

Interplay between Rac and Rho in the control of substrate contact dynamics

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Background: Substrate anchorage and cell locomotion entail the initiation and development of different classes of contact sites, which are associated with the different compartments of the actin cytoskeleton. The Rho-family GTPases are implicated in the signalling pathways that dictate contact initiation, maturation and turnover, but their individual roles in these processes remain to be defined.

Results: We monitored the dynamics of peripheral, Rac-induced focal complexes in living cells in response to perturbations of Rac and Rho activity and myosin contractility. We show that focal complexes formed in response to Rac differentiated into focal contacts upon upregulation of Rho. Focal complexes were dissociated by inhibitors of myosin-II-dependent contractility but not by an inhibitor of Rho-kinase. The downregulation of Rac promoted the enlargement of focal contacts, whereas a block in the Rho pathway not only caused a dissolution of focal contacts but also stimulated membrane ruffling and formation of new focal complexes, which were associated with the advance of the cell front.

Conclusions: Rac functions to signal the creation of new substrate contacts at the cell front, which are associated with the induction of ruffling lamellipodia, whereas Rho serves in the maturation of existing contacts, with both contact types requiring contractility for their formation. The transition from a focal complex to a focal contact is associated with a switch to Rho-kinase dependence. Rac and Rho also influence the development of focal contacts and focal complexes, respectively, through mutually antagonistic pathways.

Background

The morphology of metazoan cells spreading or moving on a natural or synthetic substrate is largely determined by two interdependent and interactive systems: the integrin-based apparatus for substrate adhesion and the actin cytoskeleton [1,2]. The actin cytoskeleton can be viewed as an assembly of integrated subcompartments, each of which has a distinct arrangement of actin filaments [2,3]. In fibroblasts, these subcompartments include contractile filament bundles, the stress fibres and two types of peripheral assemblies — lamellipodia meshworks and radial, unipolar bundles termed microspikes or filopodia.

It is now well established that, of Rho-family members, Rho activates the formation of actin stress-fibre bundles and their associated focal adhesions, whereas Rac and Cdc42 induce the formation of lamellipodia and filopodia [4]. The adhesion sites associated with these latter actin assemblies can be broadly divided into two types [5]: punctate or oblong adhesions associated with lamellipodia; and often linear adhesions associated with filopodia. It has been suggested that these latter two contact types represent precursors of focal adhesions and support the protrusive activity of the lamellipodium required for cell locomotion [5–7]. No differences in protein composition

have yet been noted between these putative precursor adhesions and focal adhesions [5–12].

Adhesion sites confined to lamellipodia and similar in morphology to one class of the precursor adhesions noted above can be induced by injecting constitutively active Rac into Swiss 3T3 cells while simultaneously suppressing Rho activity with the ribosylating enzyme C3 transferase [10]. Such sites have been termed ‘focal complexes’ [10], and similar adhesion foci have been described in rat fibroblasts treated with C3 transferase [11] and in macrophages injected with constitutively active Rac [12]. To shed light on the function of these Rac-induced focal complexes, we have investigated the dynamics of their assembly and disassembly in response to modulations in Rac and Rho activity. The results reveal separate, but also interdependent and antagonistic, roles of Rac and Rho in the initiation and maturation of substrate contacts for cell locomotion and anchorage.

Results

Rac-induced focal complexes are formed at the base of ruffling lamellipodia

To study the dynamics of Rac-induced focal complexes in relation to lamellipodia formation in living cells,

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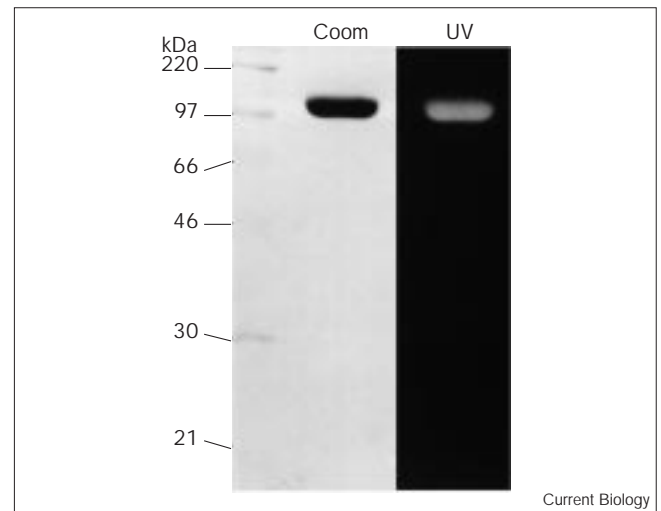
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subconfluent mouse Swiss 3T3 fibroblasts were injected with constitutively active Rac (L61Rac), C3 transferase (C3; to inhibit Rho) and fluorescently coupled turkey-gizzard vinculin, a component of focal complexes (Figure 1). Approximately 15–30 minutes after injection, the cells adopted a contact pattern identical to that described using immunolabelling [10]. Rho-dependent focal contacts were mainly dissolved and fluorescent vinculin was readily incorporated into punctate focal complexes at the cell periphery (Figure 2a,b). This result was highly reproducible, such that in a typical experiment (30 injected cells) at least 90% of cells displayed peripheral focal complexes with few or no focal contacts. Once established, the typical focal-complex pattern was stable for up to 2 hours in the presence of 5% serum. Limited and local spreading of the cell edge could still occur in this state and was accompanied by the formation of new sets of focal complexes containing vinculin (data not shown).

