Actin machinery and mechanosensitivity in invadopodia, podosomes and focal adhesions

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Journal of Cell Science 122, 3037-3049 Published by The Company of Biologists 2009
doi:10.1242/jcs.052704

Summary

The invasiveness of cells is correlated with the presence of dynamic actin-rich membrane structures called invadopodia, which are membrane protrusions that are associated with localized polymerization of sub-membrane actin filaments. Similar to focal adhesions and podosomes, invadopodia are cell-matrix adhesion sites. Indeed, invadopodia share several features with podosomes, but whether they are distinct structures is still a matter of debate. Invadopodia are built upon an N-WASP-dependent branched actin network, and the Rho GTPase Cdc42 is involved in inducing invadopodial-membrane protrusion, which is mediated by actin filaments that are organized in bundles to form an actin core. Actin-core formation is thought to be an early step in invadopodium assembly, and the actin core is perpendicular to the extracellular matrix and the plasma membrane; this contrasts with the tangential orientation of actin stress fibers anchored to focal adhesions. In this Commentary, we attempt to summarize recent insights into the actin dynamics of invadopodia and podosomes, and the forces that are transmitted through these invasive structures. Although the mechanisms underlying force-dependent regulation of invadopodia and podosomes are largely unknown compared with those of focal adhesions, these structures do exhibit mechanosensitivity. Actin dynamics and associated forces might be key elements in discriminating between invadopodia, podosomes and focal adhesions. Targeting actin-regulatory molecules that specifically promote invadopodium formation is an attractive strategy against cancer-cell invasion.

This article is part of a Minifocus on invadopodia and podosomes. For further reading, please see related articles: 'Invadosomes at a glance' by Stefan Linder (*J. Cell Sci*. **122**, 3009-3013), 'Matrix invasion by tumour cells: a focus on MT1-MMP trafficking to invadopodia' by Renaud Poincloux et al. (*J. Cell Sci*. **122**, 3015-3024) and 'Mechanisms for transcellular diapedesis: probing and pathfinding by 'invadosome-like protrusions'' by Christopher V. Carman (*J. Cell Sci*. **122**, 3025-3035).

Key words: Invadopodia, Podosomes, Actin, Mechanosensitivity, Focal adhesion

Introduction

Tumor-cell invasion across tissue barriers requires degradation of the extracellular matrix (ECM), as well as dynamic interactions between the ECM and the intracellular actin cytoskeleton that occur through organized adhesive structures. The best-characterized adhesive structures, named focal adhesions, contain clusters of transmembrane integrin receptors that are tethered at one end to the ECM and at the other to actin stress fibers, which are responsible for cell traction and ECM reorganization. However, other adhesive structures, known as podosomes and invadopodia, also exist.

Podosomes are distinct adhesive structures that are found on the ventral side of a wide range of cells, including osteoclasts, macrophages and endothelial cells. In osteoclasts, podosomes are involved in the formation of a sealing zone that establishes an isolated compartment in which bone is degraded (Gimona et al., 2008). It has also been demonstrated that lymphocytes use podosomes: they extend 'invasive podosomes' to palpate the surface of, and ultimately form transcellular pores through, the vascular endothelium (Carman et al., 2007). Invasive cancer cells and Src-transformed cells display podosome-like actin-rich membrane protrusions called invadopodia, which are primary sites of rapid actin polymerization and which represent the major sites of matrix degradation in these cells (Weaver, 2008). Invadopodia of tumor cells appear as irregular dots in the vicinity of the nucleus and in proximity to the Golgi complex. Podosomes in osteoclasts and invadopodia in Src-transformed cells share the feature of self-organizing into a ring (the so-called rosette); in the case of osteoclasts, this can expand to a belt called the sealing zone. This self-organization is crucial for efficient matrix degradation and cell invasion (Badowski et al., 2008; Saltel et al., 2006). These rosettes can be formed after stimulation with potent angiogenic factors such as vascular endothelial growth factor (VEGF) and tumor necrosis factor-α (TNFα) (Osiak et al., 2005).

Because of the significant impact of invadopodia in oncological events such as cell invasion and matrix degradation, we need more insight into the mechanisms that favor the development of invadopodia at the expense of focal adhesions. Invadopodia, podosomes and focal adhesions are all cell-matrix adhesion sites that connect the actin cytoskeleton within the cytosol to the extracellular matrix, but they differ in their architecture and dynamics despite sharing most of the same proteins (such as integrin, talin and paxillin) (Block et al., 2008) (Figs 1, 2 and 3).

Active actin polymerization, induction of membrane curvature, rapid turnover of cell-matrix adhesions and local modulation of contractile forces are all likely to play a central role in the promotion of invadopodium formation. In this Commentary, we discuss recent

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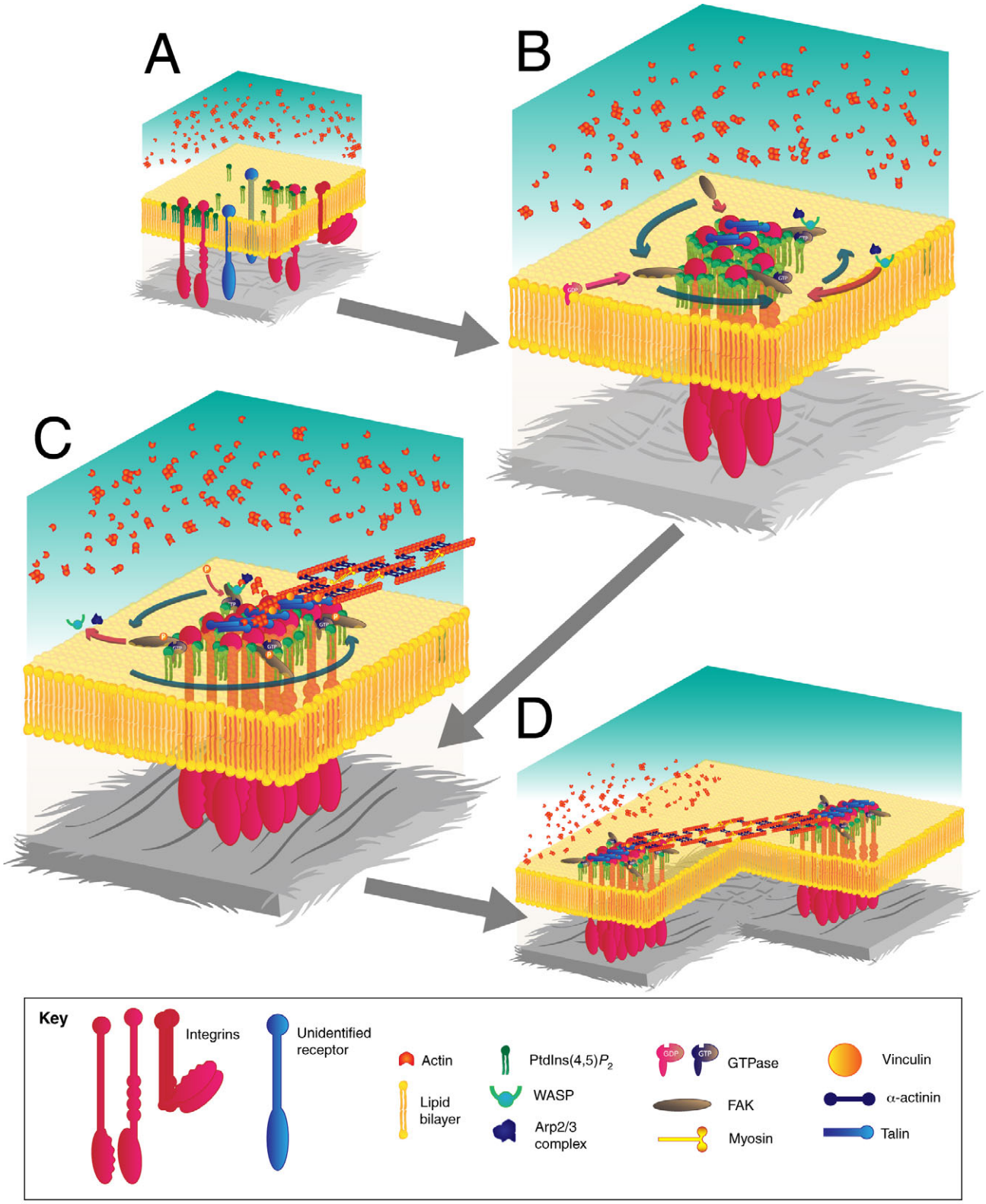

