Bidirectional signaling between the cytoskeleton and integrins

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Clustering of integrins into focal adhesions and focal complexes is regulated by the actin cytoskeleton. In turn, actin dynamics are governed by Rho family GTPases. Integrin-mediated adhesion activates these GTPases, triggering assembly of filopodia, lamellipodia and stress fibers. In the past few years, signaling pathways have begun to be identified that promote focal adhesion disassembly and integrin dispersal. Many of these pathways result in decreased myosin-mediated cell contractility.

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Abbreviations

ECM	extracellular matrix
ERM	ezrin-radixin-moesin
FA	focal adhesion
FAK	focal adhesion kinase
FN	fibronectin
GAP	GTPase activating protein
GDI	guanine-nucleotide dissociation inhibitor
GEF	guanine-nucleotide exchange factor
GFP	green fluorescent protein
IF	intermediate filament
LPA	lysophosphatidic acid
MAPK	mitogen-activated protein kinase
MLC	myosin light chain
MLCK	myosin light chain kinase
PAK	p21-activated protein kinase
PI	phosphatidylinositol
PI3K	phosphoinositide 3-kinase
PIP_2	PI 4,5-bisphosphate
PIP_3	PI 3,4,5-trisphosphate
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase

Introduction

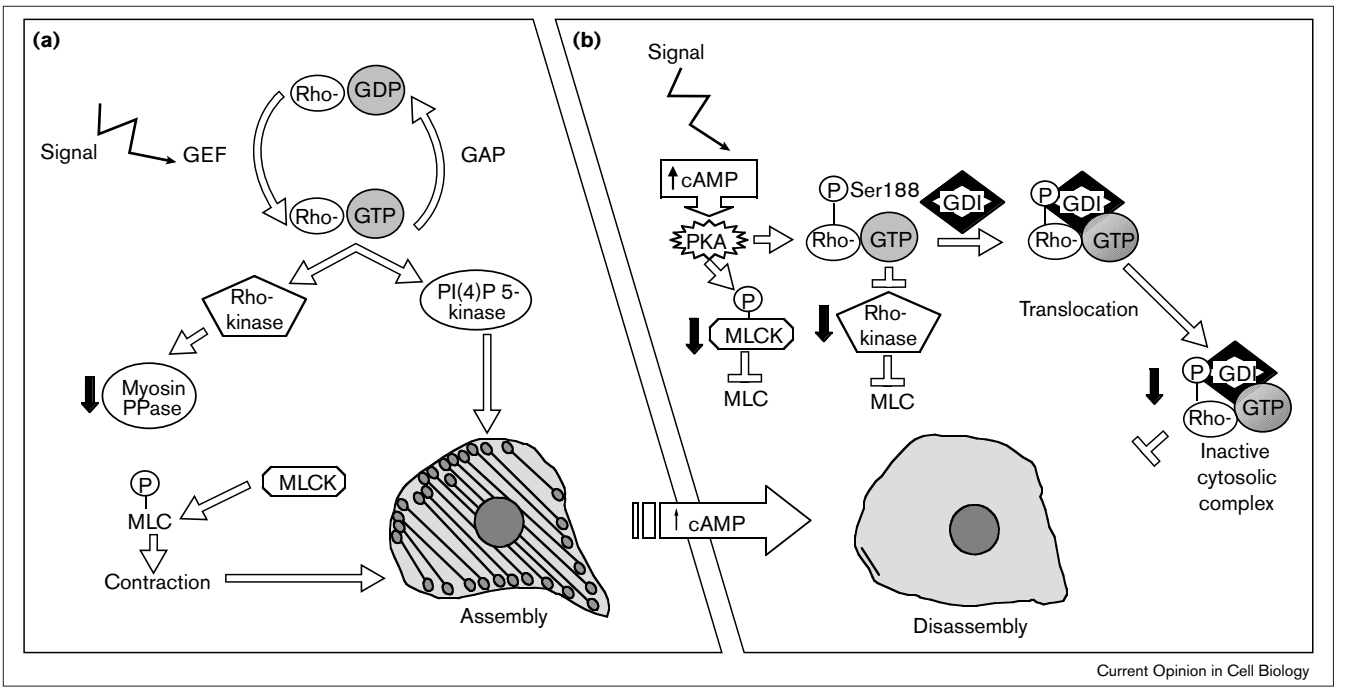
Adhesive interactions critically influence the organization of the cytoskeleton. Reciprocally, the cytoskeleton affects the organization and function of adhesive molecules such as integrins and cadherins. In this review, we focus on the relationship between the actin cytoskeleton and the organization of integrins. Integrins are receptors that form transmembrane links between the extracellular matrix (ECM) and the actin cytoskeleton. During the past decade, their importance as signal transducers from the ECM has been increasingly recognized. Integrins are frequently clustered into specialized adhesive structures, focal adhesions (FAs) and focal complexes, in which numerous signaling components are concentrated [1–3]. Many aspects of integrin biology have recently been reviewed [4•–6•]. Here we concentrate on integrin clustering and dispersal as regulated by the cytoskeleton which, in turn, is regulated by the Rho family of G proteins. We discuss signaling pathways that feedback from integrins to modulate the cytoskeleton, and consider how the state of the actin cytoskeleton controls the organization of ECM.