Time-lapse video phase-contrast and fluorescence microscopy revealed that the focal complexes present in these spread, apolar cells delineated a border that marked the base of protruding and ruffling lamellipodia formed around the entire cell periphery (Figure 2a). Ruffles typically appeared as waves moving along the cell edge, before dissociating from the lamellipodium to move randomly as phase-dense patches on the dorsal cell surface

Figure 1



Gel electrophoresis of the vinculin probe used for microinjection. The rhodamine-labelled vinculin is shown by Coomassie-blue labelling (Coom; left lane) and red fluorescence under UV excitation (right lane). The probe is homogeneous, with no breakdown products. Numbers on the left indicate apparent molecular weight, in kilodaltons (kDa).

(Figure 2c). These surface ruffles, which could be labelled (for actin) with phalloidin (Figure 2d), were not seen in

Figure 2

Induction of focal complexes in non-confluent Swiss 3T3 fibroblasts. The typical effect of co-injection of constitutively active L61Rac (800 µg/ml) and C3 transferase (10 µg/ml) together with rhodamine–vinculin (500 µg/ml) is shown. (a) Video sequence showing an enlargement of the area boxed in (b) demonstrates the stability of the focal-complex pattern and the active ruffling activity. The left-hand panels in (a) show fluorescence images and the right-hand panels show phase-contrast images. (c) Phase-contrast image of the same cell that is shown by fluorescence in (b). (d) At the end of the sequence, the cell was fixed and labelled with Cy3–phalloidin to reveal actin. Ruffling (R) is centred around the base of the lamellipodium, which is marked by the row of vinculin-containing focal complexes (compare images in (b,c,d)). The diffuse, phase-dense spots in the cytoplasm (indicated by asterisks in (a,c,d)) derive from the peripheral ruffles. Time in (a) is given in min and sec after injection. Scale bar represents 5 µm.

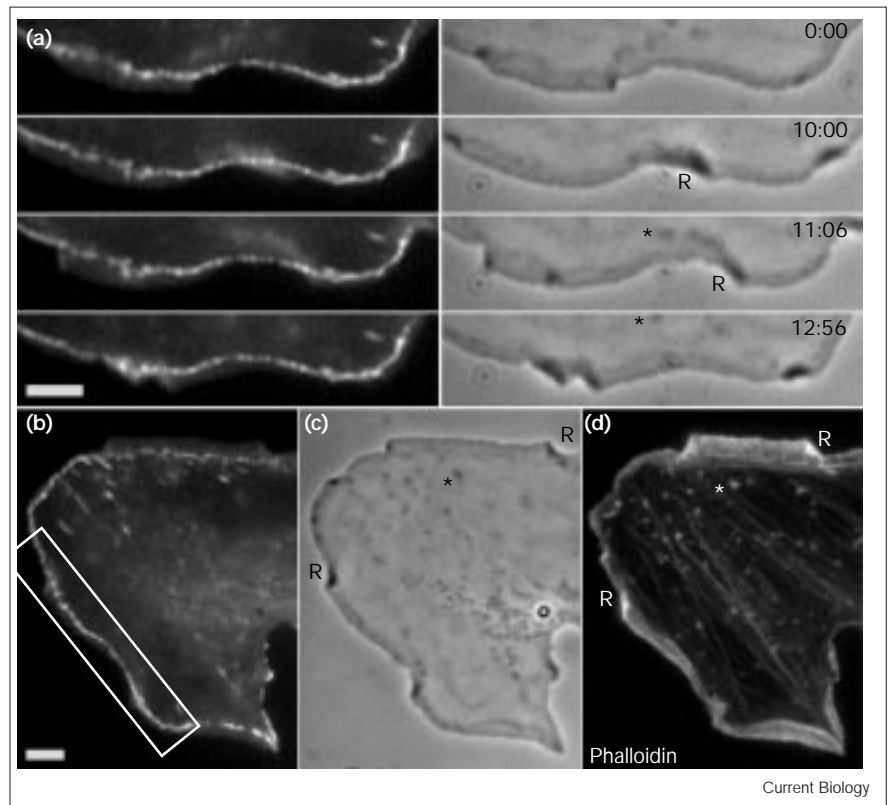
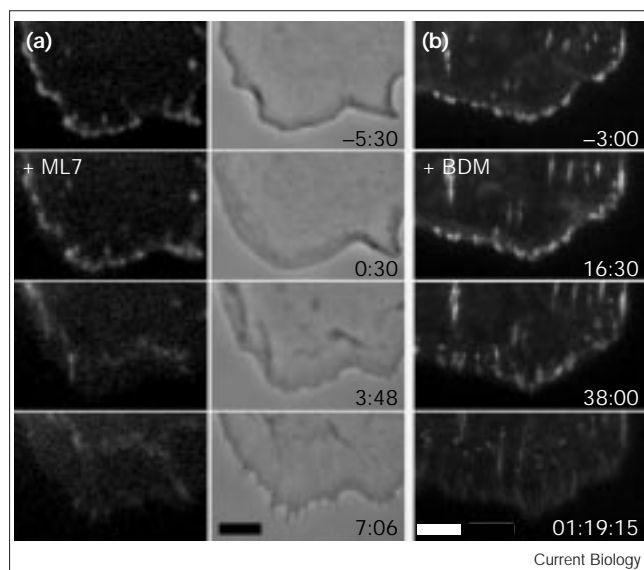


