Assembly and mechanosensory function of focal contacts

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Focal contacts, focal complexes and related extracellular matrix adhesions are used by cells to explore their environment. These sites act as mechanosensory 'devices', where internal contractile forces or externally applied force can regulate the assembly of the adhesion site and trigger adhesion-dependent signaling involving Rho-family small G-proteins and other signaling pathways. The molecular mechanisms underlying these processes are discussed.

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Abbreviations

AIACs	actin–integrin adhesion complexes
DAD	Dia-autoregulatory domain
Dia	Diaphanous
ECM	extracellular matrix
FH	formin homology
MLC	myosin II regulatory light chain
MLCK	MLC kinase
MLCP	MLC phosphatase
PAK	p21-activated kinase
p95PKL	paxillin-kinase linker
RBD	Rho-binding domain
ROCK	Rho-associated kinase

Introduction

What do we do normally when we encounter a new and unfamiliar environment? We usually recruit all our sensory skills to explore the new surroundings and find out what they are like. Cells appear to use a similar strategy and use whatever 'senses' they have in order to evaluate the chemical and physical properties of their microenvironment; they make particular use of the extracellular matrix (ECM) that they encounter. Indeed, cells gather such information at sites of ECM attachment, and these signals instruct the cells what it should do (e.g. live, die or differentiate) and where it should go. ECM adhesions can thus be regarded as highly important 'signaling centers' and as such they function as unique cellular compartments in which the external surface is physically connected to a complex of signaling and cytoskeletal proteins via transmembrane receptors of the integrin family. These adhesions are known under several names, such as focal contacts, focal adhesions, fibrillar adhesions, focal complexes and podosomes, which all share a great deal of molecular similarity [1•] and will collectively be referred to as actin–integrin adhesion complexes (in short — AIACs).

The assembly of AIACs is controlled by complex signaling pathways whose activation modulates the actin cytoskeleton (in particular, its assembly dynamics and contractility) and consequently reinforces AIAC formation. In turn, integrin-dependent signals, generated upon adhesion and assembly of AIACs, can affect these regulatory networks, forming either positive or negative feedback loops. Controlled AIAC assembly and signaling is involved in a wide variety of cellular processes during embryonic development, wound healing, inflammation and cancer invasion.

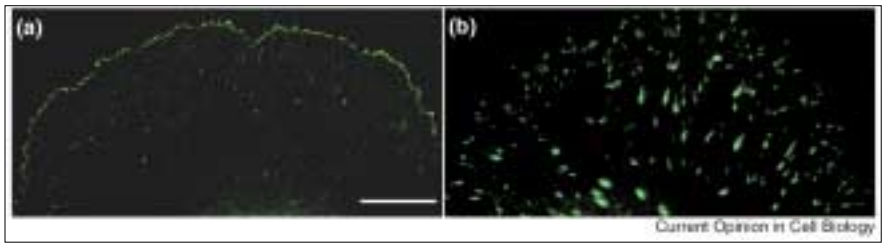
In this review we summarize some recent studies addressing the assembly and signaling activity of the best-studied example of AIACs, namely focal contacts (or focal adhesions). These studies focus on the role of small G-proteins of the Rho family in the assembly of these structures and address their dependence on tension applied by internal actomyosin contraction or by external perturbation. The unique features of focal contacts suggest that they can indeed function as mechanosensors, 'reporting' to the cells about the physical properties of the surrounding environment and participating in vivid transmembrane cross-talk between the ECM and the signal transduction system.

A brief look into the molecular complexity of focal contacts

Classical focal contacts are flat elongated structures associated with the ends of actin filament bundles (stress fibers) in a wide variety of cultured adherent cells and can be visualized by interference reflection or electron microscopy [2,3]. These structures appear to be extremely crowded with different molecules. So far over 30 distinct molecules have been reported to reside in them, and the list is still rapidly expanding (for reviews see [4–6,7•,8•]).