Cytoskeletal clustering of integrins

Integrins that are not bound to ECM ligands are generally distributed diffusely over the cell surface and appear not to be linked to the actin cytoskeleton. Association with the actin cytoskeleton is induced upon binding of ECM ligands [7–9]. Depending on the state of cytoskeletal organization, this can lead to clustering of integrins into FAs or focal complexes. FAs are large integrin aggregates found at the ends of prominent bundles of actin filaments (stress fibers). Both stress fibers and FAs are regulated by the GTP-binding protein RhoA. Focal complexes are smaller integrin clusters that occur at the tips of filopodia or lamellipodia, with these structures being under the control of the Rho family members Cdc42 and Rac, respectively [10•]. Rho family proteins function as 'molecular switches' that cycle between an inactive GDP-bound state and an active GTP-bound state. In general, these proteins have a low intrinsic GTPase activity. Cycling of nucleotides is regulated by interacting proteins, including guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). The regulators of RhoA, Rac and Cdc42 activity have been reviewed in great detail [11•,12•].

Much effort has been directed towards understanding how these Rho family GTPases organize actin and the associated distribution of integrins. More is known about how RhoA stimulates assembly of stress fibers and FAs than is known about Rac- or Cdc42-induced focal complex assembly. Separate lines of investigation have converged to reveal that RhoA stimulates actomyosin-based contractility and that this contractility contributes to the assembly of stress fibers and FAs [3,13]. RhoA•GTP binds to and activates several serine/threonine kinases. One of these, known variously as Rho-kinase, ROCKII and ROKα (closely related to p160ROCK/ROKβ), phosphorylates and inhibits myosin phosphatase, resulting in elevated myosin light chain (MLC) phosphorylation [14] (Figure 1a). In turn, MLC phosphorylation promotes both myosin filament assembly and actin-activated myosin ATPase activity [3]. These effects result in bundling of actin filaments and tension being transmitted to integrins via their associated actin filaments. Both bundling and tension will cluster integrins that are linked to actin [15]. Rho-kinase may also phosphorylate

Figure 1



RhoA-mediated assembly of stress fibers and focal adhesions, and cAMP-mediated disassembly. **(a)** Activation of RhoA stimulates the downstream targets Rho-kinase and PI(4)P 5-kinase. Activation of Rho-kinase results in phosphorylation of the myosin phosphatase (myosin PPase) [14], decreasing its activity, thereby increasing myosin light chain (MLC) phosphorylation. In addition, direct phosphorylation of MLC by Rho-kinase may occur. MLC phosphorylation promotes myosin filament formation and contractility. In turn, these result in bundling of actin filaments into stress fibers and clustering of integrins into FAs. The activation of PI(4)P 5-kinase leads to an elevation in PIP_2 levels. PIP_2 modulates the activity of several cytoskeletal proteins, including vinculin, gelsolin, profilin and ERM proteins. Together, the actions of Rho-kinase and PI(4)P 5-Kinase and possibly other RhoA targets promote FA formation. **(b)** Elevation of intracellular cAMP leads to activation of PKA. One target for PKA is MLCK, which is inhibited by PKA phosphorylation. A second target is RhoA [54]. Phosphorylation of RhoA leads to binding of the Rho guanine nucleotide dissociation inhibitor (GDI) which extracts active RhoA from its membrane location, translocating it to the cytosol, resulting in termination of the RhoA signal [54]. Elevated cAMP also inhibits nucleotide exchange on RhoA [55•], presumably through increased binding of RhoA to GDI. Furthermore, RhoA phosphorylation results in a decreased affinity for its downstream effector, Rho-kinase [56]. The combined effects of cAMP and PKA on MLCK and RhoA lead to inhibition of myosin activity and contractility. These result in stress fiber disassembly and integrin dispersal. Black arrows indicate a decrease in enzyme activity.

MLC directly [16] (Figure 1a), but some evidence supports the idea that the primary action of Rho-kinase is to inhibit myosin phosphatase (M Parizi, JJ Tomasek, personal communication). Constitutively active Rho-kinase induces the assembly of stress fibers and FAs [17,18•,19•], supporting this model; however, the organization of these structures induced by Rho-kinase is often different from that seen in normal cells, suggesting that other pathways downstream from RhoA may also contribute to their assembly and/or organization. One possibility is RhoA-activation of phosphatidylinositol (PI)-5-kinase to elevate PI 4,5-bisphosphate (PIP_2) levels [20]. PIP_2 induces a conformational change in vinculin [21,22] and ERM (ezrin-radixin-moesin) proteins [23,24] exposing binding sites for actin and other proteins that may be important in FA assembly.

The clustering of integrins into focal complexes induced by Rac and Cdc42 is less well characterized. Whether myosin is involved has not been established. One downstream target of both Rac and Cdc42 is PAK, a serine/threonine kinase implicated in the development of focal complexes [25]. PAK phosphorylates and inhibits the myosin light chain kinase (MLCK) — an enzyme that regulates myosin activity [26•] — and this might indicate that myosin is not involved in the assembly of focal complexes. A different conclusion has been drawn, however, from work with another kinase downstream of Cdc42, MRCK (myotonic dystrophy kinase related Cdc42-binding kinase), which directly phosphorylates the MLC [27].