Figure 3



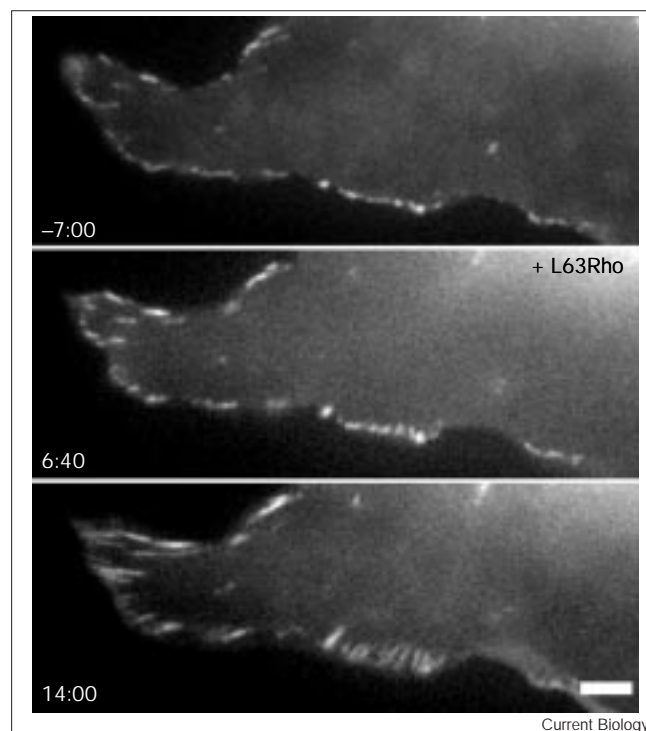
Inhibition of myosin II contractility causes disassembly of focal complexes. After induction of focal complexes as in Figure 2, (a) ML-7 (100 μ M) or (b) BDM (20 mM) was added at time 0:00. In (a), the arrest of ruffling was followed by lamellipodium protrusion at 0:30 min and inward growth at 7:06 min. The left-hand panels in (a) show fluorescence images and the right-hand panels show phase-contrast images. (b) Fluorescence images showing the disappearance of focal complexes in BDM. Time is given in min and sec after ML-7 or BDM treatment. Scale bar represents 5 μ m.

Swiss 3T3 fibroblasts under control conditions and may correspond to the actin patches described previously [13,14]. The formation of similar patches has been shown to depend on Rac activity [15], consistent with our data. Phalloidin staining also revealed a loose meshwork of relatively fine actin filament bundles, most of which appeared to terminate in, or to splay into, focal complexes at the base of the lamellipodia (Figure 2d).

Myosin-II-based contractility is required for the maintenance of focal complexes

Because focal contacts have been shown to depend on Rho-mediated contractility for their formation [16], we performed experiments to establish whether focal complexes showed the same dependence. Inhibition of myosin-based contractility by either ML-7 [17], an inhibitor of myosin light chain kinase, or by 2,3-butanedione monoxime (BDM) [18,19], an inhibitor of the actin-myosin interaction, caused the dissolution of focal complexes (Figure 3); the effect of BDM, however, occurred much more slowly than that of ML-7. Membrane ruffling was also abolished completely by ML-7, but only partially by BDM at the concentration used (20 mM). Experiments using starved cells plated onto fibronectin in the presence of platelet-derived growth factor (PDGF) to induce focal complexes [20] yielded the same result (data not shown). The injection of a

Figure 4



Focal complexes can develop into focal contacts. Following induction of focal complexes as in Figure 2, constitutively active L63Rho (1.6 mg/ml) was injected at time 0:00. Existing focal complexes then elongated into focal contacts. Time is given in min and sec after injection. Scale bar represents 10 μ m.

synthetic peptide mimicking the autoinhibitory domain of smooth-muscle myosin light chain kinase [21] also abolished focal complexes (data not shown), confirming that focal-complex dissolution could be effected through a block of the myosin-II-phosphorylation pathway.

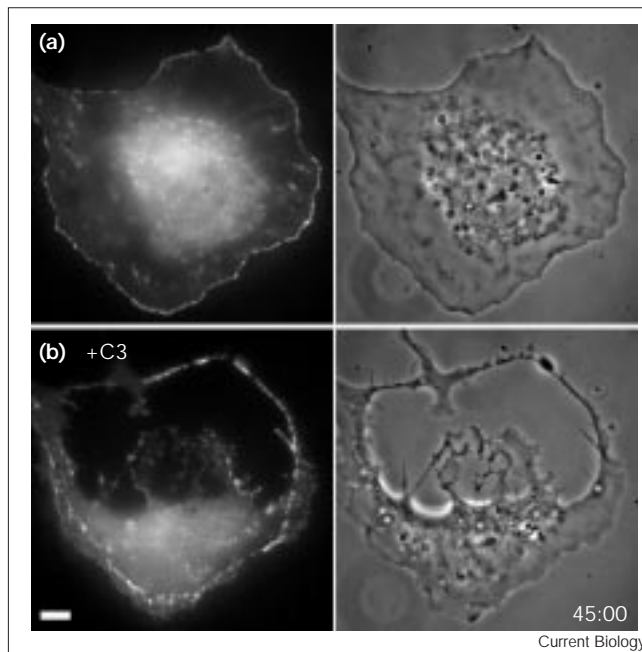
Focal complexes can be precursor sites of Rho-dependent focal contacts

The effect on focal complexes of increasing Rho activity is illustrated in Figure 4. Injection of cells with L61Rac and C3 was followed, after focal-complex formation, by injection of constitutively active L63Rho. As shown in Figure 4, pre-existing focal complexes enlarged and elongated centripetally into typical focal contacts. The same effect was observed with serum-starved cells plated on fibronectin [20]; in this case, subsequent activation of Rho by the addition of lysophosphatidic acid or serum caused the transformation of peripheral focal complexes into focal contacts in all cells (data not shown).

