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Broadening the Spectrum of Actin-Based Protrusive Activity Mediated by Arp2/3 Complex-Facilitated Polymerization: Motility of Cytoplasmic Ridges and Tubular Projections

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Abstract

Arp2/3 complex-facilitated actin polymerization plays an essential role in a variety of cellular functions including motility, adherence, endocytosis and trafficking. In the present study we employ the sea urchin coelomocyte experimental model system to test the hypotheses that Arp2/3 complex-nucleated actin assembly mediates the motility of two unusual cellular protrusions; the cytoplasmic ridges present during coelomocyte spreading, and inducible, tubular-shaped, and neurite-like projections. Our investigations couple pharmacological manipulation employing inhibitors of actin polymerization and the Arp2/3 complex with a wide array of imaging methods including digitally enhanced phase contrast, DIC and polarization light microscopy of live cells; conventional, confocal and super-resolution light microscopy of fluorescently labeled cells; and scanning and transmission electron microscopy. Taken together, the results of this study indicate that Arp2/3 complex-facilitated actin polymerization underlies the motility of coelomocyte cytoplasmic ridges and tubular projections, that these processes are related to each other, and that they have been preliminarily identified in other cell types. The results also highlight the broad spectrum of actin-based protrusive activities dependent on the Arp2/3 complex and provide additional insights into the pervasive nature of this ubiquitous actin nucleator. Furthermore we provide the first evidence of a possible mechanistic difference between the impacts of the small molecule drugs BDM and CK666 on the Arp2/3 complex.

INTRODUCTION

The Arp2/3 complex of seven proteins is a highly conserved and well-characterized nucleator of actin polymerization that is activated by the WASP family of nucleation

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promoting factor proteins. The complex generates the dendritic array of branched actin filaments that predominates in the lamellipodium (LP) – the broad, thin and fan-shaped region located at the cell periphery (reviewed by Pollard, 2007; Firat-Karalar and Welch, 2011; Ridley, 2011; Rotty et al., 2013). The highly dynamic LP undergoes rapid retrograde/centripetal flow in which the actin network and overlying membrane flow back towards the cell center, in structures that often take the appearance of ruffles. The branched network of actin filaments present in the LP feeds into a sparser array of longer, often unbranched and less dynamic actin filaments and bundles termed the lamellum (LM). Whereas retrograde flow in the LP is driven largely by actin polymerization, flow in the LM appears to be dependent on actomyosin contraction (Ponti et al., 2004; Gupton et al., 2005). Although LP actin polymerization appears dominated by the Arp2/3 complex, studies suggest that the LP is also dependent on the activity of mDia/formin class of actin nucleators that mediate the formation of elongate actin filaments (Gupton et al., 2007; Yang et al., 2007; Pollard, 2007; Koestler et al., 2013).

Numerous studies have shown that the Arp2/3 complex mediates actin-based processes beyond the LP dendritic actin network. These include its involvement in intracellular vesicle trafficking, actin membrane patch formation and associated endocytosis, generation of adherens junctions between cells, assembling the actin comet tails that provide motility for intracellular bacteria and viruses, and the formation of neurites and filopodia (reviewed in Pollard and Cooper, 2009; Rotty et al., 2013; Svitkina, 2013). The elongate actin filaments present in filopodial bundles are thought to be lengthened via polymerization from barbed-end associated mDia/formin actin nucleator proteins, however filopodia have been shown to retain Arp2/3 complex activity (Johnston et al., 2008), and filopodial formation can derive from Arp2/3 complex-branched actin filament networks (Svitkina et al., 2003; Korobova and Svitkina, 2008; Spillane et al., 2011) in a process termed convergent elongation (reviewed in Yang and Svitkina, 2011). Note that Ena/VASP proteins can play a role in filopodia formation/elongation in place of and/or complementary with formin, particularly in neurons (Bear and Gertler, 2009). Neurites are specialized extensions of neurons consisting of tubular-shaped, actin-rich extensions terminated by growth cones, the peripheries of which consists of an LP-like region containing numerous filopodia. Earlier work had suggested that the Arp2/3 complex was a negative regulator of growth cone translocation (Strasser et al., 2004), however more recent work has shown that the Arp2/3 complex is essential for neurite and growth cone formation and motility (Kordova and Svitkina, 2008). These examples make it clear that Arp2/3 complex-nucleated actin assembly has a wide range of potential applications in the cell.

We have used the sea urchin coelomocyte as a model system for investigating the multiple roles of Arp2/3 complex-facilitated actin polymerization. These cells represent an exceptional experimental model given their extraordinarily broad and Arp2/3 complex-rich LP region, exaggerated actin-based centripetal flow across the entire diameter of the cell, and extreme flatness and optical clarity allowing for direct observation of the actin cytoskeleton in unmanipulated cells. Our studies to date have indicated that Arp2/3 complex activity is crucial for structuring the LP actin network (Henson et al., 1999; 2009), for the generation of the pushing force component of retrograde flow (Henson et al., 1999; 2003), and for the closure of cytoplasmic wounds (Henson et al., 2002). In the present study we

utilized coelomocytes to test the hypothesis that Arp2/3 complex-nucleated actin polymerization underlies the generation of the prominent cytoplasmic ridges occurring during coelomocyte spreading, as well as the production of inducible, tubular-shaped, and neurite-like projections. Our investigations coupled pharmacological manipulation with a wide array of imaging methods including digitally enhanced phase contrast, DIC and polarization light microscopy of live cells; conventional, confocal and super-resolution light microscopy of fluorescently labeled cells; and scanning and transmission electron microscopy. Taken together, the results of this study provide compelling evidence that Arp2/3 complex-facilitated actin polymerization underlies the motility of coelomocyte cytoplasmic ridges and tubular projections. They also highlight the broad spectrum of processes dependent on the activity of Arp2/3 complex-nucleated actin polymerization and provide additional insights into the flexible functions of this ubiquitous actin nucleator. Furthermore we provide the first evidence of a mechanistic difference between the Arp2/3 complex inhibitory effect of the drugs BDM and CK666.

