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REVIEW

Signaling mechanisms that regulate actin-based motility processes in the nervous system

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Abstract

Actin-based motility is critical for nervous system development. Both the migration of neurons and the extension of neurites require organized actin polymerization to push the cell membrane forward. Numerous extracellular stimulants of motility and axon guidance cues regulate actin-based motility through the rho GTPases (rho, rac, and cdc42). The rho GTPases reorganize the actin cytoskeleton, leading to stress fiber, filopodium, or lamellipodium formation. The activity of the rho GTPases is regulated by a variety of proteins that either stimulate GTP uptake (activation) or hydrolysis (inactivation). These proteins potentially link extracellular signals to the activation state of rho GTPases. Effectors downstream of the rho GTPases that directly influence actin polymerization have been identified and are involved in neurite development. The Arp2/3 complex nucleates the formation of new actin branches that extend the membrane forward. Ena/VASP proteins can cause the formation of longer actin filaments, characteristic of growth cone actin morphology, by preventing the capping of barbed ends. Actin-depolymerizing factor (ADF)/cofilin depolymerizes and severs actin branches in older parts of the actin meshwork, freeing monomers to be re-incorporated into actively growing filaments. The signaling mechanisms by which extracellular cues that guide axons to their targets lead to direct effects on actin filament dynamics are becoming better understood.

Keywords: actin-depolymerizing factor (ADF)/cofilin, actin-related protein Arp2/3, ena/VASP, LIM kinase, rho GTPase.
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Cell motility is a critical function for many different types of cells. In response to infection, hematopoietic cells need to navigate through blood vessel walls and tissue spaces to reach the site of infection. Metastatic cancer cells escape their original tissues and invade distant tissues where they proliferate and cause damage. The complex process of nervous system development is also dependent upon cell motility. Developing neurons, notably cortical neurons and neural crest cells, migrate from the site where the cell was born to where the cell is destined to develop and elaborate processes. The elaboration of neurites continues this dependence on motility, as the processes by which the growth cones are guided towards a distant target involves similar processes to whole-cell motility.

The motility machinery common to migrating cells and neuronal processes requires the organized polymerization of actin filaments. Actin polymerization at the leading edge of a migrating cell or growth cone is the driving force for the forward extension of the cell membrane. Several cell structures result from different organizations of actin filaments. At the leading edge of migrating cells and growth cones is a broad, flat membrane extension called the lamellipodium. Lamellipodia are produced by the polymerization of filamentous (F)-actin underneath the cell membrane. Long, finger-like projections called filopodia are often found at the edges of the lamellipodia and are also the result of actin polymerization. In contrast to the actin found at the leading edge of a lamellipodium, actin stress fibers are long bundles of actin that traverse the cell and are linked to integrins at sites of focal adhesion.

Close examination of the lamellipodial actin of keratocytes and fibroblasts reveals that it is organized as a network of short branches, each forming off the side of an existing filament at an angle of 70°, with barbed ends oriented towards the

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Abbreviations used: ADF, actin-depolymerizing factor; Arp, actin-related protein; Cdk5, cyclin-dependent kinase 5; F-actin, filamentous actin; GAP, GTPase-activating protein; GDI, GDP dissociation inhibitors; GEF, guanine nucleotide exchange factor; NGF, nerve growth factor; robo, roundabout; SSH, slingshot; WASP, Wiscott–Aldrich syndrome protein; XAC, Xenopus ADF/cofilin.

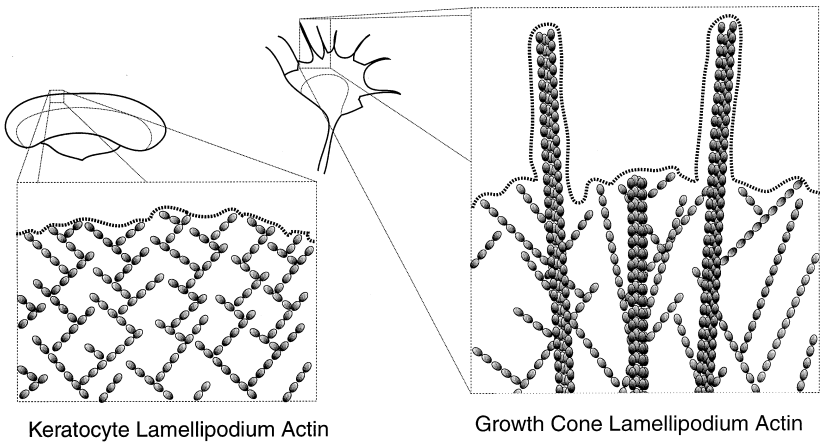

Fig. 1 Differences in the organization of actin in fibroblasts/keratocytes and neuronal growth cones. Lamellipodium actin in keratocytes and fibroblasts is organized as a meshwork of short branches. The pointed ends of filaments are oriented towards the membrane. In the growth cone, two types of actin are observed. A meshwork with less regular branching, longer filaments, and pointed ends in variable orientations fills in the spaces between long bundles of 6–12 actin filaments that radiate from the membrane.

membrane (Svitkina and Borisy 1999). The actin at the leading edge of a migrating cell is thus polymerized in an organized and localized fashion. However, the actin organization found in growth cones differs from the regular organization found in fibroblasts and keratocytes (Fig. 1). Growth cone actin occurs in two forms: a meshwork that is not as uniform as the fibroblast/keratocyte meshwork, with most but not all of the filaments having barbed ends towards the leading edge; and long bundles of 6–12 actin filaments that radiate from the leading edge towards the central zone of the lamellipodium (Lewis and Bridgman 1992). Both the meshwork and bundles are organized de novo in expanding growth cones (Welnhofer *et al*. 1997), but loss of the bundles specifically may result in growth cone collapse (Zhou and Cohan 2001).

