. The clustering of integrins and actin filaments may be mediated by myosin-based contractibility [110]. Rho plays a direct role in muscle contractibility via a calcium-dependent mechanism involving the phosphorylation of myosin light chains, a process is known as 'GTP-induced increase in calcium sensitivity' [111]. Several protein and lipid kinases are mobilized downstream of Rho. These include PI 3-kinase [112] and PI 5-kinase [113]. Experimental studies using techniques such as affinity chromatography and yeast two-hybrid screening have identified two potential direct Rho targets [114]. The Rho-binding protein p160ROCK is a 160-kDa serine threonine/kinase that contains a kinase domain in the N-terminus and a pleckstrin homology domain split by a cysteine-rich zinc finger in the C-terminus [115]. The pleckstrin homology domain could participate in membrane localization, and thus the binding of Rho to p160ROCK along with other proteins could facilitate the localization of this complex to a cellular target site. Part of p160ROCK has been detected in cytoskeletal fractions of thrombin-activated platelets [116].

p160ROCK has high homology to mytonic dystrophy kinase (MDPK) at its N-terminal. MDPK is a causative agent in myotonic dystrophy and is localized in dense plaques of smooth muscle which are structures comparable with focal adhesion sites [115]. Thus, p160ROCK has been implicated in Rho-mediated cell adhesion and focal contact formation. Microinjection of complementary DNA (cDNA) encoding ROK-$\alpha$ (a homologue of p160ROCK) with a truncated C-terminal domain into HeLa cells induced focal adhesion and actin bundle formation [117]. The myosin-binding subunit (MBS) of myosin light chain (MLC) phosphatase is a substrate for p164 Rok-K (a homologue of p160ROCK), and phosphorylation of MBS leads to a decrease in MLC phosphatase activity [118]. This leads to increased phosphorylation of MLC, increasing contractibility. This idea was supported by studies using the MLC-kinase inhibitor KT5926, which caused loss of focal adhesions and stress fibres in fibro-blasts [110, 119]. These studies concluded that the p160ROCK family of kinases were responsible for Rho-mediated enhancement of myosin contractibility.

Further investigations have shown that Rho enhancement of actin polymerization is mediated by an effector called p140mDia [120]. Studies on spreading cells have shown colocalization of p140mDia, GTP-Rho and profilin in the membrane lamellae [120]. Profilin is thought to sequester unpolymerized actin and promote actin polymerization [121]. A model of proposed action of Rho-mediator complexes has been proposed by Narumiya et al. [114], suggesting that p169ROCK, once activated by Rho-GTP, is causative in integrin binding to ECM proteins. P140mDia then acts in the membrane region, recruiting profilin which induces actin polymerization. Integrins can then attach to the cytoskeleton via actin-binding cytoskeletal proteins. Actin-associated myosin is then induced to contract by p160ROCK, which mediates the formation of focal adhesions along with ligand-occupied integrin receptors. Downregulation of Rho activity may occur by the activation of p190 Rho-GAP [102]. This protein is activated by association with the Ras-associated protein GAP, which can complex with p190 Rho-GAP via its amino terminal domain. This association causes the activation of p190Rho-GAP, inactivation of Rho and cessation of cytoskeletal assembly [122]. This phenomenon provides links between the MAPK pathway and Rho-mediated pathways, coordinating the signals received from integrins and other cell receptors such as those for growth factors. Further studies will dissect the functions and role of Rho and its associated molecules in integrin focal adhesion formation and cell adhesion functions.

## The role of phosphatidylinositol turnover in integrin-mediated signalling

A rapid increase in the level of phosphatidylinositol phosphate ($PIP_2$) is produced on integrin-mediated cell adhesion [123]. $PIP_2$ is generated by the phosphorylation of phosphatidylinositol 4-phosphate via phos-