RESULTS

Coelomocytes Display Rocketing Cytoplasmic Ridges That Contain Actin and the Arp2/3 Complex

Our typical experiments with coelomocytes require that the cells be settled onto glass coverslips for a significant period (typically >30 min) which results in well spread and highly flattened cells (see Henson et al., 1999; 2003; 2009 for examples). However, in the present study cells were examined during the process of settling and spreading using digitally enhanced phase contrast microscopy and the most striking characteristic was the presence of highly motile, phase dense linear features representative of cytoplasmic ridges (Fig. 1A–E and Supp. Movie 1; 2). These cytoplasmic ridges displayed rocketing-like motility in a range of directions, including centripetal, tangential and centrifugal, and they often underwent bending, branching and anastomosis (Fig. 1E). In some cases the linear features disappeared as the ridges folded over and were subsumed into the LP membrane of the cell that was undergoing a constant process of centripetal/retrograde flow (* in Fig. 1E, F, and Supp. Movie 1). As the cells spread, the phase dense cytoplasmic ridges often became less abundant and more restricted to the LP region (Fig. 1A–D) although they were still often present in a subset of well spread cells (Supp. Movie 2). DIC imaging demonstrated that the peripheral phase dense regions that produced the ridges gave the appearance of a raised area on the dorsal membrane of the cell (Fig. 1F). In addition, the lack of the phase contrast halo artifact near the nucleus allowed for observation of cytoplasmic ridge processes in the perinuclear region (Fig. 1G). Finally, axis-independent polarization microscopy revealed that the cytoplasmic ridge structures were positively birefringent during their period of existence on the dorsal surface of the cell and then lost this attribute after they folded over and collapsed back into the cell surface (Fig. 1H). Note that in spread cells the ridges were largely restricted to the LP region and often originated in the border between the coelomocyte LP and LM (Supp. Movie 2).

The rocketing motility of these ridges, which gave the appearance that they were surfing on the dorsal surface of the cell, was reminiscent of *Listeria* bacteria intracellular motility and

this prodded us to hypothesize that these structures were being driven by Arp2/3 complex-facilitated actin polymerization. Consistent with this hypothesis, immunofluorescent localization clearly showed that actin and Arp3 were concentrated in these processes, with the Arp3 labeling often enriched at the tips/edges (Fig. 2). The colocalization of the actin and Arp3 signals was present in peripheral (Fig 2A–F) and perinuclear cytoplasmic ridges (Fig. 2A–C), and the presence of Arp3 staining in the ridges was also seen in cells only single-labeled for Arp3 (Supp. Fig. 1A) indicating that the Arp3 signal was not the result of a possible actin staining bleed-through artifact. Phase contrast images of fluorescently labeled cells (Fig. 2G) showed that the phase dense ridges seen in the digital videos (Fig. 1) corresponded to the actin and Arp3-labeled entities. The three dimensional nature of the ridges was visible in 3D tilts of confocal Z stack through-focus projections of actin and Arp3 labeling (Supp. Fig. 1B, C) and in 3D-SIM super-resolution images of coelomocytes stained for filamentous actin using phalloidin (Fig. 2H). The 3D-SIM maximum intensity projection images highlighted the dendritic network organization of the actin cytoskeleton present in the ridges and the LP of the cell. In additional immunofluorescence staining experiments, the actin nucleation promoting factor and Arp2/3 complex activating protein WASP appeared to localize to the ridges (Supp. Fig. 1D–F), whereas microtubules did not (Supp. Fig. 1G–I).

SEM images of spreading cells demonstrated the existence of ridges on the top surface of the cells (Fig. 3A). The actin filament ultrastructural organization within these ridges was visualized using TEM imaging of critical-point-dried and rotary-shadowed platinum replicas of detergent extracted coelomocyte cytoskeletons (Figs 3B–G). The TEM images indicated that the ridges consisted of a very dense meshwork of actin filaments consistent with Arp2/3 complex-dependent dendritic arrays. Note that the density and interconnected nature of the actin filaments is most extreme at the tips of the ridges (Figs. 3D–G). This dense assemblage of interwoven actin filaments is present in nascent ridge structures (Fig. 3E) that correspond in live cells to the dark spots seen in phase contrast (Fig. 1E; Supp. Movies 1; 2) and the small swellings seen in DIC microscopy (Fig. 1F). The complexity of the actin structural organization within the ridges is no doubt partially due to the extensive superposition of actin filaments in these areas. In places where the ridges were folding over and orienting themselves parallel to the surface of the cell's membrane just prior to fusing together, the overall actin filament organization appeared similar to that of filaments at the cell edge (Fig. 3B, C).