The role of PAK in generating lamellipodia and/or filopodia is controversial. On the one hand, introduction into cells of an activated form of PAK induces Rac-type lamellipodia or ruffles, although this does not require PAK kinase activity [25]. Several studies, however, have shown that mutants of Rac or Cdc42 that fail to bind PAK *in vitro* still induce lamellipodia or filopodia, suggesting that PAK is not involved in these processes [28–30]. Recent work has offered a possible explanation for these apparently conflicting results. PAK is targeted to focal complexes and this recruitment does not require interaction with Cdc42 as it occurs in response to mutant forms of PAK that fail to

bind Cdc42 [31•]. This targeting does require PAK binding to PIX (PAK interacting exchange factor), a GEF for Rac [32•]. This suggests that there is a complex formed between Rac or Cdc42; PAK (a downstream effector) and PIX (an upstream GEF). Mutations that disrupt the Rac or Cdc42 interaction with PAK *in vitro* may not disrupt the complex *in vivo* because of the bridging interactions between PIX and PAK. These results also raise the possibility that PAK introduced into cells may induce lamellipodia by activation of endogenous Rac via PAK's interaction with PIX. Multiprotein complexes of this type may also occur with RhoA and should be considered in the analysis of RhoA mutants that fail to interact directly with particular downstream effectors. With respect to the induction of lamellipodia and filopodia by Rac and Cdc42, several other targets have also been implicated including PI 5-kinase [33], phosphoinositide 3-kinase (PI3K) [34•], POR1 [35], IQGAP [36,37] and WASP (Wiskott-Aldrich syndrome protein) [38,39]. The relative contributions of these proteins in the assembly of the actin arrays that generate lamellipodia and filopodia remain to be determined.

Rho family Rnd proteins (Rnd1, Rnd 2 and Rnd3/RhoE) [40•,41•] show closest identity to RhoA, with an identical effector domain apart from one residue. Despite this striking sequence similarity, they display a very distinct biochemical and behavioral pattern with (unlike RhoA) a very high GTP-binding capacity but little if any intrinsic GTPase activity, suggesting that they exist *in vivo* in a constitutively active GTP-bound state. Rnd microinjection into cells induces decreased adhesion (a round morphology), disassembly of stress fibers and FAs — the opposite effect of activated RhoA. Rnd3/RhoE promotes migration of MDCK cells in response to hepatocyte growth factor (HGF) [41•], consistent with antagonism of RhoA effects. The Rnd proteins appear to act as negative regulators of RhoA signaling pathways but how they are regulated and their downstream targets remain to be determined.

Integrin dispersal from FAs

Migration involves cyclical changes in local adhesive strength: decreases in adhesion can result from changes in the affinity of integrins for their ECM ligands, from disassembly of the cytoskeletal protein complex that interacts with integrin cytoplasmic domains, or from the dispersal of clustered integrins. Several factors that antagonize integrin clustering in FAs and that promote the disassembly of FAs have been identified. It is important to note that there are significant cell type differences. In epithelial cells, for example, activation of protein kinase C (PKC) by PMA leads to rapid disassembly of FAs [42], whereas in fibroblasts the same treatment either has no effect or potentiates cell spreading and FA assembly [43,44]. Agents that inhibit actin–myosin interaction, either directly [15] or indirectly through MLCK [45–47] or RhoA inhibition [48], promote disassembly of stress fibers and FAs, consistent with the idea that contractility drives the formation of these structures. Several of the factors discussed below appear to cause integrin dispersal from FAs by inhibiting contractility.

Regulation by cAMP-dependent protein kinase

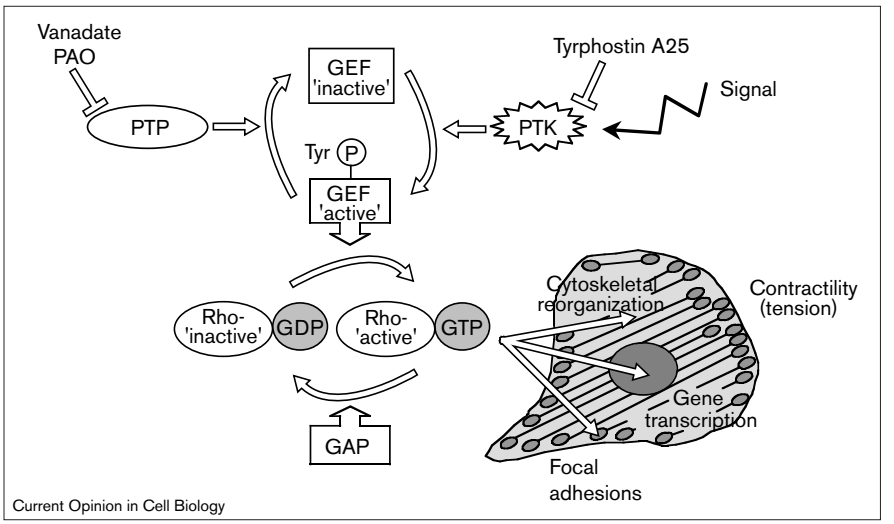
Elevated cAMP and consequent activation of protein kinase A (PKA) affects cell morphology, inducing loss of actin stress fibers and FAs, rounding of cells and detachment from the underlying substratum [45,49,50] (Figure 1b). These effects, however, are not conserved across all cell types [51]. Elevation of cAMP also decreases the phosphorylation of multiple proteins, including decreased tyrosine phosphorylation of the FA proteins paxillin [52] and FA kinase (FAK) [53]. Activated PKA phosphorylates MLCK, inhibiting its activity and causing a concomitant decrease in MLC phosphorylation [45], supporting the idea that actin–myosin interaction and contractility are critical for the assembly of stress fibers and FAs (reviewed in [3,13]).