The detailed molecular architecture of focal contacts is unknown, yet impressive 'interaction maps' depicting the potential molecular interactions can be constructed, based mainly on biochemical binding data (see [8•]). A comprehensive discussion of the molecular composition and diversity of focal contacts and other AIACs is published elsewhere [8•], therefore it suffices to indicate here that, although it appears hopeless to derive definitive structural models just from the biochemical data, some interesting features of focal contact components are noteworthy. For example, one of the characteristic properties of most focal contact proteins is their multi-domain structure. Vinculin, for example, can interact (most probably not simultaneously) with at least 10 other focal contact components, including actin, tensin, paxillin, hic-5, talin, α-actinin, vinexin, ponsin, vasodilator-stimulated phosphoprotein (VASP) and phosphatidylinositol 4,5-bisphosphate (PI[4,5]P_2), and many of these can further bind to multiple additional partners. Another noteworthy observation is that about half of the known molecular residents of focal contacts are established components of different signal-transduction pathways,

Figure 1



Focal complexes and focal contacts. Giant multinuclear SV-80 fibroblasts were stained with anti-phosphotyrosine antibody to visualize adhesion sites. A peripheral area of the lamella is shown in each case. (a) A cell treated with H-7, an inhibitor of ROCK and MLCK that blocks myosin-II-driven contractility, displays numerous dot-like focal complexes mostly accumulated at the narrow zone at the edge of lamella where lamellipodia formation occurs. Conversion of these focal complexes into focal contacts is blocked here because of contractility inhibition. (b) A cell with augmented myosin II contractility (induced here by 30 min microtubule disruption) demonstrates enhanced formation of elongated mature focal contacts, whereas the fraction of focal complexes is reduced. The bar represents 20 μm.

among them protein tyrosine kinases (e.g. focal adhesion kinase (FAK) and $pp60^{Src}$ [9]), serine–threonine kinases (e.g. p21-activated kinase [PAK] [10]), protein phosphatases (e.g. SH2-domain-containing protein tyrosine phosphatase-2 [SHP-2] and leukocyte common antigen-related tyrosine phosphatase [LAR]) and adapter proteins bearing characteristic protein–protein binding domains (e.g. SH2, SH3, PTB, LIM) [8•].

Here we will resist the temptation to address the structural and functional significance of AIACs complexity, and focus mainly on signaling proteins that play a central role in focal contact assembly, namely, small G-proteins of the Rho family. Paradoxically, these proteins, despite their major involvement in regulating focal contact formation are not physically associated with these structures [11].

Focal complexes, precursors of focal contacts

Most focal contacts grow out of small dot-like AIACs known as focal complexes, which form at the edges of lamellipodia [12–14]. The formation of focal complexes is induced by the small Rho-family G-protein Rac [12,14], whereas Rho itself is, apparently, not necessary [14]. Many of the protein components of focal contacts are also present in focal complexes, yet the two are molecularly and functionally distinct as focal complexes are enriched with activated (high-affinity) αvβ3 integrin [15•] and apply stronger traction forces to the substrate during cell migration [16••]. Paradoxically, focal complexes in stationary cells are significantly less tension-dependent than focal contacts and tend to accumulate along the cell edge following treatment with inhibitors of actomyosin contractility (Figure 1 and see below).

