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Review

Regulation of substrate adhesion dynamics during cell motility

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Abstract

The movement of a metazoan cell entails the regulated creation and turnover of adhesions with the surface on which it moves. Adhesion sites form as a result of signaling between the extracellular matrix on the outside and the actin cytoskeleton on the inside, and they are associated with specific assembles of actin filaments. Two broad categories of adhesion sites can be distinguished: (1) "focal complexes" associated with lamellipodia and filopodia that support protrusion and traction at the cell front; and (2) "focal adhesions" at the termini of stress fibre bundles that serve in longer term anchorage. Focal complexes are signaled via Rac1 or Cdc42 and can either turnover on a minute scale or differentiate, via intervention of the RhoA pathway, into longer-lived focal adhesions. All classes of adhesion sites depend on the stress in the actin cytoskeleton for their formation and maintenance. Different cell types use different adhesion strategies to move, in terms of the relative engagement of filopodia and lamellipodia in focal complex formation and protrusion and the extent of focal adhesion formation. These differences can be attributed to variations in the relative activities of Rho family members. However, the Rho GTPases alone are unable to signal asymmetry in the actin cytoskeleton, necessary for polarisation and movement. Polarisation requires the collaboration of the microtubule cytoskeleton. Changes in the polymerisation state of microtubules influences the activities of both Rac1 and RhoA and microtubules interact directly with adhesion foci and promote their turnover. Possible mechanisms of cross-talk between the microtubule and actin cytoskeletons in determining polarity are discussed.

Keywords: Actin cytoskeleton; Microtubules; Polarisation; Signaling; Rho

Contents

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1. Introduction

The adhesion of a cell to a substrate is a necessary requirement for it to spread and crawl. Studies using the technique of interference reflection microscopy (IRM) [1] were the first to show that cells do not attach uniformly to a surface but at specialised foci, the largest of which have been termed focal contacts or focal adhesions [2,3]. From the interference patterns in the IRM images it was estimated that the cell to substrate separation at focal adhesions lies in the range of 10–15 nm. The same studies [2] revealed the general immobility of focal adhesions relative to the substrate, consistent with an adhesive function. And the adhesive nature of these foci was confirmed in experiments whereby cells were mechanically sheared from the surface on which they were grown: after such treatments, focal adhesion sites were left behind, isolated and still attached to the substrate [4,5].

It is now well established that adhesion foci are complex molecular assemblies that link the extracellular matrix, via transmembrane matrix receptors (integrins) to the actin cytoskeleton [6,7]. And the identification of component proteins of focal adhesions, starting with vinculin [8] and now numbering over 50 [7] has resulted in alternative tools to visualise adhesion sites in living and fixed cells. In particular, the possibility to tag adhesion site proteins with fluorescent probes, including green fluorescent protein (GFP) has allowed the detection in living cells of adhesion complexes below the resolution offered by the IRM method [9–11]. It has also become apparent that focal adhesions are only one of a few classes of adhesion complexes observed in spreading and migrating cells. In discussing what is known about the genesis and turnover of adhesion sites during cell movement we will highlight alternative strategies of adhesion site dynamics adopted by selected cell types to move. We will then survey the current ideas about the role microtubules play in determining cell polarity, through their influence on adhesion site dynamics. And finally comment will be made on the purported pathways signaling adhesion site formation and turnover.

2. Adhesion foci and the actin cytoskeleton

In discussing the types of adhesion complexes we first note that they are all exclusively coupled to the actin cytoskeleton; and second, that the different types of adhesion complex can be conveniently classified according to the assemblies of actin filaments with which they are associated. This implicit interrelationship between adhesion site genesis and actin cytoskeleton assembly necessitates a brief description of the actin filament subcompartments generated in spreading and moving cells. These subcompartments (see also reviews [12,13]) are schematically represented in Fig. 1A and conveniently illustrated in the image in Fig. 1B of a fibroblast labelled with fluorescent phalloidin.

The first compartment is the lamellipodium and its ramifications at the advancing cell front, which include membrane ruffles (Fig. 1C). The lamellipodium is made up of a laminar meshwork of actin filaments

[14], up to about 5 μm in width and around 0.2 μm or less thick [15], [unpublished findings]. It is often punctuated by radially oriented bundles of actin filaments, ranging from 0.1 to 0.25 μm in diameter [16], termed microspikes or filopodia. The filaments of these bundles merge into the meshwork of the lamellipodium, from which they clearly arise [16] and they can extend as finger-like projections beyond the lamellipodium tip. Lamellipodia and filopodia are composed of filaments polarised with their fast growing ends directed to the cell front, consistent with a protrusive function. As protrusive "organelles" they are both engaged in cell motility. Adhesion sites in lamellipodia are commonly of a punctate or oblong nature and may be elongated beneath microspikes or filopodia that are adherent [9,17]. We will refer to these collectively as "focal complexes" [17–19].Actin filaments behind the lamellipodium are organised either into bundled arrays, or into more loose networks. At least five types of bundled arrays can be distinguished (see also [12,13]), three of which are evident in Fig. 1: (1) linear bundles, or "stress fibres" that traverse the cytoplasm; (2) concave bundles at the cell edge, either alone or at the base of lamellipodia; (3) convex, circumferential bundles at the cell edge (characteristic of epithelial cells [20]); (4) polygonal networks; and (5) dorsal arcs. In contrast to lamellipodia and filopodia, these bundles feature anti-parallel arrays of actin that contain myosin II and are therefore contractile. Dorsal arcs [12] and polygonal arrays [21] are not directly associated with the substrate and since they are inconsistent features of motile cells, will not be discussed further. Stress fibres and concave bundles are anchored to the substrate at their ends to well defined, mainly elongated adhesion sites, corresponding to the focal adhesions visible by IRM.