As the polymerization of organized actin structures is a common element for the motility of cells and neuronal processes, there has been great interest to understand the signaling pathways that control actin polymerization. A key event towards this goal was the discovery of a family of small GTPases, called the rho GTPases, which could orchestrate the formation of actin stress fibers, filopodia, and lamellipodia. Subsequently, actin-modifying proteins that function downstream of the rho GTPases were identified. While a myriad of actin-binding proteins have been identified and characterized, only a handful are required to reconstitute the organized actin polymerization used by intracellular bacteria to move around the cell. For example, Shigella and Listeria hijack the cell's actin machinery, causing polymerization to occur from a specific site on the bacteria that propels the bacteria forward as if it were a comet with an actin tail streaming behind. Carlier and colleagues (Loisel *et al*. 1999) were able to reconstitute this actin polymerization *in vitro*, with kinetics similar to *in vivo* motility, by using three key components. These were actin-related protein (Arp) 2/3 to nucleate new actin formation, a capping protein to restrict the length of growing filaments, and actin-depolymerizing factor (ADF)/cofilin to depolymerize the older parts of the network and allow for monomers to recycle to the leading edge of the network. The activities of Arp2/3 and ADF/cofilin are normally controlled by the rho GTPases. Thus, rho GTPase regulation of Arp2/3 and ADF/cofilin is probably critical to the formation of organized actin structures such as the lamellipodia found on motile cells.

With the current knowledge of how Arp2/3 and ADF/cofilin control actin polymerization and their regulation upstream by the rho GTPases, there is potential for identifying signaling cascades involved in cellular motility. These signaling cascades originate with extracellular motility cues, leading to the activation/inactivation of rho GTPases. This process, in turn, causes modifications in organized actin polymerization by Arp2/3 and ADF/cofilin. This cascade is of special interest to the development of the nervous system, not only because of the need for cortical neurons and neural crest cells to migrate during development, but also because of the complicated process of guiding neurites to their proper targets. The numerous extracellular cues that signal for neurite growth and retraction probably lead to modifications in actin polymerization at the leading edge of the growth cone via rho GTPase signaling. However, as there are differences in the architecture of the lamellipodium actin meshwork between fibroblasts/keratocytes and neuronal growth cones, there are likely differences in the molecular regulators of actin in these cells. Table 1 lists some of the key molecular regulators that could be involved in the organization of lamellipodium actin. This review will provide a broad framework for how the morphology and dynamics of the actin cytoskeleton are controlled by the rho GTPases and two of their key targets, Arp 2/3 and ADF/cofilin. A possible role for the ena/VASP proteins in the specific arrangement of growth cone actin will also be addressed. Finally, this review will survey the emerging evidence that these proteins are common mediators of numerous extracellular signals involved in neurite growth and guidance.

Rho GTPases: organizers of actin structures

The rho GTPase family, including rho, rac, and cdc42, was first identified in an Aplysia cDNA library. Rho is similar to

Table 1 Molecular regulators of lamellipodium actin organization

Rho	GTPase that stimulates stress fiber formation, focal adhesions, and contraction
Rac	GTPase that stimulates lamellipodium formation
cdc42	GTPase that stimulates filopodium formation
PAK	Serine/threonine kinase directly activated by rac and cdc42, involved in lamellipodium formation
ROCK	Serine/threonine kinase activated by rho, involved in regulation of actin polymerization and myosin
Arp2/3	Complex of 7 proteins that nucleates the formation of new actin branches off the side of existing branches
Profilin	Facilitates actin exchange of GDP for GTP
capping protein	Stops elongation of actin filament at barbed end
N-WASP	Activator of Arp2/3; binds active cdc42
Scar/WAVE	Activator of Arp2/3; indirectly activated by rac
Cortactin	Activator of Arp2/3
ena/VASP	Family of proteins that compete with capping protein; facilitate filament elongation
ADF/cofilin	Two similar proteins that stimulate pointed-end depolymerization; inactivated by LIM kinase
LIM kinase	Serine/threonine kinase that inactivates ADF/cofilin; activated by PAK and ROCK

the ras proteins in size and sequence (Madaule and Axel 1985), and purified Aplysia rho binds and hydrolyzes GTP similarly to H-ras (Anderson and Lacal 1987). Clues to the function of the rho family of GTPases came first from yeast cdc42. Cdc42 was identified as a rho-family GTPase from a temperature-sensitive *Saccharomyces cerevisiae* mutant that was defective in budding (Adams *et al*. 1990; Johnson and Pringle 1990), indicating abnormal actin polymerization. The specific roles of rho, rac, and cdc42 in actin polymerization were defined through microinjecting fibroblasts. Rho activation causes the formation of actin stress fibers. When these actin stress fibers are formed in fibroblasts, these cells become more contracted and exhibit enlarged focal adhesions (Ridley and Hall 1992). In contrast, rac causes actin polymerization that leads to lamellipodium formation and membrane ruffling (Ridley *et al*. 1992). Cdc42 causes a third type of actin-based structure, filopodia, at the edges of lamellipodia (Nobes and Hall 1995). Thus, the rho GTPases are key regulators of actin dynamics that lead to organized actin-based structures associated with the structure and motility of cells.

As it is clear the rho GTPases have important effects on the actin cytoskeleton, their involvement in nervous system development has been characterized. Rho is present in the crude membrane fraction of bovine brain (Yamamoto *et al*. 1988). RhoA, rhoB, rac1, and cdc42 are found expressed throughout the nervous system; mRNA can be found in hippocampus, cerebellum, brainstem, thalamus, and neocortex (Olenik *et al*. 1997). This widespread expression suggests a critical involvement of the rho GTPases in neuronal function.