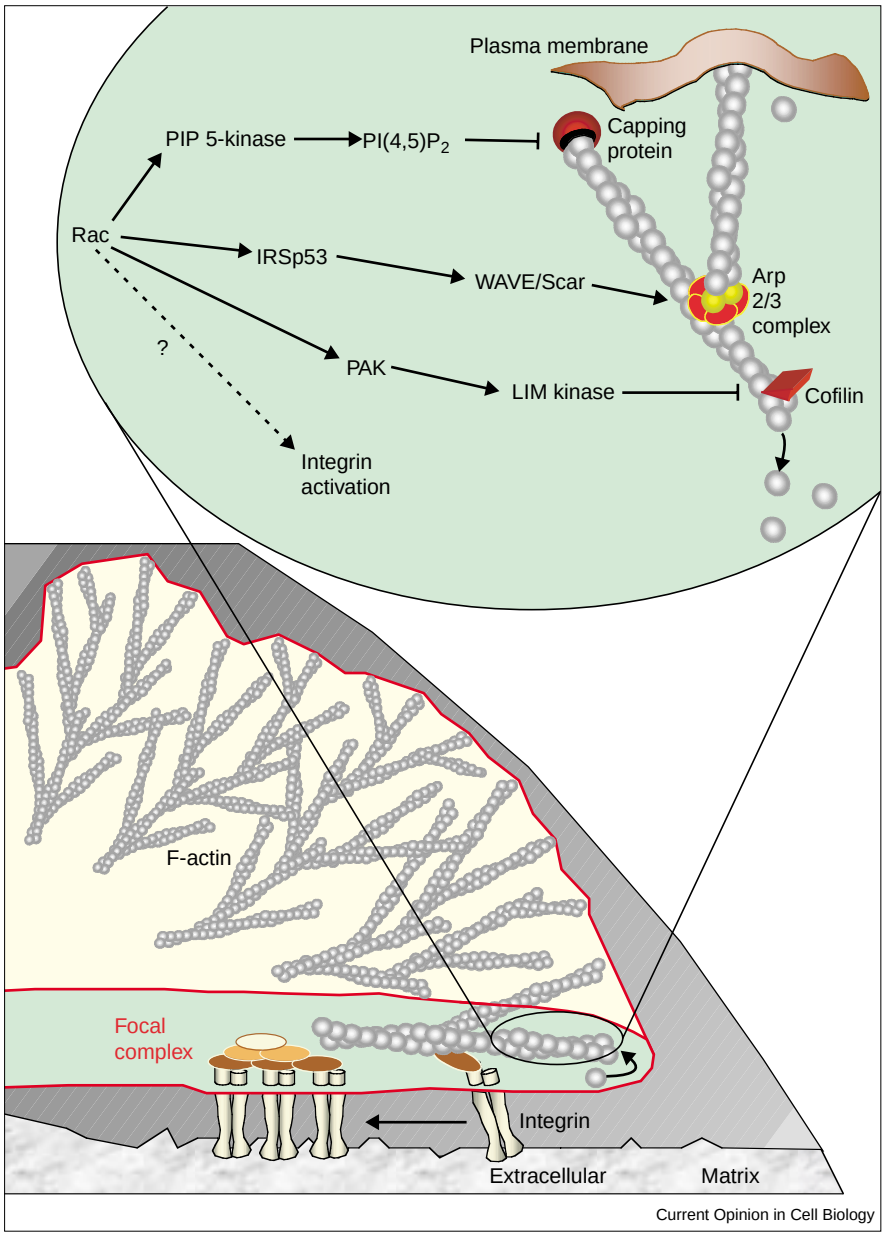
How does Rac induce focal complex formation? One possibility is that attachment to the ECM induces binding of activated Rac to the membrane [17••], particularly in the lamellipodia [18••] where it can stimulate actin polymerization and branching [19•]. Rapid actin flow in this region may, in turn, promote clustering of integrin molecules (Figure 2). There are three possible partners of Rac in mediating these effects (Figure 2): (i) type Iα phosphatidylinositol-4-phosphate 5-kinase (PIP 5-kinase α), which catalyzes $PI(4,5)P_2$ production, leading to uncapping of actin filaments [20•]; (ii) IRSp53 protein activating WAVE/Scar, which in turn activates actin filament nucleation via Arp2/3 complex [21•,22••]; and (iii) the serine–threonine kinase PAK.

PAK, which is a primary target of Rac and Cdc42 [10] might have a major role in focal complex regulation. This protein affects actin polymerization via phosphorylation and activation of LIM kinase, which phosphorylates and inactivates the actin depolymerizing proteins ADF/cofilin [23••]. Activation of this pathway seems to be essential for the Rac-induced actin reorganization [24]. Cell attachment to the ECM induces PAK translocation to the membrane [17••] and its association with integrins, possibly via paxillin and p95PKL (paxillin-kinase linker) protein [25•,26], and enhances its activation by Rac [17••]. Activated PAK undergoes autophosphorylation [27] and translocates to AIACs at the cell edges [28•]. It remains to be determined whether the membrane translocation of active Rac and its different targets and the concomitant stimulation of actin polymerization are both required and sufficient for the induction of focal complex formation.

