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NEW EMBO MEMBER'S REVIEW

The integrin–actin connection, an eternal love affair

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Integrin receptors connect the extracellular matrix to the actin cytoskeleton. This interaction can be viewed as a cyclical liaison, which develops again and again at new adhesion sites only to cease at sites of de-adhesion. Recent work has demonstrated that multidomain proteins play crucial roles in the integrin–actin connection by providing a high degree of regulation adjusted to the needs of the cell. In this review we present several examples of this paradigm and with special emphasis on the ILK–PINCH–parvin complex, which amply demonstrates how structural and signalling functions are linked together.

Keywords: actin/focal adhesion/ILK–PINCH–parvin complex/integrin/integrin signalling

Introduction

The integrin family of cell adhesion molecules mediates cellular contacts to the extracellular matrix (ECM) or cell counter receptors, thereby regulating cell motility, cell polarity, cell growth and survival (Brakebusch *et al.*, 2002). The identification and characterization of central players in integrin signalling originated from biochemical, structural and genetic studies. These studies have revealed intimate interplay between integrin and growth factor/cytokine signalling as well as mutual functional dependence between integrins and the actin cytoskeleton (Brakebusch *et al.*, 2002).

Ligand binding to integrins leads to integrin clustering and recruitment of actin filaments and signalling proteins to the cytoplasmic domain of integrins (Hynes, 2002). These specialized, ECM attachment organelles and signalling centres are called focal complexes when they are still nascent and in the process of forming, or focal adhesions (FAs) when they have matured into larger complexes. The formation of cell adhesion complexes assures substrate adhesion as well as targeted location of actin filaments and signalling components, and hence is essential for establishing cell polarity, directed cell migration, and maintaining cell growth and survival.

Recent work has revealed that the integrin–actin cytoskeleton connection is highly dynamic and subject to many regulatory processes. In healing skin wounds for example, integrin-mediated cues promote the reorganization of the cytoskeleton of keratinocytes at the wound edge resulting in directed migration and wound closure. Loss of β1-integrins on keratinocytes leads to impaired as well as non-directed migration resulting in severely delayed re-epithelialization (Grose *et al.*, 2002). Furthermore, it has become clear that the interaction between integrins and the actin cytoskeleton is differentially regulated in different locations of the cell. At the leading edge of migrating cells, integrins bind the ECM, recruit the actin cytoskeleton and initiate local reorganization of the actin network, promoting different types of membrane protrusion. At the rear of the cell, integrins detach from the ECM, dissolve the link to the cytoskeleton and are, at least partially, recycled to the front of the cell (Ballestrem *et al.*, 2001; Laukaitis *et al.*, 2001).

Signalling pathways, which depend on localized integrin activation have also been reported. For example, at the leading edge of cells, integrin signalling dissociates complexes between GTP-bound Rho-GTPases and Rho-GDI to release active Cdc42 and Rac1, resulting in membrane extension (Del Pozo *et al.*, 2002). In astrocytes, integrin binding activates Src-like kinases, which induce the formation of a complex of Cdc42, mPar6 and PKCζ (Etienne-Manneville and Hall, 2001). This complex phosphorylates and inactivates GSK-3β at the leading edge of the cell and thereby allows interaction of adenomatous polyposis coli (Apc) with the plus ends of microtubules. This is essential for the reorientation of the microtubular network and the directed movement of the cells (Etienne-Manneville and Hall, 2003). Finally, complexity is added by the fact that integrin-associated molecules are multifunctional. Integrin-linked actin binding proteins attach to signalling molecules and function as platforms, which brings kinases and substrates together. Integrin-bound signalling molecules, on the other hand, bind to actin binding proteins, enforcing the integrin–cytoskeleton connection. As it turns out for integrin-linked kinase (ILK), such adapter function might be even more important *in vivo* than the kinase function demonstrated *in vitro*.

In the present review, we will describe proteins that structurally or functionally link integrins with the actin cytoskeleton, emphasizing their multi-purpose nature (Figure 1).

Actin binding proteins as platforms for integrin-mediated signal transduction

Talin

Talin is a large protein of >2500 amino acids, consisting of an N-terminal head region of ~50 kDa and a large rod region of ~220 kDa, which contains primarily alanine-rich repeats. Via the Four point one, Ezrin, Radixin, Moesin (FERM) domain in the head, talin binds to integrin, focal adhesion kinase (FAK), phosphatidylinositol phosphate kinase type Iγ (PIPKIγ), Phosphatidylinositol (4,5) bisphosphate (PIP2), non-integrin transmembrane receptors (Horwitz *et al.*, 1986; Chen *et al.*, 1995; Borowsky and Hynes, 1998; Martel *et al.*, 2001; Di Paolo *et al.*, 2002;

