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On the Crawling of Animal Cells

Thomas P. Stossel

Cells crawl in response to external stimuli by extending and remodeling peripheral elastic lamellae in the direction of locomotion. The remodeling requires vectorial assembly of actin subunits into linear polymers at the lamella's leading edge and the crosslinking of the filaments by bifunctional gelation proteins. The disassembly of the crosslinked filaments into short fragments or monomeric subunits away from the leading edge supplies components for the actin assembly reactions that drive protrusion. Cellular proteins that respond to lipid and ionic signals elicited by sensory cues escort actin through this cycle in which filaments are assembled, crosslinked, and disassembled. One class of myosin molecules may contribute to crawling by guiding sensory receptors to the cell surface, and another class may contribute by imposing contractile forces on actin networks in the lamellae.

Ungainly in comparison to flying, swimming, or running, the crawling motions of single animal cells are nevertheless profoundly important. Countless foraging ameboid cells creep through the soil and beneath the waters, and movements of single cells are fundamental for the life of multicellular beings. After conception, selected cells of the developing mammalian zygote invade the uterine wall to establish the placenta, while the intricately programmed migration of other cells within the embryo shapes the complex form of the emerging organism. Legions of white blood cells patrol body tissues to engage hostile microorganisms. Locomotion of fibroblastic and epithelial cells heals wounds, and osteoblasts and osteoclasts crawl about as they remodel bone. The crawling malignant tumor cells that invade and disrupt normal tissue architecture account as much or more for the lethality of cancer than does uncontrolled growth. The wiring of the nervous system during fetal development takes place when peripheral segments of neuronal cells crawl, stretching out dendrites and axons; as delicate processes, the axons can extend for enormous distances. Related functionally to crawling are phagocytosis and cell spreading. The latter permits tissue macrophages and lymphocytes to grasp one another as they process antigens to program specific immune responses. Motions analogous to crawling also transform blood platelets from smooth disks to spiny spheres that plug vascular leaks after injury.

Research programs directed at describing cell crawling behavior are on a collision course with studies investigating the molecular aspects of sensory and motor machinery, but the richness of detailed information emerging from these investigations can obscure their interrelationships. This article aims to integrate these programs, based on the optimistic view that, despite large information gaps, it is reasonable to speculate on how cells crawl. Emphasized is one of the most difficult and confusing aspects of the crawling mechanism: the role of the self-assembling protein actin in the cell surface rearrangements that accompany crawling (*1*).

The author is with the Division of Experimental Medicine, Brigham and Women's Hospital, Department of Internal Medicine, Harvard Medical School, Boston, MA 02115.

Crawling

Cells initiate crawling in response to surface stimulation, usually in the form of soluble or substrate-bound extracellular molecules that act as sensory cues. The agonists, which include proteins, peptides, lipids, and small molecules, bind to specific externally disposed receptors. These sensors may be of the serpentine type coupled to heterotrimeric guanosine triphosphate (GTP)–binding protein (G protein), which pass repeatedly through the membrane, single-spanning proteins with tyrosine kinase activities in their cytoplasmic tails, or even receptors linked to the outer membrane leaflet by glycolipid anchors with no obvious direct connections to intracellular components (*2*). Less specific physical perturbations of the plasma membrane, such as exposure of cells to electrical fields, positively charged beads, or mechanical distortions, can also produce crawling behavior (*3*). The interaction of sensory influences with the membranes of cells initiates transmembrane signals that activate the complex machinery of crawling (*4*).

The crawling cell invariably has a polarity, the most conspicuous feature of which is leading lamellae, thin veillike structures that are free of organelles visible in the light microscope and that extend from the organelle-rich cell body in the direction of movement (5) (Fig. 1A). The lamellae, which may have smooth or serrated anterior edges, appear to glide forward, pulling the cell body passively behind them. As the lamellae advance, they consolidate their forward progress by transient attachment to the underlying surface. Thus, the crawling machinery requires, in addition to the restructuring of the peripheral substance of cells, reversible adhesion of externally disposed plasma membrane molecules to the substrate along which the cell crawls (6).

Cells put out variable numbers of lamellae, but only lamellae that contact the substrate on which the cells are crawling are capable of generating locomotion. Of these, usually one becomes dominant in an actively crawling cell. As the dominant lamella expands, the others withdraw coordinately. Contractions at the junction of the lamella and the cell body and also at the rear of the cell deform the cell body and

propel its internal contents forward toward the leading lamella. The flat anterior portion of the polarized cell and its narrow tail give it a shape that resembles a hand-held fan or hand mirror (*5*).

The advance of the leading lamella may proceed in different ways, depending on the cell type or the external environment. Cylindrical spikes, called filopodia, may protrude, and they sometimes coalesce to form extensions of the leading lamella. Alternatively, the protrusion takes place by the expansion of small bubbles or blebs that subsequently flatten. A typical feature of an advancing lamella is a vertical thickening that begins at the leading edge and then moves rearward. The most extreme version of this phenomenon is ruffling, in which the leading edge of the lamellar veil lifts upward from the substrate and, while continuing to protrude, bends backward to a position perpendicular to the adherent portion of the lamella. The dorsally extended veil, called the ruffle, then migrates rearward across the plane of the attached lamella toward the cell body. During its retrograde journey, the ruffle continues to fold over backward as it simultaneously retracts and eventually dissipates near the cell body (*5*).

As the lamella advances, selected external molecules on the lamellar surface diffuse randomly in the plane of the membrane. Others move forward toward the leading edge where they remain immobilized, and still others, especially those crosslinked by antibodies or lectins, migrate rearward, a phenomenon known as capping (*7*). The term "cortical flow" encompasses ruffling, capping, and related centripetal movements of lamellar substance. The lamellae of spreading cells exhibit circumferential cortical flow from the entire periphery to the cell body (*5*). Although cortical flow always accompanies cell locomotion, this process can be slower than lamellar protrusion and therefore is probably not directly responsible for it (*5, 8*).

