The Machinery of Blood Cell Movements

By Thomas P. Stossel

It is a special honor to receive an award named after E. Donnall Thomas, whose research has directly benefitted so many patients with blood disorders. My journey to this award started over 20 years ago with studies of neutrophil function; meandered through reductionist biochemistry of cellular proteins, biophysics of polymers, and molecular biology; and most recently has been searching for clinical applications. This odyssey would not have been possible without many wonderful students and independent colleagues, and it would not have even started without the initial training and support I received from Martha Vaughan at the National Institutes of Health or the encouragement and independence offered by David Nathan at Boston Children’s Hospital.

The excitement and challenge of studying cell motion derives from the fact that it encompasses many aspects of cell biology, hematology, and human physiology. My colleagues and I have focussed on the intracellular machinery that mediates cell crawling but have tried to relate this machinery to the larger picture of how it relates to instructions that activate crawling and to find ways to make these relationships useful to cell biologists and clinicians in a variety of disciplines. In this review, I provide a description of cell crawling movements, propose a minimal molecular mechanism for them, and suggest some directions where this research is going that might be of clinical importance.

DESCRIPTION OF CELL CRAWLING MOVEMENTS

Overview

Neutrophils, which I studied in the early 1970s, were an excellent starting point for a career analyzing how cells crawl, because neutrophils are the “crawliest” cells in the human body. Each day, an average-sized person produces over 100 billion mature neutrophils. These cells crawl out of the bone marrow, get a brief ride in the circulation, and then crawl again out of the vasculature for a few millimeters in search of microorganisms or necrotic tissue. One hundred billion objects moving that distance in the aggregate is equivalent to over twice around the earth. Neutrophils are also the fastest cells in the human body. They move at speeds up to 30 μm per minute, an order of magnitude faster than crawling rates of cells required for development of the embryo, wound healing, and tumor invasion (Fig 1). It is the inexorability, not the speed, of tumor cell migration that is lethal. Blood platelets do not exactly crawl in the sense of locomotion, but they undergo speedy crawling movements as they change shape from flat discs to spheres covered with multiple spines or to flattened configurations resembling fried eggs with a few spines to plug leaks in injured vessels (or occlude atherosclerotic coronary arteries). Similarly, neutrophils and mononuclear phagocytes exhibit surface crawling behavior as they engulf particulate objects.

Stimulation of Cell Crawling Movements

Cells initiate crawling movements in response to a wide variety of external stimuli. Particularly numerous are molecules that elicit neutrophil locomotion. These chemoattractants arise when inflammatory agents initiate proteolytic cascades in extracellular fluids and stimulate cells, predominantly macrophages, to secrete bioactive lipids and polypeptide cytokines. A partial list of these agents includes C5a, kinins, leukotriene B₄, interleukin-8, and N-formyl oligopeptides. C3b and C3bi and the Fc domain of IgG bind to particles induce phagocytosis by neutrophils and mononuclear phagocytes. A number of activated blood clotting factors, especially thrombin, and ADP elicit platelet shape changes. Thrombin; mitogens such as platelet-derived growth factor, epidermal growth factor, insulin-like growth factor-1, insulin, tumor growth factor-β1, and others; and extracellular matrix fragments induce crawling movements in fibroblasts, epithelial cells, and tumor cells. All of these stimuli operate by binding specific receptors that, when so engaged, turn on one or more intracellular metabolic cascades collectively designated as signal transduction reactions.

While the molecules mentioned above bind to several types of specific receptors on cell surfaces, relatively nonspecific chemical and physical perturbations can also induce crawling movements. An example of particular importance to hematology is cold-induced platelet shape change. Chilling of platelets to temperatures less than about 15°C causes platelets to alter their shape in a manner resembling agonist-induced activation. This phenomenon is paradoxical, because platelet activation is normally dependent on intermediary metabolism and the signal transduction reactions that should be depressed at lowered temperatures. Cold-induced platelet activation precludes keeping platelets produced for transfusion at refrigeration temperature, a fact that limits platelet storage to 5 days.

Cortical Protrusion

Over two centuries of observations with the light microscope has provided important inferences about the mechanism of cell crawling (Figs 1 and 2). First, crawling movements originate from protrusive activity of a special organelle-excluding region surrounding the cell periphery known as the cortex (the dark shading in Fig 1). One or more protrusions of this cortex are consistent features of crawling movements. These protrusions may be in the form of flat sheets, in which case they are called lamellae or...
lamellipodia: when they are more bulbous, they are designated pseudopodia or lobopodia. These protrusions can originate from anywhere around the cell surface, but in cells undergoing locomotion, such as neutrophils, fibroblasts, and tumor cells, they are invariably localized at the front end of the moving cell (Fig 1). In contrast, platelets and other cells that spread in preference to crawling extend protrusions circumferentially. Another important type of cell surface protrusion is filopodia, hair-like projections that provide redundant surface area to accommodate cell shape changes and are useful for pulling on extracellular structures, eg, the retraction of fibrin strands by platelets (Fig 1).