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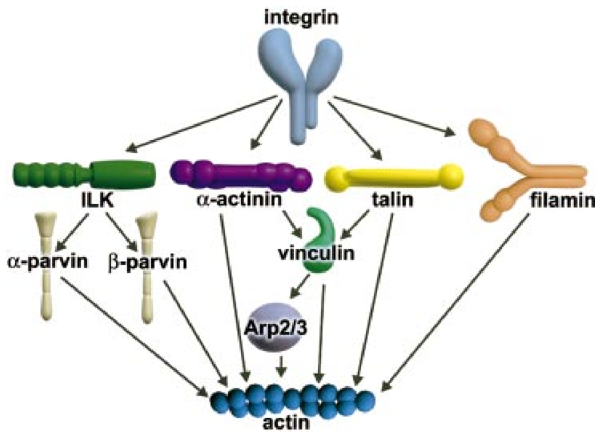

Fig. 1. Overview of different pathways by which integrin can link to the actin cytoskeleton. The molecules are not drawn to scale.

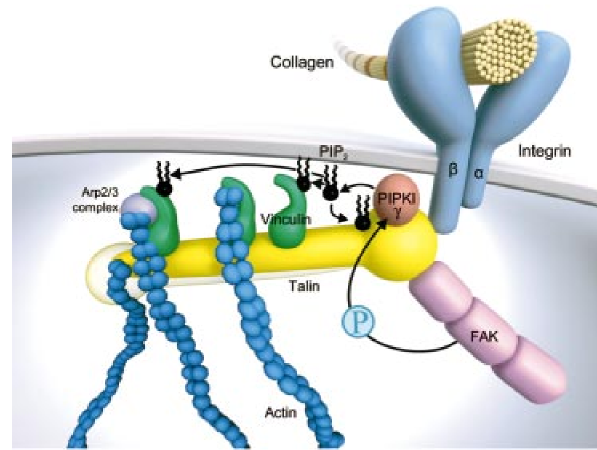

Fig. 2. Talin associates with PIPKIγ, which produces PIP2. PIP2 binds to talin, strengthening the interaction between talin and integrin. PIP2 also binds to vinculin, which then interacts with talin. PIP2 on vinculin is replaced by actin filaments. PIP2-associated vinculin can transiently bind to activated Arp2/3 complex, which nucleates actin polymerization. Talin is an antiparallel homodimer. For reasons of simplicity, binding partners of the second talin molecules are not shown. The molecules are not drawn to scale.

Ling *et al*., 2002) and weakly to actin. The rod domain contains a low affinity site for integrin (Yan *et al*., 2001), three vinculin binding sites and a major actin binding site at the C-terminus (Hemmings *et al*., 1996). Talin binding to integrin disrupts an intracellular salt bridge between the α and β-integrin subunit, leading to increased integrin affinity, which strengthens the interaction with the ECM (Vinogradova *et al*., 2002; Garcia-Alvarez *et al*., 2003).

In vivo, talin is important for the linkage of integrin clusters to the cytoskeleton. In talin-deficient flies, integrins still bind the ECM, but can neither aggregate into clusters nor connect to the cytoskeleton (Brown *et al*., 2002). These mutants develop a phenotype resembling that of flies lacking integrin βPS, characterized by a failure in germ band retraction and muscle detachment (Leptin *et al*., 1989; Roote and Zusman, 1995; Schöck and Perrimon, 2003), suggesting that integrin function is highly dependent on talin. However, talin is not essential for integrin signalling resulting in gene expression, since in the absence of zygotic talin integrin-mediated gene repression was still functional (Brown *et al*., 2002). Mammals have two highly similar isoforms of talin suggesting redundant functions. Mice lacking talin-1 form mesoderm, which fails to migrate (Monkley *et al*., 2000). Talin-1-deficient embryonic stem (ES) cells show reduced adhesion and spreading on collagen and laminin and are unable to assemble FAs or stress fibres (Priddle *et al*., 1998). After differentiation, however, vinculin-containing FAs form. Whether this is due to expression of talin-2 (Monkley *et al*., 2001) or alternative linker molecules can only be revealed by double knockouts of talin-1 and -2.

Binding of talin to integrin is increased by PIP2 (Martel *et al*., 2001). PIP2 also modulates the activity of other cytoskeletal proteins at the plasma membrane, promoting their attachment to the plasma membrane and actin filament assembly. Recent data show that talin is crucial for the localized production of PIP2 at newly engaged integrins. Upon integrin clustering, talin is recruited to focal complexes where it binds and activates the PIP2-producing enzyme PIPKIγ (Di Paolo *et al*., 2002; Ling *et al*., 2002). This leads to an increased local concentration of PIP2, which associates with talin and other proteins, facilitating the formation of FAs (Figure 2). This process is subject to regulation. First, interaction of talin with PIPKIγ is dependent on integrin and growth factor signalling (Ling *et al*., 2002). In suspended cells deprived of growth factors, talin does not associate with PIPKIγ. Secondly, tyrosine phosphorylation of PIPKIγ by FAK increases PIP2 production and the association of PIPKIγ with talin. Interestingly, high expression of PIPKIγ results in a loss of talin from FA and rounding up of the cells.