RhoA has been identified as a substrate for PKA resulting in phosphorylation on Ser188 [54]. This has been suggested to inhibit multiple aspects of RhoA activity [54,55•,56,57•,58•]. Phosphorylated RhoA displays decreased guanine-nucleotide exchange [55•], possibly due to increased affinity of RhoA for RhoGDI [54]. Binding to RhoGDI results in extraction of RhoA from the membrane and translocation to the cytosol where it is generally presumed to be inactive [54]. RhoA phosphorylation on Ser188 leads to decreased association with Rho-kinase [57•,58•]. The overall effect of cAMP-mediated phosphorylation of RhoA is to downregulate its activity. It is interesting to note that in cells under tension, sudden release of this tension causes a sharp rise in cAMP levels [59]. In this circumstance, the inhibition of RhoA activity by elevated cAMP may allow remodeling of integrin–cytoskeletal connections, thereby permitting a cell to adapt to its altered environment.

Regulation by Rac and Cdc42

Various growth factors (e.g. epidermal growth factor [EGF], insulin, platelet-derived growth factor [PDGF]) induce FA disruption [60•,61–63]. Rather little is known about the mechanism, although the effect is often dependent on the concentration of growth factor used. One possible mechanism for EGF-induced actin disruption was provided by Chang *et al.*, who demonstrated that the time course for Src-mediated tyrosine phosphorylation of p190RhoGAP correlated closely with EGF-mediated cytoskeletal disassembly [64]. Tyrosine phosphorylation of p190RhoGAP has been suggested to increase its RhoGAP activity, making it an ideal candidate for mediating a decrease in RhoA activity and leading to disassembly of FAs. Interestingly, many of the growth factors that disrupt FAs also activate Rac or Cdc42. A complex interrelationship exists between RhoA and Rac/Cdc42. In some situations, activation of Cdc42 or Rac results in subsequent RhoA activation [65,66]. Yet the functions of Rac and Cdc42 seem antagonistic to the actions of RhoA. Whereas Cdc42 and Rac promote cell extension, RhoA promotes

Figure 2



Regulation of Rho activity by tyrosine phosphorylation. A large body of evidence indicates that the assembly of stress fibers and FAs is regulated by tyrosine phosphorylation [3]. Inhibitors of PTKs (e.g. Tyrphostin A25) inhibit the activation of RhoA. Alternatively, inhibitors of PTPs (e.g. vanadate, phenylarsine oxide [PAO]) stimulate RhoA activity and induce the formation of stress fibers and FAs. In addition, there is evidence that there are critical tyrosine phosphorylation events upstream of RhoA [75]. This figure proposes a model whereby the activity of RhoA GEFs is regulated by tyrosine phosphorylation. Phosphorylation of a GEF by a PTK activates the GEF, promoting nucleotide exchange on RhoA, in turn leading to assembly of stress fibers and FAs. GEF activity is turned off by PTPase activity. RhoA GAPs could also be regulated (inhibited) by tyrosine phosphorylation but this is not shown in the figure.

contractility. Contractility can itself contribute to extension if strong adhesion exists but, when adhesion to the substratum is weak, RhoA-induced contractility will retract a leading edge and oppose the extension induced by Rac or Cdc42 [67]. This has been most clearly seen with the behavior of nerve growth cones [68,69•].

Although Cdc42 and Rac can lead to activation of RhoA, activated Cdc42 and Rac have been noted to diminish stress fibers and FAs [67,70•]. Constitutively active forms of PAK, a kinase activated by both Cdc42 and Rac, also disassemble stress fibers and FAs when introduced into cells [31•,70•,71•]. This suggests that it may be the effector responsible for the antagonism between Rac/Cdc42 and RhoA. As mentioned earlier, MLCK has been identified as a substrate for PAK, and PAK phosphorylation of MLCK inhibits its phosphorylation of MLC [26•]. These observations indicate a biochemical pathway by which Rac and Cdc42 can diminish the contractility induced by RhoA and thus favor the disassembly of stress fibers and FAs. Growth factors that activate Rac or Cdc42 typically promote cell migration. Because FAs often retard migration, the disassembly of these structures via a PAK-mediated inhibition of MLC phosphorylation would be expected to contribute to cell migration stimulated by growth factors.

Regulation by tyrosine dephosphorylation

The relationship between tyrosine phosphorylation and FA assembly is complex. The assembly of many of the signaling components in FAs depends on FAK activity generating specific phosphorylated sites that can bind other signaling proteins [72]; however, assembly of the structural components of FAs can occur in the apparent absence of tyrosine phosphorylation within FAs [73,74]. Nevertheless, many treatments that enhance tyrosine phosphorylation promote FA and stress fiber formation [3]. Conversely, agents that decrease tyrosine phosphorylation have been observed to disrupt FAs and stress fibers [3]. We conclude that although tyrosine phosphorylation promotes FA and stress fiber assembly it is not via the tyrosine phosphorylation of FA components. Evidence for upstream regulation of stress fiber and FA assembly by tyrosine phosphorylation came from Nobes *et al.* who demonstrated that there is a tyrosine kinase upstream of RhoA that regulates RhoA activation [75]. Although the mechanism by which tyrosine phosphorylation regulates RhoA activity is unclear, one possibility is through regulation of Rho GEFs (Figure 2). Work with Vav, a GEF for Rac, provides precedent for GEFs being regulated by tyrosine phosphorylation. Vav is a substrate for the nonreceptor tyrosine kinases Lck [76,77] and Syk [78•], and in platelets is tyrosine phosphorylated in response to integrin-mediated adhesion [78•,79]. This appears to regulate its GEF activity, and possibly its ability to associate with other signaling proteins [80].