The rho GTPases have emerged as common regulators of the actin dynamics that drive growth cone motility. Most of the studies of rho GTPase function in neuronal cells have

Table 2 Requirement for Rho-GTPases in neuron and growth cone motility

Cell type		Reference
Retraction/Inhibition of growth		
Rho-mediated	Xenopus tectal neurons	(Li *et al*. 2000)
	Drosophila dendrites	(Lee *et al*. 2000)
	PC12 neurites	(Sebok *et al*. 1999)
	Rat optic nerve	(Lehmann *et al*. 1999)
Rac-mediated	Chick DRG neurons	(Jin and Strittmatter 1997)
	Chick motor neurons	(Kuhn *et al*. 1999)
Elongation		
Rac/Cdc42 mediated	Cortical neurites	(Threadgill *et al*. 1997)
	Hippocampal dendritic spines	(Nakayama *et al*. 2000)
	Xenopus tectal neurons	(Li *et al*. 2000)
	Caenorhabditis elegans axon guidance	(Lundquist *et al*. 2001)
	Chick spinal neurons	(Brown *et al*. 2000)
Rho-mediated	PC12 neurons (Rho G)	(Katoh *et al*. 2000)
	Nerve growth factor-primed PC12 neurons	(Sebok *et al*. 1999)

been accomplished by expressing constitutively active or dominant negative mutants in cultured neurons and observing the effects on neurite length or branching. Endogenous rho can also be inhibited selectively by expressing the C3 exoenzyme in cultured cells. Table 2 summarizes the results of several studies: in general, rho activation is inhibitory to neurite growth, while rac and cdc42 promote neurite growth. However, the roles of rho and rac/cdc42 in neurite growth are reversed in some cases. A specific rho isoform, rhoG, causes nerve growth factor (NGF)-independent neurite growth in PC12 cells (Katoh *et al*. 2000) and when PC12 cells are primed with NGF, dominant negative rho shortens and constitutively active rho lengthens axons (Sebok *et al*. 1999). Thus the function of rho in PC12 cells appears to change based on the developmental state of the cells. In another example of role reversal, active rac appears to be required for neurite retraction in some systems, most notably semaphorin 3D-stimulated retraction (Kuhn *et al*. 2000).

How are the actin-reorganizing events controlled by the rho GTPases required for neurite growth and retraction? Growth cones are essentially F-actin rich lamellipodia, frequently decorated with filopodia, so rac and cdc42 functions could be expected in growth cone elaboration. Rac activity that leads to growth cone formation provides the tensile force for neurite elongation. If an external source of tension is applied to rac-inhibited PC-12 cells, normal neurite growth can be achieved, suggesting the activity of rac normally provides this tension via growth cone motility (Lamoureux *et al*. 1997). Rho-mediated retraction is also likely to be a tension-driven event, but via its activation of myosin proteins that generate force against the actin cytoskeleton (Narumiya *et al*. 1997). Potentially, rho could also shift the cellular actin away from lamellipodial actin and towards stress fiber formation. Loss of one of the two types of actin found in growth cones (actin bundles) is associated with growth cone collapse (Zhou and Cohan 2001), but the involvement of rho GTPases in this effect is unknown. A possible explanation for cases where rho is involved in elongation and rac causes retraction is that these proteins activate other GTPases that eventually lead to the end effect. For example, RhoG is believed to cause neurite elongation in PC-12 cells by subsequent activation of rac and cdc42 (Katoh *et al*. 2000).

Extracellular stimulants and inhibitors of growth cone motility in the nervous system signal through the rho GTPases

Numerous stimulants and inhibitors of motility and neurite growth appear to signal through the rho GTPases. In principle, extracellular guidance cues may steer growth cones by activating rac and cdc42 to attract the growth cone, or activating rho to stop neurite growth. Table 3 lists some of the agents that cause their effects on neurites through the rho GTPases. Again, the general pattern is that stimulators of neurite growth depend upon rac or cdc42 activity, whereas inhibitors of neurite growth activate rho. Some guidance molecules could operate by coordinating activation and deactivation of multiple rho GTPases. Plexin B, a semaphorin receptor, binds both GTP-rac and rhoA through different domains, suppressing rac function, but activating rho function (Hu *et al*. 2001).

Growth cone collapse stimulated by contact with members of the semaphorin group of axonal guidance molecules seems to be mediated by rac and cdc42 activation, again demonstrating that the rho GTPases can cause effects opposite of what is typically expected. Collapsin-1 (semaphorin-3D) fails to prevent neurite growth of chick

Table 3 Extracellular regulators of growth cone/cell motility and rho GTPase activity

Stimulators of neurite growth/motility – rac/cdc42 dependent	
Phorbol esters	(Altun-Gultekin *et al*. 1996)
Nerve growth factor	(Daniels *et al*. 1998)
Insulin-like growth factor-I	(Cheng *et al*. 2000)
Acetylcholine	(Kozma *et al*. 1997)
Laminin	(Weston *et al*. 2000)
Serum starvation	(Sarner *et al*. 2000)
Inhibitors of neurite growth/motility – rho dependent	
Lysophosphatidic acid	(Tigyi *et al*. 1996; Kranenburg *et al*. 1999)
Myelin	(Lehmann *et al*. 1999)
Myelin-associated glycoprotein	(Lehmann *et al*. 1999)
Prostaglandin E	(Katoh *et al*. 1998)
Eprhin	(Wahl *et al*. 2000)
Unbound p75 receptor	(Yamashita *et al*. 1999)
Frizzled/disheveled	(Winter *et al*. 2001)
Plexin B	(Hu *et al*. 2001)
Inhibitors of neurite growth/motility – rac dependent	
Collapsin-1 (Semaphorin 3D)	(Kuhn *et al*. 1999; Kuhn *et al*. 2000)

motor neurons when dominant negative rac or cdc42 is expressed (Kuhn *et al*. 1999). Dominant negative rac also prevents collapsin-induced retraction of chick DRG neurites (Jin and Strittmatter 1997).

Regulation of rho GTPase activity by guanine nucleotide exchange factors, GTPase-activating proteins and GDP dissociation inhibitors

Guidance cues and factors that control growth cone motility via the rho GTPases would have to activate signaling pathways that cause changes in rho GTPase activity. The activity of the rho GTPases is modulated by three types of regulatory proteins. Guanine nucleotide exchange factors (GEFs) facilitate the exchange of GDP for GTP, thus activating rho GTPases (Bateman and Van Vactor 2001; Vetter and Wittinghofer 2001; Zheng 2001). GTPase-activating proteins (GAPs) stimulate the rho GTPases to hydrolyze GTP and become inactive (Scheffzek *et al*. 1998; Zalcman *et al*. 1999). GDP dissociation inhibitors (GDIs) hold the rho GTPase in an inactive state by inhibiting GDP release (Sasaki and Takai 1998; Olofsson 1999). Numerous GAPs and GEFs have been identified and characterized. GAPs and GEFs are potential targets for ligand-activated signaling cascades and could serve to integrate the signals that lead to rho GTPase activity.