Fig. 1. Schematic view of signaling pathways that lead to actin organization at focal adhesions. (A) At the initial stage of adhesion formation, integrins or other unidentified receptors bind to components of the ECM (grey), leading to clustering of receptors into PtdIns(4,5)P_2-enriched areas of plasma membrane. (B) In early spreading adhesions at the cell periphery, the Arp2/3 complex and WASP are targeted to adhesions by FAK. Blue arrows represent the spatiotemporal sequence of structure assembly. Pink arrows indicate protein recruitment. (C) Autophosphorylation of FAK at Tyr397 destabilizes the Arp2/3-WASP-FAK complex. Talin is recruited to adhesions, allowing integrin-ECM linkages to be functionally coupled to actomyosin; this enables actomyosin contractility to affect adhesion reinforcement and subsequent maturation. Actin filaments can be crosslinked by α-actinin. Myosin II incorporates into the α-actinin-crosslinked actin-filament bundles. (D) The collective dynamics of focal adhesions can be imaged by the actin-stress-fiber-mediated connection of focal adhesions. Connections can be observed between focal adhesions at the front of the cell with sliding trailing adhesions at the rear of the cell.

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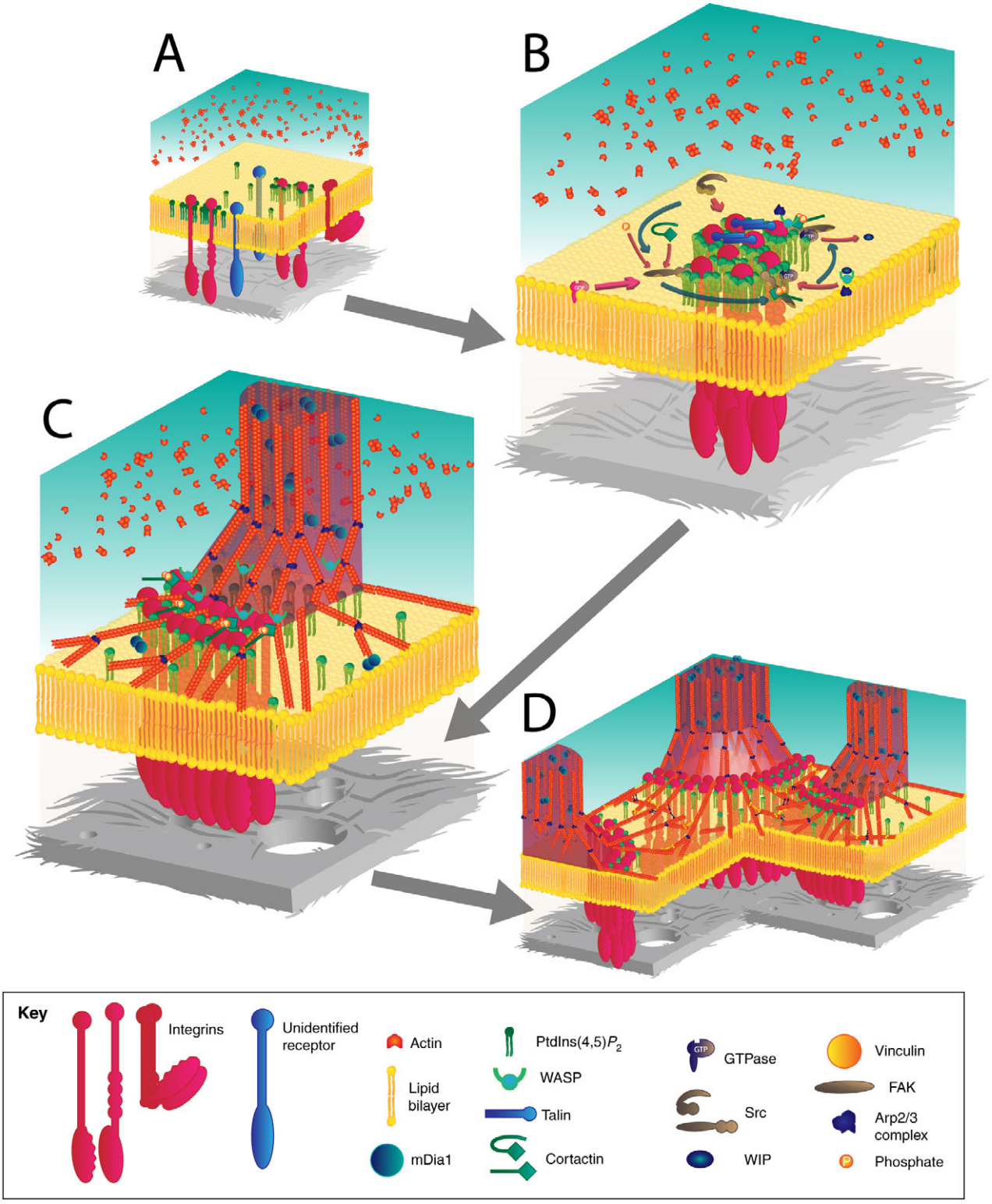

Fig. 2. Schematic view of signaling pathways that lead to actin organization at invadopodia or podosomes. (A) At the initial stage of adhesion formation, integrins or other unidentified receptors bind to components of the ECM (grey), leading to clustering of receptors into PtdIns(4,5)P_2-enriched areas of plasma membrane. (B) Recruitment of Src to adhesion sites leads to phosphorylation of several proteins such as cortactin, WASP, FAK and regulators of small GTPases. Continuous actin nucleation relies on the continuous and strong activation of the Arp2/3 complex at the membrane through the synergistic action of cortactin and WASP-family proteins. (C) DRF/mDia1 elongates actin filaments into columnar structures from the branched actin network that was previously induced by N-WASP, the Arp2/3 complex and cortactin. (D) Podosomes or invadopodia are mechanically connected through a network of radial actin filaments that lie parallel to the substratum.

insights into the actin dynamics of invadopodia and podosomes, and the forces that are transmitted through these invasive structures.

Role of F-actin in the assembly of focal adhesions, podosomes and invadopodia

Various types of directed cell motility are driven by the polymerization of an actin network that pushes the membrane forwards. During cell motility, the leading edge of the cell exhibits a range of dynamic structures such as lamellipodia, filopodia and membrane ruffles (Pollard and Borisy, 2003). Correlative fluorescence and electron microscopy show that the lamellipodium – which contains a dense network of branching actin filaments that undergo fast retrograde flow – forms a cohesive, separable layer of actin in front of a less dynamic actin network called the lamella. At the rear of the lamellipodium, the motor myosin II pulls lamellipodial actin filaments and condenses them into lamellar actin bundles, causing periodic edge retraction (as a result of mechanical breakage of the link between focal adhesions and stress fibers), as well as initiation of new adhesion sites and force generation that is accompanied by assembly of actin into stress fibers. Live-cell imaging has shown that nascent cell-matrix adhesions arise in the lamellipodium (Giannone et al., 2007) and grow and mature during the forward movement of the lamellipodium, forming focal adhesions connected to stress fibers that are tangentially oriented with respect to the ECM (Fig. 1). These focal adhesions are localized at the interface between the lamellipodial and lamellar filamentous (F)-actin networks (Hu et al., 2007). Even though the lamellipodial actin structure has been well described, the precise organization of the actin filaments that emerge from focal adhesions is poorly understood, mostly because of the absence of ultrastructural studies that show adhesion sites and the cytoskeleton at the same time.