The association of talin with vinculin, a ubiquitous cytoskeletal protein found at cell–cell and cell–ECM contacts, is also regulated by PIP2. Binding of PIP2 to vinculin unfolds the inactive molecule and exposes binding sites for talin and α-actinin in the head domain and for VASP in the tail domain (Gilmore and Burridge, 1996; Hüttelmaier *et al*., 1999). Paxillin can bind to the tail in the absence of PIP2 (Steimle *et al*., 1999). The interaction with talin facilitates vinculin binding to actin (Gilmore and Burridge, 1996), which quite likely requires dissociation of PIP2 due to overlapping binding sites (Steimle *et al*., 1999). Vinculin can also induce the polymerization of actin monomers by recruiting the Arp2/3 complex, which can initiate actin nucleation (DeMali *et al*., 2002). This interaction is transient and requires both PIP2 binding to vinculin and activation of the Arp2/3 complex by Rac1. The Arp2/3 complex can nucleate new actin filaments or induce branching of existing filaments resulting in an actin network that pushes the plasma membrane forward. Fibroblasts that lack vinculin or express vinculin mutants unable to recruit the Arp2/3 complex show reduced adhesion, spreading and lamellipodia formation. Thus, vinculin couples newly engaged β1-integrins receptors to actin polymerization and membrane protrusion.

α-actinin

α-actinin connects actin fibrils to the cytoplasmic tail of transmembrane receptors such as integrins, cadherins and ICAMs. In addition, it crosslinks actin filaments to actin bundles and networks. The actin binding domain of

α-actinin is located at the N-terminus, whereas the C-terminus consists of one or two EF hands. α-actinin dimerizes in an antiparallel fashion via interaction of the central rod domains. Four isoforms are found in humans and mice. Of the two non-muscle isoforms, α-actinin-1 is ubiquitously expressed and located primarily in FAs (Pavalko and Burridge, 1991), whereas α-actinin-4 is not found at FA or cell–cell junctions, but is present in certain types of membrane ruffles (Honda *et al*., 1998). α-actinin-4 sems to play a role in endocytosis and tumour cell motility. Muscle-specific α-actinin-2 and -3 crosslink actin filaments in the region of Z discs in striated muscles (Beggs *et al*., 1992).

Several cytoplasmic proteins interact with actinin including vinculin, zyxin, extracellular signal-regulated kinase 1/2 (Erk1/2), mitogen-activated protein/extracellular signal-regulated kinase kinase 1 (MEKK1), protein kinase N (PKN) and the p85 subunit of phosphatidylinositol-3 kinase (PI3-K), suggesting that α-actinin serves as an important scaffolding protein (Wachsstock *et al*., 1987; Crawford and Beckerle, 1991; Shibasaki *et al*., 1994; Mukai *et al*., 1997; Christerson *et al*., 1999; Leinweber *et al*., 1999). These protein–protein interactions can be modulated. PIP2 binding increases the interaction with actin, PKN and PI3-K (Fukami *et al*., 1994; Shibasaki *et al*., 1994; Mukai *et al*., 1997). PIP3, on the other hand, decreases the binding of α-actinin to integrins (Greenwood *et al*., 2000). Calcium binding to the EF-hands of non-muscle α-actinin reduces the interaction with actin filaments (Witke *et al*., 1993). Finally, FAK-dependent phosphorylation of α-actinin decreases its association with actin (Izaguirre *et al*., 2001).

In *Drosophila*, α-actinin-null mutations are lethal and are characterized by defects in muscle structure and function (Fyrberg *et al*., 1998). No α-actinin-deficient mice have been reported, but recent experiments using chromophore-assissted laser inactivation of EGFP-coupled α-actinin in Swiss 3T3 cells demonstrated that α-actinin is essential for the integrin–cytoskeleton linkage in FA (Rajfur *et al*., 2002). The integrity of the FAs themselves was not affected by the laser inactivation.

Filamin

Filamins are dimeric proteins with a head domain containing the actin binding site and a rod domain consisting of 4–24 repetitive units, which resemble the immunoglobulin fold, each consisting of ~100 amino acids (Stossel *et al*., 2001; van der Flier and Sonnenberg, 2001). They are non-covalently associated at the C-terminus. In humans, three filamin genes (filamins A, B and C) have been identified, which, by alternative splicing, give rise to several isoforms. While filamins A and B are nearly ubiquitously expressed, filamin-C is found primarily in heart and skeletal muscle.

Filamins link actin filaments in orthogonal networks or parallel bundles. *In vitro*, the type of actin organization depends on the ratio of filamin to actin. Via different repeat units in the rod, filamins associate with transmembrane proteins such as integrins (Sharma *et al*., 1995; Loo *et al*., 1998), signalling molecules such as Rho-GTPases (Ohta *et al*., 1999), MEKK (Marti *et al*., 1997) and guanine nucleotide exchange factors (GEFs) (Bellanger *et al*., 2000), and other proteins.

