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Regulation of actin polymerization and depolymerization dynamics in the cell

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Regulation of Actin Polymerization and
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by

Jody Rosenblatt

DISSERTATION

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in the

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of the

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To Mike, Nadja and Paulina

PREFACE AND ACKNOWLEDGMENTS

I would like to thank the Department of Biochemistry and Biophysics for accepting me into its program and providing a wonderful environment that is difficult to leave. I would also like to thank the people who made my time here so special. David Morgan for being a good friend and surrogate PI and resurrecting my excitement for doing science as a career. Tim Mitchison for his infectious enthusiasm for science and excellent guidance and support. The Mitchison lab for providing a friendly, fun, and stimulating environment and the best group meetings I have ever had the pleasure of going to. In particular, I thank Lisa Belmont, Linda Wordeman, Aneil Mallavarapu, and Julie Theriot for creating a fun and exciting workplace. Claire Walczak, Louise Cramer, and Peg Siebert for creating a lab Utopia. Arshad Desai for his friendship and endless amounts of help with all lab equipment. Matt Welch for inspirational work and discussions about actin and for his scientific and personal support . Jen Frazier for her friendship and support, her excellent baby sitting skills, gumption and magnificent spirit.

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The text of Chapter Two is a reprint of the material as it appears in "The Molecular Biology of the Cell". Paul Peluso did preliminary experiments on this work. The text of Chapter Three is a reprint of the material as it appears in "The Journal of Cell Biology". Brian J. Agnew, Hiroshi Abe, and James R. Bamburg contributed to this publication by providing wild type and mutant XAC clones. Matthew Welch contributed to Chapter Four by help with Arp 2/3 complex preps, providing profilin protein and antibodies and helpful discussions. Timothy J. Mitchison directed and supervised the research that forms the basis for the dissertation.

Regulation of actin polymerization and depolymerization dynamics in the cell.

Jody Rosenblatt

Abstract

Cell motility is crucial to a variety of biological events such as wound healing, immune response, tumor cell metastasis, and cell migration during development. The first step in cell migration is protrusion of the leading edge membrane, or lamellipodia. Since actin polymerization and depolymerization dynamics are required for the leading edge of a cell to move, I have analyzed how these dynamics are regulated in a cell. To do this, I have used concentrated, cellular extracts and an assay that mimics the actin polymerization and depolymerization seen at the leading edge of a cell--the intracellular, actin-based movement of the pathogen, *Listeria monocytogenes*.

The concentration of cellular, unpolymerized actin exceeds the critical concentration for polymerization by 200 to 1000-fold. Actin could be sequestered from polymerization in a cell by either by being bound to an actin-binding protein or by being bound to ADP and, thus, inherently less likely to polymerize. I isolated cellular, unpolymerized actin, analyzed its bound nucleotide and determined whether nucleotide exchange on actin was limiting. I found that the bulk of unpolymerized actin was bound to ATP and an actin sequestering protein and that nucleotide exchange was not limiting. Therefore, it is likely that actin is regulated to polymerize at specific

sites in the cell not by exchanging ADP for ATP but, rather, by releasing actin sequestering proteins from ATP-bound actin.

The actin depolymerization rates of pure actin cannot account for the rapid rates seen in a cell. I found that an ADF/cofilin protein is responsible for the fast rates of actin depolymerization in the *Listeria*/concentrated extract assay. Furthermore, actin filaments made with a non-hydrolyzible ATP analog could no longer be depolymerized in extracts or by pure cofilin. These results suggest that ATP hydrolysis within a filament may act as a marker of its lifetime and regulate the extent to which a filament is depolymerized by cofilin in a cell. Finally, I have analyzed how depolymerized actin subunits are recycled back into a polymerization-competent form by attempting to biochemically fractionate factors required for this process.

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Regulation of actin polymerization and depolymerization dynamics in the cell.

Jody Rosenblatt and Timothy J. Mitchison

Ameboid cell movement involves extension of the leading edge membrane, adhesion of this membrane to the substratum, and movement of the remaining cell body into this extension. This type of movement is typical not only to the way amoebae move but to the way a large variety of eukaryotic cells move. A few examples of ameboid cell movement include epithelial cell movement during wound closure, leukocyte chemotaxis during immune response, and tumor cell metastasis. Because cell motility is important for so many biological responses, we must understand how this movement is accomplished. My thesis focuses on the mechanics of how the leading edge membrane protrudes-the first step in cell motility.