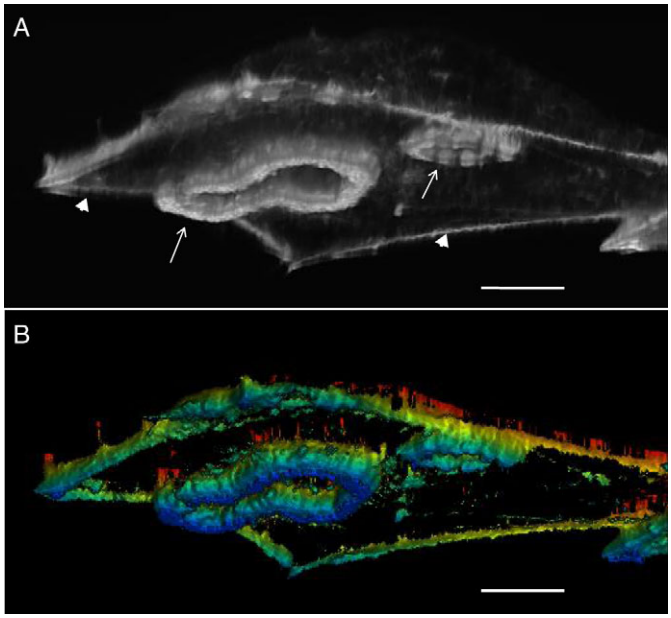

Fig. 3. Three-dimensional (3D) reconstruction of F-actin structure in a BHK-RSV cell. (A) A 3D reconstruction was derived by combining images from confocal planes viewed from the side of the basal (adherent) face. F-actin staining was carried out after fixation in 4% paraformaldehyde with TRITC-phalloidin. 3D reconstruction and rendering of the actin cytoskeleton was carried out through EDIT3D software, using grey-level images of each confocal *z*-stack (developed by Yves Usson and Franck Parazza, UMR CNRS 5525, Grenoble, France). Actin stress fibers are indicated by arrowheads, and the collective organization of podosomes and invadopodia by arrows. (B) A color scale was added, purely to indicate the relative position of the *z*-plane. The most basal plane was colored blue. Scale bars: 5 μm.

Invadopodia and podosomes differ from focal adhesions in the geometry and dynamics of their associated actin cytoskeleton (Fig. 2). Indeed, the formation of invadopodia and podosomes was historically described as a major actin-cytoskeleton reorganization that was induced by the expression of the oncogene v-Src (Tarone et al., 1985). Invadopodia and podosomes comprise an actin core containing the actin-nucleation machinery [including Wiskott-Aldrich syndrome protein (WASP), neuronal WASP (N-WASP), WASP-interacting protein (WIP), the Arp2/3 complex and cortactin] surrounded by a multimeric protein complex that consists of integrins and integrin-associated proteins such as talin, vinculin and paxillin (Mueller et al., 1992; Desai et al., 2008). Integrins and their associated proteins constitute an adhesive ring that colocalizes with a region of polymerized actin, called the 'actin cloud', between the multiple actin cores, which form a cluster, ring or belt of invadopodia or podosomes (Collin et al., 2006; Destaing et al., 2003).

Live-cell imaging of GFP-actin has revealed that podosomes undergo cycles of rapid polymerization and depolymerization, and have a life-span of 2 to 4 minutes (Destaing et al., 2003; Ochoa et al., 2000). Invadopodia are thought to have a much longer life-span of ~30 minutes but, in Src-transformed baby hamster kidney (BHK) cells, inhibition of protein tyrosine phosphatases speeds up invadopodial dynamics to give half-lives similar to those of podosomes (Badowski et al., 2008). Moreover, fluorescence recovery after photobleaching (FRAP) experiments have shown that the podosome actin core and the actin cloud undergo continuous actin polymerization and depolymerization, which is maintained throughout the podosome life span (Destaing et al., 2003). In terms of assembly, invadopodia formation is initiated by the nucleation of F-actin; these filaments are oriented perpendicularly to the substrate (Artym et al., 2006; Badowski et al., 2008); by contrast (and as described above), the assembly of focal adhesions starts with occupancy of integrins by ECM components and integrin clustering, after which actin stress fibers form (Cai and Sheetz, 2009; Vicente-Manzanares et al., 2009). Because the actin core of invadopodia assembles before the surrounding integrin-containing adhesive ring, and actin disruption is a prerequisite for invadopodium disassembly (Badowki et al., 2008), we can hypothesize that invadopodia are maintained by repetitive nucleation of actin polymerization at the invadopodium tip, followed by the rearrangement of actin filaments within the shaft.

At the molecular level, high-resolution scanning electron microscopy combined with fluorescence microscopy has resolved the molecular architecture of arrays of invadopodia and podosomes, revealing that these adhesive structures contain two F-actin networks with opposite orientations. Podosome cores are composed of bundles of actin cables that lie perpendicular to the substratum, and the cores communicate through a network of radial actin filaments that lie parallel to the substratum; these correspond to the actin cloud (Fig. 2) (Gavazzi et al., 1989; Luxenburg et al., 2007). These observations suggest the existence of tangential forces between podosome actin cores. The magnitude and direction of the resulting forces probably depends on the collective organization of a group of invadopodia or podosomes into a cluster, ring or belt.

In conclusion, focal adhesions, invadopodia and podosomes are all strongly associated with actin filaments that link neighboring structures. However, although fast-polymerizing actin seems to be

the scaffold that allows assembly of and stabilizes invadopodia and podosomes, actin stress fibers with much slower dynamics seem to be required to transmit the tangential forces that are needed for focal-adhesion maturation (Riveline et al., 2001) (Fig. 4).

How assembly of invadopodia and podosomes is coupled with actin nucleation

Understanding the structure and properties of invadopodia, podosomes and focal adhesions requires detailed knowledge of the localization and dynamics of the signaling networks that regulate actin nucleation and polymerization. For example, and as mentioned above, fast actin turnover might be important for the extension of podosomes or invadopodia, whereas slow self-renewal of actin stress fibers might be a feature of the more static focal adhesions (Fig. 4). In addition, the balance between activation and stabilization of WASP-family proteins, capping of actin barbed ends, and Arp2/3-complex-dependent branching of actin filaments seems to govern the final actin structure and to influence whether adhesive and protrusive structures form (Pollard et al., 2000). In this section, we discuss the localization and role of actin-nucleation and -elongation factors and the physical properties of the membrane in invadopodium and podosome dynamics.

