Actin cytoskeleton remodelling via local inhibition of contractility at discrete microdomains

Gerald Burgstaller and Mario Gimona*
Institute of Molecular Biology, Department of Cell Biology, Austrian Academy of Sciences, Billrothstrasse 11, 5020 Salzburg, Austria
*Author for correspondence (e-mail: mgimona@imb.oeaw.ac.at)

Accepted 19 August 2003
Journal of Cell Science 117, 223-231 Published by The Company of Biologists 2004
doi:10.1242/jcs.00839

Summary
Activation of conventional protein kinase C by phorbol ester triggers the Src-dependent remodelling of the actin cytoskeleton and the formation of podosomes in vascular smooth muscle cells. Rearrangement of actin cytoskeleton in response to phorbol-12,13-dibutyrate is characterised by the simultaneous disassembly of peripheral actin stress fibres and focal adhesions, focal de novo actin polymerisation and actomyosin contraction in the cell center, indicating a spatially and temporally segregated, differential modulation of actin-cytoskeleton stability and turnover. Taking advantage of the prominent actin cytoskeleton in A7r5 cells we show here, that the molecular basis for the local inhibition of contractility is the specific recruitment of p190RhoGAP to specialised microdomains at the focal adhesion/stress fibre interface, which are constitutively enriched in cortactin. The microdomains contain structurally altered actin filaments inaccessible to phalloidin. However, the filaments remain decorated with high molecular weight tropomyosins. Clustering of cortactin during podosome formation causes the rapid, local dispersion of myosin and tropomyosin, and interferes with the F-actin binding of h1calponin, consistent with a RhoGAP-mediated reduction of contractility. Phorbol ester-induced podosome formation is efficiently blocked by expression of constitutively active Dia1, which leads to the dispersion of cortactin. The results provide direct evidence for the spatially restricted inhibition of contractility via the recruitment and accumulation of cortactin and p190RhoGAP.

Supplemental data and movie available online

Key words: p190RhoGAP, Smooth muscle, Myosin, Cortactin, Podosomes

Introduction
Actomyosin-dependent contractile forces are involved in driving a number of cellular processes including cell polarisation, directed cell motility and muscle contraction. The structural basis for contractile events is the formation of a contractile apparatus, in which conformational changes in the actin-attached myosin heads mediate transport of cross-linked arrays of actin filaments. While contractile smooth muscle cells in tissue maintain a three dimensional array of actin and myosin filaments, they adopt a non-muscle type organisation upon culture in vitro (Worth et al., 2001). Hallmarks for this phenotypic conversion are thick stress fibre bundles arranged close to the ventral cell surface that terminate in large focal adhesions, and a banded arrangement of short segments of myosin filaments along these bundles.

The formation of actin stress fibres and focal adhesions is stimulated by the small GTPase RhoA (Ridley and Hall, 1992), and is mediated primarily via two of its immediate downstream effectors, RhoKinase and Dia (Kimura et al., 1996; Watanabe et al., 1999) (for a review, see Frame and Brunton, 2002). RhoKinase stimulates myosin II-dependent contractility in smooth muscle and non-muscle cells by inactivating myosin light chain phosphatase (Katoh et al., 2001a; Katoh et al., 2001b), whereas Dia is implicated in the regulation of actin polymerisation and the initiation of parallel arrays of actin filaments, probably through the recruitment of newly formed actin filaments to stress fibres and interactions with profilin and the Wiskott-Aldrich Syndrome protein WASp (Nakano et al., 1999; Tominaga et al., 2000). The Rho-RhoKinase-mediated increase of cellular contractility is also essential for the formation and maintenance of focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996). Conversely, reduction of contractile forces is a prerequisite for remodelling of the actin cytoskeleton including focal adhesion disassembly (Chrzanowska-Wodnicka and Burridge, 1996; Rottner et al., 1999; Riveline et al., 2001).