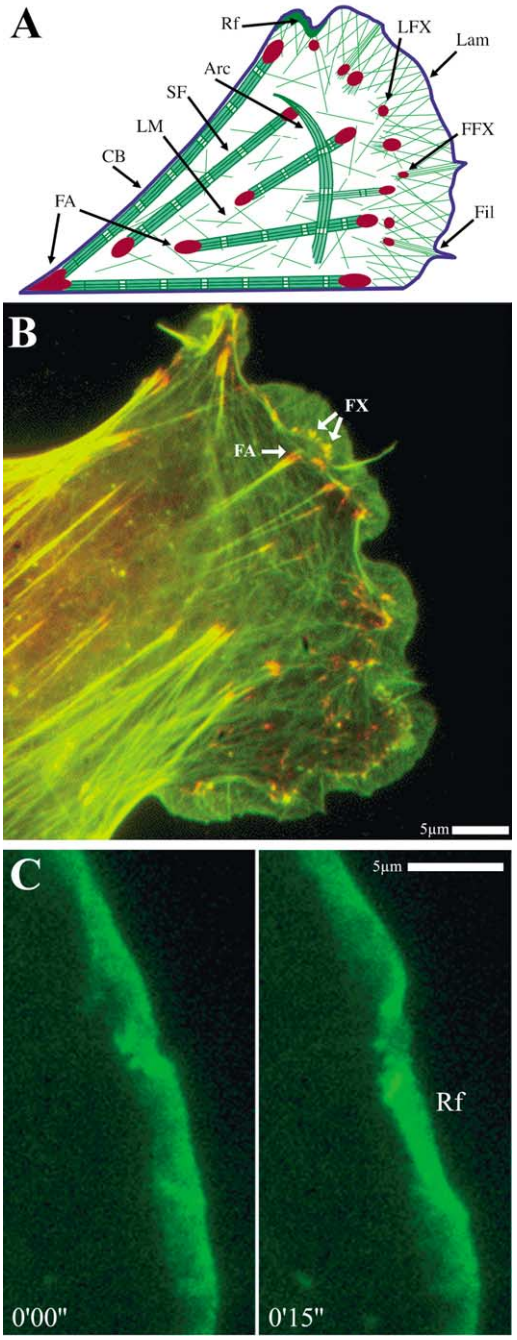

Fig. 1. Subcompartments in the actin cytoskeleton and substrate adhesion complexes. (A) Schematic representation of subcompartments in the actin cytoskeleton (green) and adhesion complexes (red). Lam: lamellipodium; Fil: filopodium (microspike); Rf: ruffle; SF: stress fibres; Arc: dorsal arc; CB: concave bundle; LM: loose meshwork; FA: focal adhesions; FFX: filopodia-based focal complexes; LFX: lamellipodia-based focal complexes. (B) Fluorescence image of a mouse Swiss 3T3 fibroblast that was fixed and then immuno-labelled for vinculin (red) and counterstained for F-actin with phalloidin (green). FA: focal adhesion; FX: focal complexes. Image was kindly provided by K. Rottner. and (C) Ruffle. Panels show sequential video frames (15 s apart) of a GFP-actin expressing B16 melanoma cell. Only the peripheral region is included to show the lamellipodium and its backfolding, to produce a ruffle (Rf). Bars: 5 μm.

3. Rho GTPases and adhesion complexes

Experiments involving the manipulation of starved cell models have established the Rho family of small GTPases as central players in the regulatory pathways signaling the assembly of the actin cytoskeleton and adhesion formation (reviewed in [22,23]). Of those studies dealing with adhesion, one or other Rho GTPase was injected into starved cells and the adhesion patterns analysed either after fixation [17,18] or directly in living cells [19]. In the present context we may note that in this experimental set-up, the cells were depolarised and non-motile, but as we shall later see, the findings are relevant also to motile cells.

To summarise: RhoA signals the formation of focal adhesions associated with actin stress fibre bundles and Rac1 and Cdc42, the formation of focal complexes in association with, respectively, lamellipodia and filopodia. At the same time it has become clear that a balance between Rho GTPase activities, influenced by mutual antagonism [19,24–26,27], is critical in determining the final patterns of adhesion and cytoskeleton organization. Different flavours of Rho GTPase activities can have profound effects on the relative proportions of lamellipodia and filopodia at the cell front and on the extent of actin filament bundle assembly [17]. Bundle assembly is further modulated by the relative activities of two downstream targets of RhoA, Rho kinase and mDia, one signaling thick, compact bundles and the other parallel arrays of fine bundles [28]. The influence of the balance of Rho GTPase activities on adhesion formation has been illustrated in living 3T3 fibroblasts in which adhesions were marked by the injection of rhodamine-tagged vinculin [19]. In these studies it was shown that focal complexes formed in lamellipodia by the injection of constitutively active Rac1 could be converted into focal adhesions by the subsequent injection of constitutively active RhoA. The same result identified focal complexes as potential precursors of focal adhesions. It could also be shown that the injection of dominant negative Rac1 into a normal, migrating fibroblast caused the suppression of membrane ruffling and focal complexes and an accompanying increase in the size of focal adhesions. And inhibition of Rho kinase in immobile 3T3 fibroblasts, expressing only focal adhesions, induced lamellipodia protrusion associated with transitory focal complexes [19]. Observations of macrophages injected with Rho GTPases [17] have generally confirmed those on fibroblasts. However, it was additionally found that membrane ruffling dependent on Rac1 was suppressed by high concentrations of constitutively active Cdc42, again illustrating the mutual antagonism between Rho family members.

4. Alternative strategies of adhesion formation in motile cells

So what types of adhesion dynamics are shown by moving cells? The early, pioneering investigations on the dynamics of the molecular components of substrate adhesions in living cells were restricted to the more prominent focal adhesions [29]. In more recent years, the development of more sensitive cameras, as well as of new fluorescent analogues of adhesion components, has opened the way for renewed analysis of the origin and turnover of adhesion sites [11,19,30–38]. For convenience, we shall illustrate the variations in adhesion dynamics during cell motility by comparing three different cell types (Figs. 2–4).