Inhibitors of Actin Polymerization and the Arp2/3 Complex Interfere with Cytoplasmic Ridge Motility

In order to confirm that the motility of the cytoplasmic ridges was actin-based, we treated spreading cells with the actin polymerization inhibitor cytochalasin D (CytoD). In the initial stages of CytoD treatment the extension of the cytoplasmic ridges was arrested, and with continued presence of the drug the ridges curled back centripetally due to the overall inward retraction of the entire cytoskeleton (Fig. 4; Supp. Movie 3). The retraction of the cytoskeleton is typical of CytoD treatment of coelomocytes and represents the result of release of the LP actin meshwork from the cell edge combined with myosin-based contraction, as we have reported previously (Henson et al., 1999; 2003; 2009). In order to

address the question of whether Arp2/3 complex activity was required for the cytoplasmic ridges, we employed two different pharmacological approaches. In our initial experiments, we treated cells with the drug 2,3-butanedione monoxime (BDM), which we have previously shown to delocalize the Arp2/3 complex from the LP edge in coelomocytes (Henson et al., 2009), a result that has also been reported in mammalian tissue culture cells (Yarrow et al., 2003). Treatment of cells with 20–30 mM BDM arrested the motility of cytoplasmic ridges that then moved centripetally in concert with the formation of a characteristic BDM-induced peripheral fringe (Fig. 5A; Supp. Movie 4). Fluorescent labeling showed that the ridges present in the BDM-treated cells retained their concentration of actin and Arp3 labeling (Fig. 5C, D). In other experiments we employed the small molecule drug CK666, a specific inhibitor of the Arp2/3 complex (Nolen et al., 2009). In contrast to BDM treatment, exposing cells to 100 μ M CK666 led to the disappearance of the cytoplasmic ridges (Fig. 5B; Supp. Movie 5) along with their associated actin and Arp3 labeling (Fig. 5E, F). CK666 treatment also resulted in a transformation of the overall actin cytoskeleton to one containing more elongate filaments, with the LP region actin dendritic meshwork being replaced by long filaments arrayed in microspikes and filopodia and/or in transversely oriented small bundles – all undergoing centripetal flow (Fig. 5B, E; Supp. Movie 5). In parallel experiments, cytoplasmic ridge motility was not affected in cells treated with 100 μ M of the inactive control small molecule CK689 (not shown).

An advantage of using either BDM or CK666 is that, unlike the drugs CytoD or the latrunculins, both are easily and quickly washed out (Danilchick et al., 2006; Henson et al., 2009; Yang et al., 2012). Interestingly, washout of either of these treatments often resulted in the reestablishment of cytoplasmic ridges in the reforming LP region of the cell (Fig. 6; Supp. Movies 4; 5). The burst of new ridge formation tended to initiate at the leading edge of the retracted dendritic meshwork of actin in both BDM and CK666 treated cells (particularly noticeable in Fig. 6A–C and Supp. Movie 4). This suggests that the leading edge of the retracted cytoskeleton maintains the Arp2/3 complex nucleation machinery in a form that can quickly reactivate upon the removal of inhibition.

Inducible Tubular Projections Generate Filopodia and Can Transpose into Cytoplasmic Ridges

In the course of treating cells with the small molecule drug BDM we noticed a peculiar phenomenon. Attached cells treated with 10–30 mM BDM followed by 1:1 hypotonic shock (hypotonic treatment has been a long-standing method for inducing the formation of filopodia in coelomocytes, as initially reported by Edds, 1979) containing 20 mM BDM developed a series of novel, phase dense, primarily cell edge-associated, and cone- to tube-shaped projections that generated filopodia at their distal tips (Fig. 7A–E). These processes arose from a phase dense and frequently indented region of the cell edge. Those that projected away from the cell typically described a curvilinear track, often bifurcated, extended above the plane of the cell, exhibited both protrusion and centripetal flow, and tended to have a finite capacity for extension (Fig. 7A–E; Supp. Movie 6). The projections' average rate of protrusion was approximately 2 μ m/min whereas the centripetal flow rate of control cells tends to be in the range of 3–4 μ m/min (see also Henson et al., 1999; 2009). Their overall morphology roughly resembled miniature neurites given their tubular

geometry and the production of filopodia. During the production of these processes the overall centripetal flow of the cell tended to slow over time. Wash-out of the hypotonic shock and BDM treatment led to immediate cessation of process formation and resumption of fast flowing, control cell-like LP array formation at the cell edge that pushed the remnants of the processes to the cell center where they were dismantled (Fig. 7E; Supp. Movie 7). On occasion, cytoplasmic ridges were evident during the wash-out and were co-located with sites of previously existing tubular projections strongly suggesting a shared mechanism of generation (Fig. 7F; Supp. Movies 7; 8). Treating cells in suspension with hypotonic shock and BDM resulted in particularly dramatic tubular projection formation with virtually the entire cytoplasm of the cell participating in the production (Supp. Fig. 2). Note that in control cells, suspension treatment with 1:1 hypotonic shock alone led to a lamellipodial to filopodial shape change (Supp. Fig. 2C) as has been previously reported for coelomocytes (Edds, 1979).