In a melanoma cell line, filamin expression increased the surface level of β1-integrin as shown by FACS analysis (Meyer *et al*., 1998). However, this decreased turnover or increased membrane insertion of integrin seems not to be due to direct interaction with filamin, since it could not be coprecipitated with β1-integrin. Beside the regulation of the surface levels of integrins, filamin controls integrin function. In CHO cells, increased filamin binding to integrins by the introduction of point mutations into the cytoplasmic domain of β1 and β7-integrins reduced membrane protrusion, cell polarization and consequently cell migration (Calderwood *et al*., 2001). Filamin can induce reorganization of the cytoskeleton by different pathways. First, filamin might stimulate changes of the cytoskeleton by providing a platform for simultaneous binding of activators and effectors, thus facilitating signalling. Trio, a GEF specifically activating Rac1, RhoA and RhoG, can bind to filamin and catalyse the transition of filamin-bound Rac1-GDP to Rac1-GTP (Bellanger *et al*., 2000). Rac1-GTP could then activate the filamin-associated effector molecule PAK1. Alternatively, filamins can activate PAK1 independently of Rho-GTPases. Treatment of MCF7 breast cancer cells with physiological signalling molecules such as heregulin and sphingosin leads to direct association of filamin with PAK1 (Vadlamudi *et al*., 2002). This results in stimulation of PAK1 kinase activity and phosphorylation of filamin by PAK1 at different serine residues near the C-terminus. The formation of a filamin–PAK1 complex and the PAK1-mediated phosphorylation of filamin is important *in vivo*, since the membrane ruffling activity of PAK1 in M2 melanoma cells is dependent on filamin expression and the phosphorylation of Ser2152 of filamin.

Deletion of the filamin orthologue in *Dictyostelium*, called gelation factor, resulted in impaired photo- and thermosensory responses (Fisher *et al*., 1997). Whether a general defect of the cytoskeleton occurs is unclear. Filamin and α-actinin seem to have redundant functions. While deletion of α-actinin in *Dictyostelium* leads only to a minor phenotype, double deletion of α-actinin and gelation factor results in reduced cell size, diminished proliferation, impaired movement and partially defective cytokinesis (Witke *et al*., 1992; Rivero *et al*., 1999). Fruit flies deficient for filamin showed female sterility and impaired actin organization of the ring canal structures (Li *et al*., 1999; Sokol and Cooley, 1999). Inactivation of the X-chromosomal filamin-A gene in humans is embryonic lethal (Fox *et al*., 1998). Heterozygous filamin-A mutations have been found in patients with periventricular heterotopia, a disease characterized by defective neuronal migration. While males usually die during embryogenesis, female patients have seizures and several non-neuronal defects (Eksioglu *et al*., 1996). Gain-of-function mutations in the filamin-A gene result in a broad range of congenital malformations, suggesting an important role of filamin-A-dependent signalling pathways in organogenesis (Robertson *et al*., 2003). Filamins might also be involved in autoimmune diseases, since high serum titres against filamin were found in patients with myasthenia gravis, graft-versus-host disease and Graves disease (Yamamoto *et al*., 1987; Leedman *et al*., 1993; Peutz-Kootstra *et al*., 2000). However, the exact role of filamin in these immune disorders remains unclear.

Signalling to the actin

After ligand binding, integrins activate signalling cascades that affect formation, turnover and linkage of actin filaments. The stimulation of Rho-GTPases is of special importance in this respect. These molecules are essential for the organization of the actin cytoskeleton and promote specialized actin structures such as stress fibres (RhoA), lamellipodia (Rac1) and filopodia (Cdc42) (Etienne-Manneville and Hall, 2002). In addition, Rho-GTPases are involved in cell proliferation, survival, polarity, vesicle transport and various other activities. Integrins can stimulate Rho-GTPases via different pathways, of which those via FAK and Src-like kinases seem to be most important (Schaller, 2001; Arthur *et al*., 2002). Recently, the integrin-associated molecules integrin cytoplasmic domain-associated protein-1 (ICAP-1) and ILK were suggested to stimulate Rho-GTPases (Degani *et al*., 2002; Rosenberger *et al*., 2003). In addition, both molecules affect the linkage of integrins to the cytoskeleton (Wu and Dedhar, 2001; Bouvard *et al*., 2003).