Some rac-GEFs promote motility and neurite growth, presumably via activation of rac. Tiam-1 causes N1E-115 cells to elaborate neurites and prevents lysophosphatidic acid-induced neurite retraction (Leeuwen *et al*. 1997). Tiam-1 is expressed in the developing CNS in migrating cortical neurons. The cerebellar granule neurons of the *weaver* mouse, which show a lack of Tiam-1 expression, fail to migrate (Ehler *et al*. 1997). Another rac-GEF, STEF, is expressed in the developing CNS and causes rac-dependent neurite growth in N1E-115 cells. Trio is a rac-GEF in the rho guanine nucleotide exchange factor family (also known as Dbl); Drosophila lacking Trio show defects in both central and peripheral axon guidance. Trio interacts with a receptor phosphatase, LAR, and potentiates the phenotype of LAR mutants (Bateman *et al*. 2000). Trio also interacts with Dock to activate rac and PAK in photoreceptor axon guidance (Newsome *et al*. 2000).

Rho-GEFs have been identified that cause neurite retraction, some of which are linked to specific extracellular signals. Ephrin-stimulated growth cone collapse occurs through activation of rho and ROCK (Wahl *et al*. 2000). A novel rhoGEF, ephexin, has been identified downstream of the Ephrin receptor and is required for Ephrin-stimulated retraction (Shamah *et al*. 2001). GEF64C, a Dbl family member in Drosophila, causes too many neurons to cross the midline when overexpressed. This phenotype mimics the loss of roundabout (robo) function. GEF64C expression can overcome robo activation. Strangely, though, GEF64C activates rho, not rac or cdc42 (Bashaw *et al*. 2001). This may be another situation where rho activation supports neurite extension.

GAP activity represents another mechanism for controlling neurite growth. Robo activation by Slot inhibits neurite growth by way of a second route. Robo activates a novel GAP, srGAP1, which inactivates cdc42. Constitutively active cdc42 can overcome robo activation (Wong *et al*. 2001). Inactivation of the p190 rhoGAP causes retraction of mushroom body axons in Drosophila, in a phenotype similar to activation of rhoA (Billuart *et al*. 2001). RhoGAP also increases neurite length in cortical neurons (Threadgill *et al*. 1997).

Figure 2 is shows schematically how extracellular factors that control neurite growth and growth cone motility could modulate the activity of rho, rac, and cdc42 by signaling through GEFs and GAPs.

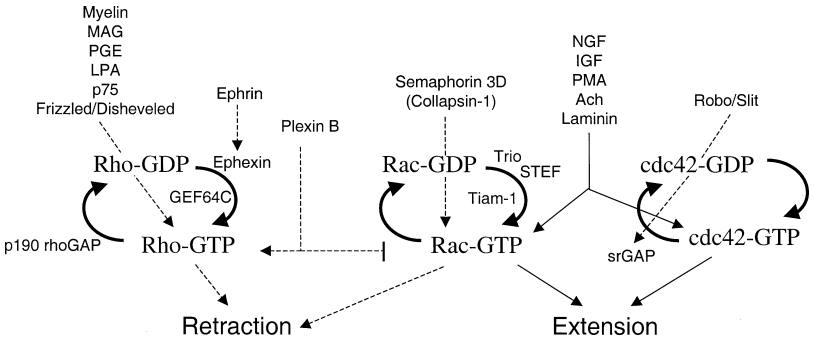

Fig. 2 Extracellular signals cause membrane extension or retraction by regulating the activity of the rho GTPases. Rac and cdc42 activity typically leads to membrane extension and rho activity usually causes neurite retraction; there are examples of the opposite effects, however (e.g. semaphorin and collapsin cause retraction via rac). Rho GTPases are active when GTP-bound and inactive when GDP-bound. Binding of GTP is facilitated by specific GEFs, which may be regulated by extracellular signals (e.g. ephrin and the GEF ephexin). GTP hydrolysis is stimulated by GAPs, which are also potential targets of extracellular guidance cues (e.g. robo/slot activates srGAP). For most extracellular signals that require rho GTPases activity, the exact mechanisms for altering the GTPase's activity state are unknown, but probably involve the functions of GAPs and GEFs.

Direct effectors of rho GTPase activation: PAK and ROCK

In order to understand how rho, rac, and cdc42 lead to changes in actin polymerization, proteins were identified that interact with the rho GTPases. A kinase with homology to yeast STE20 is present in brain and binds GTP-bound rac and cdc42. The kinase, now known as p21-activated kinase, or PAK, becomes autophosphorylated upon binding rac or cdc42. Phosphorylated PAK is then released, presumably to activate downstream signaling cascades (Manser *et al*. 1994). PAK is involved in a number of processes, including MAP kinase signaling (Marcus *et al*. 1995), leukocyte activation (Knaus *et al*. 1995), and JNK/SAPK activation (Bagrodia *et al*. 1995; Frost *et al*. 1996). A role for PAK in cytoskeletal processes is evident, as PAK localizes to membrane ruffles in response to growth factors and activated rac (Dharmawardhane *et al*. 1997). Activated PAK microinjected into fibroblasts quickly causes filopodia and membrane ruffles (Sells *et al*. 1997). LIM kinase, a downstream effector of PAK, has a direct relationship to actin dynamics and will be discussed below. PAK-independent actin polymerization downstream of rac and cdc42 is also observed, suggesting the existence of other actin-related targets of rac and cdc42 (Lamarche *et al*. 1996).

Rho-associated kinase, or ROCK, is another kinase purified from brain that interacts specifically with GTP-bound rho (Matsui *et al*. 1996). ROCK phosphorylates and inactivates myosin phosphatase, linking rho to effects on smooth muscle contraction (Kimura *et al*. 1996). ROCK is also involved in the regulation of stress fibers by rho, as microinjection of the catalytic domain of ROCK causes stress fiber formation (Amano *et al*. 1997). As with PAK, ROCK directly links to the cytoskeleton via LIM kinase. Thus, the rho GTPases probably utilize a common mechanism for regulating actin dynamics to achieve different actin-based structures.