Reversible Adhesion

After protrusion of lamellae, part of these structures must become adherent to the underlying substrate if the cell is to crawl (or retract extracellular objects centripetally). Adhesion provides a traction force that permits the cell body to be pulled forward. Thereafter, propagation of locomotion requires that the adhesion release and be re-established distally after another round of protrusion (Fig 2). Sequential bouts of protrusion, adhesion, and forward contraction of the cell body can sometimes be observed, but usually the steps are so coordinated that cells appear to glide. A large number of adhesion molecules important for cell crawling have been identified. The most extensively studied are the integrins. The first recognized clinical example of a blood cell crawling defect involving impaired adhesion was a case of genetically determined deficiency in expression of a phagocyte-specific integrin, CD11b/CD18. Inappropriate adhesion of neutrophils has also been shown to mediate catastrophic inflammatory reactions, and efforts have been directed at inhibiting cell adhesion as a strategy to abrogate inappropriate inflammatory reactions.

Sol-Gel Transformations and Gel Contraction

One of the earliest mechanistic inferences about cell crawling made by watching cells in the light microscope was that these movements were derived from changes of the cell substance between liquid-like and solid-like states. A general conceptual thread appearing in discussions of cell crawling behavior was that the body of the unstimulated cell is a liquid (a "sol") and that, upon stimulation, the extended protrusions represent solidification of this liquid material (into a "gel") that is subject to compression (contractility). "Sol-gel transformations" and gel contraction are, I believe, not mere quaint adumbrations but rather bear importantly on the mechanism of cell crawling, have been very helpful in characterizing molecular interactions of cell crawling, and may turn out to be clinically useful as well.

Sols are liquids that deform irreversibly when subjected to stress. Liquids with dense concentrations of asymmetrical solutes can have a very high viscosity, ie, it may take a lot of force to deform them, because of friction between the solute and the solvent. The internal contents of cells, which include concentrated protein solutions, membranes, and a variety of solid objects, are very viscous, on the order of 100,000 poise. Gels are also liquids, but because they are permeated with some kind of coherent network of solute, they are elastic and, when transiently deformed short of a

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**Fig 1.** Types and speeds of blood cell crawling movements.

**Fig 2.** Steps in cell crawling.
breaking point, return to their original position. Another important property of gels is their ability to impede water flow, like a sponge. Relatively small changes in the configuration of polymers within a gel can also result in large volume alterations, namely contraction and expansion.

A Mechanism for Cell Crawling

With this general information we can propose a mechanical explanation for cell crawling behavior (Fig 3). A resting cell is a liquid encased in a gel. Upon stimulation, the gel undergoes contraction; because liquids are incompressible, no shape change takes place until a focal weakening in the cortical gel, perhaps at the site of initial or strongest stimulation, permits the membrane to push outwards. This protrusion is not usually spherical like an aneurysm, because the cortical network modulates fluid flow so that the expansion is smooth. The protrusion is flattened into a lamella by formation and contraction of gel contents from subunits introduced by the flow of sol into the extending protrusion, by adhesion of the membrane to the substrate, and by contraction of the gel. Continued cycles of contraction, weakening, flow, and gel reconstruction propagate crawling. Adhesion receptors make and break connections to the extracellular matrix and to the underlying lamellar gel. This model, in general, is not new, and it incorporates others that variably emphasized contraction at the front, at the tail, or all around the cell. The challenge has been to flesh such a model out with a minimal degree of molecular definition.

THE MOLECULAR BASIS OF CELL CRAWLING

The Polymers of the Cell Periphery—Focus on Actin

When we began to work on cell crawling in the 1970s, the major polymers of the cell cortex were known (Fig 4). The model of the plasma membrane as a fluid bilayer proposed by Singer and Nicholson was well accepted. Marchesi, Branton, Steck, and others had described the spectrin-dominated two-dimensional protein network laminating the lipid bilayer of erythrocytes now known to be related to numerous hemolytic disorders, although there was no evidence at the time for such a structure in nonerythroid cells. Most compelling to those studying cell locomotion was the probable role of the muscle proteins actin and myosin, identified during the 1960s in nonmuscle cells by Hatano and Oosawa and by Adelman and Taylor. Actin was shown to represent from 5% to 20% of cell protein, representing a concentration in these cells in the millimolar range. Other cytoskeletal elements, the microtubules and intermediate filaments, were considered less likely to be directly involved in cell crawling, because, first, in contrast to actin, they were not concentrated in the cell periphery. Second, cytochalasins, implicated as specific inhibitors of actin assembly, blocked many cell crawling movements, whereas inhibitors of microtubule assembly did not have this effect.