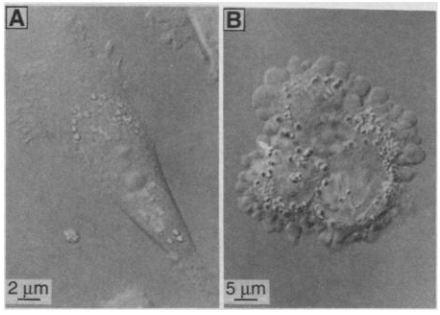

Fig. 1. Appearance in a phase-contrast microscope of (**A**) plastic-adherent mouse NIH 3T3 fibroblasts, showing the handmirror-shaped polarized morphology characteristic of crawling cells, and (**B**) a cluster of plastic-adherent human malignant melanoma cells that lack detectable actin-binding protein (ABP) (*23*). The cells were in tissue culture medium that contained serum as a source of stimuli for surface activity. Note the spherical blebs around the free circumferences of the cells.

The Lamellar Substance: Crosslinked Actin Filaments

Isolated organelle-free lamellae, which can be discharged from the bodies of crawling cells by heating, are capable of rudimentary locomotion, proving that these structures have all the sensory and motor equipment required for crawling (*9*). The leading lamella of the advancing cell and certain of the cell's appendages, such as ruffles, have elastic recoil and are sufficiently stiff to either push objects out of its way or deform them (*10*). Lipid bilayers, even when buttressed by a two-dimensional lamina composed predominantly of the fibrous protein spectrin, are too weak to account for such rigidity (*11*). This stiffness is attributable to actin filaments, rod-like double helical protein polymers that are noncovalent assemblies of monomeric subunits. The subunits within actin filaments have some degree of mobility (*12*), but, macroscopically, the filaments behave like stiff rods that resist stretching, twisting, or bending (*13*). Therefore, they can manifest bulk elastic behavior when crosslinked into a continuous matrix that invests the space within the leading lamellae (*14*).

High-resolution electron micrographs of the front end of leading lamellae (Fig. 2), from cells that lack membranes but were prepared so as to preserve their three-dimensional structure, reveal actin filaments that were initially described as kinked or bent (*15*). The branch spacing at the leading edge is often very regular, and distances of 100 nm or less have been measured between the lattice branch points (*16*). Behind the front of the leading edge, longer actin filaments crisscross at more oblique angles. In cells that collapsed during preparation for electron microscopy, these filaments appear sufficiently long to form what has been called a "peripheral weave" (*17*). Actin filament bundles traverse the relatively isotropic high-angle actin filament network that spreads blood platelets (*18*).

Bundles are also prominent in thin filopodia and along the ventral surface of substrate-adherent cells in tissue culture. In the latter, the bundles, known as stress fibers, terminate at focal contacts, where the membrane tightly adheres to the substratum. Stress fiber bundles are contractile and are more important for static attachments of cells than for locomotion (*5, 19*). Actin filament–crosslinking proteins are primarily responsible for these diverse actin filament configurations in the leading lamella (*20*) (Fig. 3).

Actin filaments tend to align spontaneously in parallel bundles (*21*), and a number of actin-crosslinking proteins stabilize such bundles (Fig. 3). Two other actin filament crosslinkers, however, counteract this natural tendency and orient actin filaments at high branch angles, accounting for the kinks and branches that dominate the peripheral substance of the leading lamella. An extensively characterized protein with this property is the large homodimer called actin-binding protein (ABP) or filamin.

Molecules of ABP spontaneously connect polymerizing actin filaments to form an elastic gel in vitro. Actin filaments in this gel branch at high angles, and the distance between actin filament branches in this gel is inversely proportional to the ABP concentration (*22*). The branch spacing within such an actin filament network in the peripheral cell lamella is consistent with the molar ratio of ABP to polymeric actin in that structure. The spacing is also sufficiently small to account for the organelle-excluding property of the lamella, yet large enough for the perfusion of water, salts, soluble proteins, and small vesicles (*15, 16*). This relatively orthogonal actin filament configuration, which has been observed in electron micrographs, is consistent with mechanical properties of such networks defined by rheological studies (*22*). It provides a large surface coverage with a minimum of actin-polymer mass, which is ideal for rapid expansion and shrinkage in any direction by peripheral assembly and disassembly.

The importance of ABP for the lamellar substance is exemplified by human tumor cells that lack ABP: they have unstable plasma membranes that bleb circumferentially rather than spread into lamellae (Fig. 1B). Expression of ABP in these cells by genetic transfection stabilizes the mem-

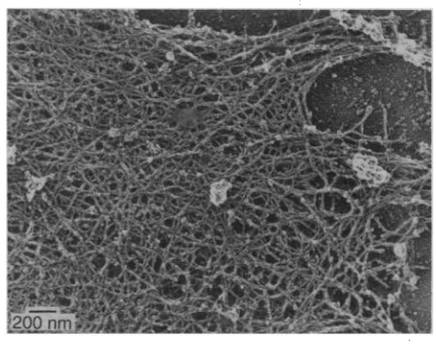

Fig. 2. Actin network investing the peripheral lamella of a rabbit lung macrophage. The lipid membranes of the cell were removed by extraction with the nonionic detergent Triton X-100. The specimen was then rapidly frozen and rotary shadowed with carbon-platinum and photographed in the electron microscope by J. Hartwig.

branes and facilitates focal spreading (*23*). A dimeric protein of *Dictyostelium discoideum* named 120 kD, which is related in primary structure to ABP, although smaller in mass, also promotes actin filament branching rather than bundle formation. Amebae genetically engineered not to express this protein form lamellae and crawl probably because they contain other proteins of overlapping function, but they have distinctive abnormalities of protrusive behavior (*24*).

In addition to linking actin filaments to one another, structural proteins bind the three-dimensional actin gel to the plasma membrane (Fig. 3) (*20*). The strength of such connections to membrane lipids and proteins varies from comparatively delicate and direct spot welds—such as those mediated by myosin I, annexin II, the spectrin–band 4.1–ankyrin complex, ABP, LSP-1, and MARCKS—to highly concentrated clusters, as found in focal contacts. In focal contacts, a sequence of protein-protein interactions involving the β subunit of integrins, talin, vinculin, paxillin, α-actinin, and others links submembrane actin filaments to the extracellular matrix or to cell-cell contacts (*20, 25*).