Although much less downstream signaling of the rho GTPases has been studied in the nervous system, PAK and ROCK are clearly involved in rho GTPase-dependent events in neuronal cells. ROCK activity is needed for agonist-induced neurite retraction in N1E-115 cells (Hirose *et al*. 1998). Injecting the PH domain of ROCK functions as a dominant-negative, and blocks prostaglandin E-stimulated neurite retraction in PC12 cells (Katoh *et al*. 1998). ROCK activity also causes growth cone formation in cerebellar granule cells (Bito *et al*. 2000). PAK promotes neurite growth in several systems. Targeting PAK to the plasma membrane, which supports PAK activation, causes NGF-independent neurite formation in PC12 cells, whereas a dominant negative PAK blocks NGF-stimulated neurites (Daniels *et al*. 1998). Dock, an adapter protein that stimulates axon growth in Drosophila photoreceptor cells, requires PAK activity to function. A novel PAK isoform, PAK5, has been identified in neurons and supports neurite growth (Dan *et al*. 2002).

Neurons contain a unique mechanism for down-regulating PAK activity. Cyclin-dependent kinase 5 (Cdk5), when partnered with a neuron-specific regulator (p35), colocalizes with GTP-rac and PAK at the growth cone leading edge. Cdk5/p35 decreases PAK activity by phosphorylating PAK on Thr212 (Nikolic *et al*. 1998; Rashid *et al*. 2001). A non-phosphorylatable mutant of PAK causes disorganization of neurites, suggesting this novel regulatory mechanism is important in controlling the neurite growth process. Interestingly, whereas ROCK tends to cause neurite retraction and PAK causes neurite growth, both signal to LIM kinase and cause the inactivation of ADF/cofilin (discussed below). How regulation of this modulator of actin polymerization can lead to different morphological outcomes is a subject of current investigation.

Arp2/3 complex, Wiscott–Aldrich syndrome proteins (WASPs), and ena/VASP proteins assemble the actin network

The Arp 2/3 complex is ubiquitous to eukaryotic cells, and was first identified in Acanthamoeba by affinity chromatography on profilin Sepharose (Machesky *et al*. 1994). The complex consists of 7 proteins: Arp 2, Arp 3, p41Arc, p34Arc, p21Arc, p20Arc, and p16Arc. The components localize to the cytoplasm of unstimulated fibroblasts, but become enriched in the lamellipodia of serum or growth factor stimulated fibroblasts (Machesky *et al*. 1997; Welch *et al*. 1997). Arp2/3 is also found in the actin tails of Listeria and Shigella (Welch *et al*. 1997; Egile *et al*. 1999) and is required for ActA-stimulated motility of Listeria (Welch *et al*. 1997). Arp2/3 and cdc42 are found together in fractions of Xenopus egg extracts that stimulate actin polymerization (Ma *et al*. 1998). Characterization of the actin-polymerizing properties of this complex led to the development of the 'dendritic nucleation' model (Mullins *et al*. 1998), which proposes how actin *in vivo* polymerizes into an organized and regular meshwork. The Arp2/3 complex is found at the branch points of the actin meshwork of fibroblasts and keratocytes (Mullins *et al*. 1998; Svitkina and Borisy 1999) where it nucleates the formation of a new actin filament off the side of an existing filament at a highly consistent angle of 70 ± 7° (Mullins *et al*. 1998). Arp2/3 caps the pointed end of the new filament and inhibits monomer association/dissociation from the pointed end (Mullins *et al*. 1998). Nucleation of new filaments happens faster if Arp2/3 is incubated with pre-existing filaments, suggesting nucleation preferentially occurs off existing filaments (Machesky *et al*. 1997). Profilin and capping protein act synergistically with Arp2/3 to promote polymerization (Blanchoin *et al*. 2000). Release of phosphate after the actin monomers at the pointed end hydrolyze their bound ATP appears to signal for disassembly of the branch point (Blanchoin *et al*. 2000).

The activity of Arp2/3 is quite low without assistance from proteins of the WASP family. These include WASP, neuronal WASP (N-WASP), and Scar/WAVE. N-WASP, though ubiquitous, was first identified in brain (Miki *et al*. 1996) and found highly enriched in neuronal cells. N-WASP contains a plekstrin homology domain for binding of phosphoinositides, a cdc42 binding domain, and a region called the VCA (for verprolin homology, cofilin homology, acidic motif) which, when mutated, suppresses epidermal growth factor (EGF)-stimulated microspike formation. The plekstrin homology and cdc42 binding domains are regulatory and binding at either site facilitates binding at the other (Prehoda *et al*. 2000). The WASP proteins stimulate actin polymerization in concert with cdc42.

In the fractionation experiments demonstrating that cdc42 and Arp2/3 together cause actin polymerization, N-WASP was identified as a third necessary component for this effect (Ma *et al*. 1998). N-WASP binds cdc42, and together they stimulate microspike formation (Miki *et al*. 1998a). The WASP proteins form a direct link from cdc42 to Arp2/3, and this became the first direct mechanism coupling the rho GTPases to actin polymerization. WASP and Scar bind to the p21Arc subunit of Arp2/3 (Machesky *et al*. 1999). WASP, N-WASP, and Scar stimulate Arp2/3′s actin-polymerizing capabilities *in vitro* (Machesky *et al*. 1999; Rohatgi *et al*. 1999; Yarar *et al*. 1999), and cdc42 and phosphatidylinositol 4,5-diphosphate are required to activate N-WASP (Rohatgi *et al*. 1999). The WASP proteins differ in the levels to which they stimulate Arp2/3 activity (N-WASP being the most potent) and in how they are regulated. For instance, Scar appears to be indirectly downstream of rac, and lacks a cdc42-binding region (Miki *et al*. 1998b; Miki *et al*. 2000). Insulin receptor substrate p53 (IRSp53) serves as the necessary binding intermediary between rac and Scar (Miki *et al*. 2000). Thus, N-WASP, WASP, and Scar may provide a way for different signals to achieve different degrees of actin reorganization by modulation of Arp2/3′s branching/nucleation activities (Fig. 3).