The first example is the epidermal keratocyte derived from amphibia or fish (Fig. 2), which so far sets the record for actin-based cell motility at up to 20 μm/min. Here, we gain insight into the minimal requirements for cell movement: a broad lamellipodium (lacking microspikes) at the cell front and a lateral arrangement of one or more actin bundles at the cell rear. Cytoplasts spontaneously derived from these cells [39] or induced experimentally [40] move at the same speed as the parent cells employing the same actin cytoskeleton organisation [40]. Adhesion dynamics in keratocytes has been visualised by simultaneous IRM and confocal fluorescence imaging of cells injected with rhodamine-conjugated vinculin [11]. Because the keratocyte moves one cell length in approximately 2 min (compared to around 1 h for a fibroblast), the maximum lifetime of adhesion sites is very short and limited to this period. Adhesion sites, as recognised by vinculin accumulation, originate as small, punctate "focal complexes" behind the front edge of the lamellipodium, beneath the actin meshwork. Vinculin, beta 1 integrin, alpha actinin and FAK have been localised in these focal complexes by immunolabelling [41].

The fate of the focal complexes in keratocytes is determined according to whether they arise in the central

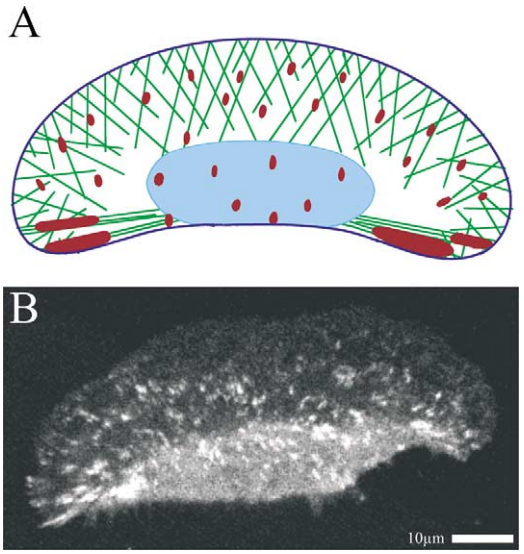

Fig. 2. Adhesion complexes in epidermal keratocytes. (A) Schematic representation of adhesion complexes (red) in relation to the actin cytoskeleton (green). The general organisation of actin in this and Figs. 3–8 is based on electron microscope observations. (B) Confocal microscope image of living, trout keratocyte that was injected with rhodamine-vinculin to label substrate adhesion sites. Image was kindly provided by K. Anderson.

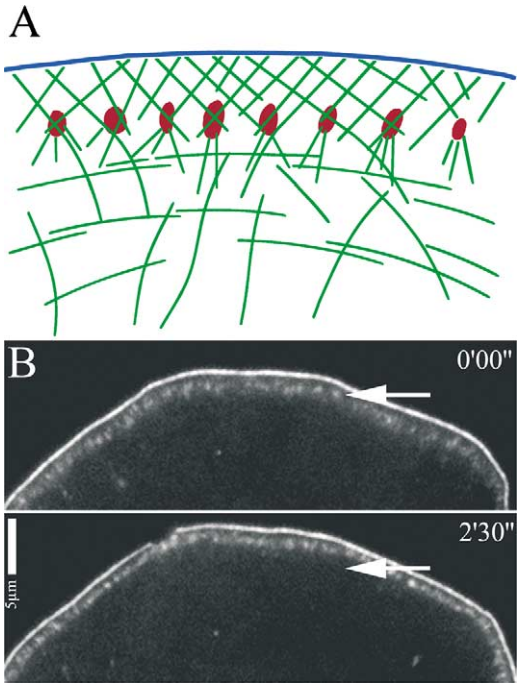

Fig. 3. Adhesion complexes in a mouse B16 melanoma cell migrating on laminin. (A) Schematic representation of adhesion complexes (red) in relation to the actin cytoskeleton (green). (B) Video frames of a live B16 cell migrating on laminin, that was transfected with GFP-VASP. VASP marks the tip of the lamellipodium as well as focal complexes at its base. Arrows indicate the equivalent positions relative to the substrate at the two times shown (min and s). Note that the focal complexes do not mature into focal adesions, but turnover at the base of the lamellipodium. Images kindly supplied by K. Rottner. Bar: 5 μm.

region, in front of the cell body, or in the flanks. In the central region, the focal complexes remain stationary relative to the substrate and either disassemble beneath the cell body as it moves over them, or are removed from the substrate as the rear edge of the cell rolls upwards [11,42]. In the regions of the lamellipodium flanking the cell body, focal complexes do not dissolve, but fuse together in the trailing edge to form larger adhesions, resembling focal adhesions. These lateral adhesions are not stationary, are short-lived and are drawn into the flanks of the cell body in a sliding motion, driven by contractility in the laterally organised bundles of actin filaments [42,43]. As well as providing the most direct example of recycling of adhesion sites, the example of the keratocyte prompts the question of whether the trailing, lateral adhesions serve a useful role in motility. Indeed they do, for without them, the lateral tension required for the traction of the cell body [42–44] could not be developed.