From focal complexes to focal contacts: the dual role of Rho

Transformation of focal complexes into focal contacts requires the activation of Rho [13,14], yet the mechanism underlying this process is still poorly understood. Active Rho has multiple targets, including several serine–threonine kinases (e.g. PKN [protein kinase N]), citron kinase, ROCK/Rho-kinase), PIP 5-kinase and several proteins without known enzymatic activity (e.g. rhothekin, rhophillin, Dia1/Dia2) [29•]. Although many of these targets were shown to modulate some functions of the actin cytoskeleton, it appears that the combined action of just two of them, namely ROCK and Dia1, is sufficient to substitute active Rho in the process of focal contact assembly (Figure 3). This is based on the capacity of these two proteins to restore stress fiber and focal contact formation in cells expressing Botulinum C3 transferase that specifically inactivates Rho [30••]. The possible function of each of these effectors will be discussed below.

Figure 2

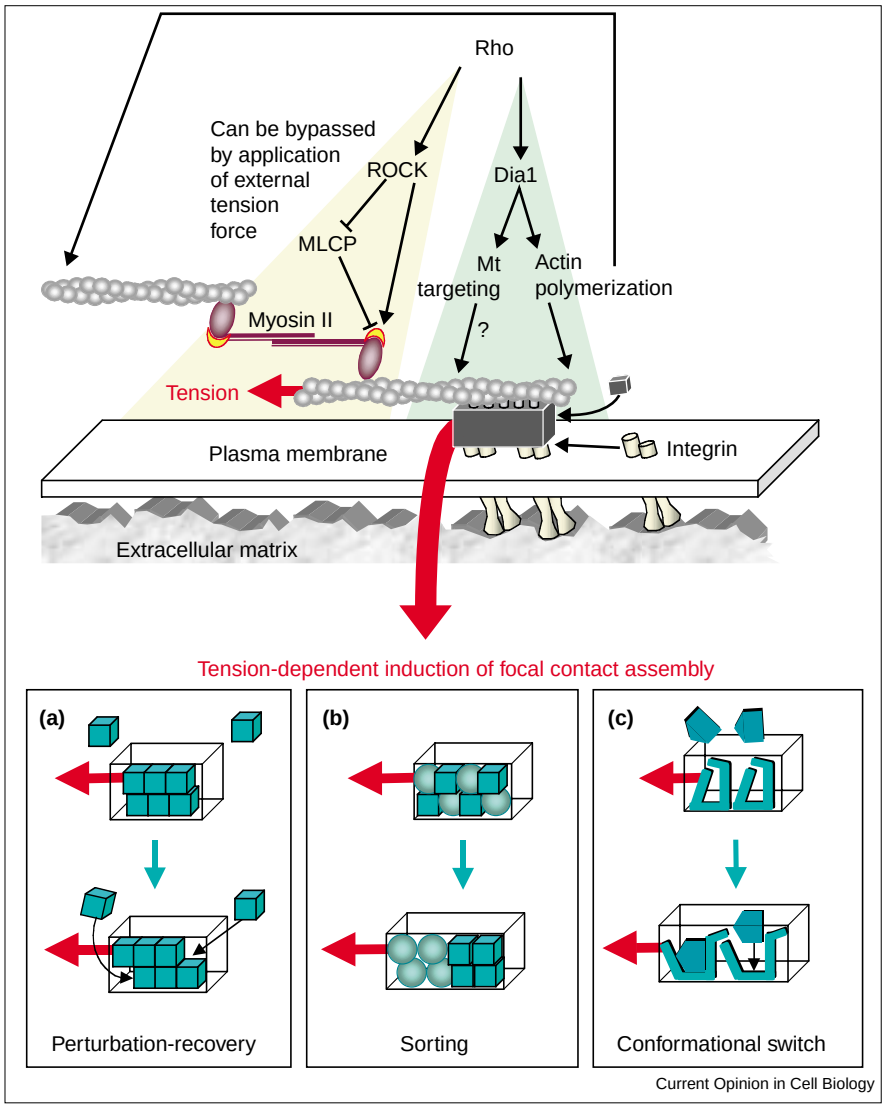


Rac-dependent formation of focal complexes. Focal complexes, small clusters of transmembrane integrin molecules and associated cytoplasmic proteins (colored ovals) are formed upon lamellipodial outgrowth on the ECM. Polymerization of actin filaments in the lamellipodium provides the driving force for lamellipodial protrusion and for the rapid centripetal actin flow that may carry the integrins and facilitate their binding to the ECM. Rac-GTP, concentrated at the leading edge of the lamellipodium, activates several pathways leading to augmentation of actin polymerization and branching via uncapping of barbed filament ends, activation of Arp2/3 complex and phosphorylation of cofilin (insert in the upper part of the figure). Some elements of these pathways, for example $PI(4,5)P_2$, may also participate in the transition of molecular components of focal complexes (e.g. vinculin) into active ('open') conformation. Other Rac-induced pathways affecting the integrin activation may also exist.

Function of Diaphanous (Dia) proteins: actin polymerization and microtubule targeting

Drosophila Diaphanous belongs to a subfamily of formin homology (FH) proteins and is necessary for cytokinesis [31]. Several homologs of this protein are involved in actin cytoskeleton regulation in budding and fission yeast [32,33] and in mammalian cells where they are known as Dia or DRF (Diaphanous-related formin homology proteins) [30••,34,35•,36••]. All Dia proteins have similar functional domains: the Rho-binding domain (RBD) at the amino terminus, several FH domains and a Dia-autoregulatory domain (DAD) at the carboxyl terminus [30••,34,37•]. Non-activated Dia proteins are believed to be in a 'closed' conformation in which the DAD binds to the RBD. Binding of Rho-GTP to the RBD 'opens' the molecule and exposes the FH domains for interaction with various downstream targets [30••,37•], such as profilin [34] and the SH3-domain-containing proteins $pp60^{Src}$ [36••] and IRSp53 [38]. Expression of activated deletion mutants of Dia1 enhances actin polymerization in cells, and the proline-rich profilin-binding FH1 domain of Dia is essential for this effect [30••]. Profilin binds monomeric actin, suppresses spontaneous actin nucleation but promotes addition of actin subunits at the barbed (fast growing) ends of actin filaments, thus enhancing actin filament assembly [19•]. It was suggested, on the basis of rather indirect evidence, that Dia promotes actin polymerization by targeting profilin and possibly by enhancing its function [34]. It was also suggested that Dia, similar to WASP, directly activates Arp2/3 [37•]. Other possible targets for Dia action are microtubules; yeast

Figure 3



Rho-dependent growth of focal contacts. Rho induces formation of the focal contacts by activating two essential pathways, ROCK-dependent (shown in yellow) and mDia1-dependent (shown in green). The main function of the ROCK-dependent pathway is to activate myosin-II-driven cell contractility owing to direct or indirect effects on the phosphorylation of myosin light chain (yellow crescent). This pathway can be bypassed if tension is applied externally. The mDia1-dependent pathway includes activation of actin polymerization (both local, in the proximity of the growing focal contact, and global, in distal regions) and possibly targeting of microtubules and subsequent microtubule-dependent delivery of certain components. If mDia1 is active, application of tension force triggers the growth of the focal contact ('black box'). The molecular mechanism of the force-induced focal contact growth is not clear. Three hypothetical models suggesting how the 'mechanosensory switch' might work are depicted in the bottom part of the figure. **(a)** The 'perturbation-recovery' model suggests that tension force applied to focal contacts perturbs the integrity of the protein meshwork associated with the adhesion sites, creating spaces that can accommodate new subunits. **(b)** According to the 'sorting' model, force might selectively displace some components from the adhesion site, changing their molecular microenvironment. **(c)** The 'conformational switch' model suggests that force applied to specific adhesion-associated molecules might induce conformational changes, leading to their transition into 'active conformation'. Mt, microtubule.