It has been generally assumed that the activity of Rho family proteins must be spatially regulated to effect changes in cell form leading to polarisation (Watanabe et al., 1997; Nabi, 1999; Arthur et al., 2000; Kaverina et al., 1999; Kaverina et al., 2000; Wittmann and Waterman Storer, 2001; Small and Kaverina, 2003). However, despite the identification of a number of factors involved in the regulation of RhoA activity (Kaibuchi et al., 1999), the molecular mechanisms underlying its local regulation remain elusive. Nevertheless, it is understood that spatially and temporally regulated modulation of RhoA activity is crucial for polarised cell function and motility. Noren et al. (Noren et al., 2003) proposed recently that GTPase-activating protein (p190RhoGAP)-mediated inactivation of RhoA is essential for the reduction of tension and contractility. In addition it has been recognised that the specific subcellular localisation of p190RhoGAP, together with its tyrosine phosphorylation, is an important determinant for its activation (Brouns et al., 2001; Haskell et al., 2001).
We and others recently showed that cultured rat vascular smooth muscle cells (A7r5) are capable of simultaneously displaying contractile activity in the cell centre and motile activity in the cell periphery. Peripheral remodelling is regulated by the dispersal of focal adhesions and the formation of dynamic podosomes at the same sites (Fultz et al., 2000; Li et al., 2001; Hai et al., 2002; Gimona et al., 2003). These findings suggested the existence of a process sufficient for reducing tensile forces in spatially restricted zones in close proximity to focal adhesions. We have more recently identified specialised zones close to the termini of stress fibres that appear to serve as foci for cytoskeleton turnover in cultured smooth muscle cells (Gimona et al., 2003; Kaverina et al., 2003). In the present work we have investigated the cellular events leading to a local reduction of contractility at these sites. We demonstrate that this involves a relocalisation of contractile proteins, including myosin, tropomyosin (TM), and h1 calponin (h1CaP) and the recruitment of p190RhoGAP and cortactin to specialised microdomains, located at the interface between stress fibres and focal adhesions.

Materials and Methods

cDNA constructs
Drs W. Wolf, T.-L. Chew and R. L. Chisholm (Northwestern University) generously supplied GFP rMLC cDNA. GFP-tagged constructs coding for GFP h1CaP or GFP α-actinin were described previously (Gimona et al., 2003).

Induction of podosome formation

The formation of podosomes in cultured A7r5 cells was induced by changing the culture medium to 1 μM phorbol-12,13-dibutyrate (PDBu) in complete growth medium as described previously (Hai et al., 2002).

Cell culture, transfection and immunofluorescence microscopy

A7r5 rat smooth muscle cells (ATCC, Manassas) were grown in low glucose (1000 mg/l) DMEM without phenol red, supplemented with 10% FBS (PAA, Austria), penicillin/streptomycin (Gibco, Canada) at 37°C and 5% CO2. For transient expression, cells were grown in 60 mm plastic culture dishes and transfected using Superfect (Qiagen, Hilden, Germany) at 70% confluence, essentially as described elsewhere (Kranewitter et al., 2001). Expression and stability of the constructs was assessed by western blotting using a monoclonal antibody against GFP (Clontech, Germany). Cells were replated onto 15 mm coverslips 16 hours post-transfection and prepared for immunofluorescence microscopy after an additional 48 hours on glass coverslips. Cells were washed three times in phosphate-buffered saline (PBS; 138 mM NaCl, 26 mM KCl, 84 mM Na2HPO4, 14 mM KH2PO4, pH 7.4), extracted in 3.7% paraformaldehyde (PFA)/0.3% Triton X-100 in PBS for 5 minutes and fixed in 3.7% PFA (Merck, Germany) in PBS for 30 minutes. Fluorescent images were captured on a Zeiss Axioscope microscope equipped with an AxioCam driven by the manufacturer’s software package (all Zeiss, Vienna, Austria) using plan-neofluar or plan-apochromat 63× oil immersion lenses.

Antibodies

A monoclonal antibody to phosphotyrosine (clone PY-99) was from Santa Cruz Biotechnologies (Santa Cruz, CA), a polyclonal antibody against non-muscle myosin from Biomedical Technologies Inc. (Stoughton, MA), monoclonal antibodies to cortactin from Upstate Biotechnologies Inc. (Lake Placid, NY), AFAP-110 and p190RhoGAP from Transduction Laboratories (Becton Dickinson, Austria), and tropomyosin (clone TM-311) from Sigma (St. Louis). Secondary horseradish peroxidase-coupled antibodies were from Amersham (Austria). Fluorescently labelled secondary antibodies and phalloidin labelled with Alexa 350 (blue), Alexa 488 (green) or Alexa 568 (red) were from Molecular Probes (Leiden, The Netherlands).

