Actin machinery: pushing the envelope

Gary G Borisy* and Tatyana M Svitkina†

The reconstitution of microbial rocketing motility *in vitro* with purified proteins has recently established definitively that no myosin motor is required for protrusion. Instead, actin polymerization, in conjunction with a small number of proteins, is sufficient. A dendritic pattern of nucleation controlled by the Arp2/3 complex provides an efficient pushing force for lamellipodial motility.

Addresses
Laboratory of Molecular Biology, University of Wisconsin, 1525 Linden Drive, Madison, WI 53706, USA
*e-mail: ggborisy@facstaff.wisc.edu
†e-mail: tsvitkin@facstaff.wisc.edu

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Abbreviations

ADF	actin depolymerizing factor
GFP	green fluorescent protein
VASP	vasodilator-stimulated phosphoprotein
WASP	Wiskott–Aldrich syndrome protein

Introduction

Progress in understanding complex phenomena has often been achieved by reconstituting elements of a system to display some functional capacity. The movement of test beads powered by the molecular motors kinesin or dynein on microtubules and myosin on actin filaments are classic examples. The protrusive activities of a cell's leading edge, however, have posed a more formidable challenge, presumably because of the greater complexity of the processes involved.

One promising approach to this problem has been developed from the 'rocket-like' motion of the microbial pathogen, *Listeria*, in the cytoplasm of infected cells, which was discovered by Tilney and Portnoy [1] to involve a subversion of the cell's actin machinery to aid the microbe's attempt to infect neighboring cells without subjecting itself to immune surveillance. Similar rocketing movements have since been reported not only for a variety of bacteria and viruses (see [2,3] for reviews), but also for endosomes [4•], external particles [5] and unidentified endogenous vesicles and structures [6,7•], suggesting that the rocketing motion reflects a normal cytoplasmic process. The finding that cell-free extracts can support bacterial motion [8] has allowed the functional assay of the molecular components involved in the rocketing system. In this review, we attempt to synthesize the results of the past year, as well as earlier contributions, and to provide a coherent overview of the molecular basis of protrusive motility.