Dia proteins, and, possibly, the mammalian homologs are involved in coordination of microtubules and actin cytoskeleton, participating in targeting microtubules to the cell cortex [33,39,40••,78]. Thus, it is possible that microtubule-dependent processes, regulated by Dia might affect focal contact assembly ([40••] and see below).

Interestingly, activation of Dia augments stress fibers and focal contact development in normal cells but fails to do so when Rho activity is blocked. Activation of Dia under those conditions can enhance the formation of thin actin fibers, which are clearly distinct from stress fibers [30••,35•], although it fails to stimulate focal contact assembly. Induction of focal contacts and stress fibers requires that Rho-associated kinase (ROCK) is activated together with Dia [30••,35•,36••]. It is noteworthy that the activation of ROCK can be substituted by external mechanical perturbation [41••].

Rho-associated kinase (ROCK) can trigger tension-dependent focal adhesion development

Two closely related serine–threonine kinases, ROCK-1 (known also as ROK beta) and ROCK-2 (known also as ROK alpha or Rho-kinase) are major targets of Rho (review: [42•]). Like Dia, ROCK is activated by transition from a 'closed' to an 'open' conformation following binding to Rho-GTP, thus exposing the amino-terminal catalytic domain [42•]. But how does ROCK control focal contact formation?

ROCK has many targets with diverse functions, including LIM-kinase, which enhances actin polymerization [43••,44–46], possibly ERM proteins (ezrin/radixin/moesin) that are membrane–cytoskeleton linkers [42•,47] and others (see [42•,48•]). However, the effect of ROCK on focal contact formation appears to involve the activation of myosin-II-driven contractility through the augmentation of phosphorylation of myosin II regulatory light chain (MLC).

The level of MLC phosphorylation is controlled by balanced phosphorylation by MLC kinase (MLCK) and dephosphorylation by MLC phosphatase (MLCP) [42•,48•,49•]. ROCK phosphorylates and inactivates the myosin-binding subunit of MLCP, leading to increased MLC phosphorylation and to enhanced myosin II contractility [42•,48•,49•]. In addition, ROCK can directly activate contractility by phosphorylating MLC just like MLCK [42•,50]. How does contractility affect the formation of focal contacts and stress fibers?

Focal contacts as mechanosensors

One of the intriguing features of focal contacts formation and stability is their strict dependence on myosin-II-driven contractility. Chemical inhibitors of MLC phosphorylation and actin-dependent myosin ATPase activity suppress focal contact and stress fibers formation [51,52]. Similar effects can be obtained by overexpression of non-muscle caldesmon, a protein that restrains the interactions of myosin heads with actin filaments [53•]. Moreover, expression of a dominant-negative deletion mutant of myosin IIA heavy chain blocks cell contractility and consequently suppresses the formation of matrix adhesions [54]. It is noteworthy that both the chemical inhibitors and caldesmon inhibit focal contact formation even when the cells express constitutively active Rho [52,53•]. Thus, myosin II acts downstream to Rho–ROCK-dependent formation of focal contacts. It should be highlighted that the sensitivity to myosin II inhibition is a characteristic feature of focal contacts discriminating them from other types of AIACs, including focal complexes and fibrillar adhesions, although in some studies high doses of inhibitors induced disruption of focal complexes [14].

The nature of the mechanical switch, which triggers focal contact and stress fiber formation, is not known, and its elucidation is a major scientific challenge (see below). We do know, however, that the force applied to focal contracts can be either endogenous (i.e. actomyosin driven) or exogenous. Specifically, when local mechanical force is applied from the outside, growth of focal contacts is achieved even if actomyosin contractility is blocked and ROCK activity is suppressed by specific inhibitors (e.g. Y-27632) [41••]. Experiments with force application by micropipette showed that tension-dependent regulation of focal contact assembly is a local process [41••]. In other experimental system, centripetally moving beads attached to the cell surface via integrin were restrained by laser tweezers, thereby inducing a mechanical force applied to the integrin receptor complex [76]. This led to a local increase in the force exerted on the bead by the cell ('reinforcement'), a process that can be explained by the force-dependent local recruitment of new components into primary adhesions [76,77].