Inducible Tubular Projections Contain the Arp2/3 Complex and Dendritic Actin Networks

Given our experience using BDM as a means of delocalizing the Arp2/3 complex from the cell edge (Henson et al., 2009), we initially reasoned that the tubular projections produced by hypotonic shock and BDM would be filled with bundles of elongate actin filaments akin to what occurs in filopodia. However, evidence from live cell imaging, immunofluorescence staining and TEM all indicated that these processes instead contained Arp2/3 complex-dependent meshworks of actin filaments. The live cell imaging was based on orientation independent polarization microscopy and revealed the expected pattern of bright linear birefringence associated with the bundles of actin filaments present in the tip-associated filopodia (Fig. 8A–E), as well as in the large filopodia generated by treating control cells in suspension with hypotonic shock (Fig. 8F). In contrast the tubular projections contained a distinct granular birefringence pattern more typical of a dense actin filament network (Fig. 8B, D, E). In support of these live cell imaging results, immunofluorescent localization using conventional fluorescence microscopy showed a dramatic concentration of Arp2/3 complex labeling in the central regions of the processes that appeared to not extend into the filopodia (Fig. 9D–I). The Arp3 staining was particularly extensive in the enlarged tubular processes present in cells treated in suspension (Supp. Fig. 2G–I). In other immunofluorescence staining experiments, the Arp2/3 complex activating protein WASP (Supp. Fig. 3A–C) and the actin filament bundling protein fascin (Supp. Fig. 3D–F) were both found to localize to the projections, with fascin staining extending into the filopodia as expected. In addition, given the ability of microtubules to influence the actin cytoskeleton and to affiliate with actin rich processes such as neurites, we examined the relationship between the coelomocyte processes and microtubules in treated cells. Double labeling of actin and tubulin did not show evidence of any association between the projections and microtubules (Supp. Fig. 3G–I).

The enrichment of the Arp2/3 complex in the tubular projections led us to hypothesize that they would be impacted by treatment with the complex's specific inhibitor, CK666. Therefore we conducted a series of experiments in which the processes were induced via treatment with BDM and hypotonic shock and then these cells were treated with either 100 μ M CK666 or its inactive analogue CK689. Treatment with CK666 led to a significant

alteration of the protrusions including the arrest of extension and centripetal flow, a general reduction and spreading out of the actin staining, and the loss of Arp2/3 complex labeling (Fig. 9J–L). Note that filopodia were retained in the projections of these CK666 treated cells and appeared larger than those seen in the induced Arp3-rich protrusions.

In an effort to obtain higher resolution images of the coelomocyte tubular projections we employed super-resolution 4Pi two-photon confocal microscopy (Fig. 10) and TEM of platinum replicas of coelomocyte cytoskeletons (Fig. 11). Computer-based 3D rendering of the 4Pi Z stacks (Fig. 10B–D) clearly demonstrated that the Arp2/3 complex labeling was enriched in the central region/core of the projections, did not extend into filopodia (labeled F in Fig. 10A, B), and that the processes could extend above the plane of the cell. The presence of actin filament meshworks characteristic of Arp2/3 complex-dependent dendritic arrays was demonstrated by TEM imaging of platinum replicas of cytoskeletons (Fig. 11). The central spine of the processes contained a very dense network of short, interconnected actin filaments whereas the filopodia (labeled F in Fig. 11C–F) had the expected bundles of elongated, unbranched filaments. In many respects the actin organization present in the core of the tubular projections (Fig. 11C–F) was similar to the dense network of actin present at the distal tips of the cytoplasmic ridges (Fig. 3D–G) and also brought to mind the concept of a tubular-shaped LP region.

DISCUSSION

In the present study we have investigated the nature of two distinct cellular processes generated in sea urchin coelomocytes and discovered that these seemingly quite different processes are nonetheless both mediated by Arp2/3 complex-facilitated actin polymerization and actually represent two related processes along the continuum of Arp2/3 complex-mediated and actin-based protrusive activity. Our results characterizing the rocketing motility of cytoplasmic ridges and the extension of inducible, tubular-shaped, and neurite-like protrusions underscores the broad spectrum of cellular processes that are driven by Arp2/3 complex-dependent branched actin filament arrays.

The coelomocyte cytoplasmic ridges (Figs. 1–6; Supp. Figs. 1, 6; Supp. Movies 1–5, 7, 8) resemble a range of processes in other cell types. The peripheral ruffles present in the LP region of many cell types are somewhat similar, however they generally lack the rocketing form of movement, the range of directions of movement and orientations relative to the cell edge, and the perinuclear as well as peripheral location seen with the coelomocyte ridges. The ridges also are reminiscent of the fin-shaped projections of concentrated actin and Arp3 seen in the fluorescent and TEM images of the LP region of non-migrating cells in which the Arp2/3 complex had been tracked using GFP labeling (Schafer et al., 1998). However, the closest similarity exists between coelomocyte cytoplasmic ridges and the intrapodia (Rochlin et al., 1999; Katoh et al., 1999; Dent and Kalil, 2001; Schaefer et al., 2002) and inductipodia (Forscher et al., 1992) present in the LP region of neuronal growth cones. These actin-rich structures on the dorsal membrane derive externally from the placement of beads applied to the surface (inductipodia), or arise *denovo* in the absence of external stimulation (intrapodia). TEM of intrapodia suggests that they are filled with a dense array of actin filaments (Rochlin et al., 1999), although it is unclear whether their formation is