Myosins

Much of the initial interest in the actin system in cell crawling focussed on myosin, which had also been identified in nonmuscle cells in general and from platelets and leukocytes, respectively, by Zucker-Franklin and by Senda and colleagues. Some of this interest arose from the fact that the first nonmuscle myosins identified resembled muscle myosin in forming bipolar filaments capable of exerting tension by sliding along actin filaments. Like muscle actin-myosin complexes, aggregates of actin and myosin from nonmuscle cells, including blood cells, demonstrated a characteristic polarity identifiable by the angle at which globular myosin head domains extending from filament-forming helical tails bound to the double helical needle-like actin filaments (Fig 5). The angle that the bound myosin head groups appeared to have in electron micrographs conferred a characteristic arrowhead appearance, and sliding actin filaments moved, concomitant with ATP hydrolysis, from the barbed to the pointed direction. Immunofluorescent micrographs of cultured fibroblasts and epithelial cells stained with anti-actin antibodies showed beautiful brightly staining actin filament bundles, resembling muscle sarcomeres, suggesting that forces for movement might arise from actin-myosin contractile interactions as in muscle. Fascination with myosin also arose from the fact that Ebashi had shown that the link between striated muscle contraction and signal transduction was calcium. Calcium ions act on troponins and tropomyosin to activate ATP hydrolysis and contractile activity in the myosin head domain.

Therefore, our first work on the biochemistry of leukocyte crawling was to confirm that neutrophils and macrophages contained myosin molecules. Adelstein and colleagues showed that smooth muscle and blood cell myosins are also regulated by calcium, but by a different mechanism than in skeletal muscle. Instead of directly regulating control proteins like troponins in muscle, calcium indirectly activates platelet and macrophage myosins for actin-activated ATPase activity and contractility by inducing phosphorylation of serine residues in the myosin head domain. Blood cells probably also contain one or more varieties of a different kind of myosin, known as "unconventional" myosin or myosin I, first discovered in an amoea species by Pollard and Korn. These myosins do not form bipolar filaments but rather use their helical tails to bind membranes.

ABP and the Cortical Actin Gel

Our focus on blood cell myosin was diverted by the discovery in 1974 that, when macrophages were disrupted in solutions containing micromolar free calcium concentrations, the cell-free extracts, which contained about 1 to 2 mg...
of actin per milliliter, remained liquid, but if the free calcium was lowered by chelation, the extracts solidified. The gelled extracts then underwent a contraction provided that ATP was present. These observations resonated with the old ideas about sol-gel transformations and contractility of gels and also with the fact that highly motile cells such as neutrophils do not form actin filament bundles but rather show diffuse actin staining in the leading lamella. We therefore began to try and understand the nature of the gelation process and determine its relevance to cell crawling movements. As a physical state transformation, the actin gelation phenomenon is complex and differs conceptually from the usual enzymatic, transport or binding reactions ordinarily studied by biologists. A variety of approaches were mobilized to address this problem.

Biochemistry. We connected the cell extract gelation observation to our earlier finding that stirred macrophage extracts precipitated large amounts of actin with few other proteins save a high molecular weight protein. Purification of this protein and adding it back to purified actin filaments efficiently precipitated the actin. This purified protein also appeared to cause actin solutions to solidify when added without stirring. We named this protein—the first new nonmuscle actin binding protein—actin-binding protein or ABP. An isoform of this protein, named filamin, was subsequently isolated from chicken gizzard. Both names have been used interchangeably, although it is now evident that the muscle and nonmuscle forms are structurally as well as functionally distinct.

Physics. More stringent evidence for the importance of ABP in the gelation of cell actin came from considerations of the physics of gelation. The coherence of a gel is achievable at low solute concentration if the solute is in the form of highly extended polymers. Because actin filaments can be many micrometers long, they can easily overlap and therefore produce the solute coherence required for gelation at very low (microgram) concentrations. Long overlapping rods cannot easily rotate and therefore can have gel-like properties. Because a few very long actin filaments will gel due to overlaps, operationally defined as cross-links, and because the length to diameter ratio of such actin filaments is very high, the individual filaments are flexible so that such a gel is not very rigid. At the concentrations of actin filaments found in cells (7 to 15 mg/mL), the filaments have a marked tendency because of entropy to align in parallel (anisotropic) bundles. Such bundling compromises their ability to fill optimally a three-dimensional space that can generate a uniform and strong gel. Moreover, for purposes of impeding water flow, a disadvantage of a gel containing aligned filament bundles is that the pore size is not uniform.