In non-motile cells a strong correlation exists between the tension applied at individual adhesion sites and the size of the corresponding contact, so that the tension per unit area (i.e. 'stress') remained constant (about 5nN/μm2). Such a correlation was observed recently in experiments using micro-patterned elastic substrates [55••], which also showed that decrease in cell contractility following 2,3-butanedione monoxime (BDM) treatment rapidly (within seconds) induced reduction in vinculin-containing adhesions. It is worth noting that these, apparently simple relationships between focal contact size and the force applied to it, may vary in different states of cells and that in motile cells, broadly distributed adhesions at the leading edge transmit strong centripetal forces [16••]. It appears that many signaling pathways can affect focal contact dynamics, and therefore experimental manipulations with such factors as PAK lead to situations where contractility and growth of focal contacts are not tightly correlated [56]. Nevertheless, the remarkable ability of focal contacts to assemble or disassemble in response to changes in the locally applied force explains the cellular response to interactions with substrates with different physical properties or motility along a rigidity gradient ('durotaxis') [57,58••].

Microtubules control focal contact formation by modulating local tension

The formation of focal contacts needs to be centrally coordinated in cells to allow for processes such as directional cell migration to take place. Indeed, dynamic microscopic analysis indicated that microtubule ends are often inserted into regions of leading lamella where focal contact formation and modulation occur [59,60]. Several mechanisms could account for this effect; for example, Dia, which acts a regulator of both microtubules and focal contacts could be involved in this targeting [30,40••,78]. Targeted microtubules presumably play a dual role in focal contact formation. They, together with appropriate motor molecules, might direct the delivery of specific components that are necessary for the development of focal contact and stress fibers [40••]. The microtubule system may also control (most likely — suppress) both global and local cell contractility (reviewed in [61]), which, in turn, can modulate the forces applied to focal contacts and thereby determine their fate. Disruption of microtubules increases tension and promotes focal contact growth, whereas growth of microtubules in the proximity of contact sites induces local relaxation of tension and suppresses focal contact growth [60,62,63,64•,65]. Thus, radial system of dynamic microtubules, together with the mechanosensitive focal contacts, might form a versatile adaptive system, which can determine the orientation of stress fibers, polarization of cell shape and directional cell locomotion [61,66].

How does the mechanosensory switch work?

The molecular nature of the mechanosensory switch at focal contacts is puzzling and invites creative speculations. One may consider several distinct mechanisms, as schematically illustrated in Figure 3. One putative mechanism is the 'perturbation-recovery' model that suggests that mechanical perturbation (stretching) of the tightly ordered submembrane plaque might create dislocation in

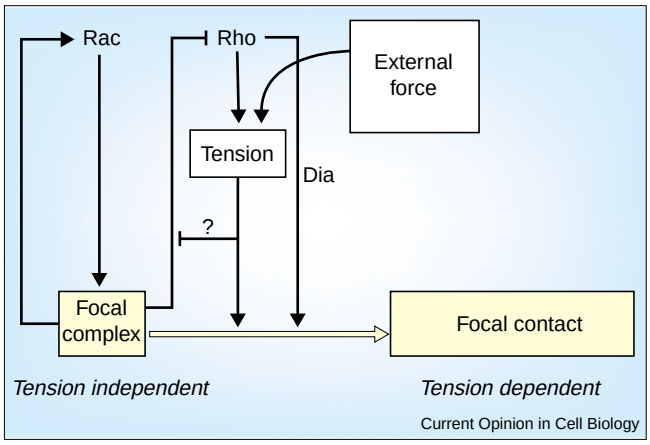
the lattice of submembrane molecules and that the gaps formed in this structure are readily 'filled-in' with anchor proteins from the soluble cytoplasmic pool. Another model ('sorting') suggests that differential responses to force of different integrins and the associated anchor proteins, induces gross reorganization of AIAC components, similar to the reorganization previously described for the exit of fibrillar adhesions from peripheral focal contacts [67•,68•]. Such sorting process might create new local molecular 'microenvironments' with distinct properties. The 'conformational switch' hypothesis suggests that mechanical forces applied at adhesion sites (in the order of ~10 nN per adhesion site [55••]) may induce conformational changes in specific components on focal contacts and change their properties, including, enzymatic activity or exposure of different binding sites. A rough calculation of the magnitude of force 'sensed' by individual molecules in the adhesion sites suggests that it might be in the range of a few pN, which is sufficient for inducing conformational changes and is below the level of force which is expected to denature proteins. Interestingly, many pivotal matrix adhesion components, including vinculin, ERM proteins, pp60^{Src} and others can be activated by conformational change from 'closed' to 'open' state [8•,69], like Dia and ROCK proteins, as discussed above. One can suggest that such conformational transitions, induced by intrinsic or external forces, applied at adhesion sites, may alter the molecular properties and composition of these sites. Finally, one should keep an open mind to the possibility that the mechanosensor might not be a cytoplasmic component of the adhesion site at all but rather an ECM component that changes its properties under tension [70] or a force-activated ion channel [71•].