The regulation of the stability of these structures is presumably important for local changes in lamellar structure. The binding of calcium-calmodulin to MARCKS or the phosphorylation of MARCKS by protein kinase C inhibits its actin filament crosslinking activity in vitro (*26*). Similar properties have been described for the neuronal protein synapsin I (*27*). Binding of immunoglobulin G to the high-affinity Fc receptor (FcγR1) of myeloid cells releases ABP from the receptor (*28*), and binding of fibronectin to integrin receptors dissociates α-actinin from focal contacts of fibroblasts (*29*). Phosphorylation reactions and guanosine triphosphate regulate the binding affinities of ankyrin for the hyaluronate-receptor glycoprotein CD44 and for the transmembrane glycoprotein CD45, which has tyrosine phosphatase activity in its cytoplasmic tail (*30*). The crosslinking of actin filaments by α-actinin may be regulated by calcium (*31*). Many of the proteins in focal contacts, such as ezrin, moesin, pp125^{FAK}, pp60^{src}, VASP, cortactin, and others, are substrates for protein kinases, have intrinsic protein kinase activity, or both. It is likely that phosphorylation reactions govern binding affinities of these components to each other and to actin. Two of these focal adhesion proteins, radixin (*32*) and tensin (*33*), can bind actin filaments at their membrane insertion sites.

Lamellar Dynamics: The Actin Cycle

A dynamic cycle of actin assembly and disassembly that remodels the actin network in the leading lamella under control of transmembrane signals is central to crawling locomotion. An actin network that is continuously undergoing assembly and disassembly can have mechanical coherence as a gel as long as filament assembly and crosslinking balance filament disassembly. Newly assembled lamellar actin filaments are relatively immobile, which is consistent with such a balance (*34*). The actin cycle operates throughout the entire lamella, although its effects are most evident at the bubbling and ruffling leading edge. Vectorial actin turnover, featuring net addition at the front and net loss at the rear, may explain directional lamellar progression (*35*).

Actin assembly and disassembly (Fig. 4). The formation of actin networks with mechanical rigidity is critically dependent on filament length and number (*14*). Monomeric actin polymerizes spontaneously by a nucleation-elongation mechanism, but the nucleation step is very inefficient relative to elongation. This kinetic behavior of pure actin favors the accumulation of a decreasing number of increasingly long filaments. At steady state, subunits derived from fluctuations in filament length, including the complete depolymerization of short filaments, add to preexisting filaments in preference to creating new nuclei. Spontaneous fragmentation of long filaments can counteract this elongation process (*36*).

In addition to the intrinsic self-association properties of actin are regulatory proteins that bind actin monomers and filaments, some of which function in response to transmembrane signaling (Fig. 5). Some of these proteins can operate both within the peripheral cell substance and at its interface with the plasma membrane to escort actin subunits on and off the lamellar actin network. The same escort protein may promote actin assembly or induce disassembly, depending on what signal it encounters.

A third level of control is the adenine nucleotide hydrolysis and exchange properties of actin that affect its assembly kinetics and its susceptibility to the actions of certain escort proteins. This hierarchical system of controls, progressing from signals to

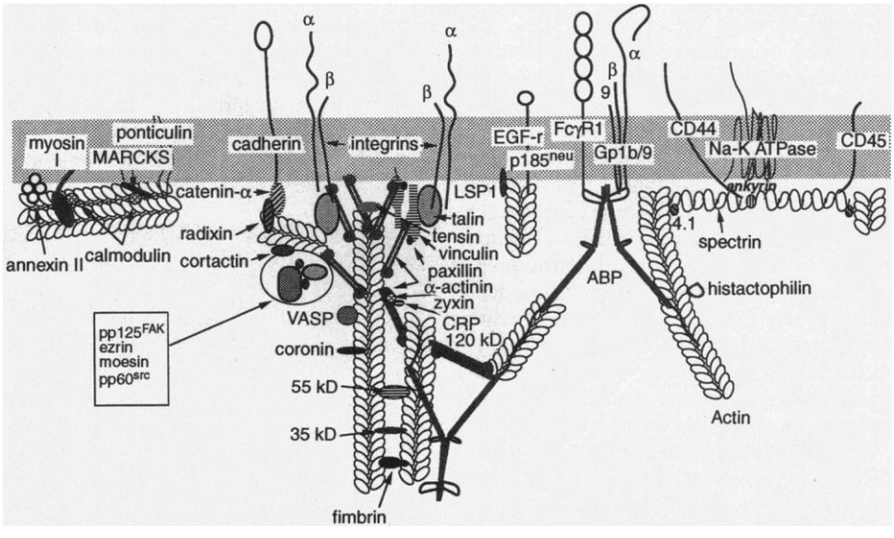

Fig. 3. Composite summary of proteins mediating connections between the plasma membrane and actin filaments and connections between actin filaments in motile cells (*20*). All of these interactions probably do not exist in all crawling cell types, and they cannot all be intact in an actively crawling cell. The figure, however, illustrates the spectrum of possible actin-membrane and actin-actin relations, which affords many points of regulation for receptor-cytoplasmic communication. Relatively low stoichiometries, relative to polymerized actin, of proteins that crosslink actin filaments at high angles (ABP and 120 kD) can interconnect large numbers of lamellar filaments. Other proteins that promote actin bundle formation tend to concentrate within those structures [α-actinin, 35 kD, 55 kD, and fimbrin (also called plastin)].