Rac-mediated focal-complex formation and membrane ruffling is independent of Rho and Rho-kinase

Given that we could overcome the inhibitory effect of C3 on Rho by injecting a constitutively active Rho mutant, the

Figure 5

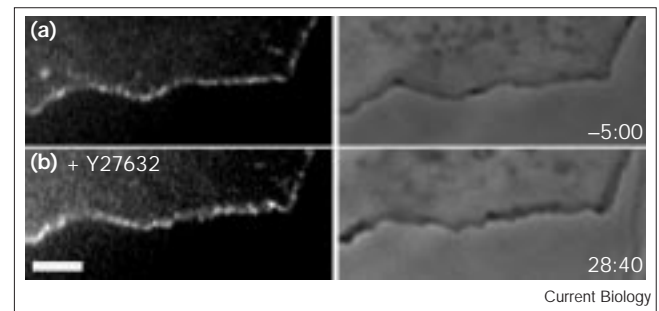


Focal complexes formed by the non-ribosylatable, constitutively active Rac construct I39L61Rac are completely resistant to C3 transferase. (a) A normal Swiss 3T3 cell after injection of I39L61Rac (800 µg/ml), rhodamine–vinculin (500 µg/ml) and 10 µg/ml C3 transferase. (b) The same cell, 45 min after a further injection of 600 µg/ml C3. The left-hand panels show fluorescence images and the right-hand panels show phase-contrast images. Note the maintenance of focal complexes even along the isolated cell edge. Scale bar represents 10 µm.

question arose as to whether C3 at the concentrations used to produce focal complexes (10 µg/ml) was in fact inhibiting all endogenous Rho activity. In other words, did the formation of focal complexes depend on residual Rho activity? To answer this question we used much higher concentrations of C3, and this led to the loss of focal complexes. Rac is also a potential substrate of C3, however, albeit a poor one [22], and the possibility had to be considered that high C3 concentrations also lead to the inactivation of Rac by ribosylation. To circumvent this problem, we constructed a mutant of constitutively active L61Rac (I39L61Rac) in which the Asn39 ribosylation site was exchanged for isoleucine. Microinjections of Swiss 3T3 cells with I39L61Rac and 10 µg/ml C3 induced the same pattern of focal complexes as that seen after injection of L61Rac. The difference, however, was that subsequent injections of C3 at concentrations ranging from 0.1 to 0.6 mg/ml had no effect on peripheral membrane ruffling or focal complexes, even when the body of the cytoplasm underwent a massive retraction (Figure 5). These data confirmed that focal-complex formation was completely independent of Rho.

Because focal complexes showed a requirement for myosin-based contractility for their formation, and Rho-kinase has

Figure 6



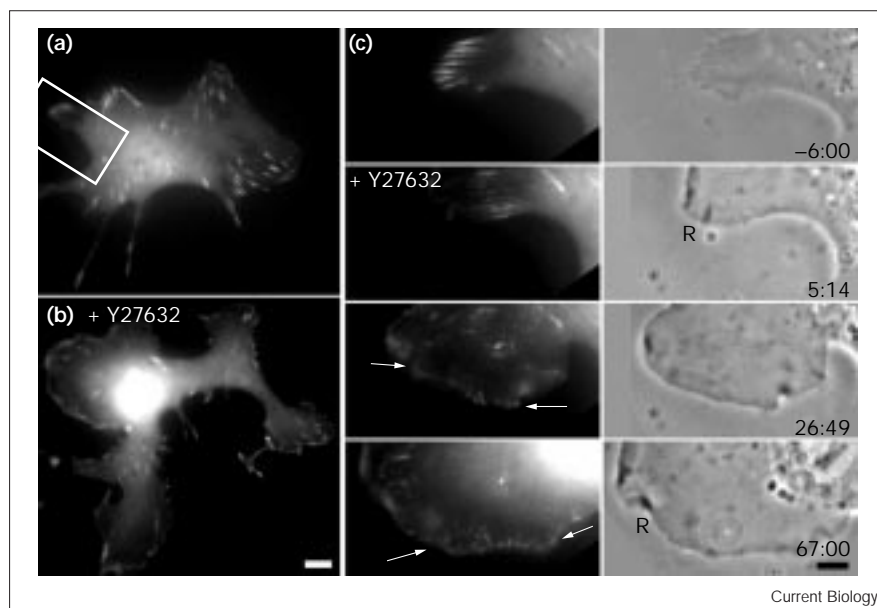
Focal-complex formation is independent of Rho-kinase. (a) Focal complexes labelled with rhodamine–vinculin formed after the cells were co-injected as described in Figure 2. (b) Focal complexes are maintained in the presence of the Rho-kinase inhibitor Y27632 (100 µM). The left-hand panels show fluorescence images and the right-hand panels show phase-contrast images. Scale bar represents 5 µm.

been shown to promote contractility by inhibiting myosin light chain phosphatase [23], it was necessary to establish whether Rho-kinase was also involved in focal-complex formation. As shown by Lamarche *et al.* [24], L61Rac interacts with Rho-kinase *in vitro*, making Rho-kinase a potential downstream target of Rac as well as of Rho (see also [25]). Experiments with the Rho-kinase (p160ROCK)-specific inhibitor Y27632 indicated that focal-complex formation was, however, independent of Rho-kinase. Thus, the treatment of L61Rac-injected cells with 20–100 µM Y27632 had no effect on membrane ruffling or on the integrity of focal complexes (Figure 6). We therefore conclude that Rac-mediated contractility is mediated by a Rho-kinase-independent pathway.