When the leading edge, or lamellipodium, of a moving cell is examined by electron microscopy, the predominant structures seen are actin filaments. Actin is a highly conserved, 43 kDa protein that binds ATP or ADP and can polymerize into filaments. Actin filaments were once thought only to be the structures that myosin motors could move along. However, blocking myosin activity by targeted mutagenesis or (DeLozanne and Spudich, 1987) with the drug butane dione monoxime (BDM) does not block membrane protrusion and suggests that another force must operate (Cramer and Mitchison, 1995; Lin et al., 1996). By contrast, drugs that block actin polymerization, such as cytochalasin D rapidly block lamellipodial extension and suggest that actin polymerization may be the force behind membrane protrusion (Forscher and Smith, 1988; Sampath and Pollard, 1991). To continue movement, a cell must also depolymerize the actin that polymerizes in the leading edge. A drug that blocks actin depolymerization, jasplakinolide, will rapidly cause a motile cell to cease moving and form abnormally large lamellipodia (Cramer, 1998). Thus, in

order for a cell to move in response to stimuli, continuous cycles of actin polymerization and depolymerization must be tightly coordinated at the leading edge.

The most direct way to study actin dynamics is through biochemistry. A long history of actin biochemistry has yielded information about how pure actin behaves in the test tube and provides a basis for how actin dynamics may occur in the cell. From these studies, we know that actin can bind ATP or ADP and can polymerize with either nucleotide. Pure actin will tend to polymerize until, at equilibrium, the actin monomer concentration reaches the critical concentration for polymerization . Since the critical concentration of ATP-bound actin is approximately ten-fold lower than that for ADP-bound actin, polymerization of the ATP-bound species is greatly favored (Pollard, 1986). Once ATP-bound actin is polymerized, the ATP within the filament is hydrolyzed and the terminal phosphate is slowly released from the filament (Wegner, 1977). Kinetic studies suggest that the interactions between ADP-bound actin subunits within the polymer lattice are weaker than those between ATP-bound subunits (Pollard, 1986). Production of ADP-actin subunits through ATP-hydrolysis, thus, favors actin filament disassembly. ATP hydrolysis also creates a difference in the critical concentrations between the two ends of an actin filament. As a consequence during polymerization experiments, polymerization will continue until the free monomer reaches a concentration that is above the critical concentration for the plus end but below the critical concentration for the minus end. At this point, the system reaches steady state and a behavior known as treadmilling occurs. Polymerization of ATP actin

continues predominantly at the plus end, balanced by depolymerization of ADP-actin predominantly at the minus end (Wegner, 1976).

While studies on the kinetics of pure actin have been informative about the intrinsic properties of actin, they have not been able to address how actin behaves in a cell. Progress on understanding actin dynamics within the cell cytoplasm had been limited to imaging experiments on live cells and the use of drugs that affect actin dynamics. Biochemical analysis of actin in cell extracts has proven difficult. Cell lysis typically causes changes in the actin concentration and/or changes in ionic conditions that affect actin behavior. Concentrated *Xenopus laevis* egg extracts address these technical problems (Murray, 1991). However, to utilize such extracts for actin research required a model system in which actin polymerization and depolymerization could be observed under physiological conditions and experimental control. One useful model system came with the discovery of the intracellular, actin-based movement of the pathogen, *Listeria monocytogenes*(Tilney and Portnoy, 1989; Dabiri et al., 1990). *L. monocytogenes is* a gram-positive bacterium that infects immunocompromised adults, infants and pregnant women. After infection, *Listeria* enters the cytoplasm of cells by phagocytosis and phagosome lysis. Then actin from the host cell cytoplasm forms a dense cloud around *Listeria* which then reorganizes into a comet tail (Tilney et al., 1992). Actin filaments are polymerized at the surface of the bacterium, become cross linked within the tail, and are then depolymerized distal to the bacterium (Theriot et al., 1992; Sanger et al., 1992). Actin polymerization in these tails is thought to be directly responsible for the movement of *Listeria* because no myosin motors have been implicated