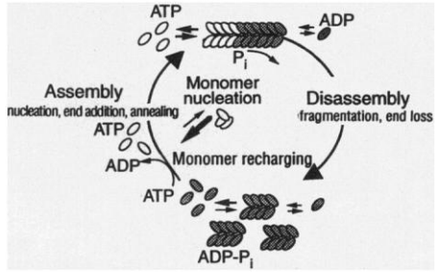

Fig. 4. Assembly and disassembly of purified actin under physiological conditions in vitro (*36*). Actin subunits with bound ATP are white, whereas those binding ADP are shaded. Monomers that fall off the pointed end contain ADP, which exchanges for ATP when it is in excess in the medium; this exchange is another potential control site for actin assembly.

escort proteins to the kinetics of actin assembly influenced by adenine nucleotides, facilitates precise regulation of the actin cycle in space and time.

Switching off actin assembly in the cell. Switching off actin assembly refers to the cessation of actin assembly, induction of active disassembly, or both. Abundant cellular actin monomer-binding proteins (*37–39*) (Fig. 5) can probably prevent nucleation of new filaments even from intracellular actin subunit concentrations estimated at hundreds of micromolar. Additionally, *Acanthamoeba* actobindin specifically attacks nuclei and disaggregates them (*40*).

Actin filaments are polarized, reflecting the structural orientation of actin monomers. This polarity is detectable by an arrowhead appearance conferred by the angle at which myosin fragments bind the actin filaments, thus defining barbed and pointed filament ends (*36*). Actin monomer exchange with the barbed end of the filament is diffusion controlled and an order of magnitude faster than at the pointed end (*36*). Barbed ends can successfully compete with most monomer-binding escort proteins for actin subunits. Thus, for actin monomer–binding escort proteins to keep actin assembly switched off, any actin filaments present must have their barbed ends blocked (capped).

The rate constant for dissociation of monomers from the barbed end, about 2 subunits per second, limits the speed of actin disassembly to the order of 0.3 μm/min (*36*), and the rate constant for dissociation from the pointed end is an order of magnitude slower. Estimates of actin disassembly rates in cells are much higher (*34*), however, and this disassembly can resist inhibitors of subunit exchange with barbed ends, indicating that depolymerization in vivo may occur primarily from the pointed ends of filaments (*41*). Fragmentation of filaments, which can create more ends for depolymerization by end loss, would be one way to accelerate actin disassembly to rates consistent with those observed in living cells.

Calcium ions and protons implicated in cellular signal transduction can activate a family of actin escort proteins that block barbed ends, and some also fragment actin filaments. Gelsolin, a protein of higher eukaryotes so named because it solates actin gels in vitro, is extensively characterized (*42*). Gelsolin and other structurally related ion-activated control proteins [*Dictyostelium* severin, the closely related *Physarum* protein fragmin (*43*), and adseverin (also called scinderin), a protein of secretory cells (*44*)] bind to the sides of filaments with high affinity. There they sever the noncovalent bonds between the monomers and remain tightly adhered to the severed barbed ends.

Another functional effect of some of these and other proteins depicted in Fig. 5 is that they aggregate actin subunits so as to promote nucleation of actin filament growth. This nucleation is, strictly speaking, assembly, because monomers convert to polymers; but in all cases, the nucleating proteins block the barbed ends of the nuclei so that the filaments grow relatively slowly in the pointed direction, especially in the presence of monomer-binding escort proteins. The net effect of these proteins, therefore, is the production of short, mechanically inactive filaments, which is equivalent to severing and capping long ones. Whether these proteins sever or nucleate actin filaments, the escort molecule remains bound to the barbed end of a short actin filament.

Calcium also increases the affinity of MCP (or gCap 39), a protein of higher eukaryotes, for binding to the barbed ends of actin filaments. This protein is similar in primary structure to the NH_2-terminal half of gelsolin, but, unlike gelsolin and other members of this protein family, it does not sever filaments (*45*). The barbed filament ends, which can be targets for this and other capping proteins, may arise from the action of ubiquitous actin subunit–binding proteins, such as cofilin and destrin. These proteins sever actin filaments but do so weakly in comparison with the proteins of the gelsolin family (*46*). Rather than severing filaments and binding them to the cut filament ends, these proteins instead appear to "nibble" monomers out of the structure of the filament. This mechanism should generate filament fragments with free barbed ends.

Switching on actin assembly in the cell. The site of actin assembly is the location where the cell relieves the restraints imposed by actin-binding escort proteins against nucleation and elongation of filaments. The concentration of monomers that are able to polymerize can then control the rate of assembly. The unpolymerized actin content of cortical cytoplasm is sufficiently high to accommodate a theoretical elongation rate on the order of several micrometers per second.

The barbed end of an actin filament is a logical candidate for the nucleus of actin assembly in the cell; short filament fragments with free barbed ends are detectable in cell extracts. The number of these fragments is larger in extracts of cells stimulated by motility-inducing stimuli, and barbed end capping activity is decreased (*18, 41*). Free actin filament barbed ends capable of