dependent on the Arp2/3 complex. A recent report on Arp2/3 complex inhibition in *Aplysia* growth cones indicates that intrapodia stain for actin and Arp3 (Yang et al., 2012). It is interesting to note that Rochlin et al. (1999) report a burst of inductipodia formation in growth cones that are washed out of cytochalasin B treatment which parallels the burst of cytoplasmic ridge formation seen in coelomocytes washed out of Arp2/3 complex inhibitor treatment (Fig. 6). In a more recent study, Vitriol and colleagues (2007) show that EGFP-chromophore-assisted laser inactivation (CALI) of actin capping protein in mammalian fibroblasts leads to the generation of numerous intrapodia/cytoplasmic ridge-like structures on the dorsal surface of the cell. These authors suggest that the appearance of these processes is driven by the sudden liberation of actin filament barbed ends in the wake of CALI-based capping protein inactivation and comment that the cytochalasin B washout-dependent burst of inductipodia effect seen in the Rochlin et al. (1999) paper may derive from the same effect. In coelomocytes it is possible that Arp2/3 complexes present near the edge of the retracted dendritic array suddenly become activated in the absence of the drug BDM or CK666 and that this rapid reduction in inhibition leads to a sudden increase of branched filament production that leads to the formation of cytoplasmic ridges. Finally, in preliminary experiments with fish keratocytes, another cell type with a broad LP region like the coelomocyte, we have noticed the appearance of phase dense, motile, cytoplasmic ridge-like structures (Supp. Fig. 4). This suggests that these structures may be a common feature of cells that contain elaborate LP regions. In terms of a possible function for the cytoplasmic ridges one possibility is that they may be involved in pinocytosis and/or phagocytosis. Coelomocytes are known to be highly phagocytic and their constant sampling of the environment is thought to play a role in immunological surveillance and defense (Gross et al., 1999; Smith et al., 2010). In support of this hypothesis our unpublished studies have determined that settling cells containing ridges take up pinocytic and phagocytic markers and concentrate them in perinuclear vesicles.

The inducible tubular projections generated in coelomocytes in this study (Figs. 7–11; Supp. Figs. 2, 3; Supp. Movies 6–8) are difficult to directly compare to protrusions in other cells. In some ways they resemble neurites due to their tubular morphology and the production of filopodia at their distal tip. The central role of the Arp2/3 complex in the generation of actin-based processes in neuronal cells has been highlighted by two studies from Korobova and Svitkina (2008; 2010) that demonstrated the importance of the complex in growth cone structure, neuritogenesis, and dendritic spine formation. However, there are a number of fundamental differences between the coelomocyte processes and those found on neuronal cells. With regards to neurites, the coelomocyte tubular processes do not contain the characteristic terminal growth cone region, often tend to extend above the plane of the cell in a curvilinear shape, and their cores contain a dense meshwork of actin filaments as opposed to the array of sparser elongate actin filaments and abundant microtubules and neurofilaments present in the shafts of neurites (Spillane et al., 2011). In addition, the absence of a crawling growth cone at the distal tip means that the coelomocyte processes are not pulled out of the cell in the manner typical with mammalian neurites, but instead the protrusions appear driven by actin assembly focused in a phase dense area at the distal tip. With regards to dendritic spines, the coelomocyte processes share the feature of having an actin network as a core structure however spines tend not to generate filopodia and are

typically smaller than the coelomocyte projections (Korobova and Svitkina, 2010). The lack of an obvious correlate to the coelomocyte processes suggests the possibility that the BDM plus hypotonic shock treatment may be inducing an essentially non-physiological condition in coelomocytes. However, in other unrelated experiments, we noticed that processes roughly similar to the inducible ones in coelomocytes were present in untreated/control HeLa cells (Supp. Fig. 5). Like the coelomocyte projections, these HeLa cell structures were small, phase dense, located at the periphery, tubular/conical in shape and generated filopodia from their distal tip. This observation suggests that the formation of these types of processes is not out of the range of normal physiological conditions.

The results seen in Fig. 7F and Supp. Movies 7 and 8 in which the tubular projections are transformed into cytoplasmic ridges during BDM and hypotonic shock wash out invites the conclusion that these two types of processes are related and share similar mechanisms of generation. It is possible that in essence the tubular process is a cytoplasmic ridge in which the Arp2/3 complex is driving dendritic network assembly whereas the filopodial formation is derived from the hypotonic shock stimulus. Note that the ability of the tubular projections to generate filopodia tends to support the convergent elongation theory of filopodial formation in which filopodia are formed from pre-existing dendritic networks of Arp2/3 complex branched filaments (Yang and Svitkina, 2011). Our current working hypothesis is that ridges and tubular projections arise from points on the membrane where there is a critical mass of activated Arp2/3 complex and that this focused activity leads to either the rocketing of a ridge or the protrusion of a tubular-shaped process.

With regards to the upstream regulation of the formation of these processes, it would be expected that they would be under the control of the Rac members of the Rho family of actin cytoskeleton-regulating monomeric G proteins. Rac activation is known to turn on the WASP family of nucleation-promoting factors that stimulate the ability of the Arp2/3 complex to mediate the formation of dendritic actin arrays (Pollard, 2007; Ridley, 2012). In preliminary experiments we have determined that the small molecule Rac1 inhibitor EHT 1864 (Onesto et al., 2008) interferes with the formation of cytoplasmic ridges in spreading coelomocytes (Supp. Fig. 6) suggesting that these processes are Rac-dependent.