and because the polymerization rate equals the rate of bacterial propulsion (Theriot et al., 1992; Sanger et al., 1992). However, the idea that polymerization pushes the bacteria forward has not yet been proven, and the coupling mechanism remains unknown (Oster and Perelson, 1992). The actin dynamics seen within *Listeria* tails are similar to those seen in the lamellipodia of motile cells and, therefore, provide a good model for studying the actin dynamics within lamellipodia. We found that *Listeria* movement can be reproduced in cell-free extracts at rates similar to those in an intact cell (Theriot et al., 1994; Appendix A). Thus, *Listeria* motility in extracts provides a biochemically manipulable system for analyzing actin dynamics in the cell. Using this system, I address several questions about the regulation of actin dynamics in the cell: How are high concentrations of unpolymerized actin maintained in the cell and how is unpolymerized actin activated for polymerization? How is actin polymerization initiated? How is actin depolymerization regulated in the cell and what protein(s) are essential for depolymerization? Once actin becomes depolymerized, how is it recycled to repolymerize?

Actin-dependent functions require that actin only be polymerized at specific sites in response to specific cues. In the cytoplasm there are a number of actin-binding proteins that help regulate cellular actin dynamics. We found that one actin-binding protein, profilin, localizes to sites where actin is polymerized and plays a role in the rate of actin polymerization (Theriot et al., 1994). This is the subject of Appendix A. *In vitro* studies on profilin show that it can enhance actin polymerization in several ways--it can increase ATP exchange on actin and thereby increase the concentration of the more polymerization-competent ATP-bound actin

species (Goldschmidt et al., 1992), it can release actin from binding the actin sequestering protein, thymosin ß4, and it can decrease the critical concentration for ATP-actin at the plus end (Carlier et al., 1993). Although profilin appears to be important for catalyzing actin polymerization *in vitro*, it was not known how profilin enhances polymerization in the cell. The work in Appendix A suggested that profilin is important for actin polymerization by *Listeria*. Other work (Marchand et al., 1995) has questioned this conclusion and suggests that further work will need to be to resolve this issue. Bound profilin and profilin binding proteins such as VASP and FH box proteins localize to important places in cells where actin dynamics are regulated (Frazier and Field, 1997). Thus the role of profilin, in *Listeria* motility and other actin-dependent processes, deserves further research.

One approach to understanding how polymerization is regulated is to examine how high concentrations of unpolymerized actin are maintained in a cell. Polymerization, then, could be controlled by reversing the mechanism by which the actin is sequestered from polymerization. The actin concentration in the cell is between 200- to 1000-fold greater than the critical concentration for polymerization. Given this high concentration, nearly all of the actin would be expected to be in polymer. Yet, most of the actin in a cell exists in an unpolymerized form. How is the actin remain unpolymerized until it is signaled to polymerize? Two possible models for how actin is sequestered from polymerization have been discussed in the literature: that it is physically sequestered by binding an actin-binding protein or that the free pool of actin is bound to ADP which is inherently less likely to polymerize (Goldschmidt et al.,

1991). Chapter two describes experiments designed to determine whether cellular, unpolymerized actin is predominantly bound to ATP or to ADP. I show that most of the unpolymerized actin in the cell is bound to ATP and an actin sequestering protein (Rosenblatt et al., 1995). This results indicates that in order to become active for polymerization, actin subunits must be released from sequestering protein. Our work leaves open the question of whether the nucleotide exchange activity of profilin is important in vivo. Conceivably this could be tested by constructing profilin mutants defective in this activity and not other functions and analyzing their effects in cells.

Once actin monomers are activated for polymerization, how is polymerization initiated? Actin subunits could be added onto pre-existing filaments or new filaments could be made by nucleation. To distinguish between these two mechanisms, one could purify the protein(s) responsible for initiating polymerization and analyze the polymerization activity *in vitro*. Welch et.al. (Welch et al., 1997) fractionated platelet extracts for an activity that could allow actin to polymerize around *Listeria monocytogenes* and purified a complex of seven polypeptides known as the Arp 2/3 complex. Analysis of the *in vitro* activity of the Arp 2/3 complex is included in Appendix B. This work shows that the Arp 2/3 complex does not recruit previously formed filaments but instead, in conjunction with the *Listeria* surface protein ActA, nucleates actin filament formation (Welch et al., 1998).