Antagonism between Rac and Rho pathways

In the course of our experiments, we found indications of a mutual antagonism between the Rac and Rho pathways in fibroblasts, as has been reported already for neuronal cells (see Discussion and [26–28]). This was first suggested by the effects of the Rho-kinase inhibitor Y27632 on normal Swiss 3T3 cells injected with fluorescent vinculin. When added at concentrations of 50 µM, Y27632 caused a shift in the contact pattern from focal adhesions to peripheral focal complexes (Figure 7). As shown in Figure 7, focal-complex formation was accompanied by a notable stimulation of ruffling activity, suggesting that inhibition downstream of Rho leads to the activation of Rac. Consistent with this, the upregulation of Rac activity by the injection of L61Rac caused a reduction in the size and integrity of existing focal adhesions (data not shown), whereas the downregulation of Rac by dominant-negative N17Rac induced the loss of focal-complex-type contacts and the growth of focal adhesions (Figure 8). Hence, the upregulation or downregulation of Rac was accompanied by a reciprocal change in Rho-like activity.

Figure 7



Transition of focal contacts to focal complexes in a normal Swiss 3T3 cell in response to Rho-kinase inhibition by Y27632. Whole cell at (a) 6 min before and (b) 67 min after Y27632 (50 μ M) addition. The boxed portion of a protruding cell front in (a) is shown enlarged in (c), at the four timepoints given, by fluorescence (left) and phase-contrast (right) microscopy. Time is given in min and sec after Y27632 addition. Arrows in (c) indicate sets of focal complexes formed sequentially at the base of the advancing lamellipodium. R, ruffle. Scale bars represent 10 μ m in (a,b) and 5 μ m in (c).

Focal complexes are involved in cell protrusion

The translocation of the leading front of a fibroblast characteristically involves phases of protrusion, ruffling and retraction [29]. The associated contact dynamics in a Swiss 3T3 cell is shown in Figure 8a,b, which correspond to insets of the cell shown in Figure 8c. In the two video frames, taken 8 minutes apart, the progress of the cell front was associated, in Figure 8b, with the development of a new set of focal complexes at the base of the advanced lamellipodium. In this example, all focal complexes that existed at the earlier time point (Figure 8a) did not disappear as the lamellipodium advanced beyond them, but either remained unchanged in size or became slightly enlarged. This latter situation differed from the scenario in the presence of the Rho-kinase inhibitor (Figure 7), where the maturation of focal complexes into focal contacts was blocked and the focal complexes were rapidly turned over.

A representative example of the effect of downregulating Rac is shown for the same cell in Figure 8c,d. Injection of N17Rac induced two prominent effects, namely the elongation of focal contacts (Figure 8c) and the suppression of ruffling activity (Figure 8d). Also conspicuous was the disappearance of some pre-existing focal complexes immediately behind the lamellipodium, confirming that these contacts depended on Rac for their existence. In an earlier study, Hock *et al.* [30] showed the elongation of peripheral contacts in cells injected with fluorescently conjugated talin; on the basis of the present results we conclude that this was due to the upregulation of Rho activity.

Discussion

Both interference reflection microscopy and fluorescently tagged contact components have been used previously to follow the assembly and disassembly of substrate contacts in living cells [7,30–33]. This is the first report, however, of the influence of Rho-family members on the dynamics of substrate contact assembly, monitored in this case using a fluorescent analogue of vinculin.

It is apparent from the present and former studies [7] that precursor contacts formed under lamellipodia and filopodia can develop into focal contacts, but the signals and detailed structural rearrangements underlying these transitions are still poorly defined. To relate these transitions to signalling pathways we chose to investigate the claim that Rac-induced focal complexes represent a distinct type of contact site that potentially supports cell motility [10]. Our aim was to shed more light on the function of focal complexes by following their dynamics in relation to changes in Rho protein activity, actin cytoskeleton architecture and the protrusive and ruffling activities of lamellipodia. Focal complexes were originally described in Swiss 3T3 cells as peripheral adhesion sites formed by the injection of constitutively active Rac and concentrations of C3 transferase sufficient to disrupt focal contacts [10]. It was supposed that these contacts were at the tips of lamellipodia, but we have now shown that they reside at the base of lamellipodia and mark the border about which ruffling activity occurs.

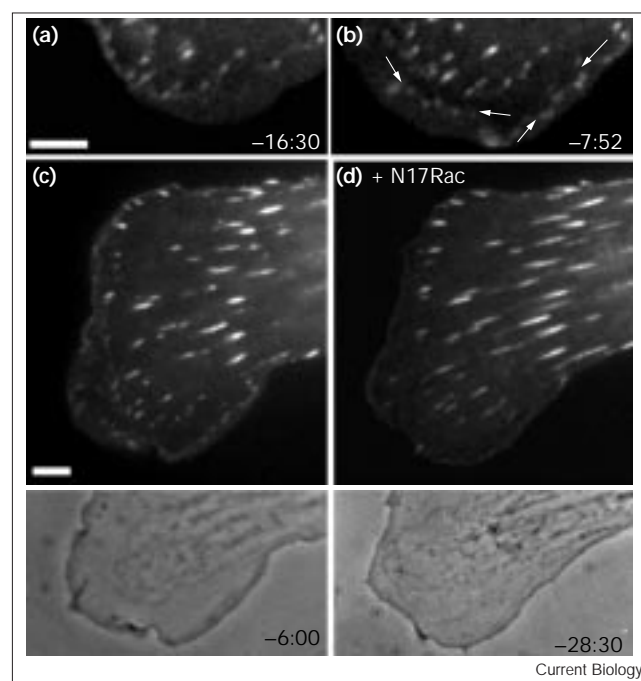
Focal-contact formation has been shown to require myosin-II-dependent contractility, which is mediated