Many conditions lead to the dephosphorylation of FA components and FA disassembly. For example, some growth factors (e.g. EGF, insulin, PDGF) induce dephosphorylation of FA proteins and this is accompanied by FA disassembly [61–63,81]. Similarly, introduction of potent protein tyrosine phosphatases (PTPs), such as that from *Yersinia*, leads to dephosphorylation of FA components and a disruption of FAs and stress fibers [82•,83,84]. The interpretation of these results has often been that the decreased tyrosine phosphorylation of FA proteins causes the disruption. In light of the previous discussion, however, we would suggest that the critical dephosphorylation is upstream of RhoA, resulting in decreased RhoA activity, and that in the case of growth factors, the dephosphorylation of FA components is a consequence rather than a cause of disassembly.

Cells deficient in the PTP Shp-2 display decreased spreading and migration, but increased FAs [85•]. With respect to the increased FAs, again our interpretation is that Shp-2

may normally regulate a RhoA GEF. In the absence of Shp-2, increased RhoA GEF tyrosine phosphorylation results in elevated RhoA activity and increased FAs. Several PTPs have been found to interact with FA components. PTP1B binds to the FA component $p130^{cas}$ (Cas) [86]. In one study, overexpression of wild-type PTP1B, but not a mutant that failed to bind to or dephosphorylate Cas, revealed decreased cell spreading and decreased cell migration [87]. These cells developed FAs, but the cells appeared to have lost their polarity, possibly indicating a suppression of Cdc42 activity. In a separate study, using different cells, overexpression of PTP1B showed no effects on spreading or morphology; however, overexpression of an inactive form of PTP1B decreased cell adhesion and decreased FAs and stress fibers [88]. In the latter work, the inactive PTP1B was considered to be acting as a dominant-negative mutant and these effects were attributed to decreased Src activity, due to increased phosphorylation of the inhibitory site on Src, Tyr527. It would be interesting to know the state of RhoA activity in these cells. Another PTP, PTP-PEST, binds to two FA components, Cas [89,90•] and paxillin [91•]. PTP-PEST has not been detected in FAs, however, and this association might only occur when the components are soluble within the cytoplasm and not concentrated within FAs. Overexpression of PTP-PEST inhibits cell migration [92•] and is associated with decreased Rac activity (SK Sastry, K Burridge, unpublished results).

A dual specificity phosphatase PTEN, has been found to dephosphorylate FAK [93•]. Overexpression of PTEN inhibited cell migration, spreading and FA assembly, whereas decreasing levels of PTEN enhanced migration. Again, the effects on FA assembly could indicate that PTEN regulates RhoA activity by acting on a tyrosine phosphorylated RhoA GEF. During the past year, however, several groups have shown that PTEN is a lipid phosphatase that dephosphorylates PI 3,4,5-trisphosphate (PIP_3) [94•–97•], antagonizing the PI3K signaling pathway. The PI3K pathway has been implicated in cell migration [34•] and so the effects of PTEN on migration, spreading and FA assembly may not be due to tyrosine dephosphorylation but due instead to PTEN's lipid phosphatase activity. Interestingly, some PTPs are closely associated with Rho family GEFs or GAPs. Thus, the PTP LAR, which in some cells is found in FAs [98], associates with Trio, which contains GEF activity for both RhoA and Rac [99]. Similarly, PTPL1 interacts with PARG1, a GAP for RhoA [100].

Regulation by nonreceptor tyrosine kinases

In the preceding section, we argued that tyrosine phosphorylation of FA proteins does not contribute to FA assembly, other than in the recruitment of signaling components. Elevated tyrosine phosphorylation of FA proteins has, however, been associated with FA disassembly and turnover. For example, in cells transformed by the oncogenic PTK *v-src*, FAs are disassembled and cytoskeletal organization is disrupted [3]. The disassembly of FAs in *v-src* transformed cells is associated with elevated tyrosine phosphorylation of multiple proteins, both structural (e.g. integrins, talin and vinculin) and regulatory (e.g. FAK, paxillin and Cas).

The tyrosine phosphorylation of many of these proteins may contribute to FA disassembly, but the involvement of FAK is noteworthy. Using temperature-sensitive mutants of Src, a correlation was found between FA disruption and FAK phosphorylation, raising the possibility that FAK functions in FA disassembly [73,101•]. Several lines of evidence support the idea that FAK has a role in FA turnover and motility. For example, cells deficient in FAK display reduced motility and increased numbers of FAs [102]. Similarly, displacement of FAK from FAs by a dominant-negative construct inhibits cell migration [74]. Conversely, FAK overexpression results in increased migration [103] and many invasive tumor cell lines with enhanced motility exhibit elevated levels of FAK activity [104]. Increased numbers of FAs in cells expressing kinase-dead v-Src [101•] or lacking FAK [102], suggest the involvement of Rho. Schwartz and coworkers have found that cells lacking FAK do indeed maintain higher levels of RhoA activity in response to adhesion (MA Schwartz, unpublished data). GRAF, a Rho GAP, found associated with the carboxyl terminus of FAK [105], may be responsible for decreasing RhoA activity in response to FAK phosphorylation.