The results of the present study highlight potential mechanistic differences between the impacts of the drugs BDM and CK666 on the Arp2/3 complex. Previous work has intimated that BDM can function like an Arp2/3 inhibitor via its ability to delocalize the complex from the leading edge (Yarrow et al., 2003; Henson et al., 2009). However, it is also clear that BDM does not inhibit all aspects of Arp2/3 complex-facilitated actin polymerization given that it does not interfere with intracellular *Listeria* motility (Yarrow et al., 2003) nor does it stop the motility of the Arp2/3 complex-dependent tubular processes in coelomocytes described in this study (Fig. 7). In contrast the Arp2/3 complex specific inhibitor CK666 blocks both the motility of intracellular *Listeria* (Nolen et al., 2009) and the extension of the coelomocyte tubular processes (Fig. 9). In addition, coelomocyte cytoplasmic ridges also display a differential response to the two drugs. BDM treatment causes an arrest of ridge motility but does not lead to a dismantling of the structures and their associated concentrations of actin and Arp2/3 complex, whereas CK666 both arrests ridge motility and causes their complete disassembly. Taken together, these results suggest that BDM is not

directly inhibiting the activity of the Arp2/3 complex and instead may be acting indirectly by influencing the function of an associated regulatory protein such as one of the nucleation promoting factors.

In the present study we have demonstrated that Arp2/3 complex-mediated actin assembly supports the motility of two distinct but related processes in sea urchin coelomocytes; cytoplasmic ridges present during cell spreading and inducible, tubular-shaped, and neurite-like protrusions. Our results broaden the spectrum of actin-based protrusive activity that is mediated by Arp2/3 complex activity and underscore the ubiquitous and flexible nature of this actin nucleator.

MATERIALS AND METHODS

Animals, Cell Preparation, Antibodies, and Reagents

Sea urchins of the species *Strongylocentrotus droebachiensis* were either collected from the near shore waters surrounding the Mount Desert Island Biological Laboratory (Salsbury Cove, ME) or obtained from the Marine Biological Laboratory's Marine Resources Center (Woods Hole, MA), and kept in either running sea water or closed artificial sea water systems at 4–15°C. Coelomocytes were isolated and maintained as described in Henson et al. (1999) with the coelomocyte culture media (CCM) consisting of 500 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, and 20 mM HEPES, pH 7.2, and cells were used within 24–36 hours of isolation. HeLa cells were purchased from ATCC and cultured in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% fetal bovine serum using standard methods. Fish epidermal keratocytes were obtained from the scales of goldfish (*Carrasius auratus*) following the methods of Svitkina et al. (1997) and cultured in DMEM plus 20% fetal bovine serum. Note that fish care, handling and keratocyte isolation were carried out in accordance with a protocol approved by Dickinson College's Institutional Animal Care and Use Committee. A monoclonal anti-actin antibody (clone C4) was obtained from MP Biomedicals, anti-WASP was from Cytoskeleton, Inc., anti-sea urchin fascin was a gift from Dr. Joann Otto (Western Washington University), fluorescently labeled phalloidin and secondary antibodies were purchased from Molecular Probes/Invitrogen/Life Technologies, TEM supplies and reagents were obtained from Electron Microscopy Sciences, CK666 and CK689 were obtained from Calbiochem/EMD/Millipore, EHT 1864 came from Santa Cruz Biotechnology, while the majority of other reagents (including 2,3-butanedione monoxime, cytochalasin D, and hexamethyldisilazane) and primary antibodies (including anti-Arp3) were purchased from Sigma/Aldrich Chemical Co.

Pharmacological Treatments

For pharmacological treatments, cells being processed for fluorescent staining or electron microscopy were treated in 35-mm Petri dishes while cells prepared for live cell imaging were treated in simple perfusion chambers. These chambers were created by placing the coverslip containing cells face-down onto either cut coverslip shims held by petroleum jelly on a slide or onto double stick tape shims on a slide. Solutions containing a drug were perfused through the chamber using the capillary action of a piece of filter paper. BDM was made up as a stock of 40–50 mM in CCM (with 10 min of vigorous stirring) and typically

used at a concentration of 10–30 mM. Cytochalasin D was made up as 1 mM stock in DMSO and used at a concentration of 500 nM – 1 μ M. CK666 and CK689 were made up as 50 mM stock solutions in DMSO and typically used at 100 μ M (following extensive vortexing in CCM to avoid precipitation). EHT 1864 was dissolved at a concentration of 50 mM in deionized water and used at concentration range between 10 and 20 μ M. Hypotonic shock treatment consisted of diluting CCM 1:1 with a modified CCM buffer containing no NaCl, resulting in a final NaCl concentration of 250 mM.

Live Cell Imaging

Cells settled onto untreated glass coverslips and assembled into perfusion chambers were viewed on a Nikon E600 or 80i microscope using either a 40X 0.75 NA phase contrast plan or a 60X 1.4 NA phase contrast planapochromatic objective lens. Digitally-enhanced phase contrast images were obtained with a Hitachi CCD camera coupled to a Hamamatsu Argus-10 real time digital image processor. Frame averaged, background subtracted, and contrast enhanced images were imported into NIH Image where additional digital contrast enhancement was performed.

For axis-independent polarization microscopy and DIC imaging coelomocytes in perfusion chambers were viewed on an LC-PolScope (Cambridge Research and Instrumentation) using a strain free 60X 1.4 NA DIC planapochromatic objective lens mounted on a Nikon Microphot SA microscope and images captured using a Q-Imaging Retiga camera and processed using PSj custom software.