The WASP/Arp2/3 mechanism for actin branch nucleation appears to be involved in neurite growth in the nervous system. N-WASP is found throughout the brain and is concentrated at nerve terminals (Fukuoka *et al*. 1997). p34Arc and p21Arc are concentrated in the actin filaments of NGF-stimulated growth cones from rat sympathetic neurons and PC12 cells (Goldberg *et al*. 2000). Mutations in the VCA region of N-WASP block NGF-stimulated neurite growth in PC12 cells and hippocampal neurons (Banzai *et al*. 2000), and an N-WASP mutant incapable of binding cdc42 also blocks neurite growth (Banzai *et al*. 2000). The Drosophila version of Scar has a role in axonal development. Mutations in Scar disrupt the normal growth and bundling of segmental axons, and mutations in the Drosophila Arp3 and ArpC1(p40) proteins also produce abnormal axon development (Zallen *et al*. 2002). Interestingly, mutations in Drosophila WASP do not lead to abnormal axon growth, but lead to problems in cell fate determination, suggesting again that the different WASP proteins regulate Arp2/3 activity under different circumstances. Another potential mechanism for Arp2/3 to be activated in neurons involves the actin binding protein cortactin. Cortactin is found in the lamellipodia and ruffles of growth cones (Du *et al*. 1998). In non-neuronal cells, cortactin binds and activates Arp2/3 via its N-terminal domain, causing both formation and stabilization of actin branches (Uruno *et al*. 2001; Weaver *et al*. 2001).

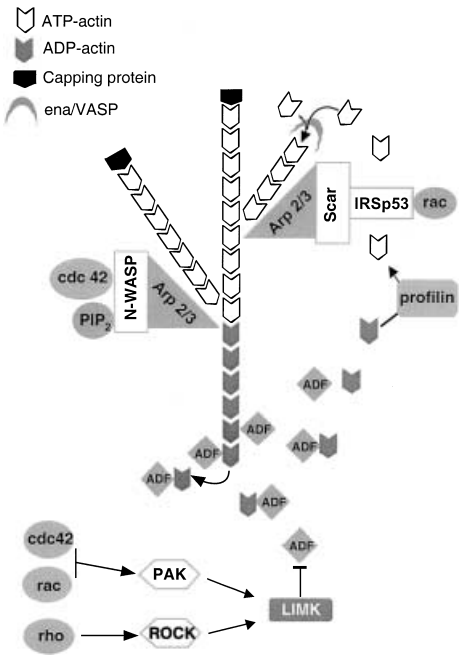

Fig. 3 Arp2/3 and ADF/cofilin work downstream of the rho GTPases to facilitate lamellipodial actin polymerization. Arp2/3 causes new branches to form on the side of existing actin branches. The new branches extend until capped by capping protein. Arp2/3 is activated by N-WASP or Scar, downstream of cdc42 and rac. Ena/VASP proteins facilitate elongation of filaments and delay capping. ADF/cofilin releases ADP-bound actin monomers from older parts of the actin meshwork, which are then recharged with ATP and are re-incorporated into growing actin filaments. ADF/cofilin is inactivated when phosphorylated by LIM kinase. LIM kinase is activated by the rho GTPases via ROCK (rho) and PAK (rac and cdc42).

While there is evidence that Arp2/3 and its activators have effects on neural development, more work is necessary to determine if the Arp2/3 components play the same or as much of a role in neurons as in keratocyte and fibroblast models of actin-based motility. Given the differences between fibroblasts and growth cones in the architecture of their actin meshworks, there must be other components besides Arp2/3 shaping actin filaments. The ena/VASP family of actin-binding proteins could contribute to these differences. Ena/VASP proteins are found at the leading edge

of lamellipodia and at the tips of microspikes in fibroblasts (Nakagawa *et al*. 2001) and are concentrated in the F-actin rich regions of NGF-stimulated sympathetic growth cones (Goldberg *et al*. 2000). In Listeria, ena/VASP binds both ActA and actin, and is thought to link the bacterium to the actin tail (Laurent *et al*. 1999). Without ena/VASP, Listeria motility is greatly attenuated (Smith *et al*. 1996). Paradoxically, ena/VASP appears to inhibit fibroblast motility. Sequestering the endogenous mena to the mitochondria by expressing a protein containing mena-binding sites and a mitochondrial targeting sequence causes fibroblasts to increase translocation speed. Targeting the endogenous mena to the cell membrane instead causes decreased motility (Bear *et al*. 2000). To understand better how ena/VASP proteins promote bacterial motility, but inhibit mammalian cell motility, the effects of these proteins on actin organization were examined. In fibroblasts with sequestered mena, the actin is highly branched and consists of short filaments with barbed ends oriented towards the membrane. When mena is targeted to the cell membrane, though, the actin filaments are much longer, less branched, and have barbed ends with less uniform orientation, more like the actin meshwork observed in growth cones (Bear *et al*. 2002). In fact, mena colocalizes with the actin bundles and filopodia of NG108 growth cones.

Ena/VASP proteins appear to function by binding to the barbed ends of filaments and competing with capping protein, allowing for longer filament extension (Bear *et al*. 2002). Whereas capping protein binds tightly to the barbed ends in the fibroblast model of dendritic nucleation, causing branches to be short, ena/VASP proteins could function to inhibit capping and allow longer filaments to form. How do longer filaments lead to inhibited motility? One mechanism is based on the observation that cells with sequestered mena show more stable lamellipodia, whereas cells with membrane-targeted mena exhibit more dynamic lamellipodia with increased ruffling. Lamellipodia that are more frequently extended and retracted may be less likely to contribute to a total forward translocation of the cell (Bear *et al*. 2002). It is early to tell where ena/VASP proteins would fit into a signaling model, but there is some evidence to suggest they function downstream of rho GTPases. In fibroblasts, IRSp53 serves as an adaptor between cdc42 and mena. Together this complex stimulates filopodia elaboration (Krugmann *et al*. 2001).