Regulation by extracellular matrix proteins

Several ECM proteins (thrombospondin, tenascin-C and SPARC) are enriched at sites of wound repair where cell migration is normally induced (reviewed in [106,107•]). These proteins stimulate migration and have been shown to promote disassembly of FAs and stress fibers [108,109]; however, it should be noted that the anti-adhesive effects of these proteins do seem to be variable and context-dependent. A role for cGMP in the action of thrombospondin and tenascin has recently been established [110], whereby this cyclic nucleotide is required for the interaction of specific regions of these proteins with cells. Recent work has implicated PI3K in thrombospondin-mediated effects on FAs and stress fibers [111•]. Decreases in adhesion are required for cells to be able to migrate; therefore, these anti-adhesive matrix proteins may contribute to efficient cell migration during wound repair.

Regulation of integrin distribution by microtubules and intermediate filaments

Most of the work studying the relationship between the Rho family of GTPases and the cytoskeleton has been aimed at understanding their control of actin organization; however, Rho has significant effects on the two other major filamentous systems, microtubules and intermediate filaments. Microtubules have long been known to affect the adhesion of fibroblasts as well as various aspects of fibroblast migration. Inhibitors of microtubule polymerization decrease the rate of fibroblast spreading [112], decrease protrusive activity of the leading edge [113], and result in a loss of polarized migration [114]. Spreading, protrusion and polarized migra-

tion are also regulated by members of the Rho family, suggesting that the state of microtubules may affect these GTPases. Consistent with this hypothesis, microtubule depolymerization enhances cell contractility and the assembly of stress fibers and FAs [115-118]. Elevated MLC phosphorylation was detected in response to microtubule depolymerization, suggesting a biochemical basis for the increased contractility [119]. This is supported by further work indicating that microtubule depolymerization activates Rho [117,120•–122•]. Significantly, GEF-H1, an exchange factor for both Rac1 and RhoA, has been localised to microtubules [123•]. It is possible that GEFs sequestered on microtubules are unable to promote guanine nucleotide exchange and that depolymerization of microtubules liberates these GEFs, allowing them to activate Rho. Whereas microtubule depolymerization has been found to activate RhoA, the converse has been found with Rac. Microtubule polymerization is associated with activation of Rac (CM Waterman-Storer, personal communication).

Microtubules in many cultured cells are highly dynamic polymers, alternating phases of growth with periods of rapid depolymerization. Some microtubules are relatively stable, however, and can be distinguished by antibodies that identify a post-translational modification, the detyrosination of tubulin to expose a carboxy-terminal glutamic acid. Cook and coworkers [124•] found that activation of RhoA increased the population of stable microtubules. One possible mechanism for this stabilization is suggested by the studies of Kaverina *et al.* [125•], who observed that FAs can capture the ends of microtubules and stabilize these structures against depolymerization. They also found that FAs can nucleate microtubule assembly under situations when cells are recovering from treatment with microtubule inhibitors such as nocodazole. The mechanism of stabilization is as yet unclear, but, as microtubules influence cell polarity, their selective stabilization may contribute to polarized migration.