Results

Cortactin clustering at specialised microdomains

Rat vascular smooth muscle cells (VSMCs) grown on glass under standard serum conditions develop a robust actin cytoskeleton characterised by thick, contractile stress fibres, and a pseudo-sarcomeric arrangement of actin-associated molecules such as myosin, TM and α-actinin. We have noticed earlier (Burgstaller et al., 2002; Kaverina et al., 2003) that a significant number of the actin stress fibres in formaldehyde-fixed and Triton X-100-extracted A7r5 cells have a region, in close proximity to focal adhesions, that appeared devoid of filamentous actin as revealed by staining with phalloidin (Fig. 1A). Double immunofluorescence microscopy, however, revealed the presence of TM throughout this region, despite the lack of phalloidin staining. (Fig. 1B-D), TM and the homodimeric F-actin cross-linking protein α-actinin are known to occupy alternating domains along the actin filament bundles. In cells transiently transfected with GFP-α-actinin to visualise
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the actin stress fibres and focal adhesions, TM localised in the gap bridging the last α-actinin periodicity and the focal adhesion (Fig. 1E-G). Taken together these results suggest that the actin filaments within this ‘microdomain’ may be structurally altered or complexed with other proteins in a way that prevents binding of phalloidin as well as some actin-associated components.

Our previous studies have shown that Arp2/3-dependent podosome formation in A7r5 cells induced by the phorbol ester PDBu originates at sites corresponding to the microdomains described above (Kaverina et al., 2003). Cortactin is a critical determinant for Arp2/3 recruitment and activation, and the stabilisation of the activated polymerisation machinery (Weed et al., 2000; Uruno et al., 2001). In A7r5 cells under serum conditions, cortactin was distributed throughout the cytoplasm, and enriched in the peripheral lamella. During podosome formation we observed an increase in cortactin clustering at the earliest detectable stage of podosomogenesis, and the clustering became more prominent during maturation of the podosomes (Fig. 2A-I). Clusters of cortactin could be found even prior to PDBu-stimulation (Fig. 2A-C). In a significant fraction of these peripheral clusters we found a striking overlap with the region of the microdomain, characterised by the lack of phalloidin staining (Fig. 2A‘-C‘). We concluded that the presence of cortactin in these microdomains could be essential for their formation and maintenance, as well as the rapid recruitment of factors involved in cytoskeletal remodelling and podosome formation upon a specific cellular signal. Notably, cortactin clustering has also been described recently in podosomes of osteoclasts (Destaing et al., 2003).

Mutually exclusive localisation of cortactin and myosin

Using cortactin as a marker for early podosome formation we observed that the clustering of cortactin interfered with the subcellular localisation of myosin filament assemblies (Fig. 3). In serum-stimulated cells the banding pattern of short myosin filaments was perpendicular to the actin bundles. Upon stimulation with PDBu, myosin II was rapidly removed from the sites of podosome formation and the localisation patterns for myosin II and cortactin were mutually exclusive (Fig. 3). We have demonstrated earlier that the initial phase of podosome formation is characterised by a highly dynamic behaviour, and the movement of podosome assemblies along actin filament bundles (Gimona et al., 2003; Kaverina et al., 2003). Frequently, streaks of myosin II-free regions that progressed along the actin tracks, could be observed (Fig. 3D‘-F‘), suggesting that the progression of podosomes is accompanied by the movement or continuous progressive assembly of cortactin clusters. Identical results were obtained with cells transiently expressing a GFP-tagged regulatory myosin light chain construct (Fig. S1, http://jcs.biologists.org.supplemental) supporting the results obtained in the antibody studies. Moreover, live video fluorescence microscopy using DsRed-tagged SM22 in combination with the above GFP-myosin construct (Fig. 3G) demonstrated that the dynamic translocation of forming podosomes coincided with the dispersion of small myosin filaments (see also Movie 1, http://jcs.biologists.org.supplemental). Thus, the mutually exclusive staining patterns of myosin and cortactin may indeed be a result of myosin filament dispersion, driven by the cortactin-containing protein complex that governs actin polymerisation and podosome formation.