As a second example, we have taken B16 mouse melanoma cells that can extend lamellipodia continuously at rates of around 2 μm/min and intermittently up to around 4 μm/min, when plated on laminin [45–47] (unpublished observations). For such motile cells, the lamellipodium is dominated by an actin meshwork, and features microspike bundles that show rapid lateral mobility [30] (unpublished data). In B16 cells transfected with GFP-VASP, small, elongated focal complexes are seen to develop in a row at the base of the lamellipodium (Fig. 3). These are short-lived, existing for around 1 min (Fig. 3B; K. Rottner, private communication) and are replaced by new sets of focal complexes as the lamellipodium advances [47]. That is to say there is an active turnover of focal complexes within the realm of the lamellipodium as it translocates. In some cases individual focal complexes may persist after the lamellipodium has advanced in front

of them and grow into longer-lived focal adhesions, together with associated stress fibre bundles of actin [47]. However, the region behind rapidly advancing lamellipodia of B16 cells is dominated by a loose network of actin filaments [45,47] that is continuous with a fraction of the filaments that make up the lamellipodium (unpublished observations). This type of loose network is likely an important component of migrating cells and can provide structural continuity in the absence of macroscopic bundles.

As a third example we take a goldfish fibroblast cell line (Fig. 4). In these cells the migrating front is dominated by filopodia that can extend 10–20 μm from the cell edge. Lamellipodia segments link adjacent filopodia, and both filopodia and lamellipodia can undergo active upfolding and ruffling activity between phases of protrusion. The notable feature of these cells is the creation of resolvable adhesion foci mainly in association with the base of filopodia (Fig. 4B). These "focal complexes" are either transitory, with lifetimes in the range of 5–15 min, or persist and differentiate into focal adhesions. In living cells, discrete adhesion sites are not recognised in the lamellipodia segments, which show two distinct actin filament organisations in the electron microscope worth mentioning (unpublished observations; Fig. 4A). In one case, the lamellipodium shows the characteristic meshwork (reviewed in [48]) with a decrease in the filament density from front to rear, with some filaments trailing off into the loose meshwork behind. In the second case, the lamellipodium is narrower and is bordered at its base by a concave bundle of actin filaments that shows continuity with the flanking filopodia. These two morphologies may be correlated respectively with phases of protrusion and retraction, whereby "hammocking of filaments" [49] between filopodia and their associated adhesions contribute to the support of the cell edge following lamellipodia retraction. In the context of Rho GTPase regulation the injection of constitutively active Rac1 into fish fibroblasts expressing GFP-VASP causes a transformation of the cell front from one dominated by filopodia to one dominated by lamellipodia and focal complexes (Kaverina, unpublished observations), resembling that seen in B16 melanoma cells These findings emphasises again that adhesion strategies are the outcome of a subtle balance in Rho GTPase activities.

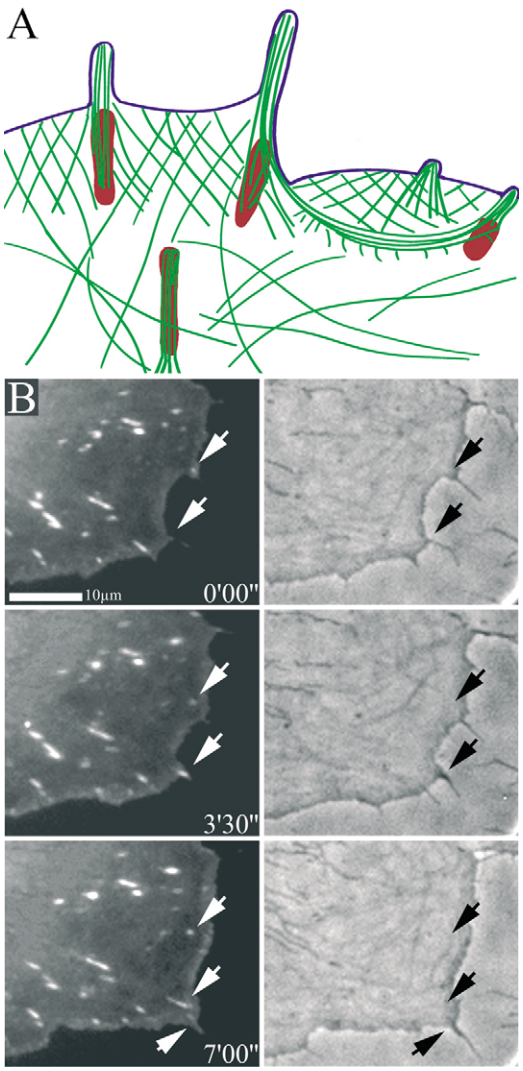

Fig. 4. Adhesion complexes in a migrating goldfish fibroblast. (A) Schematic representation of adhesion complexes (red) in relation to the actin cytoskeleton (green). (B) Paired video frames, in fluorescence (left) and phase contrast (right), of a living goldfish fibroblast expressing GFP-zyxin to mark adhesion sites. Arrows indicate equivalent positions in each video pair. Note the creation of focal complexes in association with the base of filopodia. Focal complexes can develop into focal adhesions behind the lamellipodium (central arrow at 7 min). Time (min and s). Bar: 10 μm.

Final mention should be made of the unique adhesion foci found in cells of the monocyte lineage, referred to as podosomes [50–55]. First observed in some virally transformed cells [56,57], podosomes exhibit a ring structure composed of an actin core and

adhesion proteins at the periphery. In differentiated osteoclasts, podosomes form in a prominent band at the cell periphery which appears to function as a seal around the bone resorbing zone [50]. Podosomes do not move, but they are dynamic, dissolving and then reforming in new locations, with lifetimes of 2–12 min ([50]; F. Bard, private communication). Data on the dynamics of podosomes in moving cells does not yet exist. But it appears that podosomes lie behind a narrow lamellipodium ([54,56]; F. Bard, private communication) which presumably functions in protrusion.