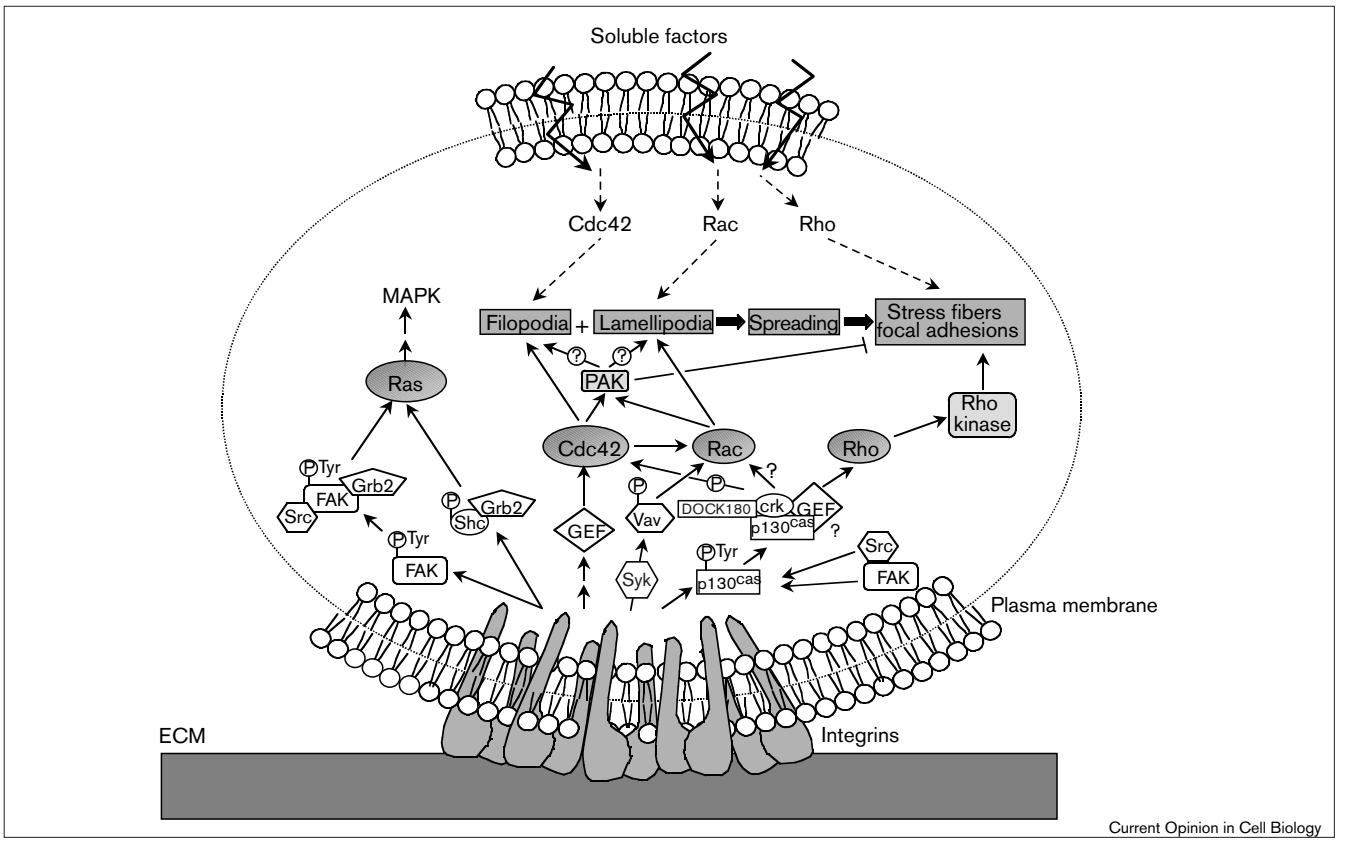
Besides stimulating stress fibers and FAs, constitutively active RhoA also causes a collapse of vimentin intermediate filaments [126]. Sin *et al.* [127•] have found that the RhoA-activated kinase ROKα (Rho-kinase) binds to vimentin and that vimentin is a substrate for this kinase. The phosphorylation of vimentin by ROKα inhibits its polymerization *in vitro*, resembling the actions of other kinases that have been shown previously to promote the collapse and disassembly of vimentin filaments [128]. Introduction of constitutively active ROKα induces the collapse of intermediate filaments, similar to their collapse induced by microtubule depolymerization [127•]. Interestingly, the collapse of vimentin filaments to a condensed aggregate next to or around the nucleus in response to microtubule depolymerization has been shown to depend on force generated by actin–myosin interaction [129]. ROKα (Rho-kinase) stimulates contractility (see above), suggesting that the collapse of the vimentin filamentous network is the result of the combined effects of vimentin phosphorylation and enhanced contractility. Vimentin phosphorylation may disconnect these filaments from their close association with microtubules, because microtubules remain extended even as vimentin condenses. The significance of the vimentin collapse in response to RhoA activation is not clear.

Integrin feedback to cytoskeletal reorganization

In studying the activation of Rho family GTPases most attention has been focused on the actions of soluble factors such as bioactive lipids, peptides and growth factors. Several studies, however, have begun to reveal that integrin-mediated adhesion can itself activate these GTPases (Figure 3). Long before Rho GTPases were identified, it was known that adhesion to ECMs induced extensive filopodia and membrane ruffling. These cytoskeletal/membrane protrusions are now recognized as the hallmarks of Cdc42 and Rac activation respectively, leading to the prediction that integrin-mediated adhesion activates these small G proteins (Figure 3). Recent work has substantiated this idea [130•,131•]. Dominant-negative mutants of Cdc42 and Rac inhibit the extension of filopodia and ruffles in response to adhesion to FN. In addition, PAK, a serine/threonine kinase activated by both Cdc42 and Rac, is stimulated by FN adhesion [131•]. Both studies found that Cdc42 activation appears to be upstream of Rac activation, consistent with the cascade that has been identified with soluble activators of these GTPases [65,66]. Several studies have also indicated that RhoA is activated upon integrin-mediated adhesion [20,130•,132•], although RhoA activation appears to be largely independent of adhesion-induced Cdc42 and Rac activation [130•,131•]. Measurement of RhoA activity using the Rho-binding domain of Rhotekin — which associates preferentially with GTP-bound RhoA — reveals that integrin-mediated adhesion causes only a modest increase in RhoGTP levels when compared to activation by soluble factors such as lysophosphatidic acid (LPA) or serum [122•].

How might integrin-mediated adhesion activate Rho family GTPases? GEFs are obvious targets because these mediate Rho family activation. With increasing numbers of GEFs being identified [12•], the challenge is to determine which ones are involved in response to integrin-mediated adhesion. In addition, it will be important to determine which signaling molecules downstream from integrins lead to GEF activation. One candidate for a role in integrin-mediated activation of Rho family GEFs in response to adhesion is Cas. Cas binds FAK [133], is a substrate for Src [134] and becomes tyrosine phosphorylated in response to integrin-mediated adhesion [135–137]. Cas also binds Crk, an SH2/SH3-containing adaptor protein that interacts with C3G, a GEF for Ras and Rap1 [138]. Cells derived from $Cas^{-/-}$ mice exhibit decreased FAs and stress fibers, suggestive of effects on Rho activity [139•,140•]. This raises the question as to whether a RhoGEF interacts with Cas or Crk. Altun-Gultekin *et al.* [141•] show that overexpression of v-Crk in PC12 cells leads to activation of Rho kinase, PIP_2 accumulation, FA assembly, stress fiber formation and cell spreading. These observations are consistent with acti-