Early, dynamic podosomes were also devoid of high molecular weight TMs (Fig. 4A-C and A‘-C‘). Although TM was found enriched in podosomes at later stages (Fig. 4D-F), the recruitment was restricted to the peripheral zone of the podosomes, away from the core at which Arp2/3 activity is high. In a previous study we observed that over-expression of the actin-binding
protein h1CaP significantly inhibits podosome formation and cytoskeletal remodelling in A7r5 cells (Danninger and Gimona, 2000; Gimona et al., 2003). Thus, the displacement of CaP from the filament is a prerequisite for this process. However, the factor(s) causing CaP dissociation remained elusive. When we employed immunofluorescence microscopy of cells expressing GFP-tagged h1CaP and counterstained for cortactin we were then able to demonstrate that the clustering of cortactin was sufficient for interference with the association of CaP with actin stress fibres (Fig. 5).

p190 RhoGAP accumulates in response to PDBu at sites of podosome formation

The activity of RhoA is inhibited both in vitro and in vivo by the association and activation of the major cellular GTPase activating protein, p190 RhoGAP (Arthur et al., 2000; Brandt et al., 2002; Noren et al., 2003). In serum stimulated cells p190RhoGAP colocalised with the actin filaments, and was enriched in the cell periphery (Fig. 6A-C). Induction of cytoskeletal remodelling by PDBu caused the rapid accumulation of p190RhoGAP at the sites of podosome formation, essentially depleting the stress fibres of the GAP (Fig. 6D-F). The localisation at peripheral actin-containing structures, however, was not affected (arrowheads in Fig. 6D-F).

Activated Dia interferes with cortactin clustering and inhibits podosome formation

Inactivation of RhoA inhibits the relaying of downstream signals via RhoA effector molecules, mostly mediated by the concerted action of RhoKinase

![Figure 3](image-url)
Local inhibition of contractility

and Dia1. Inhibition of RhoKinase by Y-27632, which is expected to reduce myosin II-dependent contractility, failed to induce podosome formation in A7r5 cells in the absence of PDBu (Fig. S2, http://jcs.biologists.org.supplemental). By contrast, ectopic expression of a truncated, constitutively active version of Dia1, mDiaΔN3, (Ishizaki et al., 2001) was sufficient to prevent podosome formation in the presence of PDBu (Fig. 7). Transfected A7r5 cells exhibited prominent parallel arrays of thin actin filaments as well as the polar clustering of vinculin-rich focal adhesions (not shown), and the formation of large, dorsal cups of ruffling membrane (Fig. 7A), similar to the effects described previously in MDCK cells (Nakano et al., 1999). Activated mDia1 is sufficient for circumventing the inhibition of RhoKinase-mediated contractility, at least when force is applied externally (Riveline et al., 2001). In A7r5 cells, the overexpression of mDiaΔN3 interfered with the subcellular mobility and clustering of Arp2/3 as revealed by staining with an antibody against the p16 subunit of the Arp2/3 complex. In PDBu-treated, mDiaΔN3-positive cells, Arp2/3 was enriched in the perinuclear region as compared to control cells (I. Kaverina and M.G., unpublished observations). In the present study, immunofluorescence microscopy on GFP mDiaΔN3-transfected cells revealed that cells expressing the activated formin were essentially devoid of cortactin clusters throughout the cell both in the presence and absence of PDBu (Fig. 7C-E).

We were also unable to clearly identify regions of discontinuous phallolidin label at the ends of the actin filaments in cells expressing activated mDia. Together with our previous work (Hai et al., 2002) these data indicate that podosome formation requires the inhibition of both Rho downstream effector pathways mediated by RhoKinase and Dia.

**Discussion**

Cytoskeletal remodelling induced by RhoKinase inhibition causes the reduction of focal adhesions via the modulation of actomyosin interactions (Amano et al., 1996). Focal adhesion stability is maintained by contractility exerted by the attached actin cytoskeleton, and the loss of contractility by myosin light chain kinase inhibition accounts for these effects (Chrzanowska-Wodnicka and Burridge, 1996; Choquet et al., 1997; Hirose et al., 1998; Rottner et al., 1999; Riveline et al., 2001; Balaban et al., 2001; Tsubouchi et al., 2002). Earlier investigations demonstrated that during PDBu-induced podosome formation in vascular smooth muscle cells, focal adhesion turnover occurs in the presence of a contractile actin cytoskeleton (Gimona et al., 2003). The loss of contractility is thus restricted to only a part of the actomyosin system. The present study suggests that microdomain regions at the ends of focal adhesions are targets for the local reduction of contractile forces, leading to focal adhesion destabilisation. The fact that such microdomains have not been previously recognised may be attributed to our use of cells with exceptionally large stress fibre bundles, which significantly facilitates their identification.