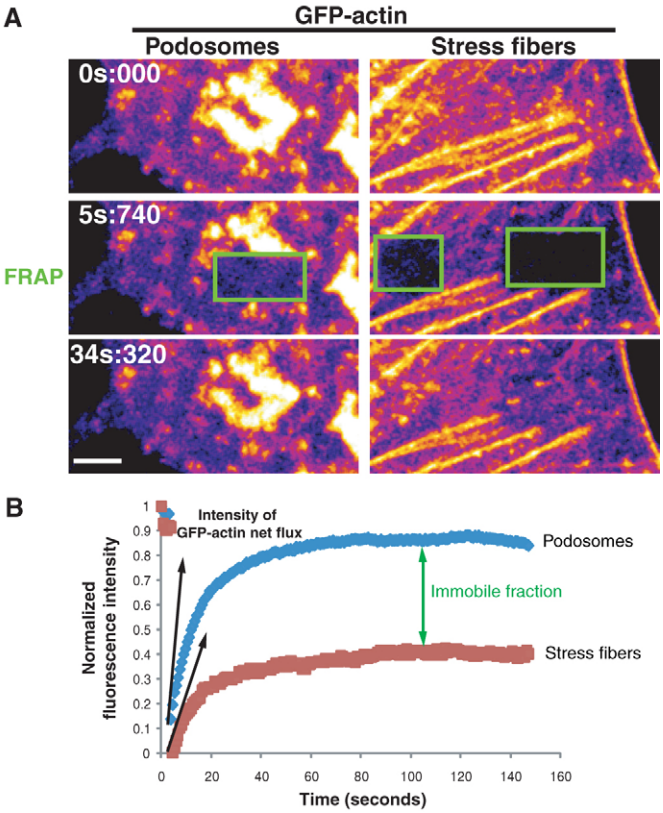

Fig. 4. Actin dynamics in stress fibers and invadopodia rosettes in mouse embryonic fibroblasts transformed with Src. (A) Recovery of GFP-actin after photobleaching (green rectangles) is faster in invadopodial rosettes than in stress fibers. Images were extracted from a time series in which mouse embryonic fibroblasts expressing Src and GFP-actin were shown to form both invadopodial rosettes and stress fibers. Imaging and photobleaching conditions were exactly the same in both conditions. (B) Analysis of normalized fluorescence intensity shows that the net flux of actin, which is determined by the tangent at the origin of the recovery curve (black arrows), is faster in podosomes than in stress fibers. The plateau of the recovery curve does not reach the same level as before photobleaching, allowing the determination of the immobile fraction in each structure. From this analysis, it seems that stress fibers are composed mostly of poorly dynamic F-actin.

Role of actin-nucleation factors in adhesive structures

Actin nucleation relies on the Arp2/3 complex, which is activated at the membrane by proteins of the WASP family (WASP is found in leucocytes, whereas N-WASP is more widely expressed). The activation of WASP and N-WASP (via opening of the closed conformation) is linked to several cooperating factors that facilitate their interaction with the Arp2/3 complex, including WASP-interacting protein (WIP), which shuttles WASP-family proteins to areas of actin assembly (Chabadel et al., 2007; Lafuente et al., 2004; Peterson et al., 2007). Cortactin (see below) promotes actin nucleation synergistically with WASP by simultaneously binding to the Arp2/3 complex and actin filaments, which has the dual effect of activating actin nucleation by the Arp2/3 complex and stabilizing the new filament branches created by the complex (Weaver et al., 2002; Weaver et al., 2001). WIP binds directly to cortactin, enhancing its ability to activate the Arp2/3 complex (Kinley et al., 2003). In addition, the activity of WASP-family proteins is probably controlled by their diffusion rate. N-WASP can interact with free barbed ends of growing actin filaments (Co et al., 2007). Recent data have shown that N-WASP is able to limit actin-filament growth by antagonizing filament-capping proteins at the barbed ends and that it thereby controls the rate of Arp2/3-complex-dependent actin-based motility of intracellular viruses (Weisswange et al., 2009).

Actin nucleators at focal adhesions

In focal adhesions, the relationship between integrins and the actin-nucleation machinery is not well understood, even though actin-polymerization activity has been reconstituted from integrin receptors and associated proteins isolated from non-adherent hematopoietic cells (Butler et al., 2006). The focal-adhesion protein vinculin can associate transiently with the Arp2/3 complex upon cell adhesion to the ECM protein fibronectin (DeMali et al., 2002), but neither Arp2/3 nor WASP has been identified in mature focal adhesions. However, the recruitment of actin into stress fibers is impaired when focal adhesion kinase (FAK), another focal-adhesion protein, is missing or cannot be phosphorylated on Tyr397 (Serrels et al., 2007). Recently, it has been established that the FERM (band 4.1, ezrin, radixin, moesin) domain of FAK interacts with the Arp2/3 complex and WASP, providing a link between integrin engagement, formation of nascent spreading adhesions and actin polymerization (Serrels et al., 2007). Arp2/3 might interact with FAK at early spreading adhesions at the cell periphery and then be released from maturing adhesion structures in nascent lamellipodia (Serrels et al., 2007). Autophosphorylation of FAK at Tyr397 destabilizes the Arp2/3-WASP-FAK complex, inhibits Arp2/3-dependent lamellipodium extension and prevents or delays stress-fiber assembly (Serrels et al., 2007; Wu et al., 2004).

Actin nucleators at podosomes and invadopodia

In contrast to the situation at focal adhesions, the Arp2/3 complex is enriched at the core of podosomes or invadopodia and throughout the length of the actin core (Baldassarre et al., 2006; Linder et al., 2000; Yamaguchi et al., 2005). Knocking down the Arp2/3 complex in osteoclasts impairs podosome formation (Hurst et al., 2004). Arp2/3-complex-dependent and N-WASP-regulated actin polymerization is essential in the early phase of podosome and invadopodium formation. Additionally, it is tempting to speculate that Arp2/3-complex-controlled actin branching has a crucial role

in the fission of podosomes to generate new daughter podosomes (Evans et al., 2003). RNA interference and dominant-negative-mutant expression analyses have revealed that N-WASP, the Arp2/3 complex and their upstream regulators Nck1, Cdc42 and WIP are needed for invadopodium formation (Yamaguchi et al., 2005). Additionally, dendritic cells and macrophages in which WASP expression is decreased or deficient (as observed in knockdown experiments, knockout mice or individuals with Wiskott-Aldrich syndrome) fail to make functional podosomes (Olivier et al., 2006), whereas WASP rescue results in the recovery of normal podosome organization (Burns et al., 2001). Within these cell types, N-WASP is not able to compensate for WASP deficiency. The reason is not fully understood, but a total rescue by N-WASP requires an additional stimulus [stimulation of protein kinase C (PKC) with phorbol myristate acetate (TPA), for instance] to form podosomes (Tatin et al., 2006). As in focal adhesions, it seems that actin polymerization is also regulated by a FAK-family member in podosomes, as revealed by the decrease of the actin net flux in Pyk2-knockout osteoclasts (Gil-Henn et al., 2007).

The role of cortactin

Another way to regulate actin cytoskeleton remodeling is the activation of Arp2/3 by cortactin, which has emerged as a key protein in the coordination of membrane dynamics. Cortactin is one of the few cytoskeletal proteins that is specifically required for the assembly of invadopodia in carcinoma cells (Artym et al., 2006). It does not accumulate into focal adhesions but rather at the edge of lamellipodia, where the polymerized actin-filament meshwork pushes the membrane of migrating cells (Bryce et al., 2005). Many studies have suggested that cortactin overexpression increases tumor aggressiveness, possibly by promoting invasion, metastasis and invadopodium formation (Artym et al., 2006; Clark et al., 2007). Deacetylation of cortactin by HDAC6 alters its association with actin, thus modulating cell motility (Zhang et al., 2003). Cortactin is also a Src-kinase substrate that is frequently overexpressed in cancer. Cortactin phosphorylation by Src enhances actin assembly and increases binding of cortactin to Nck and WIP, and might therefore favor the appearance of new podosomes (Tehrani et al., 2007). Phosphorylation of cortactin seems to be involved in its turnover and stability, possibly through its interaction with WIP, but does not trigger its localization to invadopodia. Finally, supervillin, an F-actin- and myosin-II-binding protein, was recently reported to reorganize the actin cytoskeleton and potentiate invadopodial function by acting as a mediator of cortactin (Crowley et al., 2009).