phatidylinositol 5-kinase (PI 5-kinase). PI 5-kinase complexes with Rho in a GTP-independent manner leading to its activation [113, 124]. $PIP_2$ binds to gelsolin, and profilin promoting actin filament polymerization and bundle formation [125–127]. The dissolution of gelsolin from actin filament barbed ends by $PIP_2$ may promote actin cytoskeleton assembly [128]. $PIP_2$ also binds to $\alpha$-actinin and vinculin [129, 130]. The interaction of $PIP_2$ with vinculin induces a conformational change in vinculin, leading to higher affinity binding with talin and actin [131]. Vinculin thus provides a direct link between integrin cytoplasmic tails via talin and the cytoskeleton via actin. Hydrolysis of $PIP_2$ by phospholipase C $\gamma$ (PLC $\gamma$) leads to the production of $IP_3$ and diacylglycerol (DAG). $IP_3$ induces the release of cytosolic calcium from intracellular stores, and DAG activates PLC $\alpha$, $\beta$ and $\gamma$ [132]. $PIP_2$ can also act as a substrate for phosphatidylinositol 3-kinase (PI 3-kinase), giving rise to phosphatidylinositol 3,4,5-triphosphate (3,4,5-$PIP_3$) [133, 134]. PI 3-kinase is translocated to the membrane cytoskeleton on platelet activation [135], is FAK-associated and can be phosphorylated by FAK in vitro [136]. Wortmannin, a specific inhibitor of PI 3-kinase, blocks MAPK activation by growth factors [137]. This effect may be by direct interaction between PI 3-kinase and Ras, resulting in PI 3-kinase activation [138]. As elements of this pathway interact with FAK, Rho and Ras, phosphatidylinositol turnover may coordinate cellular responses generated from integrin-mediated FAK activation, cytoskeletal assembly and regulation of gene expression.

## Integrin-mediated calcium signalling

The engagement of some integrins leads to an increase in the levels of intracellular calcium. In general, RGD-binding integrins appear to play a role in calcium signalling. RGD-containing peptides immobilized on beads or coverslips are able to induce a rise in $[Ca^{2+}]i$ in several clones of kidney epithelial cells probably via RGD binding to $\alpha v\beta 3$ and/or $\alpha v\beta 5$ [139]. There are two ways in which $[Ca^{2+}]i$ can be elevated, one via IP3-sensitive intracellular stores and a second via influx through plasma membrane channels. Melanoma cells that bind soluble type IV collagen can stimulate a pertussis toxin-insensitive, IP3-independent release of calcium stores that is not mediated by G-protein coupling that generates IP3 [140]. A large transient increase in $[Ca^{2+}]i$ occurs in platelets during aggregation. This effect closely correlates with affinity modulation of $\alpha IIb\beta 3$ [141].

The association of catalytic signalling molecules with the platelet cytoskeleton is reversible and dependent on the level of intracellular calcium [142]. The clamping of $[Ca^{2+}]i$ in CHO cells perforated with streptolysin O increased levels of adhesion to FN on elevation of $[Ca^{2+}]i$. Increased $[Ca^{2+}]i$ is required for integrin-mediated neutrophil motility [143]. $Ca^{2+}$ buffering leads to an accumulation of bound $\alpha v\beta 3$ at the rear of the cell, causing immobilization to the substrate. It seems that $Ca^{2+}$ is required for $\alpha v\beta 3$ release at the rear of the cell, allowing recycling at the front of the cell, facilitating motility. This phenomenon may be dependent on the calcineurin [144]. The leading edge of a migrating neutrophil may mediate calcium signalling, stabilizing adhesion at this site, while activated calcineurin causes dissociation of $\alpha v\beta 3$/ligand at the rear of the cell [145]. Calcium signalling can be mediated by integrin-dependent or -independent means and could in fact be the 'fine-tuning' mechanism in cell adhesion and migration activity.

## Integrin association with cell surface molecules

The characterization of integrin-associated molecules at the cell surface has been the focus of intense study over the past few years. This has resulted in numerous reports of noncovalently associated integrin complexes. The tetraspanin or transmembrane 4 family (TM4F) has been the focus of many of these studies. The TM4F proteins have four characteristic, highly conserved hydrophobic regions which are presumed to span the cell membrane, resulting in two extracellular loops [146]. Numerous studies have demonstrated a link between TM4F members and cell adhesion, motility and proliferation. At least three different TM4F members may modulate tumour cell metastasis [147, 148]. Many of these studies have involved the pertubation of TM4F proteins with specific mAbs, leading to modulation of cell behaviour. For example, anti-CD9 mAbs induce fibrinogen-mediated platelet aggregation [149, 150]. The effect of anti-CD9 mAbs on platelets was first thought to be solely due to secondary interaction with the platelet Fc receptor. However, recent studies have shown that CD9 is able to elicit platelet stimulation in the absence of Fc interaction [151]. Various studies have shown evidence of a noncovalent association between members of the TM4F proteins and integrins on a number of cell types [146]. TM4F members can also form complexes with each other in the cell membrane [146].