Fig. 4. Tropomyosin is absent from early podosomes. (A–C) Similar to the situation with myosin II, TM is rearranged and diffused at the sites of cortactin clustering during the early phase of podosome formation. Higher magnification of a selected area is shown in A′–C′. Arrowheads indicate regions of TM dispersion and podosome formation. (D–F) Only at later stages are tropomyosins found at, and overlap with, the periphery of larger podosomes. Scale bar: 20 μm.

Fig. 5. Clustering of cortactin coincides with the displacement of h1 calponin from actin stress fibres. Enlarged section from an A7r5 cell transiently transfected with GFP h1CaP (B, green), fixed and stained for cortactin (C, red) after 10 minutes of PDBu stimulation. Arrows indicate regions of cortactin clustering and depletion of calponin. Scale bar: 1 μm.
The detection of TM fibres within the microdomains verifies that the generation of the domain is not due to the artefactual removal of actin filaments during the fixation procedure. In addition, in cells transfected with GFP-β-actin such microdomains could not be observed (Gimona et al., 2003), supporting the contention that structural alterations within the filament, rather than extraction of labile F-actin is the primary cause for the lack of phalloidin association with the actin filaments. Together with the results from DesMarais et al. (DesMarais et al., 2002), the absence of actin polymerisation at the microdomains prior to activation by PDBu argues for the presence of an inactive, pre-polymerisation complex at these sites (see below).

**RhoGAP-mediated local inhibition of RhoA**

About 60% of the cell’s total GAP activity towards Rho is exerted by p190RhoGAP (Vincent and Settleman, 1999), which is activated by Src (Fincham et al., 1999; Haskell et al., 2001). Inhibition of p190RhoGAP activity initiates Rho-mediated remodelling of the actin cytoskeleton (Vincent and Settleman, 1999), and enhances cell spreading and migration (Arthur and Burridge, 2001). It has been shown previously that p190RhoGAP mediates the c-Src-dependent RhoA inactivation in PDBu-stimulated vascular smooth muscle cells (Brandt et al., 2002). Results presented here indicate that p190RhoGAP accumulates specifically at the sites of podosome initiation in response to phorbol ester.

The multiple events required for RhoA inhibition, Arp2/3 assembly, de novo actin polymerisation and focal adhesion disassembly argue for the controlled spatial and temporal assembly of one or more protein complex(es) mediating these effects. Since critical components such as p190RhoGAP and c-Src require translocation and site-specific activation, respectively, the mechanisms supporting these events need to be defined. From the results of this study we propose that the local inhibition of contractility may involve the following cascade of events. Activation of PKC by PDBu can activate the ERK1/2 signalling pathway (Zou et al., 1998). ERK activity decreases the cytosolic activity and alters the subcellular localisation of p190RhoGAP. Chen et al. (Chen et al., 2003) have demonstrated a shift of p190RhoGAP from the cytosolic pool to the Triton-insoluble cytoskeletal fraction upon activation of the Ras/Raf/MEK/ERK pathway. This shift may correspond to the translocation and accumulation of p190RhoGAP to sites of active actin polymerisation shown in this study. PKC also causes the phosphorylation of AFAP-110, and Qian et al. (Qian et al., 2002) hypothesised that phosphorylation of AFAP-110 by PKCα initiates a specific recruitment to sites of de novo actin polymerisation. Indeed, we observed that PDBu-induced activation of PKC also causes the translocation of AFAP-110 from the stress fibres to the sites of podosome formation (Fig. S3, http://jcs.biologists.org.supplemental). Work from the Flynn lab (Qian et al., 1998; Qian et al., 2000; Baisden et al., 2001) has clearly illustrated that the adaptor protein AFAP-110 activates c-Src in vitro and triggers the Src-dependent cytoskeleton modulation. Therefore, the local formation of AFAP-110 and p190RhoGAP-containing complex(es) might enable the spatially restricted suppression of RhoA via the Src-induced...