Our knowledge on the role of actin-nucleation factors at adhesive structures can be summarized as follows: actin-nucleation factors seem to localize stably at the heart of invadopodial and podosome structure, but not at mature focal adhesions (although they are transiently involved in nascent adhesions).

Membrane curvature controls actin polymerization

In contrast to focal adhesions, which are found in flat areas of the cell's ventral face, invadopodia and podosomes are protrusive structures, suggesting that they are formed through a mechanism that couples actin polymerization and membrane deformation. This process is likely to involve proteins that deform membranes, such as BAR- and F-BAR-family proteins. For instance, the F-BAR protein Toca-1 has membrane-binding and membrane-deformation activities, and generates tubular membrane structures of defined diameters (20-50 nm) by bending the membrane towards the cytosol to form positive curvature (Cory and Cullen, 2007; Takano et al., 2008). The N-WASP–WIP complex is known to be activated by Toca-1 and the GTPase Cdc42, and thereby to promote localized actin polymerization at sites of membrane curvature (Ho et al., 2004). Recently, podosome formation in macrophages was shown to require the F-BAR-domain-containing formin-binding protein 17 (FBP17). This protein recruits WASP, WIP and dynamin-2 to the plasma membrane, probably by facilitating membrane deformation (Tsuboi et al., 2009). Similarly, in NIH3T3 cells, ASAP1 [a BAR-domain protein and Arf GTPase-activating protein (ArfGAP)] was found to be a Src substrate that controls invadopodium assembly (Bharti et al., 2007). All these results show that actin polymerization can be stimulated by membrane curvature, which is triggered by spatially appropriate interactions of F-BAR proteins and the N-WASP–WIP complex (Takano et al., 2008). ASAP3, which is closely related to ASAP1, is associated with focal adhesions and circular dorsal ruffles but does not localize to invadopodia or podosomes (Ha et al., 2008). Reduction of ASAP3 expression results in fewer actin stress fibers, reduced levels of phosphomyosin, and slower cell migration and invasion. Conversely, downregulation of ASAP1 has no effect on migration or invasion. Given these new findings, it is worth noting that membrane invagination has been observed at the center of podosome actin cores (Kaverina et al., 2003; Ochoa et al., 2000).

Of equal biological importance to the generation of positive membrane curvature is the topologically opposite process – the generation of negative curvature, in which the membrane is deformed away from the cytosolic environment. The IRSp53- and MIM (missing in metastasis)-homology domain (IMD) constitutes a structural module that generates negative membrane curvature, giving rise to tubules of 80 nm diameter (Mattila et al., 2007). Whereas BAR domains stabilize membrane tubules by coating the outside of the tubules, the MIM domain binds the inside of the forming tube (Suetsugu et al., 2006). IRSp53 generates protrusions from the plasma membrane that look like actin-containing filopodia (Yamagishi et al., 2004). IRSp53 contains various Rac-binding domains, suggesting that it might also be involved in lamellipodial extension (Suetsugu et al., 2006). Indeed, IRSp53 is required, in association with the WAVE2-Abi1 complex, for some actin-mediated processes such as lamellipodium formation, but not for the formation of filopodia and podosomes (Abou-Kheir et al., 2008). However, IRSp53 also contains a Cdc42-binding motif and seems to be required for the Cdc42-induced formation of filopodia (Lim et al., 2008). This latter activity seems to depend on the stage of its association with another cytoskeletal modulator, epidermal growth factor receptor kinase substrate 8 (Eps8), which is known to be an actin-capping and -bundling protein (Disanza et al., 2006). Eps8 is not found in focal adhesions but is localized in podosomes (Goicoechea et al., 2006). All these results indicate that F-BAR proteins might have a major role in podosome formation, as might IRSp53 in lamellipodia or filopodia. An attractive hypothesis is that, depending on its partner, IRSp53 specifically induces distinct types of membrane protrusions.

Actin-elongation factors in adhesive structures

In addition to actin-nucleation factors and inducers of membrane curvature, actin-elongation factors also seem to have a role in invadopodium formation. Both Ena/VASP-family proteins and formins – which act as elongation factors – promote actin polymerization at the barbed ends of actin filaments. The emerging idea is that different formins and Ena/VASP proteins support highly

variable rates of actin-filament elongation, thus optimizing the assembly and architecture of specific actin structures. Ena/VASP proteins localize at focal adhesions, the leading edge of lamellipodia and the tips of filopodia (Gertler et al., 1996). Ena/VASP activity regulates the geometry of assembling actin-filament networks by capturing filament barbed ends and antagonizing capping proteins; this anti-capping activity involves direct binding to profilin-actin complexes and to globular (G)- and F-actin (Barzik et al., 2005; Bear et al., 2000; Ferron et al., 2007; Pasic et al., 2008). Ena/VASP proteins also bundle actin filaments (Bachmann et al., 1999; Barzik et al., 2005), and are thought to cluster filament barbed ends during filopodium formation and elongation (Applewhite et al., 2007; Svitkina et al., 2003).

Mena, a member of the Ena/VASP family, is involved in cell motility, and its upregulation in several human cancers is correlated with increased invasiveness (Di Modugno et al., 2004; Krause et al., 2003; Wang et al., 2004; Wang et al., 2007). It has been recently shown that Mena is differentially spliced in vivo in invasive tumor cells as compared with non-motile resident tumor cells. Both Mena and its invasive isoform ($Mena^{INV}$) promote actin polymerization in a cofilin-dependent manner, but the $Mena^{INV}$ isoform seems to favor invasiveness by promoting invadopodium stabilization and enhancing the matrix-degradation activity of invadopodia (Philippar et al., 2008).

Src is a key mediator of actin dynamics in podosomes and invadopodia

The non-receptor tyrosine kinase Src, which initiates podosome formation and regulates podosome structure, is a member of a family of nine closely related tyrosine kinases that is defined by a common domain structure, including a myristoylated N-terminal domain that targets Src to membranes, two Src-homology-protein-binding domains (SH2 and SH3) and the tyrosine-kinase catalytic domain. Activation of Src occurs either when Tyr527 is dephosphorylated, allowing the 'opening' of the molecule, or when the intramolecular interactions of the SH2 or SH3 domains are disrupted by intermolecular interactions with other Src-binding partners. Activation of Src leads to the autophosphorylation of Tyr416 in the activation loop of the kinase domain, which is essential for the full tyrosine-kinase activity of Src (Roskoski, 2004).

As mentioned previously, expression of v-Src, the oncogenic and constitutively active form of Src, induces a rearrangement of the actin cytoskeleton that is characterized by a switch from stress fibers to invadopodia in BHK cells (Tarone et al., 1985). Even though Src is ubiquitous, the specific link between Src and podosomes has been confirmed by the targeted disruption of the *Src* gene in mice, which leads to osteopetrosis as a result of nonfunctional osteoclasts that are unable to form a sealing zone from podosomes (Soriano et al., 1991). Indeed, bone matrix resorption by osteoclasts is known to depend on a specific organization of their cytoskeleton into a peripheral belt of podosomes. This phenotype is mostly Src-dependent because the disruption of any other Src-kinase family member failed to reproduce such a phenotype (Horne et al., 1992; Lowell et al., 1996; Sanjay et al., 2001; Soriano et al., 1991).