Actin cross-linking proteins fall into two general classes, one that stabilizes the tendency of actin filaments to form

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**Fig 4.** Polymers of the cell periphery.

**Fig 5.** Actin-myosin interaction: fundamentals.
bundles and another, of which ABP is the prototype, that counteract this bundling by causing the actin filaments to branch at high angles (Fig 6). When very dilute actin polymerizes in vitro, the filaments interpenetrate as they assemble, and as the filaments are trapped in a relatively isotropic configuration, it is possible for nearly any cross-linking protein to find overlaps and cement them in place. This experimental artifice is probably not relevant to the situation in vivo in which more concentrated actin filaments would bundle if not prevented from doing so first by the actin cross-linking proteins specifically designed for this function.

All of the actin filament cross-linking proteins are bivalent in that they have two actin filament binding sites, and they bind actin filaments with approximately equivalent affinities in the micromolar range. What distinguishes them is the structure conferred by the amino acid sequences separating the two actin binding sites. When the distance between these sites is small or the intervening structure is rodlike, the cross-linking protein is better designed to stabilize bundles; when the spacing between the sites is large and when it contains limited flexibility, the protein is capable of promoting isotropic branching of the actin filaments. ABP, for example, is a large dimer with a short self-association site at one end, the carboxy terminus, separated by 160 nm from two actin binding sites. The spacer arms of the ABP subunits contain 23 beta sheet repeats interrupted in two places by random coil sequences designated hinges. The beta sheet repeats presumably confer rigidity on the subunit arms while the hinges provide for strategic flexibility that permits the ABP dimer to function as a leaf spring to hold actin filaments at near perpendicular angles to one another.

The sol-gel transformation of linear polymers represents the formation of a single giant coherent molecule from dispersed subunits and occurs at a critical degree of polymer cross-linking. As a critically determined transition, gel formation is analogous to the boiling or freezing of liquids and is thus a particularly adept mechanism for regulating cell structure—small changes in actin composition or configuration can have profound effects on cell consistency. The transition from sol to gel can be monitored by an abrupt change in the consistency of the actin solution as ABP is added; at the same critical ABP concentration, an actin solution effectively inhibits solvent flow. The orthogonal branching of actin filaments by ABP was inferred from the fact that ABP induces an abrupt sol to gel transformation of an actin solution at the lowest concentration compared with other actin filament cross-linking proteins. This concentration can be as low as nearly one ABP dimer per actin filament, equivalent to a molar ratio of one ABP per thousands of actin subunits.

Ultrastructure. Electron micrographs prepared by a variety of techniques of actin filaments cross-linked by ABP support the conclusion derived from physical studies that ABP promotes the high-angle branching of actin filaments. The extended lamellae of macrophages, moreover, contain orthogonal actin filament networks consisting of 1-μm-long filaments branching every 100 nm at close to right angles. A similar actin network is visible in the spread lamellae of activated blood platelets. These networks resemble actin filament gels assembled with low ABP concentrations in vitro, and ABP molecules have been localized histologically at the branch points between actin filaments.

Actin Gelation, Contraction, and the Mechanism of Cell Crawling

Returning to the general model proposed above for cell crawling movements, we can now envisage the cortical gel as some kind of network of filamentous actin (Fig 7). Cell activation induces contraction of the network, presumably by inducing the phosphorylation of myosin molecules through transient increases in cytosolic calcium (although see below). The hydrostatic force imposed by network contraction forces the membrane bilayer outwards at a region where it becomes detached from the underlying protein network. Simultaneously at the site of membrane expansion, actin filaments assemble from monomeric subunits into relatively short (1 μm) linear filaments. These filaments are cross-linked by ABP and other cross-linking proteins into a three-dimensional uniform network. This network of actin filaments is dynamic, but nonetheless it is coherent, and it
modulates the flow of sol, provides strength to the forming protrusion, and serves as an anchor for adhesion molecules welding the protrusion to the underlying substrate. In addition, the cross-linking of initially freely diffusing actin filaments into an immobilized state imparts contractility on the solvent included within the gel, and the squeezing action on the solvent can provide additional force to protrude membrane forward at the leading edge. When the membrane is tightly affixed to the underlying extracellular matrix, and is thereby prevented from advancing, the contractile force can produce waves on the dorsal surface of the leading lamella, and these waves move back to cell body, a phenomenon known as "cortical flow."  