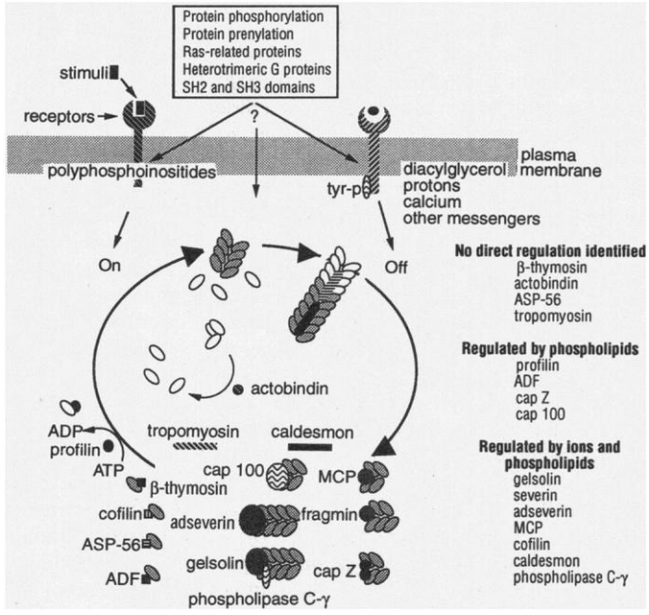

Fig. 5. Regulation of the actin cycle in crawling cells by receptor-mediated on and off switching of actin assembly. Polyphosphoinositides switch on actin assembly by desequestration of actin subunits complexed to profilin (*37*), cofilin, and ADF (or destrin) (*51*) and by uncapping the barbed ends of actin filaments blocked with Cap Z (*49*), gelsolin (*42*), fragmin (*43*), MCP (*45*), adseverin (*44*), or Cap 100 (*50*). Profilin also catalyzes adenine nucleotide exchange on actin subunits (*55*). Tropomyosin and caldesmon bind to and stabilize assembled actin filaments. Caldesmon's binding to actin is inhibited by calcium (*47*). Calcium, protons, and hydrolysis of polyphosphoinositides switch off actin assembly (promote disassembly) by inducing capping of actin filament barbed ends and monomer sequestration (*47*). Thymosin (*38*) and a platelet actin monomer–sequestering protein, ASP-56 (*39*), appear to buffer the polymerizability of actin subunits by mass action, as they are not known to be directly regulated. Actobindin disaggregates spontaneously, forming actin nuclei (*40*). Phospholipase C-γ binds complexes of actin filaments and gelsolin, which can then possibly target the enzyme to its polyphosphoinositide substrate (*57*). Ligation of certain receptors leads to phosphorylation on tyrosines in the cytoplasmic tail of the receptor, and the modified receptor enables phospholipase C-γ to compete profilin and possibly other proteins off the polyphosphoinositides (*60*). The spatial and temporal control of the actin cycle arises from segregation of switching in space and time, which distributes signals, and from changes in the intrinsic susceptibility of actin to interact with actin-binding molecules, depending on whether their bound adenine nucleotide is ATP or ADP.

elongation localize at the growing margin of activated cells (*18, 35*).

Theoretically, regulatory proteins can induce actin nucleation by three mechanisms. In one, a protein aggregates actin subunits by attaching to the side of the incipient filament so that both barbed and pointed ends are free. Ponticulin, an integral membrane protein of *Dictyostelium* amebae (*47*), and talin, a peripheral membrane protein (*48*) (Fig. 3), work this way in vitro. Diacylglycerol, which accumulates in membranes of agonist-activated cells (*4*), enhanced spontaneous actin nucleation activity of an isolated *Dictyostelium* ameba membrane preparation (*47*), raising the possibility that this lipid indirectly induces actin assembly. Second, a protein could cluster actin subunits and remain bound to the pointed end of the new filament, leaving the barbed end free for elongation. There is, however, little evidence for this alternative.

Evidence from studies of actin assembly in vitro implicates a third, more complex mechanism in which the nuclei originate after removal of the escort proteins that are tightly bound to the barbed ends of the short actin oligomers created by fragmentation or nucleation as described above (*40–43, 49, 50*). This uncapping is caused by the binding of the escort proteins that are complexed to the barbed ends to aggregates of membrane polyphosphoinositides that undergo metabolism in response to agonists (*4*) (Fig. 5). Cap Z (*49*), *Dictyostelium* cap 100 (*50*), and members of the gelsolin family (*40–43*) are polyphosphoinositide-regulated capping proteins.

Polyphosphoinositides can also regulate the polymerizability of actin subunits. Profilin (*37*), cofilin, destrin (also called actin depolymerizing factor or ADF), *Acanthamoeba* actophorin, and sea urchin depactin are proteins that bind actin monomers and inhibit their nucleation and addition to the barbed ends of actin filaments. Polyphosphoinositides lower the affinity of most of these actin subunit–binding proteins for actin filaments (*51, 52*). Some of the many signals implicated in the control of events in stimulated cells (*4*), such as G protein activation, protein phosphorylation cascades, and protein prenylation reactions, could hypothetically induce synthesis of these phospholipids, whereas the nonspecific physical events that also initiate crawling (*3*) may promote clustering of the phospholipids in the plasma membrane.

The accumulation of appropriate lipids at the leading edge or other sites may provide the on signal for actin assembly. The simultaneous uncapping of the barbed ends of actin filaments and desequestration of monomers (mediated by the accumulation of polyphosphoinositides around an activated receptor) can provide for rapid filament growth at precisely the membrane sites where instructions for assembly are likely to originate and where assembly is to occur. The control of an on signal in two dimensions, in which lipids are used to convey such instructions, is greater than that of a signal in three dimensions, accomplished with a diffusible messenger. An additional advantage of nucleating actin growth by uncapping barbed ends at the membrane is that the elongating filaments will, at least initially, have the polarity required for the myosin-mediated functions described below, relative to membrane protrusion and vesicle traffic toward the leading edge. Hydrolysis of the polyphosphoinositides can help switch off assembly by increasing the availability of membrane-bound escort proteins at the membrane for actin. Thus, as a cycle, actin assembly and disassembly are interrelated: disassembly can provide ingredients—actin monomers and filament fragments as a source of barbed ends—for subsequent assembly.

As the filaments elongate, they are incorporated into a three-dimensional network by filament crosslinking molecules. In addition, the proteins tropomyosin and caldesmon bind to the groove of the actin filament helix. These proteins and others that crosslink actin filaments into parallel bundles can stabilize the filaments in the growing network against spontaneous or induced fragmentation (*53*).

Actin as a clock in the coordination of switching on and off. An inherent bias built into growing actin filaments by adenine nucleotide metabolism may help them resist occasional inappropriate signaling instructions to disassemble. Actin is an adenine nucleotide–binding protein. In living cells, adenosine triphosphate (ATP) concentration usually exceeds that of adenosine diphosphate (ADP), which favors the binding of ATP to actin subunits. During actin assembly, ATP bound to actin hydrolyzes to ADP and inorganic phosphate (P_i), and because the hydrolysis is relatively slow and dissociation of P_i even slower relative to actin assembly, rapidly growing filaments have subdomains containing ATP, ADP-P_i, or ADP, in order from the barbed to the pointed end of the filament (Fig. 4) (*36*). If slow ATP hydrolysis and P_i release in actin filaments leads to even slower conformational changes of actin subunits within the filaments, actin filaments can be considered as clocks that vary in susceptibility to interactions with other proteins with filament age. For example, new actin filaments near the leading edge may resist and older filaments near the cell body may be susceptible to breakdown by the various severing proteins within the cell (*54*).