Src tyrosine-kinase activation is both necessary and sufficient for podosome and invadopodium formation. Because actin-core polymerization is a prerequisite for the assembly of podosomes and invadopodia, Src has the capacity to regulate (through phosphorylation) and/or recruit (through interactions with its SH2 and SH3 domains) specific substrates that regulate actin polymerization and architecture. Re-expression of Src mutants in *Src*-null osteoclasts has shown that its tyrosine-kinase activity is essential for podosome regulation and is not compensated for by its adaptor function; however, Src needs to be properly localized by either its SH2 or SH3 domains, as its membrane localization is not sufficient (Destaing et al., 2008). In osteoclasts, Src regulates rearrangements in the actin cytoskeleton by stimulating Rac1 GTPase via a protein complex that also includes the Syk kinase, αvβ3 integrin and the Rac guanine-nucleotide exchange factor Vav3 (Zou et al., 2007). Src also downregulates the activity of the GTPase ARF6, via the GTPase-activating protein GIT2, to maintain sealing zones and osteoclast polarity during bone degradation (Heckel et al., 2009).

Among the identified substrates of Src is Tks5 (Fish), a scaffold protein that binds to members of the ADAM family of membrane-spanning proteases, to WASP-family proteins and to cortactin (Abram et al., 2003; Seals et al., 2005; Seals and Courtneidge, 2003). In the initial stages of podosome formation, phosphatidylinositol (3,4)-bisphosphate [PtdIns(3,4)P_2] plays an important role in anchoring Tks5 to the plasma membrane (Oikawa et al., 2008). Tks5, which is essential for podosome formation, forms a complex with Grb2 at adhesion sites in a Src-dependent manner. Furthermore, N-WASP binds to all SH3 domains of Tks5, which facilitates the formation of podosome rings. Podosome precursors are formed around focal-adhesion-related adhesions, and this might be in response to a change in the phosphorylation status of proteins (such as Tks5) and in the composition of phosphoinositides on the plasma membrane (Oikawa et al., 2008). Thus, the N-WASP–Arp2/3-complex signal might accumulate on the platform of the Tks5-Grb2 complex at focal adhesions, which is stabilized by PtdIns(3,4)P_2. These data provide the molecular basis for the transformation of focal adhesions into podosomes and/or invadopodia.

AFAP-110 is another Src-associated protein with a role in podosome formation. In response to activation of PKCα, Src is activated in an AFAP-110-dependent fashion, and this signaling pathway is responsible for PKCα-induced podosome formation; by contrast, AFAP-110 colocalizes with actin stress fibers in quiescent cells (Flynn et al., 1993; Gatesman et al., 2004). AFAP-110 contributes to the aggressiveness and growth of tumors (Zhang et al., 2007). This protein might have an important role in the regulation of podosome stability and lifespan as an actin-filament cross-linking protein and as an adaptor protein that relays PKCα signals to activate Src (Dorfleutner et al., 2008). It is also possible that AFAP-110 controls podosome half-life through its ability to regulate cell contractility and cross-link stress fibers (Burgstaller and Gimona, 2004).

Activation of the endoprotease calpain-2 by extracellular-signal-regulated kinases 1 and 2 (Erk1/2) (Glading et al., 2004) might result from the Src-dependent phosphorylation of paxillin (Badowski et al., 2008; Petit et al., 2000). Previous studies have established a role for calpain-2 in the turnover of the focal adhesions of migrating cells; calpain-2 is thought to mediate talin proteolysis in focal adhesions and thereby to trigger disassembly (Franco et al., 2004). Another study revealed that calpain-mediated cleavage of the integrin chain β3 at Tyr759 switches the functional outcome of integrin signaling from cell spreading to retraction (Flevaris et al., 2007). Calpain-2 is also thought to increase invadopodium dynamics by acting both downstream and upstream of Src, through the proteolysis of specific Src effectors such as cortactin (to promote invadopodium disassembly) and through proteolysis and activation of the Src activator PTP1B (to promote invadopodium assembly)

(Cortesio et al., 2008). The protein tyrosine phosphatase PTP1B can promote Src activation through the direct dephosphorylation of the inhibitory phosphotyrosine 529 (Bjorge et al., 2000; Cortesio et al., 2008; Liang et al., 2005).

In conclusion, Src is a major player in the assembly and dynamics of invadopodia and podosomes, acting at multiple levels from actin nucleation to the activity of integrin-associated complexes. High Src activity seems to be essential for its role at invadopodia, but leads to diminished focal-adhesion assembly.

Invadopodia, podosomes and focal adhesions are mechanosensitive structures with distinct characteristics

Adhesive structures and environment sensing

A cell can sense and respond to a wide range of external chemical and physical signals. It can integrate this information through its adhesive structures and, as a consequence, can change its morphology, dynamics and behavior. In addition, cells can adapt their adhesive structures to external constraints such as the rigidity, density or topography of the substrates (Fig. 5A,B). Several physical parameters might contribute to the strength of adhesion between a cell and its environment, including ligand surface density, number of adhesion receptors, affinity of integrins for their respective ligands, strength of receptor linkages and organization of the receptors at the cell surface (Gallant et al., 2005; Gupton and Waterman-Storer, 2006; Huttenlocher et al., 1996; Palecek et al., 1998). The more rigid the matrix, the higher the intracellular-tension and cell-traction forces (Paszek et al., 2005; Wang et al., 2000). In terms of cell sensitivity to extracellular stiffness, experiments and theory have shown the role of two important parameters: adhesion-site dynamics and cytoskeleton tension (Fereol et al., 2009). Augmented surface density of matrix ligands induces faster focal-adhesion assembly. It has been recently shown that matrix-density sensing depends on the focal-adhesion regulator ICAP-1 (Millon-Fremillon et al., 2008). Matrix topography is also important, because the distance between individual integrin molecules modulates adhesion: the distance between individual integrins must be no more than 55 nm to reinforce adhesion (Arnold et al., 2004; Selhuber-Unkel et al., 2008) and allow recruitment of vinculin to adhesions (Cavalcanti-Adam et al., 2006).

An emerging concept is that the functions of some proteins can be up- or downregulated by stretching; thus, the proteins act as mechanosensors to convert mechanical cues into chemical signals, in a process called mechanotransduction. This property has been demonstrated for extracellular proteins such as fibronectin and intracellular proteins such as zyxin, talin and p130Cas [a Src-family