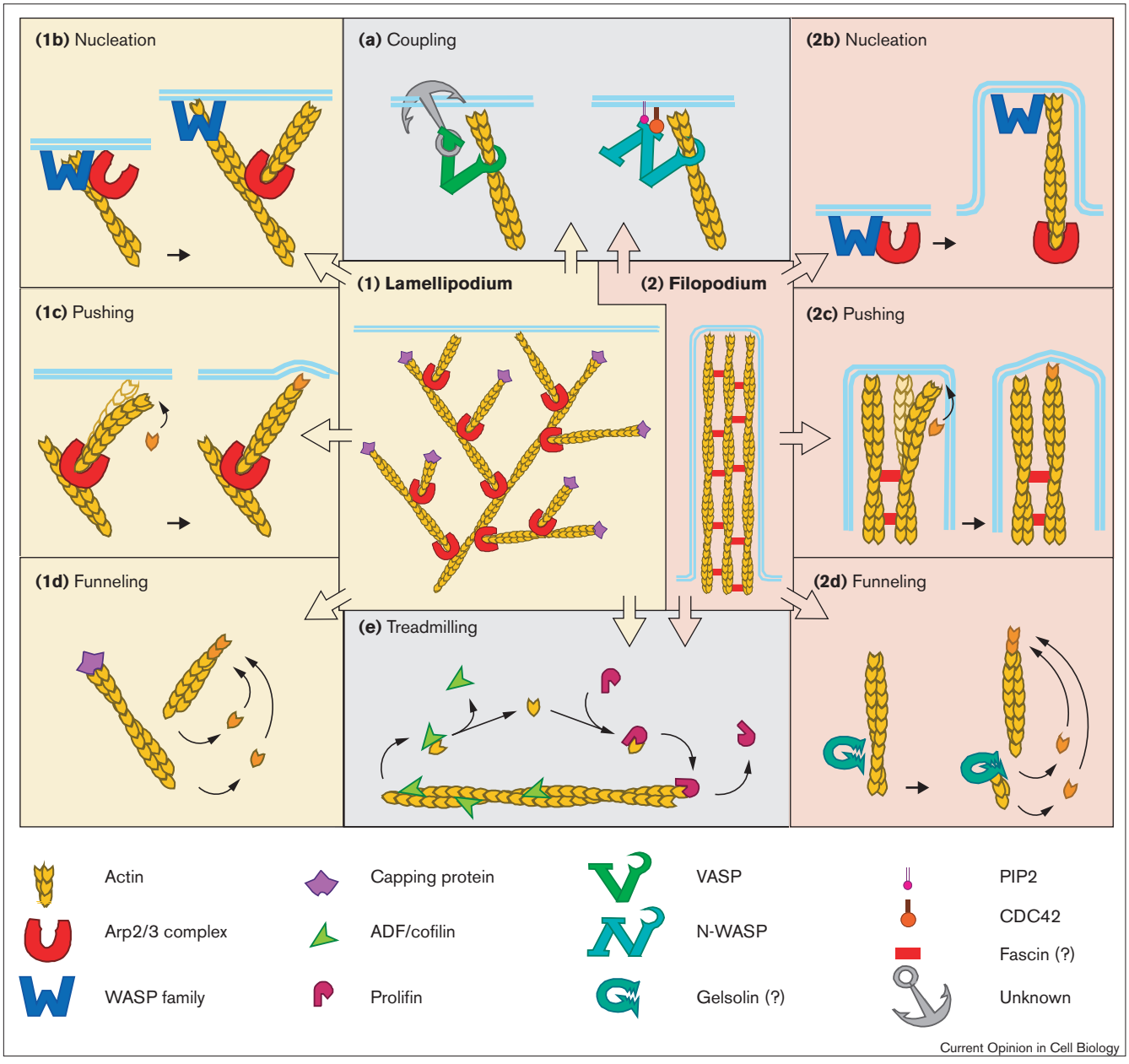
The molecular players

Through a combination of biochemical, genetic and cell biological approaches, an expanding cast of characters has been identified to be involved in actin filament nucleation, including components of a signaling cascade leading from small GTPases through members of the WASP family to the Arp2/3 complex [9••–13••] (reviewed by Mullins (pp 91–96) in this issue). Supporting players are molecules that modulate the dynamic properties of actin filaments by coupling them to the surface (Ena/Mena/VASP family), capping their ends (capping protein, Arp2/3 complex), crosslinking them (α-actinin, fascin, filamin), severing them (gelsolin, ADF/cofilin), sequestering subunits (thymosin β4), promoting dissociation from the pointed end (ADF/cofilin) and regulating association at the barbed end (profilin) (see reviews by Cooper and Schafer (pp 97–103) and by Bartles (pp 72–78) in this issue). Further, when microbial pathogens arrive by means of the actin machinery at the cell surface and begin to protrude beyond the cell or into neighboring cells, an additional set of characters may be called into action — ones involved in the interaction of actin filaments with the cell membrane (ezrin/radixin/moesin) [14,15,16•].

Different species of microbial pathogens apparently have individualized strategies, taking over the actin-based machinery by intervening at different steps in the overall process. Remarkably, the molecules involved in microbial rocketing have proved also to be involved in lamellipodial and filopodial protrusion. These sheet-like and spike-like processes, respectively, represent two different components of protrusive activity of crawling cells. Thus, the rocketing motion may be significant not only for understanding an apparently novel system of intracellular transport but also for understanding a key mechanism of crawling motility. Cell-free extracts are powerful analytical tools [17]; nevertheless, their complexity imposes limitations on the strength of the conclusions that can be drawn. Consequently, the holy grail in this area has remained the full reconstitution of motility with purified proteins.