Another outstanding question in cellular actin polymer dynamics is how is actin depolymerization regulated? This question has been Do actin filaments depolymerize in the cell as they do *in vitro*, by treadmilling, or do other mechanisms operate? One would predict that rapid rates of actin

depolymerization in the cell would have to accompany the rapid rates of actin polymerization, if polymerization is to continue. Yet, the *in vitro* rates of depolymerization by spontaneous subunit dissociation from the minus-end are too slow to account for the depolymerization rates seen for actin filaments in the lamellipodia or in *Listeria* tails (Pollard, 1986; Theriot and Mitchison, 1991; Theriot et al., 1992). This discrepancy led to the 'nucleation-release' model which proposed that subunits may depolymerize off the plus end and that they could do this at a rate that would account for the rates seen in the cell (Theriot and Mitchison, 1992). The nucleation-release model, however, was based upon the rate of disappearance of marks made into lamellipodial filaments assuming that the filaments were no longer than 0.2 μ (Tilney and Portnoy, 1989; Tilney, 1990). Once filaments were found to be much longer-as much as 5 μ in lamellipodia (Small et al., 1995) and in *Listeria* tails (Sechi et al., 1997), it became clear that the intrinsic depolymerization rates could not account for those seen *in vivo* . Thus, actin depolymerizing enzymes must be invoked to allow for the rates seen in the cell. The most important part of my thesis work was to identify such enzymes in a physiological context. In Chapter three, I demonstrate that a member of the ADF/cofilin family of proteins is responsible for depolymerizing actin filaments in the tails of *Listeria monocytogenes* (Rosenblatt et al., 1997). Although it is controversial whether this family of proteins depolymerizes by severing pieces of filaments or by end-wise removal of subunits (Maciver et al., 1991; Carlier et al., 1997), I show that this ADF/cofilin protein depolymerizes specifically the ADP-containing subunits of an actin filament (Rosenblatt et al., 1997). This activity suggests a model where actin filament depolymerization in

the cell is regulated by ATP hydrolysis as it is during treadmilling, but actin depolymerization in the cell is assisted by actin depolymerizing proteins.

Finally, to complete the cycle of actin polymerization and depolymerization, depolymerized subunits must be recycled into a polymerization-competent form. Actin polymers are not recruited to sites of actin polymerization to form nuclei for further polymerization (Welch et al., 1998). In Chapter four, I show that simply depolymerizing actin is not sufficient to recycle it. I also examine how the depolymerized subunit is recycled into polymer by fractionating extracts to isolate protein(s) that can recycle actin subunits in a microscopic assay. I was not able to solve the recycling problem during my thesis yet I think the issue remains interesting and worthy of future investigation.

The results within this thesis should give some insight into how actin polymer dynamics are regulated within a cell. I have approached these studies with a view to an underlying thermodynamic principle--how is the energy of ATP consumption translated into the force production of lamellipodial protrusion or bacterial propulsion? ATP exchange or hydrolysis could be used for controlling polymerization or depolymerization, respectively. In chapter two, I determine whether ATP consumption is important for regulating actin polymerization in the cell. ATP hydrolysis is not required for actin to polymerize but production of ATP-bound actin could be important for enabling polymerization. My findings show that most of the cellular, unpolymerized actin is ATP-bound, so nucleotide exchange on actin does not appear to be an important control point for regulating actin polymerization.

Although production of ATP-bound actin does not appear to be important for controlling actin polymerization, ATP hydrolysis within the actin filament may be important for controlling actin depolymerization. The results in Chapter three show that only the ADP-bound subunits within an actin filament are susceptible to depolymerization and suggest that ATP hydrolysis directs the lifetime of a filament. Actin polymerizes at the barbed end near the surface of *Listeria monocytogenes* (or the plasma membrane) and after the filaments reach a certain length, dictated by ATP hydrolysis, they are depolymerized. Thus, the energy from ATP consumption during actin filament dynamics is used to restrict actin polymer to a specific location. The use of ATP in cellular actin dynamics reflects its use in regulating the dynamics of pure actin. ATP consumption does not appear to be important for polymerization since either ATP-or ADP-containing actin subunits can polymerize. ATP hydrolysis, however, is important for creating the differences in the two ends of a filament that account for polymer treadmilling. While ATP is used to regulate treadmilling of actin filaments in the cell just as in the test tube, the main difference between actin depolymerization in a cell versus a test tube is that actin depolymerizing enzymes are available to speed up actin filament turnover. By combining the spatial regulation of actin polymerization and depolymerization so that polymerization only occurs at the cell tip and depolymerization only occurs on the older, more cell-central filaments, polymerization can proceed in one direction and provide a net force for membrane protrusion in this direction.