Some of the best evidence for the validity of this model has come from the opportunity to ask the question, what happens if actin filaments cannot be efficiently cross-linked into a uniform network? The opportunity arose when we were provided with permanent cell lines cultivated from malignant melanomas excised from seven unrelated patients. The cells of four of these lines exhibit typical features of crawling cells in culture. When stimulated by factors present in serum, the cells acquire a polarized morphology with one or more protruded lamellae, and they are capable of directed migration in response to a gradient of these factors. All of these cells express ABP at levels about 1 dimer per 500 actin molecules. In contrast, cells from the other three lines express barely detectable quantities of ABP protein and mRNA. The gene for ABP, which is on the X chromosome, however, is present in these cells. These ABP-minus cells respond to serum factors by extending spherical projections, designated blebs, from around the entire cell circumference. The blebs expand rapidly to about 10 μm in diameter, stabilize, and then gradually retract. These cells move poorly if at all toward a chemoattractant gradient. Restoration of stable ABP expression to one of the ABP-null by transfection of the full-length ABP cDNA causes the cells to cease constitutive blebbing on stimulation, and to acquire the ability to crawl directionally in proportion to the expression level of ABP (Fig 8). At the ratio of ABP to actin found in wild-type cells, the formerly ABP null cell lines have rates of chemotaxis indistinguishable from wild-type cells. Interestingly, cells expressing supranormal ABP amounts have somewhat slower chemotactic activity.  

The findings with these melanoma cell lines demonstrate unequivocally the importance of ABP for efficient stabilization of cell cortical actin. In the ABP null cells, stimulation induces cortical contraction and focal weakening as in the ABP-expressing cell. However, the former lack the ability to modulate solvent flow, and the membrane blows out in an aneurysmal fashion. Actin assembles at the leading edge of the protruded membrane but is not efficiently linked into a uniform network. Eventually sufficient actin assembles to stabilize the structure by filament interpenetration, and contractile forces squeeze the bleb back into the cell body as blowouts elsewhere reduce the hydrostatic pressure in the initial blebs.

**Stimulus-Response Coupling, Actin Assembly and Disassembly, and Cell Crawling**  

As mentioned above, cells respond to myriad stimuli with crawling movements. A simple and reproducible assay capitalizing on the specific binding of fluorescent phalloidin to actin filaments has permitted investigators to document that net actin polymerization sometimes followed by depolymerization accompanies cell stimulation. Many signal transduction mechanisms, eg, calcium transients, pH changes, protein phosphorylation reactions, phospholipid turnover, lipoxygenase activity, GTP-binding proteins, and others, have been proposed to mediate these changes. The large number of candidates and the curious problem of nonspecific stimuli such as cold-inducing actin assembly make the problem of signal-response coupling confounding at face value. To address
this difficulty, it is helpful to consider two peculiarities about the assembly of actin (Fig 9).

**Actin filament assembly from subunits.** The first pecu-
liarity is the fact that the spontaneous assembly of actin
subunits into filaments requires a nucleation step in which
two or three monomers aggregate. This nucleation reaction
is highly unfavorable; the critical concentration at which
such nucleation is likely to occur is in the near millimolar
range. The second is based on the “barbed” and “pointed”
end polarity of actin filaments first recognized by myosin
binding. The barbed ends of actin filaments serve as nuclei
onto which actin subunits can rapidly add when present at
concentrations more than a few micromolar. Cytochalasins
compete for actin subunits to form filaments, explaining in part the inhibitory effect of these
compounds on cellular actin assembly. A nearly 10-fold
higher subunit concentration is required to promote addition
of subunits to the pointed ends. The lower critical concentra-
tion for addition to the barbed end depends on the presence
of ATP bound to the actin subunits that hydrolyzes to ADP
and orthophosphate during assembly.44

Cells capitalize on these characteristics of actin assembly
by using two general types of control proteins (Fig 10A).
One of these, of which β-thymosin and, to a lesser extent,
profilin are the principal representatives, binds unpolymer-
ized actin subunits to inhibit their spontaneous assembly.45
The affinity of thymosin for actin is in the micromolar range,
however, such that, should free barbed ends be present, they
can compete effectively for actin subunits; actin assembly
might be inhibited somewhat, but nevertheless would occur
efficiently. Because about half of the total actin in resting,
unstimulated cells is polymeric, plenty of barbed ends theo-
retically would be present to cause most of the actin to be
polymerized. This contradiction is solved by the second type
of control protein that blocks subunit exchange on the barbed
ends of actin filaments. Two general types of these so-called
capping proteins have been identified: the gelsolin protein
superfamily (Table I) and a highly conserved capping pro-
tein, also named capZ. Uncapping of actin filament barbed
ends by removal of these proteins can determine where and
when actin filament elongation occurs.46