5. Tension, adhesion and retraction

Chrzanowska-Wodnicka and Burridge [58] have shown that the formation of focal adhesions is dependent on the development of tension in the actin filament cytoskeleton, through actin–myosin interactions (reviewed in [58]). Likewise, focal complexes rely on actomyosin tension for their formation and integrity [19] and, like focal adhesions exert traction on the substrate [36]. The dependence of focal adhesion development on tension has been elegantly illustrated by the mechanical manipulation of cells with microneedles [37]: these experiments have shown that externally applied forces can induce adhesion site growth in the same way as intracellular contractility. An interesting difference in this case was the requirement for mDia activity downstream of RhoA, but not Rho kinase [37]. Using the same approach, we have been able to show that the restraint of the cell body in migrating B16 melanoma cells induces the formation of actin filament bundles from the loose network behind the lamellipodium, and the transformation of focal complexes into focal adhesions (Kaverina, unpublished observations).

In a migrating cell, tension in the actin cytoskeleton is necessary for adhesion at the front, illustrated by the retraction induced by myosin inhibitors [32,59] as well as for the retraction of the trailing cell body in the last phase of motility [60,61]. Using flexible substrates, Beningo et al. [36] and Balaban et al. [62] have recently quantitated the forces exerted at adhesion sites in living cells. Whereas, Balaban et al. [62] found that larger adhesions exerted more force per unit area, Beningo et al. [36] found the opposite. This difference may partly be explained by the analysis of stationary cells in one study [62] and motile cells in the other [36]. In motile fish fibroblasts, the anterior focal complexes and early focal adhesions exert more stress than mature focal adhesions. This finding is in line with decreased substrate deformation around the trailing tail of fibroblasts [63] and the observed sliding of trailing contacts during cell edge retraction [30,32,34,64]. Taken together, these studies underline tension as a central factor in the development and dissociation of adhesion complexes, an aspect that we will return to below.

6. Cross-talk of microtubules with adhesion foci

In fibroblasts, the depolymerisation of microtubules leads to the depolarisation of cell shape [65], an increase in the contractility of the cytoskeleton [66] and an amplification in the size of focal adhesions [58]. This response is paralleled by the activation of RhoA [67,68]. Conversely, the repolymerisation of microtubules following the disassembly is associated with the activation of Rac1 [69]. A direct correlation therefore exists between microtubule polymerisation dynamics and the activity of the Rho GTPases proteins that direct actin cytoskeleton organisation and substrate adhesion dynamics.

A link between microtubules and adhesion sites was independently illustrated in another context. Thus, observations of living cells in which microtubules and adhesion components were labelled with fluorescent probes revealed that microtubules specifically target adhesion foci as they polymerise towards the cell periphery [31] (Fig. 5). This interaction is close range, since focal adhesions are able to capture microtubules and stabilise them temporarily against depolymerisation by nocodazole [31]. And the closeness of this association has more recently been corroborated by total internal reflection fluorescence microscopy of cells expressing GFP-tubulin and GFP-zyxin ([70]; unpublished observations), whereby microtubules over adhesions were seen to dip into the exciting evanescent wave formed within 150 nm of the substrate [70]. Interestingly, the assembly of podosomes in macrophages is microtubule-dependent [54] and microtubules are required for stabilisation of the belt of podosomes at the periphery of osteoclasts (F. Bard, private communication).

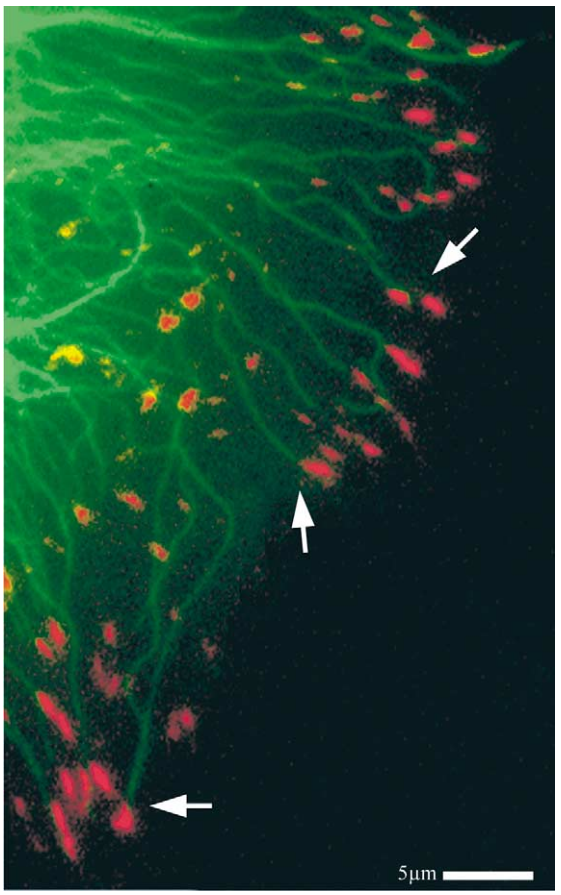

Fig. 5. Targeting of focal adhesions by microtubules. Figure shows superimposed video frames of a fish fibroblast that was expressing GFP-tubulin (green channel) to label microtubules and injected with rhodamine-tagged vinculin to label adhesion sites (red channel). The time separation between the two channels was less than 2 s. Arrows indicate typical targeting events. Bar: 5 μm.

Such an intimate cross-talk between microtubules and adhesion foci must serve a function, and other data indicate that this function is to modulate adhesion site dynamics [32,33]. Accordingly, experiments on living cells have demonstrated that the targeting of focal adhesions or focal complexes by microtubules either retards the growth of adhesions or promotes their disassembly [32]. Similar dynamics of disassembly associated with microtubule targeting could be mimicked by the local application of inhibitors of myosin contractility to a cell edge [32]. And taking this observation one step further, it was demonstrated that depolarised cells lacking microtubules could be polarised and induced to move by the asymmetrical application of the same myosin inhibitor [33]. It was therefore concluded that microtubules exert their influence on cell polarisation by modulating adhesion site turnover through the point delivery of signals that antagonise myosin contractility at adhesion foci. Bershadsky et al. [68] have also attributed a role of microtubules in the general or local suppression of contractility. They showed that microtubule disruption in serum starved cells induces focal adhesions and stress fibre formation, and that this effect was prevented by the inhibition of cell contractility modulated via RhoA.