through the phosphorylation of the myosin regulatory light chain [23]. The dependence of this process on Rho has been attributed to the engagement of the Rho-binding protein p160ROCK or Rho-kinase, which acts to suppress myosin light chain phosphatase, thereby facilitating light-chain phosphorylation by myosin light chain kinase (reviewed in [23,34]). As we show here, focal complexes can also be disassembled by inhibitors of both myosin contractility and myosin light chain kinase, but notably not by the inhibitor of Rho-kinase, Y27632 [35]. Consistent with our findings, Uchata *et al.* [35] showed that the expression of dominant-negative p160ROCK blocked the formation of focal contacts and stress fibres, but did not suppress Rac-induced membrane ruffling. Focal complexes thus depend on myosin-II contractility, but this must be stimulated through a pathway not involving Rho-kinase. This alternative pathway remains to be clarified, as well as what contractility means in the context of the filament organisation of a focal complex. It may be, however, that as with focal contacts [36], the development of tension is important in the organisation of the extracellular matrix that goes hand-in-hand with development of substrate contacts.

The results of an initial series of experiments suggested that focal complexes might be dependent on a level of Rho activity below that required to form focal contacts. Thus, a second injection of C3 transferase into cells previously injected with L61Rac and C3 caused the dissolution of focal complexes (data not shown). As C3 can also ribosylate and inactivate Rac *in vitro* [22], we could not exclude the possibility that in addition to further inhibition of Rho activity, increased C3 concentrations also suppress Rac activity *in vivo*. Injection of a Rac mutant lacking the C3-ribosylation site resolved this question. Using this mutant, we showed not only that high C3 concentrations can inhibit Rac activity *in vivo*, but that focal complexes are completely independent of Rho. These findings confirm focal complexes as a distinct type of adhesion site initiated by way of the Rac pathway, as originally proposed [10]. They also indicate, contrary to recent claims [37], that membrane ruffling is totally independent of Rho.

In contrast to previous suggestions [38] we found no evidence for the activation of Rho through Rac in Swiss 3T3 cells. Instead, our results pointed to a mutual antagonism between the two. To explain the results with starved cells [38] we conclude that there is enough residual Rho activity to initiate the development of focal complexes, albeit slowly, into focal adhesions. An antagonism between the Rac and Rho pathways has previously been proposed from studies on neuronal cells [26–28], for which neurite extension is dependent on Rac and Cdc42 and retraction is Rho-dependent. In these cells, downregulation of Rho or Rho-kinase promoted neurite extension [26,28], whereas upregulation of Rac prevented neurite retraction in

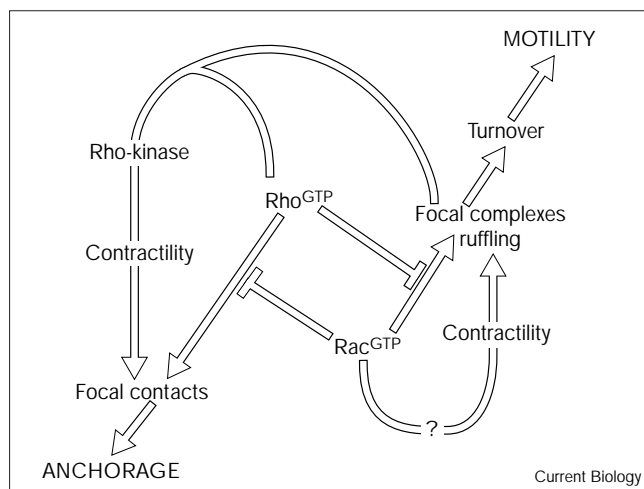
Figure 8



Effect of Rac down-regulation on focal complexes, focal contacts and membrane ruffling. Focal complexes and focal contacts are labelled with rhodamine–vinculin. (a,b) Fluorescence images showing two frames of part of the cell shown in (c,d) before injection of N17Rac. Focal-complex formation is accompanied by cell protrusion. New sets of focal complexes (between arrows in (b)) are formed as the cell edge advances. The arrowhead marks the same contact at the two timepoints, for reference. (c,d) Downregulation of Rac potentiates growth of focal contacts. The same cell as in (a,b) is shown by fluorescence (upper panels) or by phase-contrast (lower panels). N17Rac was injected at time 0:00. Note the elongation of pre-existing focal contacts and the cessation of ruffling (lower panels). Time is given in min and sec after injection of N17Rac. Scale bars represent 5 μ m.

response to the activation of endogenous Rho [27]. More recent observations on actin filament reorganisations in fibroblasts transfected with different Rho mutants have indicated that downregulation of either Rho, Rac or Cdc42 leads to the activation of the other two [39], and that high Cdc42 levels in macrophages antagonise the formation of lamellipodia [12]. The present studies, confined to Rac and Rho, put their antagonism in the context of dynamic changes observed in single manipulated cells. Observation of the same cells before and after treatment with the Rho-kinase inhibitor Y27632 revealed a dramatic stimulation of ruffling activity, which accompanied dissolution of focal contacts. And the downregulation of Rac, characterised by the suppression of membrane ruffling, led to a conspicuous increase in the size of focal contacts. Whether or not these antagonistic effects are due to reciprocal increases in Rho partner activities, or to the availability or modification of their downstream targets, is currently unknown. One possibility is that the Rac-dependent influence on focal

Figure 9



Proposed scheme for substrate-contact dynamics in Swiss 3T3 cells based on the present study. Rac induces membrane ruffles and focal complexes. Motility is associated with the creation of new focal complexes beyond the pre-existing sets. Focal complexes can develop into focal contacts for anchorage on engagement of the Rho pathway and this involves a switch from Rho-kinase-independent to Rho-kinase-dependent contractility. Rac and Rho also exert mutually antagonistic effects at some point in their respective pathways.

contacts may involve p21-activated kinase (Pak) [40], whose activation has recently been shown to suppress myosin light chain kinase activity [41]. In this case, however, Pak must be specifically targeted to focal adhesions, because focal complexes are also dissociated by inhibitors of myosin light chain kinase.