Actin polymerization has long been known to be essential for lamellipodial and filopodial protrusion and for rocketing motion. The more challenging issue has been whether actin polymerization is the driving mechanism for movement. Basic thermodynamics and force calculations indicate that the free energy of ATP hydrolysis that accompanies actin polymerization provides sufficient energy to do the work of lamellipodial protrusion [18]. Further, biophysical modeling has proposed a plausible 'elastic Brownian ratchet' mechanism by which the energy may be transduced into motion [19], and quantitative analyses are consistent with the view of an 'actin nanomachine' at the leading edge [20•]. However, it has hitherto not been possible to exclude an alternative mechanism in which a member of the myosin family is responsible for driving

Figure 1



Functional steps for the two major protrusive structures of crawling cells, lamellipodia (1) and filopodia (2). Both mechanisms require the barbed ends of actin filaments to be held close to the surface being pushed. **(a)** VASP is involved with coupling with both structures, through an as yet unidentified molecule. An additional coupling pathway is provided by N-WASP, which binds PIP2 and is triggered by Cdc42. Members of the WASP family activate the Arp2/3 complex and nucleate formation of actin filaments on pre-existing filaments. **(1b)** In lamellipodia, activation and nucleation are repeated to generate a dendritic array of filaments; **(2b)** in filopodia, activation and nucleation need only occur once. Actin filaments are thought to push against the surface by an elastic Brownian ratchet mechanism **(1c, 2c)**. Nucleation followed by capping of barbed ends in lamellipodia **(1d)** or severing, followed by capping of barbed ends in filopodia **(2d)**, produce an excess of free pointed ends compared to barbed ends, leading to a more rapid growth of remaining barbed ends (known as funneling). The intrinisc low rate of treadmilling of actin filaments is accelerated by the synergistic action of cofilin and profilin **(e)**.

protrusion, with actin polymerization merely 'keeping up' and providing the substrate on which force is exerted.

In a landmark result, the holy grail has been claimed: the reconstitution of rocketing motility at physiologically significant velocities has now been achieved from purified components [21••], and the findings provide definitive evidence for several important conclusions. Motility has been reconstituted for two systems. One is *Listeria monocytogenes*, for which the only essential

bacterial protein is ActA; the other is *Eschrichia coli* expressing IcsA, the only essential bacterial protein for motility of *Shigella*, which have been transfected into *E. coli* as a non-pathogenic substitute. *Listeria* bypasses most of the cellular signaling cascade, and through ActA, directly activates the Arp2/3 complex [22••] and, thus, actin polymerization [23], whereas *Shigella* (*E. coli* IcsA), intervenes upstream in the signaling pathway and recruits N-WASP [24•], which then activates the Arp2/3 complex [11••]. Consequently, *E. coli* expressing IcsA were coated with N-WASP before reconstitution. Remarkably, besides actin, motility required only the Arp2/3 complex, ADF/cofilin and capping protein [21••]. VASP, although not essential, was very important for *Listeria* movement, increasing its velocity ten-fold; profilin, also not essential, increased velocity two- to three-fold; and α-actinin, although not affecting velocity, improved the regularity of motility. These results demonstrate that a relatively simple actin polymerization machinery is sufficient to drive rocketing motion. No myosin motor is required. The definition of a set of minimal components for motility provides a foundation for considering the specific role of each component within the motility process and for determining how the rocketing motion of microbes relates to the cellular activities of lamellipodial and filopodial protrusion. We will consider the roles of the individual components in turn (Figure 1).

Coupling the actin machinery to the surface

For both microbes and cells, it is necessary to target the motile machinery to the proper sites. Bacteria solve this problem by expressing special proteins on their walls (ActA, IcsA) that activate the machinery and physically couple it to the bacteria. The bacteria can be substituted by synthetic beads coated with either ActA [25•] or WASP [13••] which exhibit motility in cell extracts. Because VASP can bind both ActA and actin, it has been proposed to serve as a sliding connector between ActA and an actin filament to keep the growing barbed end near the bacterium wall and thus increase the efficiency of pushing [26•]. In *Shigella* (or *E. coli* IcsA), the connection between the bacterium and the tail is mediated by N-WASP, which binds IcsA, F-actin and the Arp2/3 complex, and which may function like VASP to keep barbed ends close to the bacterial surface [27••]. In cells, VASP family proteins may play a role similar to that proposed for *Listeria*, as they have been found at the tips of filopodia in the growth cone [28•] and the extreme leading edge of lamellipodia and filopodia in fibroblasts in amounts correlating with the rate of protrusion [29•]. Yet, the mechanism of VASP interaction with the membrane is uncertain. One possible intermediate is zyxin, which interacts with VASP [30]; however, recent results on expression of green fluorescent protein (GFP)–zyxin gave clear localization at focal adhesions but not at the leading edge [31•].