Fluorescent Localization and Imaging

For fluorescent localization of filamentous actin with phalloidin, coelomocytes were fixed with 0.25% glutaraldehyde plus 0.1% Triton X-100 in Buffer A (75 mM KCl, 2 mM $MgCl_2$, 320 mM sucrose, 20 mM EGTA, 20 mM PIPES, pH 7.0) for 10 min, rinsed in PBS, and then stained with AlexaFluor 488 or 568 conjugated phalloidin (Molecular Probes/Invitrogen). For immunofluorescent staining of Arp3, fascin, WASP, and/or actin coelomocytes were prefixed in 0.00025% glutaraldehyde in CCM for 2–3 min (the appropriate drug treatment was added to this prefix to prevent recovery), fixed in 1% formaldehyde or paraformaldehyde and 0.5% Triton X-100 in Buffer A for 5 min, and then postfixed in 100% methanol at $-20^{\circ}C$ for 5 min. Following a rinse with phosphate buffered saline (PBS), cells were blocked with PBS plus 1% bovine serum albumin and 2% goat serum, and stained with primary antibodies followed by the appropriate fluorescently labeled secondary antibodies. Cells were mounted in Prolong antiphotobleach containing DAPI (Molecular Probes/Invitrogen) and epifluorescence microscopy was performed on a Nikon E600 or 80i microscope using a 60X 1.4 NA planapochromatic phase contrast objective lens and digital images were captured using a Photometrics CoolSnap Cf camera (Roper Scientific). Confocal microscopy was performed on an Olympus Fluoview 500 instrument using a 60X 1.4 NA planapochromatic DIC objective lens. Super-resolution 3D Structured Illumination Microscopy (SIM) was performed on a Nikon N-SIM instrument using a 100X 1.49 NA apochromatic TIRF objective lens. For 4Pi two photon super-resolution microscopy quartz coverslips were used for settling cells and imaging was performed on a Leica TCS 4Pi microscope using two opposed 100X 1.35 NA glycerol

immersion objective lenses. Three dimensional renderings of 4Pi Z stacks were generated using Amira software.

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

Preparation of coelomocytes for SEM followed the methods of Nicholson-Dykstra and Higgs (2008) in which cells were fixed with glutaraldehyde, post-fixed in osmium tetroxide, dehydrated sequentially in ethanol, dried using hexamethyldisilazane, and then gold sputter coated. Digital SEM images were then obtained using a JEOL JSM-5900 instrument operating at an accelerating voltage of 5–10 kV. TEM imaging of critical point-dried and rotary-shadowed replicas of coelomocyte cytoskeletons followed methods described in Henson et al. (1999) and Svitkina (2007). Briefly, coelomocytes settled onto naked glass coverslips were prefixed in 0.0001% glutaraldehyde in CCM (plus appropriate drug treatments) and then extracted in 0.5% Triton X-100 in Buffer A. After extraction, the cells were then fixed in 2.5% glutaraldehyde in the same buffer and then treated with aqueous tannic acid followed by aqueous uranyl acetate. Then the cells were dehydrated in a graded ethanol series, critical-point-dried, and rotary shadowed with platinum and carbon. Metal replicas were separated from glass coverslips using hydrofluoric acid, mounted on Formvar-coated grids, and observed and digitally photographed on a JEOL JEM-200CX TEM operating at 100 kV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Spreading coelomocytes contain cytoplasmic ridges that display rocketing motility
Digitally-enhanced phase contrast microscopy (A–E) shows that the ridges become concentrated at the periphery and diminish over time in spreading cells (A–D) and that their motility displays a range of directions from centrifugal to tangential to centripetal (arrows in E - each image separated by 10 sec) and there is frequent branching and anastomosis (resulting in the circles apparent in E–G). DIC microscopy (F, G) reveals peripheral ridges (arrows in F - each image separated by 40 sec) and perinuclear ridges (N in panel G marks the nucleus). In panels E and F the asterisks (*) mark ridges that have folded over and are subsumed into the LP region. Polarization microscopy (H) demonstrates that ridges display positive birefringence (note that white corresponds to 1 nm retardance). Bars = 10 microns.

Figure 2. Cytoplasmic ridges concentrate actin and the Arp2/3 complex

Fluorescent labeling of actin (A, D) and Arp3 (B, E) indicates that each are concentrated in the peripheral ridges (present in the top cell in panels A–C) and perinuclear ridges. These correspond to the phase dense linear elements seen in live cells (compare panel G with Fig. 1). Note that in fully spread cells not containing ridges (bottom cell in panels A–C) the peripheral Arp3 staining is restricted to a cortical band. Super-resolution 3D SIM imaging (a maximum intensity projection of a Z series) of phalloidin labeled actin filaments (H) highlights the three-dimensional nature of the ridges as well as the predominance of the dendritic network form of actin filament structural organization. Bars = 10 microns.

Figure 3. Electron microscopic imaging of cytoplasmic ridges

SEM imaging of spreading coelomocytes (A) demonstrates the presence of cytoplasmic ridges in the cells similar to those seen in light microscopy (Fig. 1, 2). TEM of platinum replicas of coelomocyte cytoskeletons (B–G) shows that ridges consist of a dense meshwork of actin filaments similar to that seen at the cell edge (box in B corresponds to panel C, and panel D is a magnified view of the ridge present at the top of panel C). High magnification images of the distal tips of ridges (D, G) as well as areas corresponding to newly generated ridges (E, similar to the structure pointed out by the arrow in the live cell in the third frame of Fig. 1F) show that they contain a very dense meshwork of apparently interconnected short actin filaments. Bars in A, B, F = 5 microns; bar in E = 0.5 microns.