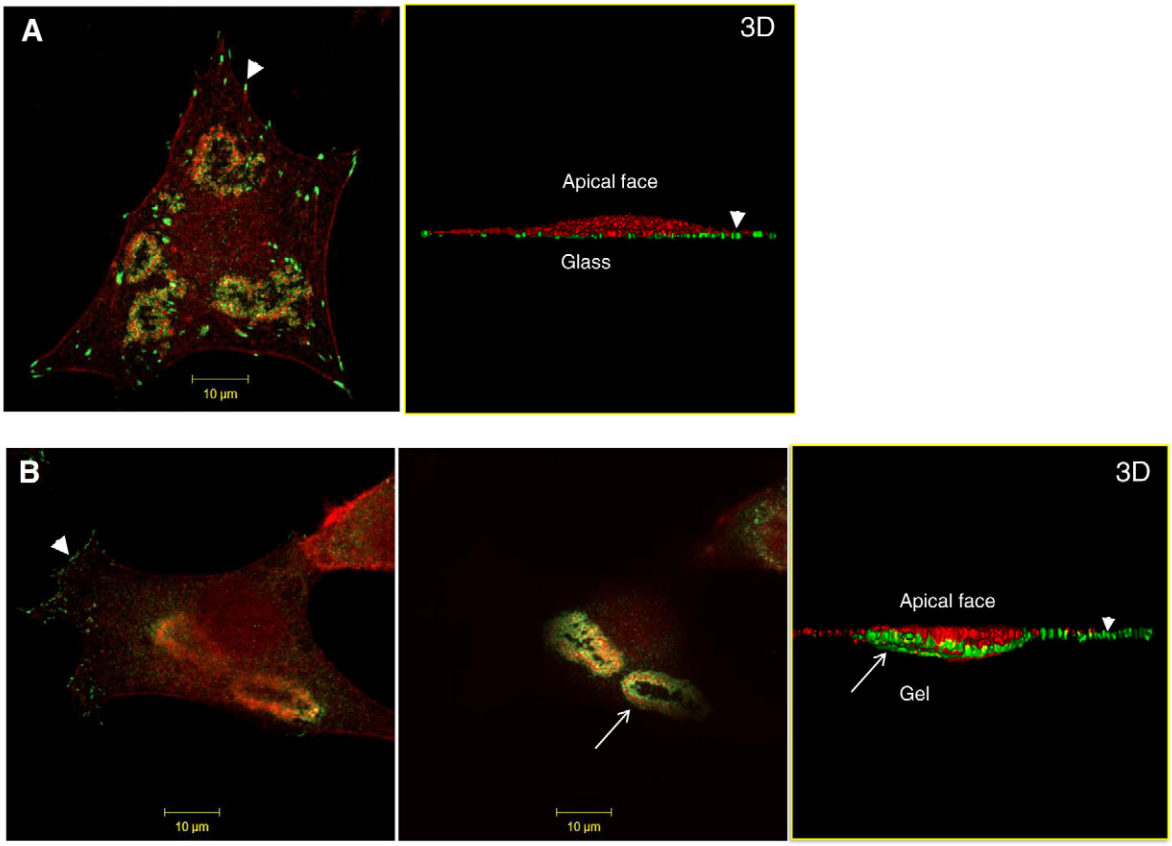

Fig. 5. Visualization of actin structure and paxillin in mRFP-actin-transfected BHK-RSV cells. (A,B) Rigid (A) and flexible (B) substrates were coated with vitronectin. Staining for paxillin (green) was carried out after fixation in 4% paraformaldehyde with anti-paxillin antibodies. 3D reconstruction (right-most images) was carried out using EDIT3D software as in Fig. 3. (A) On the left is an image from a single confocal plane of a BHK-RSV cell adherent on glass (rigid) substrate. The right image shows a 3D reconstruction. Arrowheads indicate focal adhesions. (B) Left and middle panels show images from two confocal planes of a BHK-RSV cell adherent on hydrogel made of polyacrylamide (flexible) substrate. Confocal planes were from the top of the gel (left) and inside the gel (middle). Note that focal adhesions (arrowheads) are smaller in size on the flexible substrate (B) than on the rigid one (A). (B) The 3D reconstruction (right) shows that collective organizations of podosomes or invadopodia (arrow) seem to 'push' the gel, hauling the whole cell body.

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kinase substrate that is involved in various cellular events such as migration, survival, transformation and invasion (Defilippi et al., 2006)]. For instance, direct application of a piconewton force stimulates the mechanical extension of p130Cas, unmasking a Src substrate domain and thereby allowing its phosphorylation by Src-family kinases (Sawada et al., 2006). Force-induced conformational changes in talin lead to the exposure of a binding site for vinculin (del Rio et al., 2009), and force can also modify extracellular fibronectin to alter integrin adhesion (Hirata et al., 2008; Hoffman et al., 2006; Yoshigi et al., 2005). The forces generated by cellular adhesion not only stretch but also partially unfold fibrillar fibronectin (Baneyx and Vogel, 1999; Smith et al., 2007). The stretching of matrix fibers not only increases their rigidity but is also thought to make new binding sites available. Below, we discuss the role of mechanosensing in focal-adhesion assembly and maturation, and describe new data that indicate a mechanosensing role for podosomes and invadopodia.

Interplay between actin polymerization and myosin in focal-adhesion assembly and maturation

Mechanical forces are required for the assembly and maturation of focal adhesions. Nascent adhesions form at the base of the lamellipodium in a myosin-II-independent manner, and their assembly rate is proportional to the rate of lamellipodial protrusion. Nascent adhesions seem to be different from other adhesive sites known as focal complexes (the precursors of focal adhesions), which are two times larger, induced by active Rac1, dependent on myosin II and appear mainly at the lamellipodium-lamellum interface (Choi et al., 2008; Nobes and Hall, 1995). At the rear of the lamellipodium, nascent adhesions either disassemble or mature through a sequential mechanism that is coupled to myosin-II-induced tension. At focal adhesions, there is active polymerization of actin filaments that can be crosslinked by α-actinin. Myosin II incorporates into the α-actinin-crosslinked actin-filament bundles and displaces α-actinin. This allows the contraction of actin bundles, generating tension. The overall process is extremely dynamic (Cai and Sheetz, 2009; Vogel and Sheetz, 2009).

Stress fibers are anchored to focal adhesions, which grow in response to contractile force. Traction forces generated by stress fibers are in the order of several hundred nanonewtons (nN). Analyses of actin dynamics in stress fibers indicate that preformed actin filaments are added to the adhesion sites and enable the rapid turnover of actin in stress fibers (Hotulainen and Lappalainen, 2006). Maturation of focal adhesions from nascent adhesions can occur along an α-actinin–actin template that elongates centripetally from nascent adhesions (Choi et al., 2008). Maturation and growth of focal adhesions involve force reinforcement that is dependent on talin (Zhang et al., 2008) (Fig. 5A,B). These internal tensions can be mimicked by application of external forces (Riveline et al., 2001). Maturation into focal adhesions is mediated by Rho kinase and mDia1, both of which are effectors of the small GTPase RhoA (Kimura et al., 1996; Watanabe et al., 1999); Rho kinase stimulates myosin-II-dependent contractility by inactivating myosin-light-chain phosphatase (Katoh et al., 2001), whereas mDia1 is involved in actin nucleation and in the elongation of parallel arrays of actin filaments (Burridge and Chrzanowska-Wodnicka, 1996; Rottner et al., 1999). Myosin II exerts a force of 5.2 $nN/\mu m^2$ on focal adhesions (Schwarz et al., 2002). Maturation of focal adhesions is a slow process that can take up to 60 minutes (Zamir et al., 1999) and corresponds to a seven-fold force reinforcement (Gallant et al., 2005).

Mechanics of podosomes and invadopodia

Whereas mechanosensing is well documented for focal adhesions, little has been known about whether this occurs in invadopodia and podosomes. However, recent reports provide compelling evidence that invadopodia and podosomes are also major sites through which cells sense mechanical forces (Collin et al., 2008). It seems that, in contrast to focal adhesions, intracellular tensions are not required for podosome assembly. However, podosome lifespan, the mean distance between podosomes, their collective organization into a rosette, and expansion of the rosette all depend on the flexibility of the substrate (Collin et al., 2006). These findings suggest that intracellular constraints have a role in the collective dynamics of these podosome rings. Recent data have demonstrated that a rosette of invadopodia in Src-transformed BHK cells can exert traction forces with a magnitude of 200 Pa, which is comparable to that generated underneath focal adhesions (Collin et al., 2008). Therefore, rosettes can be thought of as mechanosensory structures that can sense and transmit mechanical forces.