Another possibility for the targeting of actin polymerization to the membrane is N-WASP, which, in addition to the Arp2/3 complex, also interacts with the small GTPase Cdc42 and with phosphatidylinositol (4,5) biphosphate (PIP2), and thus may link actin filaments to the membrane through these molecules [11••]. Another WASP family member, Scar, appears to have a critical role in targeting the Arp2/3 complex to lamellipodia [9••]. The intracellular parasite, Vaccinia virus, intervenes in the actin-polymerizing machinery earlier than other known parasites. Tyrosine-phosphorylated viral protein, A36R, recruits adaptor protein Nck and N-WASP to the actin–tail-virus interface [32•], suggesting that more membrane-targeting proteins may yet be discovered.

How does actin really push?

Given that the actin machinery is coupled to a microbial or cellular surface, how does polymerization actually generate a pushing force? All models require the polymerizing actin to be crosslinked in some way or to be anchored to the substratum; otherwise, the force of polymerization would drive the filaments rearward instead of the surface forward. A problem for polymerization models to solve is how can a subunit elongate a filament abutting a surface?

A solution to this problem is the 'elastic Brownian ratchet' model [19] which envisages the actin filament as a spring-like wire that is constantly bending because of thermal energy. When bent away from the surface, a subunit can 'squeeze' in, lengthening the wire. The restoring force of the wire straightening against the surface actually delivers the propulsive force. From the measured stiffness of actin filaments, Mogilner and Oster [19] calculated that the length of the 'pushing' actin filament (that is, the 'free' length beyond the last crosslinking point) must be quite short — in the 30–150 nm range. Beyond this length, thermal energy would be taken up in internal bending modes of the filament and pushing would become ineffective. These considerations are important because the motility reconstitution results [21••] demonstrate that the crosslinker, α-actinin, is not essential *in vitro*, and, moreover, its absence does not even diminish velocity. This result was unexpected because a previous study using an inhibitory protein indicated that α-actinin was essential for movement *in vivo* [33]. Perhaps, α-actinin is required *in vivo* but not *in vitro* because the resistance to motion is greater in cytoplasm than in buffer. Even in buffer, however, the theoretical considerations indicate that a crosslinker is required. If this conclusion is correct, the reconstitution results tell us that a component other than α-actinin must be serving the crosslinking function. This component is likely to be the Arp2/3 complex.

Arp2/3 complex mediates dendritic nucleation

The Arp2/3 complex [34•,35•] combines several properties that are consistent with a role in coupled nucleation and crosslinking of actin filaments — a process called dendritic nucleation ([36••]; see [37•] for a review).

When activated by members of the WASP family or the bacterial equivalent ActA, the Arp2/3 complex binds to the side of a pre-existing filament, nucleates a new actin filament and caps its pointed end, resulting in a Y-junction with a characteristic angle of ~70°. After nucleation, the activator protein (a WASP family member or Act A) dissociates from the Arp2/3 complex and is available to activate another nucleation event. Consistent with this idea, the Arp2/3 complex in cells localizes to the branch point of Y-junctions between actin filaments in lamellipodia [38••]. The Arp2/3 complex is also localized along the length of microbial rocket tails [23], suggesting the existence of a similar branched structure. However, existing information on the supramolecular structure of rocket tails is contradictory. The original microscopic analysis [39], as well as subsequent structural studies [16•,40] indicated that actin filaments are short and oriented at an angle to the tail axis, which is consistent with a branched structure, but a later analysis [15] suggested that a significant fraction of the filaments were long and co-axial with the tail. It will be important to carry out further structural analysis to resolve this issue and determine whether a dendritic organization occurs in rocket tails as it does in lamellipodia.