References:

Carlier, M. F., Jean, C., Rieger, K. J., Lenfant, M., and Pantaloni, D. (1993). Modulation of the interaction between G-actin and thymosin beta 4 by the ATP/ADP ratio: possible implication in the regulation of actin dynamics. Proc Natl Acad Sci U S A *90*, 5034-8.

Carlier, M. F., Laurent, V., Santolini, J., Melki, R., Didry, D., Xia, G. X., Hong, Y., Chua, N. H., and Pantaloni, D. (1997). Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: implication in actin-based motility [see comments]. J Cell Biol *136*, 1307-22.

Cramer, L. (1998). manuscript in preparation.

Cramer, L. P., and Mitchison, T. J. (1995). Myosin is involved in postmitotic cell spreading. J Cell Biol *131*, 179-189.

Dabiri, G. A., Sanger, J. M., Portnoy, D. A., and Southwick, F. S. (1990). *Listeria monocytogenes* moves rapidly through the host-cell cytoplasm by inducing directional actin assembly. Proc. Natl. Acad. Sci. USA. *87*, 6068-6072.

DeLozanne, A., and Spudich, J. A. (1987). Disruption of the *Dictyostelium* myosin heavy chain by homologous recombination. Science (Wash.) *236*, 1086-1091.

Forscher, P., and Smith, S. J. (1988). Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. J Cell Biol *107*, 1505-16.

Frazier, J., and Field, C. (1997). Actin cytoskeleton: are FH proteins local organizers? Curr Biol *7*, R414-7.

Goldschmidt, C. P., Furman, M. I., Wachsstock, D., Safer, D., Nachmias, V. T., and Pollard, T. D. (1992). The control of actin nucleotide exchange by thymosin beta 4 and profilin. A potential regulatory mechanism for actin polymerization in cells. Mol Biol Cell *3*, 1015-24.

Goldschmidt, C. P., Machesky, L. M., Doberstein, S. K., and Pollard, T. D. (1991). Mechanism of the interaction of human platelet profilin with actin. J Cell Biol *113*, 1081-9.

Lin, C.-H., Espreafico, E. M., Mooseker, M. S., and Forscher, P. (1996). Myosin drives retrograde F-actin flow in neuronal growth cones. Neuron *16*, 769-782.

Maciver, S. K., Zot, H. G., and Pollard, T. D. (1991). Characterization of actin filament severing by actophorin from Acanthamoeba castellanii. J Cell Biol *115*, 1611-20.

Marchand, J. B., Moreau, P., Paoletti, A., Cossart, P., Carlier, M. F., and Pantaloni, D. (1995). Actin-based movement of Listeria monocytogenes:

actin assembly results from the local maintenance of uncapped filament barbed ends at the bacterium surface. J Cell Biol *130*, 331-43.

Murray, A. W. (1991). Cell cycle extracts. Methods Cell Biol *36*, 581-605.

Oster, G., and Perelson, A. (1992). Cell protrusions. .

Pollard, T. D. (1986). Rate constants for the reactions of ATP- and ADP-actin with the ends of actin filaments. J Cell Biol, 2747-54.

Rosenblatt, J., Agnew, B. J., Abe, H., Bamburg, J. R., and Mitchison, T. J. (1997). Xenopus actin depolymerizing factor/cofilin (XAC) is responsible for the turnover of actin filaments in Listeria monocytogenes tails [see comments]. Journal of Cell Biology *136*, 1323-32.

Rosenblatt, J., Peluso, P., and Mitchison, T. J. (1995). The Bulk of Unpolymerized Actin in *Xenopus* Egg Extracts Is ATP-bound. Mol. Biol. Cell *6*, 227-236.

Sampath, P., and Pollard, T. D. (1991). Effects of cytochalasin, phalloidin, and pH on the elongation of actin filaments. Biochemistry *30*, 1973-80.

Sanger, J. M., Sanger, J. W., and Southwick, F. S. (1992). Host cell actin assembly is necessary and likely to provide the propulsive force for intracellular movement of *Listeria monocytogenes*. Infect. Immun. *60*, 3609-3619.