However they are regulated, Ena/VASP proteins probably have profound effects on neuronal motility. Ena/VASP proteins are highly expressed in developing cortical plate cells (Goh *et al*. 2002). In order to characterize ena/VASP function during development, pregnant rats were treated with a retrovirus encoding the mitochondria-targeting construct before the first stages of cortical neuron migration. Early born neurons that express this construct end up migrating too far, reaching the superficial layers of cortex normally inhabited by later-born neurons (Goh *et al*. 2002). This result nicely demonstrates that proper regulation of actin polymerization and organization is critical for normal neuronal development. A protein that directly affects actin architecture is required to keep neurons from migrating too far. It will be interesting to discern how ena/VASP proteins in neurons are regulated (i.e. are they up-regulated by inhibitory cues), or if they function generally to slow the rate of cell motility.

Cycling of actin monomers through the polymerized network is controlled by ADF/cofilin and LIM kinase

ADF and cofilin are actin-binding proteins of similar size (~18 kDa), sequence and function. ADF was originally purified from chick brain and found to increase the actin monomer pool (Bamburg *et al*. 1980). Cofilin was purified from porcine brain and increased the actin monomer pool, but also bound F-actin monomers with a 1 : 1 stoichiometry (Maekawa *et al*. 1984). Members of the ADF/cofilin family are ubiquitously expressed in eukaryotic cells and are similar, but distinguishable in function, and able to be interchanged across phyla. (For a thorough review of the distinguishing features in ADF/cofilin expression and function, see (Bamburg 1999).) ADF preferentially binds ADP-bound actin monomers and has two functions, both of which probably contribute to lamellipodium extension. First, ADF causes the depolymerization of actin filaments from the pointed end, increasing the G (monomeric)-actin pool. Profilin binds the released monomers and facilitates ADP/ATP exchange, providing new monomers for addition to the leading edge of the actin meshwork. By increasing the turnover of actin monomers, ADF is largely responsible for the *in vivo* kinetics of actin treadmilling observed in bacterial actin comets or lamellipodia (Carlier *et al*. 1997; Rosenblatt *et al*. 1997). ADF also severs actin filaments, increasing the number of uncapped barbed ends that may undergo polymerization. Epidermal growth factor induces ADF-dependent increases in free barbed ends in MTLn3 cells (Chan *et al*. 2000). While there is currently debate about which function of ADF is key to its facilitation of actin polymerization and membrane extension, the evidence indicates that ADF activity is required for actin-based motility processes.

ADF activity is subject to inactivation by phosphorylation on serine 3. This inactivation is carried out by the serine-threonine kinases LIM kinase (LIMK) and testicular protein kinase (TESK 1 and 2). TESK 1 and 2 are highly expressed in testes, but low levels of both are detected in nervous tissue and thus may have functions pertinent to neural development (Toshima *et al*. 2001a; Toshima *et al*. 2001b; Toshima *et al*. 2001c). LIMK, however, is highly expressed in brain (Mizuno *et al*. 1994; Cheng and Robertson 1995). While searching for a function for LIMK, it was noted that over-expressing LIMK causes an increase in phalloidin-stained F-actin structures, to the point that F-actin filaments form large clumps. The similarity of this phenotype to the

phenotypes of yeast and Drosophila with inactive cofilin led to the discovery that cofilin was the substrate for LIMK (Arber *et al*. 1998). LIMK is the regulatory link between rho GTPase signaling and cofilin activity. Inactive LIMK blocks rac- and insulin-induced lamellipodium formation (Yang *et al*. 1998), as well as rho-induced stress fibers and cdc42-induced filopodia (Sumi *et al*. 1999). Rac and cdc42 signal to LIMK via PAK, which phosphorylates and activates LIMK on threonine 508 (Edwards *et al*. 1999). Rho, via ROCK, similarly activates LIMK by phosphorylation at the same site (Maekawa *et al*. 1999; Ohashi *et al*. 2000). Thus, LIMK and ADF/cofilin are potential effectors of any of the signaling mechanisms that utilize rho GTPase activity to modify actin architecture and dynamics (Fig. 3).

While earlier studies of LIMK indicated that loss of its activity (and thus, increased cofilin activation) inhibits rac and cdc42 actin polymerization (Yang *et al*. 1998; Sumi *et al*. 1999), there is also evidence that LIMK activity/cofilin inactivation inhibits lamellipodium extension (Zebda *et al*. 2000). Considering that ADF/cofilin activity is required for actin dynamics (Carlier *et al*. 1997), the function of LIMK in this process, especially downstream of pro-polymerization regulators like growth factors, rac and cdc42, is less clear. ADF/cofilin activity may be spatially regulated within the actin architecture, with LIMK inactivating ADF/cofilin in particular regions where its activity would interfere with membrane extension. Or, the turnover rate of ADF/cofilin may be regulated by lamellipodium-activating signals, utilizing LIMK to inactivate ADF/cofilin, and a regulated or continuously activated phosphatase to activate ADF/cofilin. There is evidence to suggest this occurs in response to growth factors (see below). Several potential activating phosphatases have been identified, including PP1, PP2A, and PP2B (Meberg *et al*. 1998; Ambach *et al*. 2000). Slingshot (SSH) is a recently discovered family of phosphatases that have apparent specificity for ADF/cofilin (Niwa *et al*. 2002). The details of how these phosphatases are regulated are unknown, but it is possible they may be responsive to activating signals. A model for regulation of actin dynamics that includes both LIMK and cofilin activities would suggest that coordinated activation/inactivation of cofilin is important for membrane extension.