In contrast, the Arp2/3 complex is not found along the length of filopodia [38••], suggesting that it does not play a role in filopodial elongation, even though it may be critical for their initiation. This is compatible with the axial organization of actin filaments in filopodia. It should be noted that some microbes, namely, *Rickettsia*, may have developed a filopodial mode of intracellular transport. Their tails have linear, co-axial actin filaments and Arp2/3 is absent from their length [16•]. Actin dynamics in *Rickettsia* tails are significantly slower than in *Listeria* [41•], and more similar to the dynamics in filopodia [42•,43•] as opposed to lamellipodia. The similarities and differences of microbial transport with lamellipodia and filopodia reinforce the idea that they may be considered as model systems for understanding each kind of cellular protrusion.

ADF/cofilin and profilin accelerate steady-state treadmilling

Another issue is to distinguish between transient and steady-state protrusion. The rocketing motion of microbes and the gliding of keratocytes are steady-state processes, whereas the response of fibroblasts to growth factor [44,45•] or the activation of platelets [46,47] or the chemotactic response of neutrophils [7•] clearly show important transient responses. A polymerization-driven motility mechanism must be capable of accounting for steady-state movement at observed velocities of 5–10 μm min^{-1} as well as transient responses. Steady state is an important consideration because the balance of reactions required by the steady-state process puts severe constraints on available mechanisms. The high velocities are important because they indicate that the actin filaments are highly dynamic — a typical keratocyte lamellipodium of 10 μm depth can completely turn over within 1–2 minutes. The rocket motility reconstituted *in vitro* [21••] approached these velocities, attaining speeds in the range 2–3 μm min^{-1}. In the filament treadmilling model [48], steady state is achieved by a balance of growth of actin filaments at the barbed end and shortening at the pointed end. Growth is determined by the concentration of available subunits and can be made indefinitely high. The problem is that, in the steady-state process, mass balance requires that growth at the barbed end will ultimately be limited by dissociation of subunits from the pointed end and this process for pure actin filaments is slow, 0.2 s^{-1}, which corresponds to 0.04 μm min^{-1} — approximately two orders of magnitude slower than the observed motility. Thus, additional components are needed to accelerate the pointed-end dissociation.

ADF/cofilin and profilin [49,50,51•,52•] are two components that apparently work together to speed up treadmilling [53•]. ADF/cofilin binds to actin-ADP filaments and, in the steady state, increases dissociation from the pointed end 25-fold and speeds propulsion of *Listeria* in extracts [54]. Under other experimental conditions, ADF/cofilin reduces the length of rocket tails [55]. Both results demonstrate that ADF/cofilin is responsible for accelerating the turnover of actin filaments [56]. ADF/cofilin also can sever actin filaments [57,58•], but it should be noted that this activity *per se* cannot speed up the treadmilling of pure actin filaments in the steady state because for every pointed end that can depolymerize, severing also creates a barbed end that can grow. The effect of ADF/cofilin on dissociation may be even stronger. Because dissociation of ADF/cofilin from the pointed end is a reversible reaction, the net dissociation in the steady state is the algebraic balance of microscopic dissociation and association reactions. A factor that suppressed the association reaction would have the effect of promoting net dissociation. Such a factor is profilin.

Profilin is a cytoplasmic protein whose primary binding partner is soluble actin [59•]. The profilin–actin complex has the unique property that it can bind to and elongate the barbed end but not the pointed end. Profilin binds more strongly to ATP-actin than to ADP-actin. In the presence of steady high levels of ATP favoring nucleotide exchange, profilin will compete successfully with ADF/cofilin for actin subunits [53•,60•], thus suppressing the back association of actin at the pointed end and resulting in a higher net dissociation rate. At the resulting higher actin concentrations, spontaneous assembly becomes energetically more favored. Profilin also has the desirable property of suppressing spontaneous nucleation. Thus, profilin provides a pool of actin subunits capable of adding only to the barbed end and thus acts synergistically with that of ADF/cofilin, leading to a 75–125-fold increase in treadmilling [53•]. These

activities alone raise the treadmilling rate of actin filaments close to physiological speeds; but there is one more component yet to be considered — capping protein ([61]; reviewed by Cooper and Schafer (pp 97–103) in this issue).