Ishizaki *et al.* [42] have shown that a dominant-negative mutant of Rho-kinase, when co-expressed with constitutively active Rac, inhibited the formation of focal contacts but not of focal complexes. We now confirm these findings by showing the transition in real time from focal contacts to focal complexes in response to Rho-kinase inhibition. The independence of focal complexes of Rho-kinase thus distinguishes their signalling pathways from those of focal contacts. According to this criterion, the differentiation of a focal complex into a focal contact is associated with a switch from Rho-kinase-independent to Rho-kinase-dependent contractility. We suggest that this switch parallels the spatial transition from a 'Rac domain' (the lamellipodium and ruffling membrane) into the 'Rho domain' behind the lamellipodium. In a rapidly moving cell edge, only a minority of focal complexes may develop into focal contacts; the rest are dissociated, and replaced by a new set of complexes that form ahead of the preceding set. This dynamic pattern of contact formation is dominant in fibroblasts treated with the Rho-kinase inhibitor, which promotes localised cell motility, and is typical in B16 melanoma cells migrating on laminin (K. Rottner,

unpublished observations). With the reservation that focal complexes are not the only contact type used to support cell locomotion, we propose that Rac is required for the initiation of new contact sites, whereas Rho serves in the maturation of preformed contacts into focal contacts. Further work will be required to relate the relative roles of Rac and Cdc42 in early contact formation.

Conclusions

According to the scheme shown in Figure 9, the general conclusions of this work are summarised. Focal complexes are a category of early contact sites formed in association with the induction of ruffling lamellipodia by Rac and can support the motile advance of the cell front. Focal complexes may be short-lived, or can develop into focal contacts under the influence of additional downstream effectors in the Rho pathway. Myosin-dependent contractility is required by both focal complexes and focal contacts for their formation, but is activated through different routes: the Rho pathway depends on Rho-kinase to stimulate contractility, whereas the Rac pathway does not. The mutual antagonism between Rac and Rho reflects their opposing roles in motility and anchorage and may allow rapid domination of one or the other in response to signalling cues.

Materials and methods

Cells

Swiss 3T3 fibroblasts were maintained in DMEM with 10% fetal bovine serum (FBS; Sigma), penicillin and streptomycin (Life Technologies) at 37°C in the presence of 5% CO₂. For microinjection experiments, cells were subconfluently replated onto fibronectin-coated glass coverslips in DMEM containing 5% FBS for 12–24 h. Coverslips were acid-washed and coated with 50 µg/ml fibronectin in PBS overnight at 4°C. Fibronectin (Boehringer Mannheim) was stored as a stock solution in 2 M urea at 4°C.

Microinjection

Injections were performed with sterile Femtotips (Eppendorf) held in a Leitz micromanipulator with a pressure supply from an Eppendorf microinjector 5242. Cells were injected with a continuous outflow mode from the needle under a constant pressure of between 20 and 80 hPa.

Proteins for microinjection and drugs

Vinculin from turkey gizzard was kindly provided by M. Gimona and conjugated to 5-TAMRA (carboxytetramethylrhodamine succinimidyl ester; Molecular Probes) essentially according to the manufacturer's instructions. After addition of 2 mg sucrose per mg protein, small aliquots were snap-frozen in liquid nitrogen and stored at –70°C. Before use, the fluorescent vinculin was dialysed against 2 mM Tris-acetate pH 7.0, 50 mM KCl, 0.1 mM dithioerythritol (DTE). Control experiments showed that the contact pattern of living cells microinjected with fluorescent vinculin was identical to the contact pattern of the same cells labelled with anti-paxillin antibodies after fixation.

Recombinant L63Rho, L61Rac, N17Rac and C3 transferase (C3) were expressed as glutathione-S-transferase (GST) fusion proteins in *Escherichia coli* and purified as described [10,38]. The purity of the proteins was confirmed on Coomassie-stained SDS-polyacrylamide gels and protein concentrations were determined using the Bradford assay (Biorad). The proteins were dialysed into 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTE for microinjection [10]. Asn39 in L61Rac

was exchanged for isoleucine by site-directed mutagenesis. After sequencing, the mutant I39L61Rac was purified and microinjected as described above for the recombinant Rho proteins. For induction and visualisation of focal complexes in living cells, we microinjected L61Rac (or I39L61Rac), C3 and fluorescent vinculin in a mixture (concentrations: L61Rac, 800 µg/ml; I39L61Rac, 800 µg/ml; C3, 10 µg/ml; vinculin, 500 µg/ml). In several cases, the initial injection with this triple mixture was followed by a further injection of C3 or a Rho protein (see Results). Control of the second amount injected was facilitated by including FITC-dextran in the injection mixture. In other experiments, the initial injection was followed by treatment of the cells with inhibitors. ML-7 (Alexis Corporation), the inhibitor of myosin light chain kinase, was used at a concentration of 100 µM; the actomyosin inhibitor BDM at 20 mM and the inhibitor of p160ROCK, Y27632 ([35]; provided by Yoshitomi Pharmaceutical Industries) at concentrations between 20 and 100 µM (see Results). Suitable drug concentrations for video microscopy were determined from control experiments on fibroblasts cultured on coverslips, fixed after different time periods and labelled for vinculin and actin.