Figure 3



Integrin-mediated adhesion activates Rho family GTPases. Attachment of cells to FN leads to activation of both Cdc42 and Rac [130•,131•]. Both Rac and Cdc42 activate multiple downstream targets, including the kinase PAK, which has been implicated in cytoskeletal reorganization, including the disassembly of stress fibers [31•,70•,71•]. Rho is also activated by integrin-ligation [122•,132•], leading to the formation of stress fibers and FAs [132•]. It should be noted, however, that the activation of RhoA by integrin ligation is less robust than its activation by soluble factors. The GEFs responsible for Cdc42, Rac and RhoA activation following integrin engagement are not known, but one candidate is Vav, a Rac GEF that becomes tyrosine phosphorylated and activated in response to integrin-mediated adhesion in platelets [78•,79]. Another candidate for a role in Rac and RhoA activation is $p130^{cas}$ in complex with the adapter protein Crk [139•,141•–143•], and the Crk-binding protein DOCK180 [144•–147•].

vation of RhoA and possibly Rac. Other work strongly supports the activation of Rac via Cas.

As mentioned earlier, overexpression of FAK promotes cell migration [103]. Exploring the basis for this, Cary and coworkers [142•] found that this enhancement of motility was mediated by Cas. Tumor cells with enhanced migration displayed elevated Cas tyrosine phosphorylation and overexpression of Cas was found to promote migration of cells on ECM [143•]. Mutations that prevented Cas tyrosine phosphorylation blocked the migratory response; furthermore, cell migration was promoted by expression of either Cas or Crk, whereas cytokine-stimulated migration was inhibited in cells expressing mutations in Cas or Crk that inhibited their interaction. Significantly, migration in response to Cas or Crk was prevented by dominant-negative Rac, suggesting that the Cas–Crk complex leads to Rac activation [143•]. A likely protein involved in Cas-Crk signaling to Rac is DOCK180 which binds to Crk in response to integrin-mediated adhesion [144•,145•] and also binds Rac [146•,147•]. Although it does not appear to be a conventional Rac GEF itself, DOCK180, has been reported to enhance nucleotide exchange and when overexpressed in cells promotes elevated Rac•GTP levels [147•].

Cytoskeletal regulation of matrix assembly

There is a close relationship between the organization of the ECM and the actin cytoskeleton. This has been most studied with fibronectin (FN). Not only is there a parallel distribution of FN fibrils on the cell surface with submembranous bundles of actin filaments and FA proteins but disruption of microfilaments with agents such as cytochalasin leads to a parallel disruption of the fibrillar FN matrix on the outside [148]. Clues as to why ECM organization depends on cytoskeletal integrity have been provided recently. FN matrix assembly is promoted by serum and Mosher's group has identified the critical ingredient in serum as LPA [149]. LPA is a lipid that stimulates RhoA activity, as well as initiating other signaling pathways. Inhibiting RhoA activity or actin–myosin interaction was

shown to prevent FN matrix assembly leading to the suggestion that it is the tension generated by the cytoskeleton that contributes to the assembly of a FN fibrillar matrix [120•,150•]. Several studies have provided evidence for cryptic self-assembly sites within FN [151–153] and recent work has demonstrated that at least some of these sites can be exposed by mechanical stretching of FN [150•]. Dramatic evidence that cells exert tension on FN fibrils has been provided through the use of green fluorescent protein (GFP)-tagged FN. These experiments demonstrate that live cells can impose sufficient tension on FN fibrils to generate considerable stretch. Upon detachment, FN fibrils under tension were observed to contract rapidly to a quarter of their stretched length [154•].

Tension generated by the actin cytoskeleton affects the assembly and organization of the ECM and, in turn, the organization of the ECM influences many aspects of cell behavior. This has been illustrated in studies showing that cell growth is diminished for cells adhering to an abnormal FN matrix assembled from truncated FN [155•] or for cells in which FN fibril assembly has been blocked [156•].

Conclusions

Much has been learned about how RhoA stimulates integrin clustering into FAs and the role of myosin-mediated contractility in this process. In contrast, much less is known about the clustering of integrins into focal complexes in response to Rac and Cdc42. The crosstalk between Rho family GTPases appears more and more complicated. In some situations, Cdc42 and Rac activate RhoA, but the actions of RhoA are often antagonistic to Rac and Cdc42. Downstream effectors for Rac and Cdc42 have been identified that inhibit the development of stress fibers and FAs induced by RhoA. Understanding the functions of the many effectors of these GTPases will be a challenge for the future, as will be the unraveling of their complex interactions.

Many different factors have been identified that promote disassembly of FAs and dispersal of integrins. For some of these factors, progress has been made identifying the signaling pathways involved. Interestingly, several of them converge on the regulation of myosin activity and result in inhibition of contractility. Not only do Rho family proteins regulate the state of the actin cytoskeleton but recent work has demonstrated that these proteins also regulate microtubules and vimentin intermediate filaments. In addition, the state of microtubule polymerization affects the activity of RhoA.

An exciting development in the field has been the recognition that integrin-mediated adhesion itself triggers activation of Cdc42, Rac and RhoA. With increasing numbers of GEFs being identified for Rho family GTPases, another challenge will be to identify which GEFs are activated in response to integrin ligation. We anticipate that adhesion-mediated regulation of Rho family GTPases will play an important part in the complex process of cell migration.

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