**Actin filament disassembly.** Capping the barbed ends
of elongating filaments can stop further filament growth. But,
because exchange of subunits with the pointed filament end
is slow (the off-rate constant is 1 per second or slower),
capping is not an efficient mechanism for disassembly. To
achieve disassembly rapidly, cells use proteins that sever or
nibble actin filaments, which simultaneously shortens them
and increases their number, providing more ends from which
depolymerization can take place (Fig 10B). Some members
of the gelsolin family bind to the sides of actin filaments,
sever the noncovalent bonds holding subunits together in the
filaments, and remain firmly affixed to the severed barbed
ends, thereby preventing reassnelling of the broken filaments.
A second class of actin disassembling proteins, examples of
which are cofilin, actin-depolymerizing factor (ADF), depuc-
tin, and actophorin, nibbles actin monomers out of filaments,
possibly at places where there are defects or kinks, which
Actin Assembly in Cells

I. Actin subunit binding by (•) β-thymosin inhibits the spontaneous nucleation of actin subunits.

II. Actin subunit binding proteins have lower affinities for actin than free barbed filament ends.

III. Thus, together with reversible barbed end capping, they regulate actin assembly.

Actin Disassembly in Cells

I. Barbed end capping terminates assembly -- free subunit-binding protein concentration increased

II. Actin filament severing and nibbling + barbed end capping creates many pointed ends for depolymerization.

Table 1. The Gelsolin Family

<table>
<thead>
<tr>
<th>Protein</th>
<th>Distribution</th>
<th>Cap</th>
<th>Sever</th>
<th>Nucleate</th>
<th>Cross-Link</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelsolin</td>
<td>Wide; abundant in plasma, platelets, and leukocytes</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td>Villin</td>
<td>Brush border epithelial cells</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td>✔</td>
</tr>
<tr>
<td>Fragmin</td>
<td>Physarum polycephalum</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severin</td>
<td>Dictyostelium discoideum: rat Lewis carcinoma cells</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cap-G</td>
<td>Macrophages, fibroblasts</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protovillin</td>
<td>Dictyostelium discoideum</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adseverin</td>
<td>Adrenal and platelets</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flightless</td>
<td>Drosophila, humans</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig 10. Control of intracellular actin assembly and disassembly. Not shown is that profilin promotes actin assembly by catalyzing adenine nucleotide exchange on actin subunits, and that some capping proteins also work by nucleating actin subunits and then remaining bound to the barbed end of the oligomers formed.

also results in increased numbers of shortened filaments. These proteins are much less efficient than the gelsolin-related proteins in shortening actin filaments, but they are abundant proteins and, together with capping proteins, could play an important role in filament shortening.

Control of actin assembly by signal transduction. The actin filament severing protein gelsolin has been characterized in considerable detail. Its original discovery came from our efforts to purify the factor responsible for calcium's prevention of cytoplasmic extract actin gelation described above. Gelsolin's cDNA was cloned in 1986, and part of its three-dimensional crystal structure in a complex with actin solved last year. This last study has shed important insights into the severing mechanism. Research on gelsolin has also connected cell signal transduction to actin assembly. Micromolar calcium and more recently proton concentrations have been shown to activate gelsolin for actin filament binding, severing, and capping.

However, removal of calcium or protons is insufficient to
cause gelsolin to dissociate from actin filament barbed ends. The only non-denaturing condition shown to effect such dissociation is the binding of gelsolin-actin complexes to a class of membrane lipids, the polyphosphoinositides implicated in signal transduction (Fig 11). The action of these phospholipids, which also dissociate actin subunits from profilin, requires that they have two acyl chains, a glycerol backbone, and phosphates in the 4 or 5 positions of an inositol ring. The physical chemistry of presentation of the lipid with the protein is also important, and indirect evidence suggests that phospholipid clusters are involved. It is now appreciated that nearly all of the known actin filament barbed end capping proteins are inhibited by polyphosphoinositides.

This information provides for a unifying picture linking cell surface stimulation to actin assembly and disassembly (Fig 12). During receptor-mediated cell stimulation, reaction cascades lead to the breakdown or synthesis of polyphosphoinositides. The former are coupled to release of calcium (and possibly protons) from intracellular compartments, and the resulting effect of these ions on gelsolin and related proteins predictably results in actin filament disassembly. Conversely, reactions leading to the local synthesis or clustering of polyphosphoinositides would promote actin assembly by inducing the uncapping of actin filament fragments capped by gelsolin and other capping proteins. Actin assembly and disassembly occur in different parts of the cell at different times, and it is therefore reasonable for the cell to use different signals for the two processes. The intermediary of phospholipids in promoting actin assembly may explain the basis of the apparently nonspecific stimuli inducing actin assembly. Physical and mechanical perturbations may cause perturbations in the configuration of membrane lipids, promoting clustering of membrane polyphosphoinositides.
It has been difficult to obtain unambiguous evidence in favor of the circuitry shown in Fig 12 for controlling actin assembly in cells on the basis of correlating changes in signal transduction intermediates with net changes in cell actin assembly. The relative proportions of assembled and disassembled actin in human neutrophils has been shown to be roughly inversely proportional to the free calcium concentration, although neutrophil locomotion can take place on certain surfaces with intracellular free calcium levels clamped at nanomolar concentrations. Because of the probable redundancy of signalling mechanisms (eg, both calcium and protons activate gelsolin), compartmentation of signalling reactions, and ambiguities in the experimental approaches, the failure to achieve clean correlations is not surprising.