ADFs are likely key modulators of actin dynamics in neuronal cells. ADF travels with actin monomers in the slow component of axonal transport, perhaps functioning in a transport capacity by complexing with the monomers (Bray *et al*. 1992). ADF is expressed in cerebellar neurons, but shows distinct localizations depending upon cell type. ADF is expressed in the dendrites of Purkinje cells, but the axons of mossy and climbing fibers (Lena *et al*. 1991). ADF phosphorylation state is regulated in neurons by second messenger systems and growth factors (Meberg *et al*. 1998). Increases in intracellular calcium and cAMP levels cause a rapid dephosphorylation of ADF in cortical neurons. This dephosphorylation appears to be affected by different phosphatases – protein phosphatase 2B in the case of calcium, and protein phosphatase 1 for cAMP. NGF and insulin also stimulate ADF phosphorylation and ADF localization to ruffles, where its activity is probably required to promote the actin reorganizations associated with lamellipodium formation. LPA, which causes neurite retraction, stimulates ADF phosphorylation, potentially via rho activation of LIMK. Interestingly, growth factors also activate pathways that cause ADF phosphorylation – probably the pathway from the rho GTPases to LIMK. This would suggest that the regulation of ADF/cofilin activity is via phosphate turnover, and that ADF/cofilin needs to be in both activated and de-activated states at specific spatial or temporally distinct points in the dynamic reorganization of the actin cytoskeleton.

The effects of ADF on neurite extension were studied by expressing different mutants of Xenopus ADF/cofilin (XAC) in rat cortical neurons (Meberg and Bamburg 2000). Expression of wild-type XAC increases neurite growth, whereas an inactive XAC shows no increase in neurite extension. A non-phosphorylatable XAC causes a slight increase in neurite length. Neurons expressing wild-type XAC do not exhibit increased F-actin. Instead, combining an inhibitor of actin polymerization with expression of XAC leads to a decrease in F-actin. Together, these observations support the model that ADF facilitates neurite extension by increasing actin turnover, rather than increasing actin polymerization per se.

ADF may also have a unique function in several neurodegenerative processes that lead to a loss of axons and dendrites without cell death (Minamide *et al*. 2000). Neurodegenerative conditions, including ATP depletion, peroxide, and glutamate, cause the formation of 'rods' in axons and dendrites. These rods consist of ADF, cofilin, and actin. Rod formation is induced simply by over-expressing active ADF/cofilin. When large enough, the rods disrupt the microtubules of the neurite and cause distal degeneration.

LIMK may also play a role in growth cone guidance. Semaphorin 3A causes first phosphorylation, then dephosphorylation, of cofilin, suggesting that LIMK may be activated downstream of semaphorin 3A. A cell-permeable peptide fragment of cofilin that inhibits LIMK activity *in vitro* blocks semaphorin 3A-induced growth cone collapse, as does dominant-negative LIMK (Aizawa *et al*. 2001). LIMK activity might be involved in synaptic maturation, as it becomes highly expressed in synaptic terminals during postnatal week 2, long after neurites have formed (Wang *et al*. 2000). Finally, LIMK is one of the genes lost in patients with Williams' syndrome (Donnai and Karmiloff-Smith 2000). Williams' syndrome is caused by a deletion at 7q11.23 spanning several genes, and leads to a number of developmental abnormalities, including cognitive impairment. Potentially, loss of LIMK may lead to problems in

cortical cell migration or axon/dendrite growth, but whether this occurs in Williams' syndrome remains unclear.

Conclusion

Rho, rac, and cdc42 mediate changes in the actin cytoskeleton that affect cell motility and neurite extension. The rho GTPases act downstream of extracellular guidance cues that stimulate motility by organizing actin or that form lamellipodia to drive the cell or neurite forward. Extracellular signals that cause neurite retraction also signal through rho GTPases, often by activating rho, or by inactivating rac/cdc42. Signals from extracellular cues could reach the rho GTPases through a number of GEFs and GAPs. The rho GTPases indirectly affect actin through a number of downstream mediators that control actin polymerization and depolymerization, including Arp2/3 and ADF/cofilin.

The Arp2/3 complex nucleates the formation of new actin branches, and thus initiates the step that causes forward extension of the actin meshwork and subsequent extension of the membrane at the leading edge. Arp2/3 is activated by WASP proteins, some of which receive signals from cdc42 and rac. Extracellular stimulants of neurite extension and motility that activate rac and cdc42 could cause forward extension of the cell membrane via activation of a WASP protein and Arp2/3.

Significant differences exist in the arrangement of actin filaments in fibroblasts/keratocytes and neuron growth cones. Conceivably, there are differences in the regulatory proteins that organize actin between different cell types or between whole-cell lamellipodia and growth cones. The differences in regulation are currently unknown, but ena/VASP proteins are capable of causing actin arrangements that are similar to that observed in growth cones.

ADF/cofilin depolymerizes actin from the pointed end of filaments, and also severs filaments. ADF/cofilin is critical for maintaining the dynamics of actin treadmilling by releasing monomers from the older parts of the meshwork, which recycle to actively growing filaments. Severing of filaments may also lead to forward extension by exposing uncapped barbed ends, where polymerization can take place. ADF/cofilin is inactivated by LIMK, which functions downstream of the rho GTPases, and is potentially activated through dephosphorylation by a number of phosphatases whose regulation is not understood at this point. Since ADF/cofilin activity is required for maintenance of the dynamic actin meshwork, the role of its inactivation downstream of LIMK is unclear. LIMK inactivation of ADF/cofilin may occur at specific locations within the actin meshwork where stabilized actin is required (for instance, right at the leading edge where polymerization is predominant). Or, activity of ADF/cofilin may be coordinately regulated through simultaneous activation by phosphatases and inactivation by LIMK. The localization of LIMK and ADF/cofilin phosphorylation within the lamellipodium and timing of ADF/cofilin inactivation/activation requires further study to understand how regulation of ADF/cofilin activity by the rho GTPases contributes to lamellipodium extension.

The actin motility machinery is common to many cell types and subject to regulation by numerous extracellular signals. In the nervous system, signals that stimulate neurite growth and guide the neurites as they head for their proper targets could be integrated to control the actin machinery. Lamellipodial actin polymerization could be increased when a growth cone detects an attractant, or decreased when an inhibitor cue is contacted. One can imagine that these events occur locally within a growth cone that encounters attractants and repellents simultaneously from different directions, such that multiple signals are spatially integrated to cause localized actin polymerization that subsequently steers an axon along a specific path to a target.

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