A striking correlation has also been observed between the increase in the matrix-degrading activity of invadopodia in breast carcinoma cells and the increase in rigidity of the ECM (Alexander et al., 2008). This proteolytic activity is linked to the phosphorylated form of mechanosensing proteins such as p130Cas and FAK (Alexander et al., 2008). It has also been shown that, in Src-transformed BHK cells, rosette formation is required for efficient ECM degradation and transmigration through a HeLa-cell monolayer (Badowski et al., 2008; Saltel et al., 2008; Saltel et al., 2006). A tempting conclusion is that the birth of podosomes or invadopodia does not require tensile forces, whereas collective organization of invadopodia or podosomes into rosettes is controlled by applied external-force anisotropy, which is not yet well characterized.

Indeed, in contrast to what is observed with focal adhesions, podosomes or invadopodia seem to be promoted by a decrease in local cellular contractility (Burgstaller and Gimona, 2004). In line with this idea, it is noteworthy that assembly of individual invadopodia often occurs in the center of the ventral surface of cancer cells, where traction forces are lower than at the cell periphery (Cai et al., 2006; Cai and Sheetz, 2009; Dembo and Wang, 1999; Dubin-Thaler et al., 2008; Pelham and Wang, 1999; Tan et al., 2003). Indeed, podosome assembly is correlated with the local dispersal of contractile proteins, including myosin, tropomyosin and calponin, and the recruitment of p190RhoGAP to podosome sites (Lener et al., 2006). Also, the adhesion-associated ion channel TRPM7 plays a role in relaxing cellular contractility in response to mechanical forces, through phosphorylation of myosin-II heavy chain (Clark et al., 2006). Activation of TRPM7 induces the transformation of focal adhesions into podosomes through a kinase-dependent mechanism, an effect that can be mimicked by pharmacological inhibition of myosin II. Inhibition of RhoA or Cdc42 abolishes invadopodium formation (Sakurai-Yageta et al., 2008; Yamaguchi et al., 2005); however, this effect might not be because of a diminution of contractility, but rather because of the requirement for other Rho effectors such as DRF/mDia1 (human/mouse) formins for the formation and activity of invadopodia in two- and three-dimensional systems (Lizarraga et al., 2009).

Recent data show that podosome rosettes develop torsional tractions that can deform the underlying matrix underneath podosome rings (Collin et al., 2008). Short-term treatment with the myosin inhibitors ML7 or blebbistatin, or with the ROCK inhibitor Y27632 (which all decrease contractility) leads to transient

dissipation of podosome rosettes, but the supramolecular structures are still present in cells subjected to these inhibitors for longer treatment periods (M.R.B. and C.A.-R., unpublished). Some myosin II has been observed at the periphery of the core domain of podosomes in Src-transformed cells, and also in association with the sealing zone on osteoclasts (Saltel et al., 2008; Tanaka et al., 1993), but myosin II is noticeably absent in invadopodia or podosomes so contractility does not seem to occur within these individual structures.

The dynamic assembly of invadopodia or podosomes is still not well understood and the molecular motors associated with this process remain to be identified. All published observations indicate that actin polymerization might be sufficient to promote podosomal or invadopodial protrusion, which in turn would trigger tangential forces through actin radial arrays around podosomal or invadopodial actin cores. Indeed, N-WASP activation has been visualized at the base of invadopodia, suggesting that Arp2/3-complex-mediated actin nucleation is confined to this area (Lorenz et al., 2004). Subsequently, DRF/mDia1 might act on the branched array induced by N-WASP, the Arp2/3 complex and cortactin, and elongate actin filaments to trigger invadopodial protrusion. This process, combined with the tangential forces developed through actin radial arrays around invadopodial actin cores, should permit podosome rosettes to protrude from the cell if the surrounding environment is sufficiently flexible (Fig. 5). Undoubtedly, the spatiotemporal measurements of force-field displacements of podosome rosettes will resolve this point, and will add a mechanical element to our understanding of the fast invasion of cells expressing podosomes or invadopodia. Future research will also include investigating how the physical ECM environment affects cellular invasiveness, because cancer-associated breast tissue is much stiffer than normal tissue (Boyd et al., 2005; Samani et al., 2007). Some reports already suggest a role for tissue rigidity in promoting both the formation and invasiveness of tumors, possibly by increasing invadopodial activity (Alexander et al., 2008; Parekh and Weaver, 2009).

Conclusions and perspectives

Our increasing knowledge of invadopodial involvement in tumor-cell invasion and metastasis makes these structures very attractive targets for cancer therapy (Stylli et al., 2008). The mode of invasion that is induced by invadopodial structures requires fast assembly of adhesions, ECM proteolysis and a dynamic actin cytoskeleton (to allow fast changes in supramolecular structures and the development of traction forces). Tissue invasion is most efficient when these cellular processes are combined. This Commentary emphasizes the role of many actin-cytoskeleton-associated proteins as major players in the Src-mediated organization of adhesive interactions such as podosomes and invadopodia. The combination of actin nucleators and elongation factors, each with distinct mechanisms and modes of regulation, allows the versatility that is required to construct actin networks with specialized architectures and functions. Moreover, there is growing evidence that actin-binding proteins have multiple roles in tumorigenic and metastatic processes of various human tumors. Indeed, the balance between actin regulators might determine the type of adhesive structure that is formed and eventually account for the differences in shape and dynamics of invadopodia and podosomes.

Force and membrane tension generated by intracellular motors (such as myosin) and regulated by substrate viscoelasticity might affect actin polymerization, and in turn the formation of invadopodia or podosomes and their collective organization into rosettes. The urgency of defining these mechanisms is particularly obvious as growing evidence indicates that alterations in cellular mechanoresponses are involved in many diseases. Theoretical physical models also need to be developed to address the physical mechanisms that underlie the formation of invadopodia and podosomes, and the maintenance of their collective organization.

We apologize to those authors whose important work we were unable to cite owing to space restrictions. We thank the members of the laboratory for all their input and helpful discussions. Artwork was done by Olivier Ali. We thank Laurent Blanchoin for critical reading of the manuscript. Work in the Albiges-Rizo laboratory is supported by grants from CNRS, INSERM, the Ligue Nationale Contre le Cancer, the Association de la Recherche pour le Cancer and the Région Rhône-Alpes.

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Commentaries and Cell Science at a Glance

JCS Commentaries highlight and critically discuss recent and exciting findings that will interest those who work in cell biology, molecular biology, genetics and related disciplines, whereas Cell Science at a Glance poster articles are short primers that act as an introduction to an area of cell biology, and include a large poster and accompanying text.

Both of these article types, designed to appeal to specialists and nonspecialists alike, are commissioned from leading figures in the field and are subject to rigorous peer-review and in-house editorial appraisal. Each issue of the journal usually contains at least one of each article type. JCS thus provides readers with more than 50 topical pieces each year, which cover the complete spectrum of cell science. The following are just some of the areas that will be covered in JCS over the coming months:

Cell Science at a Glance

Collective cell migration at a glance *Peter Friedl*
Wound repair at a glance *Paul Martin*
Dynamins at a glance *Jenny Hinshaw*
Desmosomes at a glance *Kathleen Green*
mTOR signalling at a glance *David Sabatini*
The primary cilium at a glance *Peter Satir*

Commentaries

Collective cell migration in development *Cornelis Weijer*
ABL-family kinases *Tony Koleske*
Tail-anchored protein biogenesis – the beginning for the end? *Stephen High*
Cytoskeletal dynamics in axon guidance *Phillip Gordon-Weeks*
Apical trafficking in epithelial cells *Enrique Rodriguez-Boulan*
Matrix elasticity, cytoskeletal forces and physics of the nucleus *Dennis Discher*

Although we discourage the submission of unsolicited Commentaries and Cell Science at a Glance poster articles to the journal, ideas for future articles – in the form of a short proposal and some key references – are welcome and should be sent by email to the Editorial Office (jcs@biologists.com).

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