Sechi, A., Wehland, J., and Small, J. (1997). The isolated comet tail pseudopodium of Listeria monocytogenes: a tail of two actin filament populations, long and axial and short and random. J Cell Biol *137*, 155-67.

Small, J. V., Herzog, M., and Anderson, K. (1995). Actin filament organization in the fish keratocyte lamellipodium. J Cell Biol *129*, 1275-86.

Theriot, J. A., and Mitchison, T. J. (1991). Actin microfilament dynamics in locomoting cells. Nature *352*, 126-131.

Theriot, J. A., and Mitchison, T. J. (1992). The nucleation-release model of actin filament dynamics in cell motility. Trends Cell Biol. *2*, 219-222.

Theriot, J. A., Mitchison, T. J., Tilney, L. G., and Portnoy, D. A. (1992). The rate of actin-based motility of intracellular *Listeria monocytogenes* equals the rate of actin polymerization. Nature (Lond.) *357*, 257-260.

Theriot, J. A., Rosenblatt, J., Portnoy, D. A., Goldschmidt, C. P., and Mitchison, T. J. (1994). Involvement of profilin in the actin-based motility of L. monocytogenes in cells and in cell-free extracts. Cell *76*, 505-17.

Tilney, L. G., Connelly, P.S., and Portnoy, D.A. (1990). Actin Filament Nucleation by the Bacterial pathogen, Listeria monocytogenes. J. Cell Biology *111*, 2979-2988.

Tilney, L. G., DeRosier, D. J., Weber, A., and Tilney, M. S. (1992). How Listeria exploits host cell actin to form its own cytoskeleton. II. Nucleation, actin filament polarity, filament assembly, and evidence for a pointed end capper. J Cell Biol *118*, 83-93.

Tilney, L. G., and Portnoy, D. A. (1989). Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, listeria monocytogenes. J. Cell Biol. *109*, 1597-1608.

Wegner, A. (1976). Head to tail polymerization of actin. J. Mol. Biol. *108*, 139-150.

Wegner, A. (1977). The mechanism of ATP hydrolysis by polymer actin. Biophys Chem *7*, 51-8.

Welch, M. D., Iwamatsu, A., and Mitchison, T. J. (1997). Actin polymerization is induced by Arp2/3 protein complex at the surface of Listeria monocytogenes. Nature *385*, 265-9.

Welch, M. D., Rosenblatt, J., Skoble, J., Portnoy, D. A., and Mitchison, T. J. (1998). The human Arp2/3 complex is composed of evolutionarily conserved subunits and is localized to cellular regions of dynamic actin filament assembly. Science *281*, 105-8.

Chapter Two

The bulk of unpolymerized actin in *Xenopus* egg extracts is ATP-bound.

Jody Rosenblatt, Paul Peluso, and Timothy J. Mitchison
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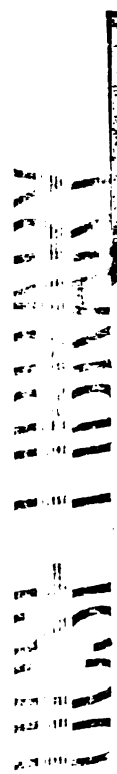

The Bulk of Unpolymerized Actin in *Xenopus* Egg Extracts Is ATP-bound

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Non-muscle cells contain 15–500 μM actin, a large fraction of which is unpolymerized. Thus, the concentration of unpolymerized actin is well above the critical concentration for polymerization in vitro (0.2 μM). This fraction of actin could be prevented from polymerization by being ADP bound (therefore less favored to polymerize) or by being ATP bound and sequestered by a protein such as thymosin β_4, or both. We isolated the unpolymerized actin from *Xenopus* egg extracts using immobilized DNase 1 and assayed the bound nucleotide. High-pressure liquid chromatography analysis showed that the bulk of soluble actin is ATP bound. Analysis of actin-bound nucleotide exchange rates suggested the existence of two pools of unpolymerized actin, one of which exchanges nucleotide relatively rapidly and another that apparently does not exchange. Native gel electrophoresis of *Xenopus* egg extracts demonstrated that most of the soluble actin exists in complexes with other proteins, one of which might be thymosin β_4. These results are consistent with actin polymerization being controlled by the sequestration and release of ATP-bound actin, and argue against nucleotide exchange playing a major role in regulating actin polymerization.