FAK

FAK is a tyrosine kinase that is present only in FAs. Based on binding of FAK to peptides of the β1 cytoplasmic domain and on co-precipitation experiments, FAK is thought to bind integrins directly (Schaller *et al*., 1995; Chen *et al*., 2000). However, since the 'integrin binding region' of FAK is not required for localization of FAK to the FA and the 'FAK binding region' of β3-integrin is not required for FAK activation, it could be that the *in vivo* integrin–FAK interaction is indirect (Tahiliani *et al*., 1997; Shen and Schaller, 1999). This is conceivable since FAK binds strongly to talin and paxillin, which directly and indirectly bind integrin (Chen *et al*., 1995; Hildebrand *et al*., 1995). Upon cell attachment to the ECM, FAK becomes autophosphorylated at Tyr397 either directly by integrin clustering or after phosphorylation of tyrosines 576 and 577 by Src, which enhances the catalytic activity of FAK (Schaller, 2001). It is possible that both pathways occur. Src itself might be activated before binding to FAK by a tyrosine phosphatase such as PTP 1B, SHP-2 or PTPα. Activated FAK can bind and phosphorylate a range of different substates, which allows further recruitment of adaptor and signalling molecules. Different regions of the FAK molecule are involved in protein–protein interactions. The phosphorylated Tyr397 can be bound by SH2-domain-containing proteins such as Src-like kinases, Grb7, PI3-K, Shc and PLCγ (Schlaepfer and Hunter, 1997; Han and Guan, 1999; Reiske *et al*., 1999; Zhang *et al*., 1999). Grb2 binds the phosphorylated Tyr925. SH3-domain proteins like p130Cas and Rho-GAP protein GRAF interact with proline-rich sequences close to the C-terminus. FAK binds to paxillin and talin via the focal adhesion targeting sequence (FAT) (Chen *et al*., 1995; Hildebrand *et al*., 1995).

Rho-GTPases can be activated by FAK through several mechanisms. First, a p130Cas–Crk–DOCK180 complex can activate Rac1, which promotes lamellipodia formation (Kiyokawa *et al*., 1998). Second, PI3-K can stimulate Rho-GTPase-activating GEF molecules via PIP3 production, which in turn stimulates Rho-GTPases (Das *et al*., 2000). Third, FAK can directly or indirectly interact via paxillin with the adapter GIT1 and with GEFs of the Cool/PIX family, which activate Rac1 and Cdc42 (Turner *et al*., 1999; Zhao *et al*., 2000). Finally, Src-like kinases can activate GEFs through phosphorylation (Crespo *et al*., 1997; Han *et al*., 1997; Teramoto *et al*., 1997). In short, integrin activation triggers the formation of various phosphoprotein complexes that can modify the actin cytoskeleton particularly by activating Rho-GTPases.

ICAP-1

ICAP-1 was identified in yeast two-hybrid screens as an integrin binding protein of 200 amino acids containing many putative phosphorylation sites (Chang *et al*., 1997; Zhang and Hemler, 1999). ICAP-1 binds β1-integrin, but no other integrin β subunits. Overexpression of wild type ICAP-1 increased cell migration on fibronectin and strongly reduced spreading. ICAP-1 is phosphorylated upon attachment to fibronectin. Mutation of Thr38 to aspartic acid, mimicking phosphorylation, reduced cell spreading while mutation to the non-phosphorylatable alanine increased spreading of CHO cells on fibronectin, suggesting that phosphorylation of ICAP-1 modulates integrin function (Bouvard and Block, 1998). Recently, it was shown that ICAP-1 might function as a Rho-GDI by binding Cdc42 and Rac1 and sequestering them in an inactive form in the cytosol (Degani *et al*., 2002). It was suggested that ICAP-1 binding to β1-integrin releases the inhibition of the Rho-GTPases, thus promoting cell spreading and migration. An alternative model suggests that ICAP-1 binding to β1-integrin prevents talin association and dissolves FAs. This is supported by the observation that ICAP-1 is never found in the FA (Bouvard *et al*., 2003), rather, prominent ICAP-1 expression occurs at the ruffling edges of migrating cells. These observations suggest that ICAP-1 is a negative regulator of β1-integrin avidity, prevents talin-mediated connection to the cytoskeleton and talin-mediated signalling events. ICAP-1-deficient mice, though viable, develop several defects that could be caused by diminished integrin activity (D.Bouvard and R.Fässler, unpublished data).

The ILK–PINCH–parvin complex as large docking station for actin-regulating proteins

Is the kinase activity of ILK essential in vivo?

ILK was described in 1995 as a Ser/Thr kinase that binds to the cytoplasmic tails of β1, β2 and β3-integrin subunits (Hannigan *et al*., 1996). It was suggested that ILK stimulation might be crucial for the activation of various integrin signalling pathways. Although the kinase catalytic domain lacks certain amino acids that are usually highly conserved in other Ser/Thr kinases, ILK was shown to readily phosphorylate peptides and model substrates such as myelin basic protein (MBP) *in vitro* and to induce phosphorylation of the protein kinases PKB/Akt and GSK-3β in cells overexpressing ILK (Delcommenne *et al*., 1998). Whether ILK also phosphorylates other targets, including the cytoplasmic domains of integrins, was never convincingly shown.