Capping protein decommissions ineffective barbed ends

Capping protein is an essential component for the reconstituted motility of microbial rockets [21••], an apparently paradoxical result as it seems to be antagonistic to the driving force for motility, which is polymerization at barbed ends. Visualization in living cells by tagging with GFP indicates that capping protein is enriched in active lamellipodia in which Arp2/3 is present [62•]. The function of capping protein can be understood as to control where actin filaments 'push'. Only those filaments at the microbial (or lamellipodial) surface are effective in generating propulsive force. Barbed ends elsewhere would non-productively consume actin subunits and compete with effective barbed ends. Thus, the rationale of polymerization-driven movement is to cap barbed ends globally while permitting uncapped barbed ends locally [63].

This differential capping carries several implications. First, in terms of the elastic Brownian ratchet model which requires short 'free' filaments, it provides a mechanism for taking out of commission those filaments that grow too long and therefore lose effectiveness. Second, by maintaining most barbed ends in a capped state, the nature of the treadmilling steady state is altered. In the classical treadmilling condition, both ends of a filament are free (and therefore present in equal numbers) and the steady-state concentration is intermediate between the critical concentrations for the two ends. With barbed ends capped (except for the privileged few) free pointed ends will outnumber free barbed ends, creating a 'funneling' effect in which many depolymerizing pointed ends feed subunits to a few growing ends [64,65]. The free barbed end will grow rapidly because the combined effects of ADF/cofilin and profilin allow a steady-state concentration of subunits greatly in excess of the intrinisic critical concentration for the barbed end.

The array treadmilling model

The funneling condition, however, cannot be maintained without auxiallary hypotheses. The few growing filaments will elongate at the expense of the depolymerizing capped filaments, which will ultimately disappear. Thus, the true steady state for funneling requires the continuous production of new filaments. This could be achieved by severing old filaments, as proposed by the 'treadsevering' model [66]. In this model, as the privileged growing filaments elongate, their distal ends are severed, creating new barbed ends which become capped and new pointed ends which depolymerize. In support of this model, breakage of filopodial bundles from the rear and depolymerization of the resulting fragments has been observed as a calcium-induced response of neurons [67]. The severing activity of ADF/cofilin in combination with the barbed end capping activity of capping protein could accomplish treadsevering. Another possibility is that gelsolin, by being both a strong severing molecule and barbed-end capper, could serve this function. Although not necessary for *in vitro* rocket motility [21••,55] studies on gelosin-null cells indicate that it makes a contribution in a cellular context. Dermal fibroblasts showed reduced ruffling and motility [68] and, in growth cones of neurons, retraction of filopodia was impaired [69].

An alternative to treadsevering is continuous nucleation such as has been proposed in the dendritic nucleation and array treadmilling model [36••,37•,38•]. In this model, new filaments are continuously being nucleated (by Arp2/3 complex) on pre-exisiting filaments forming Y-junctions and on short filaments effective at pushing. A key issue here is what makes a few filaments privileged in contrast to many others that become capped by abundant capping protein. One possibility is that nascent filaments are protected from becoming capped by a Cdc42-dependent mechanism [70•]. Protection may be afforded by their interaction with VASP or members of the WASP family or other yet unknown proteins. The strong proportionality between GFP–VASP levels at the leading edge and the rate of protusion [29•] suggests that a VASP–actin filament interaction is rate limiting. This interaction may be equivalent to 'protection' because the overall rate of protrusion is thought to depend on the sum of pushing forces exerted by individual actin filaments [19]. Protection of filament barbed ends may also be dependent upon their stiffness, which is related to their terminal 'free' length. As the filaments grow longer, they lose protection, become capped and drop behind the leading edge to be replaced by newly nucleated filaments. Each nucleation event creates both a barbed and a pointed end. The pointed end is initially thought to be capped by the Arp2/3 complex at a Y-junction. However, in the steady state, debranching (or 'pruning') must ultimately occur permitting depolymerization from the pointed end and recycling of the Arp2/3 complex. The details of 'pruning' remain to be worked out. It could occur by ADF/cofilin-induced depolymerization [71•], by spontaneous dissociation of Arp2/3 from a Y-junction, or by regulated dissociation of Arp2/3. As no regulatory enzymes are present in the reconstituted motility system, however, the activity of ADF/cofilin is probably sufficient for the pruning.