INTRODUCTION

Actin-dependent cell movements, such as cytokinesis or locomotion toward a chemoattractant, require precise temporal and spatial regulation of actin polymerization (Korn, 1982; Pollard, 1986a,b; Cooper, 1991). Although several proteins have been identified that might be important for this regulation, the exact mechanisms for control of actin polymerization at specific sites in cells have remained elusive. In vitro, actin polymerization is regulated by monomer concentration and the bound nucleotide. Under conditions considered physiological, the critical concentration for polymerization of ATP-actin is 0.2 μM whereas that of ADP-actin is 2 μM. (Pollard, 1986a). Because the concentration of unpolymerized actin in most cells (8–250 μM) is well above the critical concentration for actin polymerization, it has long been realized that cells must possess a mechanism for stabilizing unpolymerized actin (Korn, 1982).

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A number of actin monomer binding proteins have been implicated in actin polymerization regulation. Recent studies have highlighted two proteins that may be important for actin polymerization control, thymosin β_4 and profilin. Thymosin β_4 is an abundant, 5-kDa polypeptide that is thought to be the major actin-sequestering protein in many higher eukaryotic cells (Safer *et al.*, 1990; Cassimeris *et al.*, 1992; Yu *et al.*, 1993). Thymosin β_4 binds ATP-actin with a 50-fold higher affinity than ADP-actin (Carlier *et al.*, 1993), inhibits actin nucleotide exchange when bound (Goldschmidt *et al.*, 1992), and inhibits actin polymerization in vitro. Microinjection or overproduction of thymosin β_4 in fibroblasts can induce extensive loss of actin stress fibers (Sanders *et al.*, 1992; Yu *et al.*, 1994) supporting its role as a negative regulator of actin filament formation. Profilin is a 15-kDa protein that binds actin monomer (Carlsson *et al.*, 1977) and phosphatidylinositol 4,5-bisphosphate (PIP_2) in a mutually exclusive manner and is thought to be regulated by multiple cellular signal transduction pathways (Vojtek *et al.*, 1991; Sohn, 1994). This link between profilin and signal transduction at the plasma membrane makes pro-

filin an attractive candidate for a key regulator of actin polymerization. Certain studies show that profilin can act as an actin-sequestering protein and inhibit actin polymerization (Carlsson *et al.*, 1977; Ozaki and Hatano, 1984; Haugwitz, 1994;). However, other work contends that profilin can also be a promoter of actin filament formation. Genetic studies have shown that profilin is important for actin filament assembly and stability in a variety of organisms (Haarer *et al.*, 1990; Cooley *et al.*, 1992; Magdolen *et al.*, 1993; Balasubramanian *et al.*, 1994; Finkel *et al.*, 1994). In addition, profilin is localized at the cell membrane (Carlsson *et al.*, 1977; Buss *et al.*, 1992) and at the end of *Listeria* (Theriot *et al.*, 1994) where actin polymerization occurs. In vitro studies have described two activities that could explain how profilin could act as a promoter of polymerization in vivo. One activity of profilin is its ability to act as a nucleotide exchange protein for actin (Korn, 1982; Goldschmidt *et al.*, 1992) and the other is its ability to release actin from sequestration by thymosin β_4 and to lower the critical concentration for ATP-actin at the barbed ends of actin filaments (Pantaloni and Carlier, 1993). To date, the role profilin plays in controlling actin polymerization in vivo is unclear and the balance between its role in sequestering actin and promoting polymerization may vary in different cells.

To understand the key regulatory step in actin polymerization, we must examine how unpolymerized actin can exist at levels well above the critical concentration for polymerization in vivo. Specifically, the high monomer concentration requires either that unpolymerized actin is sequestered by monomer binding proteins and/or that the bound nucleotide is ADP. To examine the potential roles of these two mechanisms for actin sequestration in vivo, we have developed a protocol for isolating unpolymerized actin from cell cytoplasm and analyzing its bound nucleotide.