The reaction rates of certain escort proteins with actin monomers are faster when the monomers are bound to ADP compared with ATP. This situation favors the binding of control proteins to subunits dissociating from old rather than new filaments and spares ATP-containing monomers for the elongation of growing filaments. In addition to its activity as a monomer-sequestering protein, profilin catalyzes adenine nucleotide exchange on actin monomers (*55*). The metabolic state of the cell, defined as the ATP/ADP ratio, may affect the actin cycle, as has been observed (*56*). The functional activities of most of the actin-binding proteins depicted in Fig. 5 are not responsive to ions or other diffusible signals identified thus far. Because these proteins bind actin with much lower affinity than the ion-activated proteins, such as gelsolin, the energy state of the actin alone may control its susceptibility to destabilization by these proteins.

Binary Switch Hypothesis for the Actin Cycle

Assume, as a simplified approach to the explanation of the phenomena associated with crawling, that agonists ligating receptors at the cell surface switch on actin assembly immediately beneath the receptor and also set in motion processes that turn the switch off. The steps in switching assembly on and off may include receptor activation followed by desensitization as well as all of the downstream consequences of the different signals generated by the receptors that affect the various escort proteins. Additional control of switching arises from escort proteins, which provide feedback for receptors, and from the exchange and hydrolysis of adenine nucleotides on actin itself. The switching on and off of actin assembly introduces ideas that explain polarity, protrusion, cortical flow, adhesion, and capping.

Polarity and persistence. A sessile cell initially encountering soluble stimuli exhibits protrusive activity over its entire surface that is accompanied by a burst of net actin assembly. Subsequently, in cells that migrate, surface activity and most of the polymerized actin localize on one pole of the cell where the leading lamella extends. Once the cell is crawling, the total amount of polymerized actin falls back to the baseline value (*57*), although the steady-state rate of actin assembly and disassembly, the actin cycle, turns over rapidly (*34*). The binary switch hypothesis predicts that the initial protrusive response of newly activated cells is a relatively synchronous switch on around the cell perimeter followed by a switch off.

A cell usually encounters stimuli asymmetrically because of random fluctuations in agonist concentration, especially if there is a concentration gradient. If the actin assembly in the on state favors subsequent

assembly or limits disassembly, the region of the cell first engaged by stimuli might tend to accumulate polymerized actin. This localized triggering might account for the appearance of a leading lamella and for the directional loss of organelle exclusion after forward movement stops. Such feedback between sensory reception and structural remodeling also explains episodic lamellar protrusion at a constant velocity as well as the persistence of cell crawling behavior in the face of chaotic and unstable gradients of stimuli (58).

Once assembled into a three-dimensional network and interactive with selected plasmalemmal proteins, actin and the components involved in maintaining actin assembly may concentrate in the region of initial network growth, at the leading edge of the cell. The actin gel components might also reciprocally act on the membrane so as to inhibit receptor desensitization or concentrate receptors that are switched on. Therefore, the gelation of actin by actin-filament crosslinkers explains in part persistence of the leading edge. In support of this idea, ABP-deficient cells are virtually incapable of locomotion and do not normally form lamellae, but rather protrude and retract blebs all around their circumference in response to agonist stimulation (Fig. 1B). Repletion of ABP by stable genetic transfection of the deficient cells to concentrations found in motile cells provides the transfected cells with the capacity to polarize a lamella and undergo locomotion (23).

Hydrolysis of polyphosphoinositides by stimulus-activated phospholipase C activity could release the actin-regulating polyphosphoinositide-binding proteins from the plasma membrane for subsequent sequestration of monomers, as well as for capping, severing, and nucleating filaments in the pointed direction, all reactions that promote disassembly. That polyphosphoinositide-metabolizing enzymes associate with cellular actin and that some of the proteins controlling the actin cycle bind or inhibit phospholipase C suggest additional controls that could bias switching to the on state (59, 60).

Another factor considered important for the determination of polarity in cell crawling is the recycling of plasma membrane in general and of receptors in particular between the surface and the cell interior. Translocation of chemoattractant and adhesion receptors from internal vesicles to the leading edge of the lamella by exocytosis has been documented, as has the internalization of the receptors, preferentially near the junction of the lamella and the cell body and also at caps at the rear of moving cells (61).

Contributing to the polarity of receptor trafficking are microtubules that radiate from an organizing center near the nucleus of interphase cells and terminate near focal contact sites (62). Although pharmacologic disruption of these polymers does not usually prevent short-term cell crawling, some cells without assembled microtubules become less able to maintain polarity and persistence of movement in a given direction. In cell types that normally spread radially rather than crawl, however, microtubule dissolution paradoxically induces cell polarity and ameboid locomotion (63), indicating that microtubules may either increase or decrease positional bias of receptor trafficking from the center of the cell to the periphery. The centriolar microtubule-organizing apparatus may determine whether microtubular orientation enhances or diminishes polarity. Motor molecules of the myosin I type attached to receptor-containing vesicles represent another possible mechanism for the promotion of polarity, if they move the vesicles along actin filaments in the lamella in the direction of the leading edge (Fig. 6) (19, 64).