We should note how the array treadmilling model compares and differs with an earlier 'nucleation-release' model [72]. Both models stipulate nucleation and release from coupling molecules at a surface; however, in the earlier model, filaments are nucleated individually and become connected to each other later. Their ends are free and depolymerization is allowed to occur from either end, perhaps primarily from the barbed end. In the array treadmilling model, new filaments are 'born' connected

to the array and remain connected after release from the surface. Barbed ends become capped after decoupling from the pushing surface and remain capped. Pointed ends are initially capped and depolymerization occurs from pointed ends after their uncapping.

In summary, in the array treadmilling model [37•,38••], each of the components critical to the *in vitro* motility system [21••] has a specific role which is coming into focus. Steady-state protrusion is the result of a cycle in which the Arp2/3 complex upon activation gives birth to a new filament branch and caps its pointed end; the filament elongates at the barbed end by addition of actin or profilin–actin complexes; the nascent filament's barbed end is initially protected from being capped by an as yet undetermined mechanism with VASP or a WASP family member keeping the barbed end close to the surface; ineffective barbed ends are decommissioned by becoming capped by capping protein; debranching and recycling of Arp2/3 complex occur with uncapping of the pointed end; and the resulting free pointed ends, catalyzed by the action of ADF/cofilin, shorten at physiological velocity, providing subunits for further growth. Finally, it should be noted that the treadsevering and array treamilling models are not mutually incompatible and each mechanism may have its own role. Dendritic nucleation and array treadmilling are likely to be the primary mechanisms for lamellipodial protrusion. Treadsevering may be more important for the turnover of filopodia. It remains to be determined which mechanism operates under which conditions in a cellular context.

Conclusions

Many pieces in the puzzle of motility have been fitted together in the past year, and now seems to be a good time to step back and look at the picture. We have attempted to do so in this review. Although the puzzle remains incomplete and some of our suggestions may be wrong in detail, the benefit of an overview is that it provides a conceptual framework in which to evaluate the pieces yet to come.

Although this account has focused exclusively on the mechanism of actin polymerization-driven protrusion, we do not wish to imply that this is the sole mechanism of protrusive motility. The reconstituted motility system demonstrates that actin polymerization is sufficient to drive the rocketing motility of microbes. Actin polymerization is also a plausible mechanism for driving lamellipodial and filopodial protrusion. However, these results do not exclude other mechanisms from contributing to cellular protrusion. The complexity of cellular protrusion indicates that molecules in addition to the *in vitro* minimal set are almost certainly involved. Although no myosin motor is required for microbial motility *in vitro*, myosins may be involved in cellular protrusion, either directly in generating force or in delivery of components needed at the leading edge. The recent exciting discovery that myosin VI, unlike all other myosins, has a force polarity towards the pointed end ([73••], see the review by Hammer (pp 42–51) in this issue) raises all sorts of new opportunities for cellular traffic. Besides, lamellipodia and filopodia are not the only kind of cell protrusions. Some cells such as pollen tubes and certain amoebas extend themselves by cylindrical processes with hemispherical fronts. Explanations for this kind of protrusion have included hydrostatic or osmotic pressure as the driving force [74]. Finally, cogent arguments have been advanced for lipid flow resulting from membrane recycling itself being the driving force [75]. It is not at all unreasonable to suppose that multiple mechanisms for cellular protrusion exist, with certain ones being dominant in particular cellular contexts. The diversity of cellular behavior suggests that we should not keep our eyes wide shut.

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