7. Rho GTPases and microtubule engagement

As already indicated, RhoA, Rac1 and Cdc42 act in three signal-transduction pathways regulating the assembly of actin stress fibre bundles, lamellipodia and filopodia respectively. Rho GTPases signal to diverse effectors to initiate a downstream response. Each of these GTPases act as a molecular switch, cycling between an active GTP-bound, and an inactive GDP-bound, state. Guanosine nucleotide exchange factors (GEFs) facilitate the exchange of GDP for GTP, and GTPase-activating proteins (GAPs) increase the rate of GTP hydrolysis of Rho GTPases [71]. Here, we discuss the possible pathways linking regulators and effectors of Rho GTPases to microtubules (Table 1).

7.1. RhoA

As we have seen, highly dynamic focal complexes formed in association with lamellipodia are dependent on Rac1, while those formed beneath filopodia are initiated via Cdc42. Both types of complexes can mature into focal adhesions in a process that requires RhoA. At which regulatory stage microtubules exert their influence on contractility is not yet known. However, existing data suggest that Rac1-dependent focal complexes can turnover and disassemble independently of microtubules ([46]; Kaverina unpublished). In the same context, we are reminded that keratocytes polarise quite happily without microtubules [39] as so do some primary fibroblasts [72]. Common to these examples is the absence of established stress fibre bundles and focal adhesions. We are prompted

Table 1
Regulatory proteins implicated with microtubules

Protein	Relation to actin cytoskeleton	Relation to microtubules	Reference
Rac1	Promotes lamellipodia and associated focal complex formation	Activated during microtubule re-polymerisation	[17–19,69]
RhoA	Promotes stress fibre formation and focal adhesion maturation	Activated upon microtubule depolymerisation	[17–19,73]
Cdc42	Promotes filopodia and associated focal complex formation. Important for polarised motility		[17–19,92,94]
P190RhoGEF	Rho-specific GEF	Binds microtubules in vivo and in vitro	[74]
Lfc	GEF, activates Rho in vitro. Overexpression leads to stress fibre and ruffle formation	Binds microtubules in vivo and in vitro	[75]
GEF H1	Activates Rac and Rho in vitro	Binds microtubules	[76]
mDia	Rho effector Promotes formation of parallel arrays of fine actin bundles	Over-expression promotes the formation of "Glu" microtubules	[28,77]
Pak	Rac and Cdc42 effectors; can modulate actomyosin contractility downstream from Rho		[83,84]
ASEF	Rac specific GEF	Binds APC and could be delivered by microtubules	[85]
RhoG	Rac and Cdc42-activating GTPase	Localisation and activity depends on microtubule integrity. Binds kinectin and could be transported by kinesin	[86,88]
Trio	GEF for Rho and RhoG/Rac/Cdc42	Localisation and activity depends on microtubule integrity	[87]
CIP4 and WASP	Cdc42 effectors; involved in actin dynamics	Bind to microtubules	[93]
mPar6/PKCzeta complex	Cdc42 effector	Involved in MTOC polarization	[92]
Src	Involved in RhoA activation cycle; important for mDia bundle formation	Binds Tau and MAP2 colocalises with microtubules when inactive	[96,98–102]
Ras	Involved in regulation downstream of RhoA	Ki-Ras localisation depends on microtubule integrity	[104,107]
MAPKs: MLK-2, JNK, ERK	Involved in Ras-Rho interplay	MLK-2 and JNK localise to microtubules	[104,105]

to conclude that below a certain threshold of stress at adhesion sites, cell asymmetry can be induced and maintained by the actomyosin system [40] and that above this level microtubules are required to promote the adhesion disassembly to control cell shape. This stress threshold may be determined by the engagement of RhoA to promote the maturation of adhesions initiated via Rac1 and Cdc42. According to these considerations microtubules most likely exert their influence selectively on the RhoA pathway.

RhoA-GTP pull down assays confirm the findings already cited (Section 6) that RhoA is strongly activated upon microtubule depolymerisation [73]. But, how is RhoA activity influenced by microtubules? RhoA itself does not bind microtubules nor to microtubule-binding proteins and it's intracellular localisation is not microtubule-dependent. Some of RhoA upstream regulators, however, show microtubule-binding activities (Fig. 6). An interesting candidate, P190RhoGEF binds microtubules in vivo as well as in vitro. This exchange factor is specific for RhoA and elevates RhoA activity when overexpressed. Interestingly, in cells in which microtubules were disassembled, overexpression of p190RhoGEF failed to amplify RhoA activation, indicating that p190RhoGEF is involved in the microtubule-dependent upregulation of RhoA [74]. Similar functions could be attributed to other related Dbl-like microtubule-binding GEFs, such as Lfc, which activates RhoA in vitro and, when overexpressed, stress fibre and ruffle formation in vivo, consistent with activation of both Rac1 and RhoA [75]. Also, GEF H1 binds microtubules and activates both Rac1 and RhoA in vitro [76].

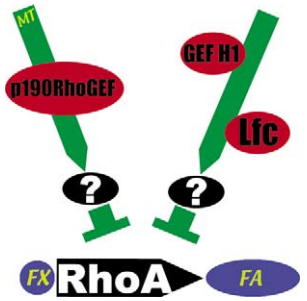

Fig. 6. Potential pathways linking microtubules to the regulation of RhoA. MT: microtubule; FX: focal complex; FA: focal adhesion. See text for details.