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**Fig. 6.** Accumulation of p190RhoGAP. PDBu treatment induces the rapid translocation of p190RhoGAP from actin stress fibres (A-C) to the sites of podosome formation (D-F). Note the almost complete displacement of p190RhoGAP following PDBu stimulation (E), despite the presence of prominent actin stress fibres in the centre of the cell (D,F). The localisation of p190RhoGAP with membrane ruffles remains unchanged (arrows). Scale bar: 20 μm.

**Fig. 7.** Activated Dia interferes with cortactin clustering. Ectopic expression of active GFP-mDiaAN3 suppresses podosome formation induced by PDBu (A,B), and causes the dispersion of cortactin with the concomitant failure to form cytoplasmic clusters (C-E). Scale bar: 20 μm.
Muscle calponin variant efficiently suppressed by high expression levels of the smooth filaments by the dispersion of TM. Providing a mechanism for the protection of branched antagonistic model further supports the above hypothesis by actin filaments (Weaver et al., 2001), an effect that appears to cortactin has also been shown to inhibit the de-branching of dispersion of Arp2/3 and p190 RhoGAP (2). The reduction of contractile forces by the suppression of RhoA activity and the displacement of tropomyosin, calponin and myosin II causes the actin filaments to shrink and gradually reduce attachment to the focal adhesion sites. Further progression, growth and maturation of late podosomes (l.p.) along the existing actin trails causes further displacement of myosin filaments and the total separation of actin filaments from peripheral adhesions. The total loss of stress fibre/focal adhesion attachment leads to complete elimination of contractility and the subsequent disassembly of the focal adhesions, while regions of stable actomyosin interaction remain contractile.

Several studies have suggested a direct relationship between reduced levels of CaP expression and the alterations in actin cytoskeleton stability in a number of smooth muscle-derived tumours such as leiomyosarcoma (Horiiuchi et al., 1998; Horiiuchi et al., 1999). We observed that clustering of cortactin also interferes with the colocalisation of h1CaP with actin filaments, and in A7r5 VSMCs under normal culture condition the localisation of cortactin and h1CaP were mutually exclusive. We thus hypothesise that the progression of the cortactin-associated molecular complex(es) includes the displacement of CaP from the stabilised filaments prior to actomyosin disassembly and p190RhoGAP-mediated reduction of contractile forces.

Phosphorylation of p190RhoGAP. Inhibition of RhoKinase and Dia downstream of Rho then causes the uncoupling of actomyosin interactions resulting in the dispersion of the myosin II-containing thick filaments and the disassembly of actin stress fibres. The local inhibition of contractile forces together with the suppression of Dia function subsequently stimulates the disassembly of focal adhesions.

Cortactin-containing complexes modulate actin binding proteins

Cortactin has been suggested to function as a scaffolding protein in various large protein complexes used to regulate adhesion and actin assembly (Weed et al., 2000; Olazabal and Machesky, 2001; Weed and Parsons, 2001; Destaing et al., 2003) (reviewed by Weaver et al., 2003). Thus, the observed clustering and activation of cortactin at the focal adhesion/stress fibre interface microdomain may serve as a nucleus for the further assembly, stabilisation and activation of the actin polymerisation machinery. Recruitment of the Arp2/3-complex at the microdomain within the region of reduced contractility (see Fig. 8) allows rapid polymerisation of new actin filaments. The actin-binding function of cortactin may play a pivotal role in the progression of the Arp2/3-containing polymerisation machinery along the actin stress fibres towards the contractile domain. At this point, a (hypothetical) ‘polymerisation complex’ (Arp2/3, WASp, WIP, profilin, monomeric actin and SM22), and a separate, ‘contractility-inhibiting complex’ (cortactin, p190RhoGAP, and possibly c-Src, AFAP-110 and PKCα), may move together to increase the region of low contractility and to enhance cytoskeleton remodelling, and the dispersion of h1CaP, myosin and TM. It is notable that cortactin has also been shown to inhibit the de-branching of actin filaments (Weaver et al., 2001), an effect that appears to counteract the branch-inhibiting function of TM. This antagonistic model further supports the above hypothesis by providing a mechanism for the protection of branched filaments by the dispersion of TM.