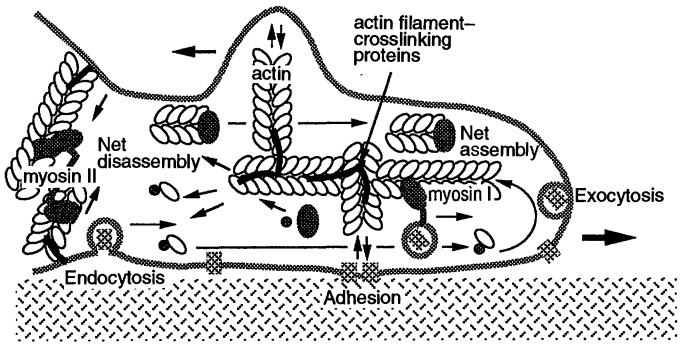

Fig. 6. Operation of the actin cycle in the lamella of a crawling cell. Actin monomers and filament fragments, respectively sequestered and capped by escort proteins, diffuse to the leading edge where, in response to on signals, they dissociate from their escort proteins. Monomers add to the barbed ends of uncapped filament fragments. Actin filament–crosslinking proteins incorporate growing filaments into a three-dimensional mechanically coherent network. Filaments growing up and down from the center of the network mediate cortical flow and adhesion, respectively. Some escort proteins fragment actin filaments at the rear of the leading lamella, and monomers dissociate from ends of the fragmented filaments and bind monomer-sequestering escort proteins. Individual subunits and filament fragments bound by the escort proteins are recycled to the front of the lamella. Shuttling of chemoattractant receptors contributes to the promotion of actin assembly at the leading edge. Vesicular traffic may also provide a continuous supply of substrate adhesion receptors that collect in adhesion plaques on the ventral surface of the lamella. These receptors may be removed from the surface by endocytosis. Myosin I molecules may participate in this receptor movement by motoring vesicles toward the leading edge. In this scheme, bipolar myosin II filaments contract actin filaments at the interface between the leading lamella and the cell body.

Protrusion. The fluidity of the plasma membrane precludes its having a primary mechanical role in stabilizing the cell surface, but it is ideal for facilitating protrusion and, with its receptors and signaling machinery, for guiding the directional growth of the underlying actin network. A prerequisite for the protrusion of the leading lamella, irrespective of the driving force, is that the plasma membrane bilayer must detach transiently from its many submembrane actin filament connections (Fig. 4) or the membrane-bound actin filaments must loosen from their neighbors by localized solation of the underlying actin network.

Although the association between actin polymerization and membrane protrusion is well established (65), the actual driving force for membrane extension of the leading lamella is unclear. One proposed explanation is the "solation-expansion" hypothesis, which invokes cycles of brief and localized osmotic swelling in a transiently disrupted actin filament gel at the leading edge; this expansion powers outward movements of the membrane (66). The swelling has directionality because neighboring lateral and interior cortical domains where the gel remains intact have hydraulic as well as elastic resistance. Subsequent actin assembly and crosslinking, leading to reconstitution of the disrupted gel, causes solidification of the cortical gel in the position of the osmotically driven advance. Changes in membrane ion pump and channel activities accompanying cell stimulation (67) may also be involved in the relevant osmotic changes. The blebbing and transient thinning of the actin cortex observed frequently at the leading edge of migrating cells support this mechanism for lamellar progression (5, 68). Solation expansion also allows for the separation of the membrane from the underlying skeleton.

Increased osmolarity of the medium retards lamellar protrusion, which is consistent with the solation-expansion theory (69). Elevations in extracellular osmolarity, however, have the opposite effect on some filopodia, enhancing their extension rate (69). This result favors a different mechanism, the "Brownian ratchet" model, for protrusion of those structures. The Brownian ratchet is a simple explanation for membrane protrusion in which the fluid planar polymer at the leading edge, once free from constraints, fluctuates as a result of Brownian motion. Its outward excursions provide space for actin subunits to add to

actin filaments at the membrane-cytoplasm interface. Increasing the osmolarity of the extracellular medium may therefore enhance the rate of filopodial extension by concentrating actin subunits within that structure (*70*).

Another concept to explain filopodial protrusion is that membrane-associated myosin I molecules move antegrade along immobilized actin filaments and push the membrane at the leading edge in the direction of advance. The extension of membrane could create a space for the addition of actin monomers to the ends of filaments that previously abutted the membrane (*71*). This model requires that the myosin molecules attach to something more solid than the fluid lipid-membrane bilayer to exert force.

Coordination. Building blocks consisting of sequestered actin monomers and oligomers with capped barbed ends cycle from the back to the front of the advancing lamella, where they are available for new actin filament assembly after interaction with signal phospholipids at the leading edge (Fig. 6). As the lamella enlarges by forward advance, the distance for diffusion of these subunits from the rear to the front increases. If steric hindrance or binding of subunits, particularly oligomers, within the actin filament gel impedes this diffusion, enlargement of the lamella would slow the advance and compromise its persistence. This kind of impediment to lamellar extension coupled with diminution in intensity of the signals for on switching at the established lamella or with increased on switching elsewhere around the cell surface could put a new site of protrusion at an advantage for extension relative to the old one. These events might explain the observed coordination between rapid emergence of a new lamella coupled with the disappearance of an old one (*5*).

Cortical flow. If actin assembly induced at the front of the dorsal surface of the leading lamella switches on sequentially from the leading edge rearward and then switches off in the same sequence, actin filaments perpendicular to the plane of the lamella would assemble and then disassemble correspondingly, in response to the rearward progression of switching on and off. These hypothetical events might result in waves of actin filaments that move from the front to the back of the lamella; these waves could constitute the substance of and driving force for retrograde movement of cortical thickenings, such as ruffles. The intensity of the signals for on switching might attenuate progressively toward the rear of the lamella, as dilution and endocytosis reduce the concentration of activated receptors. A diminution in signal could account for the decrease in height, and eventual disappearance, of waves. The shape of the construction of orthogonal branches and control of the timing of assembly and disassembly could provide for the bends in ruffles and still maintain their mechanical coherence and stiffness. This wave hypothesis does not require, although it does not rule out, that some component of cortical flow involves retrograde movement of intact actin filaments, powered possibly by myosin motors.

A role for surface waves in cortical flow has been considered before (*72*) and, although very speculative, is consistent with the global turnover of actin filaments in the leading lamella (*34*). It also fits with recent observations that a crawling fish scale cell observed from above has vigorous actin assembly at the midpoint of the leading edge that attenuates radially in a graded manner to a minimal value at the widest extent of the leading lamella (*73*).