The identification of PKB/Akt and GSK-3β as ILK targets provided an explanation of how integrin-mediated cell proliferation (anchorage-dependent growth) and cell

survival could be executed. GSK-3β is a negative regulator of *Wnt* signalling (Cohen and Frame, 2001), which is inactivated by phosphorylation mediated by *Wnt* signals or ILK. This leads to the stabilization and increase of β-catenin levels, which permits translocation of β-catenin into the nucleus where it associates with Lef-1/Tcf transcription factors to activate genes including cyclin D1 and c-*myc*. The formation of Lef-1/Tcf–β-catenin complexes and increased cyclin D1 expression are observed in ILK-overexpressing cells and explain their anchorage-independent growth (Novak *et al*., 1998) and the formation of tumours, although with long latency, in mice overexpressing ILK in mammary glands (White *et al*., 2001). PKB/Akt is a Ser/Thr kinase implicated in cell proliferation, survival and growth factor signalling. Full activation of PKB/Akt requires phosphorylation on residues Thr308 and Ser473. The Thr308 kinase is called 3-phosphoinositide-dependent kinase 1 (PDK1) and is well characterized (Lawlor and Alessi, 2001) while the PI3-kinase-dependent kinase that phosphorylates Ser473, termed PDK2, is less well characterized. Several experiments reported by Dedhar and colleagues suggested that ILK is PDK2 (Persad *et al*., 2001).

However, genetic studies in *Drosophila*, *Caenorhabditis elegans* and mouse have cast doubt upon the kinase activity of ILK. Both flies and worms lacking ILK expression show severe muscle abnormalities (Zervas *et al*., 2001; Mackinnon *et al*., 2002), but neither patterning defects typical of impaired *Wnt* signalling nor increased apoptosis typical of impaired PKB/Akt activity were observed in ILK-deficient flies (Zervas *et al*., 2001). More importantly, the defects in mutant flies and worms could be fully rescued with ILK genes that lacked kinase activity. We made mice lacking ILK expression and found that they die shortly after implantation. Therefore, we established fibroblastoid cell lines in which ILK is deleted *in vitro* using the Cre/loxP system. These cells have severe adhesion and proliferation defects but normal phosphorylation of PKB/Akt and GSK-3β (Sakai *et al*., 2003). Similarly, a chondrocyte-specific deletion of ILK in mice did not affect the phosphorylation levels of PKB/Akt and GSK-3β (Grashoff *et al*., 2003) indicating that in fibroblasts as well as in chondrocytes ILK is not required to phosphorylate PKB/Akt and GSK-3β. Finally, biochemical studies with mammalian cells revealed that ILK is part of a multi-protein complex without intrinsic kinase activity for PKB/Akt (Hill *et al*., 2002). Whether ILK is functioning as a kinase *in vivo* is difficult to answer as long as the kinase activity has not been selectively abrogated in mice. Clearly, however, the kinase activity is neither necessary for invertebrate development nor required for PKB/Akt and GSK-3β signalling in several vertebrate cells.

ILK drives actin reorganization/dynamics in vivo

If ILK has no essential kinase activity *in vivo*, how can ILK exert its function? Answers came from a combination of genetic studies in flies, worms and mice and screens for ILK interactors using mammalian cells, which point to a prominent role of ILK in modulating the actin cytoskeleton. The absence of ILK expression in *Drosophila* leads to a severe defect in integrin-mediated adhesion. Both ILK- and βPS integrin-deficient flies exhibit muscle detachment, but for different reasons (Zervas *et al*., 2001). In muscles lacking βPS integrin expression, the plasma membrane detaches from the ECM, but still anchors actin filaments. In ILK-deficient muscle the plasma membrane is attached to the ECM but fails to connect to actin filaments, suggesting that ILK is a structural component that links the cytoskeleton and the plasma membrane at sites of integrin-mediated adhesion. In *C. elegans* the homologue of mammalian ILK is found in body-wall muscles where it concentrates together with β-integrin/PAT3 at FA-like muscle attachment sites (dense bodies). Loss of ILK expression leads to an embryonic-lethal phenotype characterized by muscle detachment. The phenotype is called PAT4 (Paralysed and Arrested elongation at the Two-fold stage) and resembles the loss-of-function phenotype of β-integrin/PAT-3 (Mackinnon *et al*., 2002). Since the actin cytoskeleton has not been analysed in β-integrin/PAT-3 mutant worms, it is not known whether abnormal F-actin attachment to adhesion sites is causing the muscle phenotype.