Whatever the elusive mechanosesor is, tension-induced structural reorganization of focal contacts may affect focal contact growth indirectly, via integrin signaling and in particular, via the modulation of Rho activity. Although prolonged integrin stimulation by fibronectin leads to Rho activation, initially (during the first 10–15 min) Rho activity in stimulated cells decreases [72••,73••]. Interestingly, this decrease can be observed without attachment to the substrate, in suspended cells treated with RGD peptide engaging the integrin receptors [74••]. The signaling components that are involved in the integrin-dependent suppression of Rho were shown to be FAK [73••] and pp60^{Src} [74••]. One can speculate (Figure 4) that mechanical tension applied to nascent focal contact blocks this negative regulatory pathway at some point, which should locally abolish the integrin-dependent inhibition of Rho activity and induce a burst of Rho-dependent assembly of mature focal contacts. It is interesting to mention, in this connection, that directional motility based on cellular mechanosensitivity ('durotactic' ability) depends on FAK [75].

Conclusions

The recent findings described here, although still rather incomplete, start to outline the basic rules that regulate the fate of a focal contact. Initially, binding of integrins to their ECM partners takes place at the leading edge. Then, Rac is activated and drives the formation of focal complexes by activating the assembly of dynamic actin network in the lamellipodium. Rho is next in the picture and drives the maturation of focal contacts by the activation of both Dia and ROCK; the latter is responsible for the generation of myosin-II-dependent tension applied to the growing contact sites, and the former may be involved either in actin polymerization or microtubule targeting of adhesion sites. Pulling on these complexes promotes tension-dependent incorporation of new components into the nascent adhesion site, leading to its growth. Finally, continued pulling leads to the molecular sorting ('maturation') of different forms of AIACs, including 'classical' focal contacts and fibrillar adhesions [67•,68•]. Integrin-dependent signaling, activated upon focal contact assembly can provide a feedback that may, in principle, be also regulated by tension. Thus, AIACs appear to function as unique organelles, 'sensing' and 'exploring' both the chemical and physical nature of the ECM in the cell's microenvironment and regulating the long-range adhesion-mediated signaling response.

Figure 4



This figure depicts the possible feedback loops controlling the formation and growth of focal contacts. Integrin engagement upon formation of focal complexes creates a signal leading to activation of Rac, which forms a positive feedback loop providing continuous formation of new lamellipodia and focal complexes. At the same time, Rho activity is suppressed (possibly via a FAK/Src-dependent mechanism), which delays the development of contraction until the cell successfully spreads. Activation of Rho by external stimuli like LPA or sphingosine phosphate (not shown here) overcomes this suppression and induces formation and growth of the focal contacts. Rho activates Dia and stimulates cell contractility via Rho kinase. Tension created by cell contractility, as well as by external mechanical force, activates the 'mechanosensory switch' in the focal complexes inducing their growth. One can suggest that tension abolishes integrin-dependent suppression of Rho function.

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