An oscillating switch mechanism also accommodates ligand capping. If switching transmembrane receptors on causes them to adhere to proteins contained in the actin gel beneath the membrane-submembrane lamina but switching them off releases them from these constraints, the receptors could diffuse in the plane of the membrane when switched off but would immobilize when switched on, provided that the underlying matrix is in an assembled state. The state of such assembly in moving submembrane actin-gel waves could affect the drift velocity of the surface receptors and, by a diffusion-trapping mechanism (*7*), carry crosslinked external ligands to the back of the lamella.

Reversible adhesion. If events similar to those described above on the top of the lamella take place on the bottom as well, a wave of reversible actin assembly in the direction of the substrate should progress from the leading edge of the lamella to the rear. As the growing filaments encounter the ventral surface of the membrane, they become potential nucleation sites for transmembrane adhesion complexes (Figs. 3 and 6). The existence of multiple components in some types of adhesion complexes, such as the ones bridging the space between the cytoplasmic tails of the integrin class of cell-substrate adhesion receptors and intracellular actin filaments (*25*), provides opportunities for combinatorial regulation of cell adhesion to the extracellular matrix and to other cells. Transmembrane signaling by integrins ligated to extracellular matrix molecules or other ligands (*74*) could regulate the clustering of adhesion-complex proteins with actin filaments to create an attachment contact as the wave advances. Actin disassembly followed by disaggregation of the adhesion complex would allow the retreating wave to detach from the substrate. The stability of adhesion complexes may be under the regulation of polyphosphoinositide metabolism in the vicinity of the integrin receptors. Perturbation of this metabolism might account for dramatic effects on actin assembly of activated small GTP-binding proteins, which have not been shown to bind directly to actin or to actin escort proteins (*4*). The adherent patch on the ventral surface presumably keeps the lamella tacked to the substrate and permits retraction of the rear of the lamella and the cell body.

Contractions. Calcium induces the phosphorylation of the structural tails and regulatory subunits of hexameric myosin II by activation of myosin kinases. The phosphorylation reactions regulate myosin's reversible assembly into bipolar filaments and the contractile activity of these aggregates with actin filaments. Contraction of membrane-bound actin filaments powered by myosin may also account for the compression of the crawling cell's lamella, at its junction with the cell body, and tail. It may also contribute to solation of the actin network at the rear of the lamella and in retracting lamellae (*19, 75*). Myosin oligomers may impose additional forces on the assembled actin to create the traction forces that have been observed in the leading lamellae of some advancing cells (*76*).

Tests of the binary switch hypothesis. A pharmacologic strategy that switches actin assembly off is the treatment of cells with toxins that inhibit actin assembly in vitro. Most extensively studied are cytochalasins and related compounds that inhibit actin monomer addition to filament barbed ends. They also induce ATP hydrolysis by actin monomers (*77*). The ADP-containing monomers are less polymerizable than ATP-containing monomers and, as explained before, are more susceptible to binding by escort proteins (*54*). Some bacterial toxins ADP-ribosylate actin subunits and thereby make them act as caps of barbed ends (*78*). The effect of these agents on cells is to inhibit their protrusive activity and to cause actin in previously extended processes to disassemble, leaving a thin rim of evidently more stable actin filaments, which may represent part of a two-dimensional membrane lamina (*41*).

Calcium directly activates some of the actin escort proteins that disassemble actin. Therefore, preventing intracellular calcium increases, associated with the signal transduction that leads to cell crawling, is an indirect test of the importance of these proteins in the mechanism of cell crawling behavior. Activating agents transiently raise the intracellular concentration of free calcium in blood platelets and cause severing of the actin filaments that support the discoid shape of the resting cell. This severing is an important step that leads to subsequent lamellar extension. Preventing

the calcium transient by intracellular calcium chelation blocks the fragmentation of actin filaments and the protrusion of lamellae (*18*). Various other cell types, however, exhibit lamellar protrusion when loaded with intracellular calcium chelators, which keep free calcium in the cytosol at nanomolar levels (*4*). Protons may replace calcium as messengers in the chelated cells (*42*), but these inconsistencies demand further research and caution that despite general themes, the details of crawling mechanisms are customized among different cell types.

Changing the expression of actin escort proteins in cells can be a more direct test. Increasing the concentration of β_4 thymosin in fibroblasts by microinjection increases the amount of unpolymerized actin in the cell and causes cell rounding, which is consistent with enhancement of off switching (*79*). Stable overexpression of human profilin in cultured hamster cells by genetic transfection caused the cells to stabilize their actin filaments, which fits profilin being an on-switch promoter in this setting, either by its catalysis of adenine nucleotide exchange on actin subunits or by its inhibition of phospholipase C-γ, which decreases calcium transients that activate off-switch effects of other escort proteins (*80*). Overexpression by stable genetic expression in murine fibroblasts of human gelsolin, an escort protein that promotes both on and off switching, depending on what signal it receives, increased agonist-induced locomotion of the overexpressing cells in proportion to the amount of gelsolin produced (*81*).

Prospects

Removal of membrane lipids from a crawling cell with detergents leaves its lamellar actin network sufficiently intact to retain the shape of the crawling cell. This dilution-resistant actin cytoskeleton is a remnant of a crawling cell and contrasts with the profound lability of actin networks in living cells (*34*). The stability of actin filaments in the detergent-extracted lamella, despite the removal of unpolymerized actin and other cytoplasmic constituents, suggests that removal of the lipid membrane freezes many of the barbed ends of actin filaments in a capped state (*19, 46*). A promising avenue of investigation, therefore, might be to reconstitute the missing ingredients that existed in the membrane, restore filament end–uncapping activity to the extracted actin networks, and then elucidate the components responsible for this activity.

Aside from an intrinsic interest in understanding how cells crawl, the demonstrated ability to enhance or inhibit cell crawling by tinkering with the intracellular machinery of the actin cycle and with the control of actin filament architecture may mean that the research devoted to cell crawling is approaching new ways of modifying this important cell function for enhancement of normal physiology and for the amelioration of disease. Examples of such interventions include hastening the healing of wounds, blunting the invasion of the white blood cells that cause inflammation, and retarding the invasive crawling behavior of cancerous cells.

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