Figure 4. Inhibition of actin polymerization arrests the motility of cytoplasmic ridges
 Digitally-enhanced phase contrast imaging of a cell (time in seconds appears at the bottom of the images) treated with the actin polymerization inhibitor cytochalasin D (+CytoD at 200 sec) shows that this drug arrests the motility of ridges (arrows) and causes them to curl back towards the cell center in concert with the retraction of the entire cytoskeleton due to the release of the LP actin meshwork from the cell edge combined with actomyosin contraction. Bar = 10 microns.

Figure 5. Arp2/3 complex inhibition arrests the motility of cytoplasmic ridges

Live cell imaging shows that addition of the drug BDM stops the motility of the ridges (A), and immunofluorescent staining indicates that actin (C) and Arp3 (D) staining patterns of the ridges are retained. In contrast, treatment with the drug CK666 not only stops the ridge motility but also leads to their disappearance (B), with an associated loss of their actin (E) and Arp3 (F) staining. Bars = 10 microns.

Figure 6. Cytoplasmic ridges undergo a burst of reformation upon wash out of Arp2/3 complex inhibition

Coelomocytes washed out of either BDM (A–C) or CK666 (E, F) treatment can display a burst of cytoplasmic ridge reformation in the periphery of the cell (B, C, F) often occurring at the leading edge of the retracted dendritic meshwork of actin (arrows in A). Fluorescent phalloidin staining of actin (D) shows that the wash out (WO)-induced ridges (left-hand cell in D) are similar in actin staining to those present on control cells (Fig. 2), and also indicates that coelomocytes not exhibiting LP-like actin network recovery (right-hand cell in D) do not generate ridges. Bars = 10 microns.

Figure 7. Induction of tubular-shaped projections in coelomocytes that protrude from the cell edge and generate filopodia at their tips

Substrate attached control cells (A) develop a characteristic cleared peripheral fringe when treated with BDM (B), and when this treatment is followed with BDM plus a 1:1 hypotonic shock the cells generate unusual, phase dense, and tubular-shaped projections at their edges (arrows in C, D, and E). These projections form filopodia at their distal tips, have a curvilinear pattern of protrusion, often bifurcate (arrows in D), extend above the plane of the cell, and can be quite long (arrows in E). Wash out of the BDM plus hypotonic treatment results in the reestablishment of control LP organization at the edge and the loss of tubular projections (WO in E). Close examination of tubular projections upon wash out (F) shows that they can directly transpose into cytoplasmic ridge-like structures. Images in panel E are 30 sec apart while those in panel F are 50 sec apart. Bars = 10 microns.

Figure 8. Polarization microscopy indicates that the tubular projections contain actin networks instead of bundles

DIC (A, C) and LC-PolScope (B, D, E, F) images of live cells treated with BDM+Hypo (A–E), or +Hypo only (F). Panels A–D depict the same cell at different focal planes. The tubular projections contain strong linear birefringence within their short filopodia, similar to that seen in the large filopodia generated by hypo treatment alone (F), however the core of the projections has a granular pattern of birefringence (inset in D and panel E). This suggests that the actin structural organization in these processes is not bundles of long actin filaments but instead a network of short filaments. Note that in the polarization images that white corresponds to 1 nm retardance. Bar = 10 microns.

Figure 9. Tubular projections have cores rich in actin and the Arp2/3 complex and are altered in cells treated with CK666

Fluorescent staining of actin (red) and Arp3 (green) and DNA (blue) in control (A–C) and BDM+Hypo (D–I) treated cells. In control cells the peripheral band of Arp3 punctate staining codistributes with the LP actin meshwork (A–C), whereas in BDM+Hypo treated cells actin and Arp3 both are highly concentrated in the tubular projections (D–F). Higher magnification images (G–I) indicate that Arp3 does not extend into the filopodia (arrows in G) present on the projections. Treating BDM+Hypo cells with CK666 results in an alteration in the projections in which the Arp3 core staining is lost and the projections spread out, although filopodia are retained (J–L). Bars = 10 microns.

Figure 10. Super-resolution microscopy of tubular projections

4Pi two photon confocal images of BDM+Hypo treated cell labeled for actin (red) and Arp3 (green). A through-focus projection of a Z-series (A) and 3-D renderings of this same series from three different perspectives (B–D) clearly demonstrate that the Arp3 staining concentrates in the core of the projections and does not extend into filopodia (labeled F in panels A, B), and that the projections can protrude above the plane of the cell (C, D). Bar = 5 microns.

Figure 11. TEM imaging reveals dendritic networks of actin filaments in tubular projections
 TEM of platinum replicas of coelomocyte cytoskeletons from control (A) and BDM+Hypo treated (B–F) cells. Control cells contain the expected dendritic meshwork in the peripheral LP region (A) whereas the edge of BDM+Hypo treated cells contains sparse bundles of transversely-oriented actin filaments interspersed with the regions of tubular projections (B). The indicated projections in panel B (arrow) are shown at higher magnification in panels C and D whereas projections from other cells are depicted in panels E and F. In all of these images the core of the processes contain a dense and dendritic network of short filaments whereas the filopodia (arrows labeled F in C–F) consist of bundles of elongate filaments. Bars in A and B = 5 microns; in C–F = 0.5 micron.