In mouse, loss of ILK expression leads to peri-implantation lethality. Around implantation the primitive endoderm develops on the surface of the inner cell mass (ICM) of the blastocyst and lays down a basement membrane (BM) that is required for adjacent ICM cells to polarize and establish the columnar epiblast (primitive ectoderm), and for the remaining ICM cells to undergo apoptosis resulting in the establishment of the proamniotic cavity. Cell differentiation in embryoid bodies (EBs) closely mimics the events occurring at peri-implantation (Li *et al*., 2003) and therefore, we used ILK-null EBs to delineate the reason for the peri-implantation lethality caused by loss of ILK. In ILK-deficient EBs the primitive endoderm differentiates and produces a BM but the polarization of ICM cells to form columnar epiblast cells and subsequent cavitation is severely impaired. This defect is different from β1-integrin-deficient mice, where the primitive endoderm does not express laminin α1 and hence cannot form a BM, resulting in peri-implantation lethality (Aumailley *et al*., 2000; Li *et al*, 2002). If this defect is rescued by exogenous addition of laminin, the β1-null EBs lay down a BM, develop a normal epiblast and form cavities (Li *et al*., 2002). These data indicate that β1-integrins can act independently of ILK, and ILK independently of β1-integrins.

The underlying cause of the polarization defect of ILK-deficient epiblast is abnormal F-actin reorganization (Sakai *et al*., 2003). Normal ICM cells adjacent to the endodermal BM distribute F-actin evenly beneath their plasma membrane. During elongation and polarization of the epiblast cells the F-actin locates to the apical side facing the cavity. In ILK-null EBs, F-actin accumulates in ICM cells at sites of integrin attachment to the BM already prior to polarization. In few EBs, a few ICM cells gain columnar shape with some F-actin in an apical belt while a significant amount, however, remains at the basal side where integrins attach the polarized epiblast cells to the BM. These findings indicate that a major function of ILK in epiblast cells is to prevent F-actin accumulation at integrin adhesion sites to the BM. A similar requirement of ILK to reorganize F-actin is also evident in ILK-deficient fibroblasts and chondrocytes, which accumulate abnormal

aggregates of F-actin beneath the plasma membrane and display several other abnormal actin-based processes such as diminished cell spreading and delayed formation of stress fibres and FAs (Grashoff *et al*., 2003; Sakai *et al*., 2003).

Loss of ILK expression leads to a severe actin-based defect in fly and mouse epiblast as well as fibroblasts and chondrocytes. While mammalian cells accumulate abnormal amounts of F-actin at sites of integrin adhesion, ILK-deficient muscle cells in flies fail to link actin filaments to integrin adhesion sites in muscle cells resulting in a detachment from, rather than an accumulation of F-actin at, the cell membrane (Zervas *et al*., 2001). Although this is a striking difference, the F-actin accumulation at the membrane might also occur during muscle development. When contraction starts, however, the F-actin will be detached from the membrane due to weak interaction with for example, intergins. Therefore, F-actin distribution should be determined prior to muscle cell contraction. In addition, F-actin localization should be analysed in ILK-deficient wing epithelial cells that detach from the ECM, which results in blister formation. However, it cannot be excluded that ILK exerts different or opposing functions in different cell types.

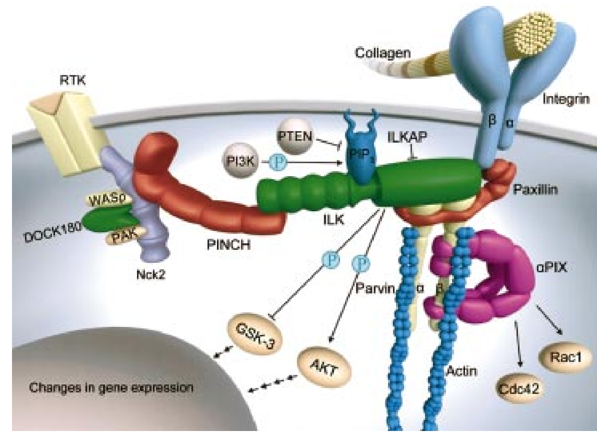

Fig. 3. ILK recruits several adaptor proteins that modulate actin dynamics or actin attachment to the integrin adhesion site either directly or indirectly. In addition, ILK can be located to the plasma membrane through interactions with phospholipids. Membrane binding activates the kinase function, subsequent to the phosphorylation of Akt and GSK3. RTK, receptor tyrosine kinase; ILKAP, ILK-associated phosphatase. The molecules are not drawn to scale.

Binding partners of ILK

Much progress has been made in identifying ILK-interacting proteins and surprisingly, each of them can modulate actin either directly or in an indirect manner (Figure 3). In addition to the C-terminal putative kinase domain, ILK is composed of an N-terminal array of four ankyrin repeats and a pleckstrin homology (PH) domain. While it has not been determined whether the PH domain interacts directly with phospholipids, the C-terminal region has been shown to bind paxillin (Nikolopoulos and Turner, 2001), and the N-terminal region has been shown to bind the double zinc finger domain (LIM)-only adapter proteins PINCH-1 and PINCH-2 (Zhang *et al*., 2002a; Braun *et al*., 2003), which interact with the SH2/SH3-containing adaptor protein Nck2 (or Grb4; Tu *et al*., 2001a). Nck2 can bind through its SH2 domain phosphorylated tyrosine residues of receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) or platelet-derived growth factor (PDGFR) and thereby possibly linking integrin and growth factor signalling. In addition, the SH3 domains of Nck2 can recruit actin-regulatory proteins such as WASp (Wiskott–Aldrich Syndrome protein), DOCK180 (180 kDa protein downstream of Crk) and PAK (p21-activated kinase) suggesting a role for actin reorganization/actin dynamics.