We have shown earlier that podosome formation is efficiently suppressed by high expression levels of the smooth muscle calponin variant h1CaP (Gimona et al., 2003), and

Inhibition of cortactin clustering by Dia activity

The regulation of podosome formation seems to involve the antagonistic activities of RhoKinase and mDia (Sahai and Marshall, 2002) which, in collaboration with Src, regulate stabilisation and disassembly of the actin cytoskeleton (Tominaga et al., 2000; Ishizaki et al., 2001). Nakano and colleagues (Nakano et al., 1999) have demonstrated that activated mDia can also counteract the phorbol-ester-induced disassembly of stress fibres in MDCK cells, and the work of Riveline et al. (Riveline et al., 2001) further showed that mDia activity is sufficient for contact formation, while myosin II activation via RhoKinase is dispensable for this process. Together with the established direct association of mDia with Src these data point towards a crucial involvement of mDia in the formation of podosomes. Inhibition of podosome formation by active mDia is probably mediated by the interference with Arp2/3 translocation in the cell. Indeed, A7r5 cells overexpressing the constitutively active mDiaAN3 mutant were unable to form podosomes in response to PDBu, and Arp2/3 failed to accumulate at sites of actin polymerisation at the cell periphery (I. Kaverina and M.G., unpublished observations). This can be explained by the dispersion of cortactin observed in mDiaAN3-expressing A7r5 cells. Thus, a possible mechanism by which formin activity can bypass the formation of podosomes and cytoskeletal remodelling may involve the inhibition of cortactin cluster formation.

Fig. 8. Schematic representations of cytoskeletal remodelling events during the induction and formation of podosomes. Stress fibres align with the perpendicular arrangement of myosin and are anchored in vinculin-rich focal adhesions (1). The cortactin-rich microdomain residing at the stress-fibre/focal adhesion interface initiates the formation of early podosomes (e.p.; hatched orange), enriched in Arp2/3 and p190 RhoGAP (2). The reduction of contractile forces by the suppression of RhoA activity and the displacement of tropomyosin, calponin and myosin II causes the actin filaments to shrink and gradually reduce attachment to the focal adhesion sites. Further progression, growth and maturation of late podosomes (l.p.) along the existing actin trails causes further displacement of myosin filaments and the total separation of actin filaments from peripheral adhesions. The total loss of stress fibre/focal adhesion attachment leads to complete elimination of contractility and the subsequent disassembly of the focal adhesions, while regions of stable actomyosin interaction remain contractile.
Microtubule-actin cytoskeleton interactions at the microdomains

Activation of signalling molecules in vivo must be spatially organised. Indeed, subcellular localisation at specialised compartments, as well as the exclusion of certain components from such locations is essential for the correct assembly and function of macromolecular complexes (Schafer et al., 1998). Blanchion et al. (Blanchion et al., 2001) reported that the actin binding activity of TM interferes with branch formation and WASp-mediated nucleation activity of Arp2/3, and exclusion of TM from sites of de novo actin filament polymerisation has been observed by DesMarais and colleagues (DesMarais et al., 2002) in the protruding lamellipodia of migrating cells. Arthur and Burridge (Arthur and Burridge, 2001) have also shown that p190RhoGAP activity is necessary to prevent the premature formation of actin stress fibre bundles during cell spreading. Together these observations support a mechanism of site-specific inhibition and reorganisation of components interfering with Arp2/3-dependent actin polymerisation upon initiation of cytoskeletal remodelling.

The intriguing studies from the Small lab (reviewed by Small and Kaverina, 2003; Small et al., 2002) have demonstrated that the sites of cell anchorage may serve as a potential interface between the actin and the microtubule cytoskeleton, and the authors suggested that signals delivered by microtubules locally regulate actin turnover and focal adhesion stabilisation. Together with the work of Chrzanowska-Wodnicka and Burridge (Chrzanowska-Wodnicka and Burridge, 1996) our work supports the existence of specialised microdomains that can serve to initiate cytoskeletal remodelling by regulating the spatially and temporally restricted reduction of contractility, cytoskeletal rearrangement and focal adhesion disassembly.

We thank Ulrike Tischler for technical support, Toshimasa Ishizaki and Shuh Narumiya for mDia GFP cDNA constructs, and W. Wolf, T.-L. Chew and Rex L. Chisholm for the generous gift of the GFP rMLC construct.

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