As TM4 protein expression has an effect on integrin-mediated cell functions such as adhesion and migration, it was proposed that TM4F proteins are able to modulate integrin function. Analysis of degranulated platelets has shown that modulation of CD9 by anti-CD9 mAbs gives rise to increased serine/threonine phosphorylation of pleckstrin and MLC probably via PKC and MLCK

activation [151]. Perturbation of CD9 leads to a similar pattern of phosphorylated proteins as seen on $\alpha$IIb$\beta$3-fibrinogen binding. Tyrosine phosphorylation on stimulation of CD9 was not affected by the Rho inhibitor endotoxin C, thus demonstrating an independent pathway for signalling via CD9. Inhibition of PKC inhibited the level of tyrosine-phosphorylated proteins in CD9 perturbed platelets. CD9 is able to transduce similar signals to $\alpha$IIb$\beta$3 and may be involved in $\alpha$IIb$\beta$3 modulation via intracellular signalling pathways. Recent evidence suggests a novel link between PI 4-kinase, the TM4F proteins, CD63 and CD81, and $\alpha$3$\beta$1 [152]. This relationship was not detected with $\alpha$5$\beta$1, suggesting integrin subtype specificity and did not appear to be adhesion-dependent.

Recent studies have shown that CD9 binds to regions of the ECM protein FN (D. A. Wilkinson et al., unpublished observations). CD9 may not only modulate integrin function by intracellular pathways, but may also compete with integrins for binding FN. CHO cells transfected with CD9 have shown that CD9 regulates many cellular processes involving cell adhesion, spreading and FN matrix assembly (G. A. Cook et al., unpublished observations). This effect could be due to competion for FN binding with $\alpha$5$\beta$1 or by intracellular modulation of integrin function. Integrin-associated protein (IAP50) also associates with $\beta$3 integrins [153]. Anti-IAP mAbs do not stimulate platelets [154], but IAP50 interaction with the cell-binding domain of thrombospondin (TS-1) causes a dramatic platelet response [155]. An IAP50 agonist peptide (4N1K) derived from the CBD of TS-1 causes $\alpha$IIb$\beta$3 activation and stimulates platelet aggregation [156]. 4N1K binding selectively induced the phosphorylation of Lyn and Syk and their association with FAK. The phosphorylation of Syk was blocked by pertussin toxin, implicating a role for hete-rotrimeric G proteins. A complex between IAP50 and c-Src has been detected by immunoprecipitation [156]. CD9, $\alpha$IIb$\beta$3 and IAP50 have been detected in complex on platelets, and this association may be dependent on platelet activation (C. M. Longhurst et al., unpublished observations). The role of integrin membrane-associated complexes in cell function and their role in integrin-mediated cell signalling is yet to be fully appreciated.

## Conclusion

Integrins play a major role in the coordination of cellular processes and provide a unique means of bidirectional communication between the inside and outside cellular environments. The cellular responses to integrin activities are particular to various cell types, and thus the general pathways defined to date do not allow for individual integrin-mediated cell responses. Recent studies with integrin cytoplasmic associated molecules such as $\beta$3-endonexin have shown some integrin subtype specificity, but the role of these proteins is yet to be defined. The role of serine/threonine kinases in integrin-mediated signalling pathways needs to be defined, particularly in light of recent studies of paxillin phosphorylation. The role of integrin-associated molecules such as the TM4F proteins in integrin-mediated signalling events also needs clarification. The dissection of integrin signalling pathways in vivo is further complicated by the cellular responses to agents such as growth factors, which can mediate similar intracellular responses as integrin-ligand interactions. Further advances in understanding these areas will provide the potential for therapeutic interventions in cancer therapy, wound healing, thrombosis and inflammatory responses.

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