The putative kinase domain of ILK can recruit a new family of F-actin binding proteins. This family has three members and was identified independently in several laboratories and has multiple names. Wu and colleagues identified the calponin homology-ILK binding protein (CH-ILKBP; Tu *et al*., 2001b), which is identical to actopaxin described by Nikolopoulos and Turner (2000) or α-parvin identified by Olski *et al*. (2001). In addition to α-parvin, Olski *et al*. (2001) described the primary sequence of β-parvin, which is identical to the ILK-binding protein affixin (Yamaji *et al*., 2001), and γ-parvin, which was not tested for ILK binding. We will call the family members α-, β- and γ-parvins throughout the review to underline their structural similarity.

Overexpression of mutant forms of ILK, PINCH or parvin, which are unable to interact with each other, revealed that the formation of a ternary complex of ILK, PINCH and parvins is essential for their recruitment to FAs (Zhang *et al*., 2002b). The significance of the ILK–PINCH–parvin complex for cell adhesion, cell spreading, FA assembly and stress fibre formation has been shown in several cell culture studies. The ILK–parvin interaction, for example, was investigated in a myoblast cell line, in which disruption of the ILK–α-parvin complex retarded formation of stress fibres as well as FAs and delayed cell spreading (Tu *et al*., 2001b). It seems that the formation of the ILK–PINCH–parvin complex, however, is not sufficient for FA localization and that interactions with additional proteins are necessary for FA recruitment. A possible candidate for such an additional interactor is paxillin, which binds integrins, ILK and α-parvin (Nikolopoulos and Turner, 2002). Besides the recruitment of the ILK–PINCH–parvin complex into FAs, paxillin may also further reinforce F-actin interactions through recruitment of vinculin. Overexpression of a mutant parvin defective for paxillin binding impairs cell adhesion and cell spreading, supporting the hypothesis that the recruitment of parvin and hence the entire complex to integrins is important for actin filament assembly/recruitment at cell adhesion sites (Nikolopoulos and Turner, 2000). Recent yeast two-hybrid studies identified the guanine exchange factor αPIX [Pak-interacting exchange factor, also called Cool2 (cloned out of library) or ARHGEF6 (Rosenberger *et al*., 2003)] as a new β-parvin-interacting protein. αPIX contains a Dbl homology domain, which can activate the small GTPases Rac and Cdc42.

Altogether these reports show that the ILK–PINCH–parvin complex can modulate the actin cytoskeleton at several levels. First, proteins such as the parvins can recruit F-actin either directly or indirectly through paxillin and vinculin. Secondly, the recruitment of

Nck2 to PINCH can modulate actin polymerization through WASp and the Arp2/3 complex. Thirdly, Nck2 or parvin can bind GEFs such as DOCK180 or αPIX and effector proteins of small GTPases such as PAK and thereby regulate actin turnover. In this respect it is interesting to note that the expression of a dominant-negative Rac in MDCK epithelial cells leads to inverted deposition of F-actin (O'Brien *et al*., 2001). Instead of apical distribution, F-actin is often found at the basal site of the cells. This phenoype resembles the defects that we have observed in the ILK-null EBs where F-actin is present at the integrin–BM adhesion site (Sakai *et al*., 2003).

The availability of mutant mice and cells, GFP-tagged proteins that allow imaging of live cells *ex vivo* and *in vivo*, and specific antibodies for biochemistry should make it possible now to unravel the many remaining mysteries of the ILK–PINCH–parvin complex. In particular, they should enable us to determine whether the ILK–PINCH–parvin complex is 'just' an actin modulator that affects integrins indirectly, like each actin modulator would do, or whether it is indeed used by the integrins to transduce messages into and receive messages from within cells.

Concluding remarks

More than 20 molecules are currently described that interact directly with the intracellular domain of integrins. Many more are indirectly linked and participate in the formation of big multiprotein complexes that form around the integrins, linking them to the cytoskeleton and inducing cytoskeletal rearrangement. Such protein complexes allow a highly graduated response of the cell to external stimuli, which is crucial for cell polarity and migration. Furthermore, multiprotein complexes enable many levels of regulation with respect to complex composition, protein–protein interaction and activation status of individual components. Via such regulatory processes information from other signalling pathways can also be integrated. This high degree of regulation allows many cell-specific responses.

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