In another scheme, stabilised "Glu" microtubules have been attributed a role in polarisation, involving the RhoA effector, mDia [77]. Over-expression of mDia was reported to promote the formation of "Glu" microtubules oriented towards the wounded edge in a fibroblast monolayer, but the preferred polarisation of this subset of microtubules was not compelling. Furthermore, Glu microtubules are capped, non dynamic [77] and far removed from the advancing front as compared to dynamic microtubules [31]. It is therefore difficult to accept the idea of microtubule stabilisation as a "key event" in polarisation [77].

Further, since contact growth upon tension application requires mDia [37], and mDia is likely to be important for microtubule interaction with the cell cortex [78] Geiger and Bershadsky [79] have suggested a dual role for microtubules in adhesion regulation. One involves signaling disassembly and the other foresees the delivery of specific components (such as members of the mDia pathway) that are necessary for the development of focal contacts and stress fibres. In this context, a balance between the two could determine adhesion site turnover.

7.2. Rac1

Waterman-Storer et al. [69] observed that Rac1 is activated during microtubule re-polymerisation after drug-induced disassembly. This result prompted the suggestion that polymerising microtubules mediate the activation of Rac1 at the cell front, to induce protrusion, whereas depolymerising microtubules at the cell rear mediate the activation of RhoA, leading

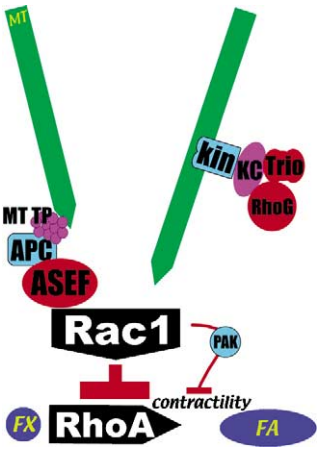

Fig. 7. Potential pathways linking microtubules to the regulation of Rac1. Rac1, in turn, promotes focal adhesion turnover via its antagonism of RhoA. MT TP: microtubule tip proteins; kin: kinesin; KC: kinectin. See text for details.

to contractility and retraction [80,81]. Lamellipodia advance can however occur without microtubules [33,39,46,82] and the spatial arrangement of microtubules in moving cells does not locally correlate with lamellipodium protrusion events. There is also no evidence that microtubule depolymerisation is enhanced at the cell rear as compared to the cell front. If Rac1 is involved in the regulation of polarisation via microtubules, it most likely acts at the level of focal adhesions. In this case, the antagonism between the Rac1 and RhoA pathways may play a role. Hence, Rac1 via Pak could reduce actomyosin contractility downstream from RhoA ([33], reviewed in [34]), (Fig. 7).

A good candidate for microtubule-dependent Rac1 stimulation is ASEF [35]. It binds APC and activates Rac1 in an APC-dependent manner. Since APC can be transported on the growing tips of a subset of microtubules in a complex with other microtubule tip proteins, ASEF could be delivered by microtubules to certain adhesion sites.

Another candidate pathway potentially involved in the microtubule-dependent regulation of Rac1 is the Rac1 and Cdc42-activating GTPase RhoG and it's exchange factor Trio. Trio is a multifunctional GEF that contains 2 exchange domains, one RhoA specific, another specific for RhoG and less active on Rac1. Microtubule disruption prevents localisation of RhoG to the plasma membrane and inhibits its activity

[36]. The N-terminal part of the Trio molecule which specifically activates RhoG also localises to the membrane and activates Rac1 and Cdc42 and all these activities are blocked by microtubule disruption [37]. RhoG was reported to bind kinectin, a link suggested as essential for the transport of this GTPase to the cell periphery by kinesin [86,88]. Inhibition of kinesin transport by antibody injection, as well as inhibition of kinectin–RhoG interaction, is sufficient to block RhoG-induced ruffling [88]. RhoG might then be delivered in a complex with Trio, since Trio localisation also depends on microtubules (Fig. 7).

7.3. Cdc42

In line with the role of Cdc42 in the polarisation of yeast [89] some evidence is now emerging for an involvement of this Rho family member in the polarisation of vertebrate cells [90]. In particular, Nobes and Hall [91] showed that inhibition of Cdc42 in a wound healing assay inhibited polarisation of cells into the wound. However, the involvement of microtubules on the activity of Cdc42 was not analysed. Since an asymmetric distribution of Cdc42 was also found necessary to transduce it's effect on polarity [92] it is not unlikely that microtubules function to provide this asymmetry.

The influence of Cdc42 on overall adhesion dynamics in motile cells is not yet clear, but one can speculate that this GTPase establishes contact asymmetry in collaboration with microtubules. In fish fibroblasts, focal complexes associated with filopodia are targeted by microtubules and intense targeting results in focal complex disassembly (Kaverina, unpublished results). This finding indicates that microtubules could intervene in the Cdc42 pathway leading to focal adhesion assembly. A potential regulator of Cdc42 linked to microtubules could again be RhoG, which may be transported along microtubules in a kinesin-dependent manner through its ability to bind kinectin [86,88].

Alternatively, regulatory molecules downstream of Cdc42 may collaborate with microtubules. Some specific Cdc42 effector molecules, involved in actin cytoskeleton regulation, such as CIP4 and WASP, have been shown to bind to microtubules in a regulated way [93]. Other Cdc42 effectors are involved in polarized localization of the microtubule organizing centre (MTOC) in cells moving into an artificial wound [92,94], and in astrocytes the mPar6/PKCzeta complex was identified as such an effector [92]. The relocalization of the MTOC is interestingly dependent on dynein. However, we have recently found that dynein activity is not required for polarised motility of fibroblasts (Krylyshkina, unpublished). Taken together, these data suggest separate signalling routes from Cdc42: to the polarisation of the MTOC via mPar6/PKCzeta; and to the polarized distribution of actin structures responsible for motility (including adhesions), probably via CIP4 and WASP. Also, mDia-dependent formation of polarized stable microtubule arrays is regulated independently of Cdc42 [94]. Clearly, there is still a lot to do to establish how Cdc42 influences cell polarity in coordination with other GTPases.