Video microscopy

Cells were injected and observed in an open heated chamber (Warner Instruments) maintained at 37°C on an inverted microscope (Axiovert 135TV; Zeiss) equipped for epifluorescence and phase-contrast microscopy. Injections were performed at an objective magnification of 40× (NA 0.6 LD Achroplan or NA 1.3 Plan Neofluar) and video microscopy with a 100×/NA 1.4 Plan-Apochromat objective with or without 1.6 optovar intermediate magnification. Filters blocking wavelengths below 590 nm were used for phase-contrast illumination in order to avoid excitation of the fluorescent probe. Tungsten lamps (100 W) were used for both transmitted and epi-illumination. Data were acquired with a back-illuminated, cooled CCD camera from Princeton Research Instruments driven by IPLabs software (both from VisiTron Systems) and stored as 16-bit digital images. The microscope was additionally equipped with shutters (Optilas) driven through a home-made interface to allow separate recordings of video sequences in phase-contrast and fluorescence channels. The sequences were analysed and processed on a Macintosh Power PC 7100/80 using IPLabs and Adobe Photoshop 2.5.1 software.

For phalloidin staining, cells were extracted and fixed with a mixture of 0.25% Triton X-100 and 0.5% glutaraldehyde in cytoskeleton buffer (CB; 10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, 5 mM MgCl₂, pH 6.1) for 1 min followed by 1% glutaraldehyde in CB for 10 min. Cy3-phalloidin was a kind gift of H. Faulstich (Heidelberg) and was used at a concentration of 0.2 µg/ml for 30 min at room temperature. Pictures of cells which were fixed and stained on the microscope were taken in 100 mM DTE in CB to avoid photobleaching.

Supplementary material

Videos showing the formation and characteristics of focal complexes in Swiss 3T3 cells are published with this paper on the internet.

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Supplementary material

Interplay between Rac and Rho in the control of substrate contact dynamics

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Supplementary results

Movie 1

Induction and characteristics of focal complexes (the movie corresponds to Figure 2). The movie shows a Swiss 3T3 cell co-injected with rhodamine–vinculin (500 µg/ml), L61Rac (800 µg/ml) and C3 transferase (10 µg/ml). Typical focal complexes are formed at the cell periphery and focal adhesions are essentially absent. Cells in this fully spread state contain stable focal complexes, which mark the base of a ruffling lamellipodium encircling most of the cell perimeter. Ruffles are typically propagated along the cell edge and sometimes pinch off and move onto the dorsal surface of the main cell body.

Movie 2

Dissociation of focal complexes by ML-7, an inhibitor of myosin light chain kinase (the movie corresponds to Figure 3a). The movie shows a cell injected as described in Movie 1 to produce focal complexes. The cell was then treated with ML-7 (100 µg/ml), at the timepoint indicated. Note the release and inward withdrawal of focal complexes after ML-7 addition.

Movie 3

Dissociation of focal complexes by BDM, an inhibitor of the actin–myosin interaction (the movie corresponds to Figure 3b). The conditions used were the same as those described in Movie 2, except that BDM (20 mM) was added at the timepoint indicated.

Movie 4

The up-regulation of Rho induces the transition of focal complexes into focal adhesions (the movie corresponds to Figure 4). The movie shows a cell injected as described in Movie 1 to produce focal complexes. The cell was then injected with constitutively active L63Rho (1.6 mg/ml) at the timepoint indicated. Note the elongation of individual focal complexes into typical focal adhesions.

Movie 5

The formation of focal complexes is completely independent of Rho (the movie corresponds to Figure 5). The movie shows a cell co-injected with rhodamine–vinculin (500 µg/ml), I39L61Rac, a non-ribosylatable mutant of constitutively active Rac, (800 µg/ml) and C3 transferase (10 µg/ml). At the timepoint indicated, the cell was injected again with C3 at a 60-fold higher concentration (600 µg/ml) than that used in the initial injection. Note

the retention of focal complexes at the cell periphery, even as the body of the cell retracts.

Movie 6

Focal complexes are not dependent on Rho-kinase for their formation (the movie corresponds to Figure 6). The movie shows a cell injected as described in Movie 1 and then treated with the Rho-kinase inhibitor Y27632 (100 µM) at the timepoint indicated. Note the retention of focal complexes in the presence of the inhibitor.

Movie 7

Rho-kinase inhibition leads to the disruption of focal adhesions and the induction of focal complexes (the movie corresponds to Figure 7). The movie shows a Swiss 3T3 cell that was injected with rhodamine–vinculin and then treated with the Rho-kinase inhibitor Y27632 (50 µM). Note that the disappearance of focal adhesions after Y27632 addition is accompanied by the induction of protrusive and ruffling activity at the cell periphery, which is indicative of the up-regulation of Rac. The protrusion of the ruffling cell edge (inset) is associated with the formation of consecutive sets of focal complexes.

Movie 8

The effect of down-regulation of Rac on focal complexes, focal contacts and membrane ruffling (the movie corresponds to Figure 8). The first part of the movie shows a motile Swiss 3T3 cell that was injected with rhodamine–vinculin; the second part shows the response to the subsequent injection, at the timepoint indicated, of dominant-negative N17Rac (700 µg/ml). Note that the formation of focal complexes in the first part of the movie is associated with the advancing and ruffling lamellipodium. Injection of N17Rac causes rapid suppression of ruffling activity and the disappearance of focal complexes. Furthermore, there is a notable elongation of existing focal adhesions, indicative of a simultaneous up-regulation of Rho-like activity.