The best evidence in support of this proposed mechanism has come from studies of platelet activation. First, recent work building on earlier studies by White, Bainton, Boyles, Fox, Nachmias, and others has established that the resting blood platelet has a spectrin-based submembrane lamina resembling the red blood cell cytoskeleton (Fig 13). The platelet skeleton is not only attached to transmembrane proteins as in erythrocytes but also to an internal gel of actin cross-linked by ABP. The actin filaments of the internal skeleton are linked to the membrane directly to spectrin and indirectly to the glycoprotein Ib/IX complex by ABP. Activation of platelets by thrombin or glass leads to rapid severing of the actin filaments at the periphery of the internal skeleton that mobilizes the membrane and its underlying skeleton that expands. The large area of the open canalicular system of the platelet provides excess membrane to accommodate this expansion. Thereafter, two types of actin filament assembly occur. One leads to the formation of one or more actin filament bundles that invest long filopodia. The other consists of growth of actin filaments onto the severed fragments in the platelet periphery, and this assembly leads to the expansion of a circumferential lamella.

The initial fragmentation of actin filaments during platelet activation is absolutely dependent on a transient increase in intracellular free calcium. In fact, if platelets activate with their calcium clamped to nanomolar concentrations with an intracellular chelator, they become progressively distorted by the internal growth of enormously long actin filaments that coil around the cell periphery. All of these actin assembly events are inhibited by cytochalasin B, indicating that they represent barbed end growth. Gelsolin molecules can be localized at the barbed ends of actin filaments in resting platelets, and new filament growth off of the barbed ends of filaments in activated platelets has been demonstrated by electron microscopy. All of this evidence strongly implicates uncapping of capped barbed actin filaments, some of which had been severed, as the intermediary steps of platelet activation. However, additional work is required to prove unequivocally that polyphosphoinositides are the final signalling step leading to actin filament barbed end uncapping in vivo.

An important aspect of control of cell crawling by the actin system is the formation and breakage of specific connections between membrane proteins and actin filaments. The mechanism for disconnecting the membrane from the underlying actin skeleton involving severing of actin filaments described in the previous section concerning initial platelet activation is relatively crude. More complex and precise interactions, involving the formation and breakdown of multicomponent complexes between the extracellular surface and the intracellular actin gel, however, probably mediate many connections between the actin network and the extracellular matrix, and these are subject to regulation by protein phosphorylation and other modifications.

**CLINICAL IMPLICATIONS OF THE MACHINERY OF CELL CRAWLING MOTIONS**

The usual reasons given to justify research on cell crawling is that if we could understand the mechanisms of it better, we could modulate crawling for physiologically useful purposes—speed it up in, for example, wound healing, and slow it down in the setting of inflammation or tumor metastasis. Proof of the concept that such modification might be possible was provided when we showed that murine fibroblasts made to overexpress modest quantities of human gelsolin by
stable transfection had increased chemotactic responsiveness in proportion to the amount of gelsolin overexpressed. Interestingly, it was not possible to increase gelsolin expression by more than twofold in these cells, except by transient transfection methodology. In the latter case, the cells rounded up as their actin filament architecture disaggregated.99 Thus, as described above for ABP transfection experiments, there appears to be an optimal ratio of regulatory proteins to actin with a relatively limited window for altering cell function by changing this ratio.

Recently, we have engineered mice by homologous recombination that express no gelsolin.61 Although apparently healthy and able to breed normally in captivity, these mice have a long bleeding time and a delayed inflammatory response to intraperitoneal irritation. These physiologically altered responses correspond to slow activation of platelets and blunted chemotactic responsiveness of neutrophils in vitro. The findings imply that gelsolin is required for rapid crawling responses but that other members of the gelsolin family and other capping proteins compensate against major impairment in cell function. Platelets, for example, contain at least one other member of the gelsolin gene family, adseverin.62 Why is there such redundancy? The explanation lies in the analogy that an automobile engine with multiple cylinders runs much more smoothly than a lawn mower with one cylinder. The different capping proteins also provide for specific responses to specific stimuli. The existence of superficially healthy gelsolin-null mice with blunted inflammatory and thrombotic responses also points to gelsolin and other components of the cell crawling machinery as potential pharmacologic targets. Such targets are at the final steps of cell shape change and motility, distal to stimuli, receptors, and signal transduction processes, and therefore should be broadly effective sites of intervention.