Some alternative ideas of how microtubules may determine the polarity via interfacing with Rho GTPases have been recently reviewed by Wittmann and Waterman-Storer [81].

8. Other potential regulators linked to microtubules

8.1. Src

Different lines of evidence suggest that Src kinase activity is involved in regulating focal adhesion turnover. The v-Src temperature-sensitive mutant (as well as c-Src in its active conformation [95]) translocates to focal adhesions at the permissive temperature [96] and the kinase activity of v-Src leads to eventual focal adhesion disassembly upon phosphorylation and degradation of FAK. FAK-containing focal adhesions also grow faster in Src−/− cells in comparison with wild type cells [97], resulting in the suppression of cell motility. The link between the Src- and RhoA-dependent pathways regulating focal adhesions is, however, unclear. On the one hand, Src kinases can downregulate RhoA activity via phosporylation of P190RhoGAP (a major Src and Fyn substrate) [98]. On the other hand, RhoA activation in starved cells results in the translocation of Src to focal adhesions, a translocation that is blocked by myosin inhibition [99]. Interestingly, the localisation of kinase-dead v-Src to focal adhesions blocks their turnover and causes them to enlarge, unless Src kinase is activated [90]. Additionally, the RhoA downstream

effector mDia was found to interact with Src, and inhibition of Src blocked mDia-dependent stress fibre formation [100]. There is no direct evidence for microtubules-binding Src, but Src kinases have been shown to bind the microtubule-binding proteins Tau and MAP2 in neurons [101,102]. And the localisation of inactive c-Src as well as v-Src mutant at restrictive temperature has been shown to correspond with zones of high microtubule density [99]. If there is any interaction it may be speculated that microtubules act as transitory docking sites for inactive c-Src.

8.2. Ras

Nobes and Hall [91] have reported that inhibition of Ras by antibody injection induces large focal adhesions and blocks cell motility, presumably by disrupting focal adhesion dynamics. A temporal association of active Ras with focal adhesions has been observed and several possibilities exist for the interplay between Ras and Rho GTPases on the molecular level, which could be essential for actin cytoskeleton regulation (reviewed in [103]). For example, it has been recently shown that the lack of stress fibres in Ras-transformed cells is a result of a functional uncoupling of RhoA from Rho kinase, dependent on the ERK-MAP kinase pathway downstream of Ras [104]. Interestingly, activity of the MAP kinase pathway members has long been known to be influenced by microtubule-specific agents. Of special interest is the finding that such kinases as MLK2 and JNK are localised in punctate structures along microtubules in fibroblasts [105]. MLK2 activates ERK and therefore can be involved in down-regulation of the RhoA downstream effect on stress fibre and focal adhesion formation. In a two hybrid, screen MLK2 associated with KIF3, a kinesin superfamily motor, suggesting that it might be one of the regulators delivered by kinesins to adhesion sites [105].

It is not excluded that the intracellular localisation of Ras itself could be defined by microtubules. Ki-Ras 4B appears to be the isoform of Ras most important for cell motility [106] and it has been shown that the functionally essential membrane targeting of Ki-Ras depends on microtubule integrity and dynamics [107]. This Ras isoform associates with microtubules upon prenylation and when microtubules are stabilised and disorganised by taxol it fails to be transported to the plasma membrane. Thus, microtubules could potentially influence focal adhesion dynamics by modulating delivery of active Ki-Ras to adhesion sites (Fig. 8).

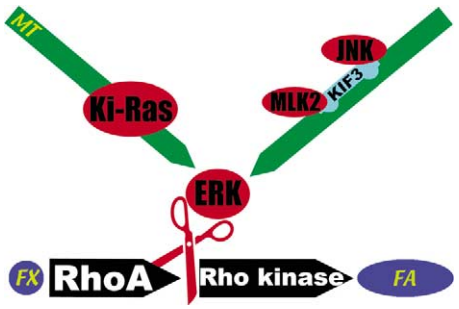

Fig. 8. Potential pathways linking Ras to focal adhesion turnover. Scissors indicate uncoupling of RhoA from Rho kinase by Erk. See text for details.

9. Delivery of components to the cell front

Alternative ideas of how microtubules may influence cell polarity have been discussed by Nabi [108]. These hinge in the main on the delivery of membrane via vesicle traffic along microtubules. We cannot rigorously exclude the possibility that structural components of adhesions are delivered to or removed from adhesion sites by microtubules. In this connection, paxillin was found to bind alpha- and gamma-tubulin, as well as to co-localize with microtubule organising centres in lymphocytes [109]. We consider this unlikely, however, since adhesion site formation per se is not microtubule-dependent.

It has also been suggested that integrins and other membrane components of adhesion plaques are delivered to the sites of adhesion site assembly via microtubule-driven membrane traffic [110]. In support of this idea, a block in vesicle transport was found to inhibit cell spreading [111] and delivery of integrins to the cell membrane. The question of how membrane is replenished at the cell front is an interesting one that remains to be clarified and could involve members of the Rab family of small GTPases [112,113]. We only note that cells can spread in the absence of microtubules and for this do not depend on microtubule-linked vesicle trafficking.

10. Concluding remarks

Much has still to be learned about adhesion site dynamics during motility. Not least is the problem of adhesion site composition, which is far from complete [7], as well as the temporal association of the component molecules with adhesion sites. Here we are only just beginning to scratch the surface [79,35,114,115]. Other questions include the localisation of regulators and regulatory complexes, which can only properly be defined in living cells, requiring probes and instrumentation that are just now becoming available. Added to this is the question of how the movement of cells in vitro relates to migration in vivo. Steps in this direction are promising [116,117] and set the stage for further progress.

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