Because no specific gelsolin inhibitors are currently known, a more immediate clinical direction has come from another angle. In the 1980s, it was determined that extracellular fluids of humans contain actin depolymerizing activity. Subsequent work showed that this activity was the result of two proteins. One of these, Gc globulin or the vitamin D-binding protein, is present in blood at a concentration of 8 μmol/L and binds specifically to actin subunits with high affinity. The other is a gelsolin isoform, synthesized by the same gene as cellular gelsolin on chromosome 9 and processed for export by alternative mRNA splicing. Blood plasma contains about 3 μmol/L gelsolin. A mutant form of this plasma protein is the cause of an uncommon autosomal dominant genetic disorder, Finnish-type (Meretoja) amyloidosis, characterized by late onset cranial neuropathy and corneal lattice dystrophy caused by deposition of gelsolin fragments in the affected tissues.59 Collectively, these two proteins bind actin released into the extracellular space by injury or cell death and represent what has been called the extracellular actin scavenger system.64

Extracellular actin complexed to Gc protein or to gelsolin has been detected along with partial depletion of the scavenger proteins in pregnancy, in fulminating hepatic failure, in hemolytic falciparum malaria, in adult respiratory distress syndrome, in septic shock, and in burn wound fluid.64,65 In addition to preventing fluid stasis due to buildup of long actin filaments, the scavenger system may ameliorate other toxic effects of actin. These include activation of blood platelets by ADP bound to actin subunits66 and interference with fibrin formation and fibrinolysis.67,68 It has been proposed that depletion of actin scavenger proteins might be clinically beneficial.64

Evidence in support of this contention has come from an unexpected source. Cystic fibrosis, the most frequent genetic disorder of caucasians, is caused by a defect in a chloride transport regulator. This defect somehow results among other effects in the early inflammation of airways and colonization by bacteria that exacerbates the inflammation. The inflammatory response leads to thick airway mucus that is difficult to expectorate, and the obstruction furthers the inflammatory insult. Intense neutrophilic infiltration is the hallmark of this inflammation that ultimately destroys airways, and irreversibly damages lung function.

The high consistency of cystic fibrosis sputum is not unique. Sputum of high viscosity is generally associated with purulence—intense neutrophil infiltration. Sputum is a complex material comprised of mucus glycoproteins, serum proteins, and, when purulent, the contents of degenerating inflammatory cells. Because DNA was first discovered in pus, it has been widely assumed that the high viscosity of purulent sputum is due to DNA polymers released from the nuclei of deteriorating neutrophils. On the basis of this belief, bovine pancreatic deoxyribonuclease I (DNase I) was used as a mucolytic in the 1950s until adverse reactions led to its abandonment. Recently, DNase I has been revived as a therapeutic agent in the form of human recombinant protein69 and was recently approved by the Food and Drug Administration for use in cystic fibrosis.

DNase I, in addition to being a DNA-hydrolyzing enzyme, is also an actin-subunit binding protein70 that depolymerizes actin by preventing subunits dissociating from actin filament ends from polymerizing back onto filament.71 This fact suggested that some of DNase I's mucolytic activity might result from its actin depolymerizing activity. We documented the presence of filamentous actin in cystic fibrosis sputum.72 Because the viscosity of actin filaments is proportional to the fifth power of actin filament length,73 shortening of actin filaments ought to have a marked effect on cystic fibrosis sputum viscosity if actin filaments are important contributors to this physical property.

In a direct test of this hypothesis, we showed that human plasma gelsolin diminished the viscosity of cystic fibrosis sputum at relatively low (<100 nmol/L) concentrations in vitro. DNase I also decreased the sputum viscosity, but higher (>500 nmol/L) amounts were required. These results were consistent with DNase I's effect on the sputum being mediated by slow actin depolymerization by subunit sequestration that would require large amounts of DNase I compared with gelsolin, which instantaneously severs actin filaments72 (Fig 14). Because gelsolin is a normal human extracellular protein that can be produced by recombinant technology, it is a candidate for mucolytic therapy in cystic fibrosis and other diseases characterized by purulent sputum. Presumably, gelsolin normally enters inflamed airways but in insufficient quantities to disaggregate the very large actin burden present.
If gelsolin is shown to be effective by appropriate clinical investigation, this research will have come full circle: from neutrophils back to neutrophils (albeit from live neutrophils to dead neutrophils) after a digression into reductionist studies of actin gelation and its regulation. There are many reasons to hope that gelsolin therapy will be useful in cystic fibrosis and other airway disorders, but in particular it would support the Donnall Thomas tradition of hemologic research eventuating in benefit for patients.

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