Isolation of actin from cells usually requires extensive dilution of the cytoplasm with buffer, which can cause depolymerization of actin filaments and thus potentially generate artificially high levels of ADP-actin. We have chosen *Xenopus laevis* egg extracts (Murray, 1991) to perform these experiments because these appear to recapitulate cytoplasmic regulation of actin polymerization and have the advantages of a cell-free system. Specifically, *Xenopus* egg extracts maintain a high level of unpolymerized actin, and this actin can be induced to polymerize by addition of a physiological desequestration/nucleating site, the surface of a *Listeria monocytogenes* bacterium (Theriot *et al.*, 1994). Furthermore, *Xenopus* egg extracts must be capable of rapidly depolymerizing actin filaments because the *Listeria* tail actin turns over with normal kinetics (Theriot and Mitchison, 1992). In this paper we quantitate the level of unpolymerized actin in these extracts and further document their filament depolymerizing activity. We then investigate the mechanism that maintains this large concentration of unpolymerized actin by analyzing the actin's bound nucleotide and potential sequestering proteins.

To isolate actin with its bound nucleotide we used immobilized DNase 1 because this protein not only binds specifically to actin, but also blocks the nucleotide on actin from exchanging once bound (Hitchcock, 1980; Mannherz *et al.*, 1980). This reagent allowed us to analyze the nucleotide content of unpolymerized actin as well as to study the extent and rate of actin nucleotide exchange in a model system for cellular actin dynamics. In addition, we used a native gel system (Safer, 1989) to determine whether the unpolymerized actin is complexed with any monomer binding proteins. Our results are inconsistent with nucleotide exchange playing a key regulatory role for actin polymerization and are more consistent with release of ATP-actin by a sequestering protein such as thymosin β_4.

MATERIALS AND METHODS

Materials

Frozen rabbit skeletal muscle was obtained from Pelfreeze (Rogers, AR). ATP and dithiothreitol (DTT) were obtained from Sigma Chemical Co. (St. Louis, MO). Biogel P-6, Affigel-10, and low molecular weight standards were obtained from Bio-Rad Laboratories (Richmond, CA). Ten-kilodalton cut-off filters were obtained from Millipore Co. (Bedford, MA). DNase 1 was obtained from Boehringer Mannheim Biolabs (Indianapolis, IN). DNase 1 beads were made by coupling DNase 1 to Affigel-10 using 50 mM HEPES, pH 7.7, and 80 mM $CaCl_2$ as the coupling buffer. Typically 20 mg of DNase 1 was bound per milliliter of Affigel. ^{32}P-α ATP was from NEN (Boston, MA). One milliliter of Mono-Q HR 5–5 column was obtained from Pharmacia (Piscataway, NJ). Mouse anti-actin monoclonal antibody was obtained from Amersham (Arlington Heights, IL). Alkaline phosphatase-conjugated rabbit anti-mouse antibody was obtained from Promega (Madison, WI). *N*-hydroxysuccinimidyl 5-carboxytetramethyl rhodamine was obtained from Molecular Probes Inc. (Junction City, OR)

Protein and Extract Preparation

Rabbit skeletal muscle globular actin (G-actin) was prepared as described (Spudich and Watt, 1971) and centrifuged at 436 000 × *g* for 15 min at 4°C. The supernatant was stored at 4°C and used within 10 days.

Concentrated meiotically arrested cytoplasmic extracts from *Xenopus laevis* eggs were prepared as described (Theriot *et al.*, 1994). A partially purified fraction of thymosin β_4 was made by boiling *Xenopus* egg extracts for 10 min, centrifuging 10,000 × *g* for 10 min, and centrifuging the 10,000 g supernatant at 436,000 × *g* for 15 min. A purer thymosin β_4 fraction was made by concentrating the boiled supernatant using a 3000 molecular weight cut-off Centricon unit, desalting with a biogel P-6 column, and filtering through a 10,000 molecular weight cut-off Centricon unit.

Fluorescent Microscopy of Actin Filaments

Polymerized rabbit skeletal muscle actin at 30 mg/ml was mixed with a twofold molar excess of *N*-hydroxysuccinimidyl 5-carboxytetramethyl rhodamine (Molecular Probes Inc.) as previously described (Kellogg *et al.*, 1988). The final G-actin was dialyzed

against G-buffer (5 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM DTT, 0.2 mM ATP), clarified by centrifugation at 100,000 × *g* in a TLA Beckman centrifuge (Palo Alto, CA) for 15 min., frozen in aliquot in liquid nitrogen, and stored at −80°C. The stoichiometry of labeling was ~0.125 rhodamine molecules per actin monomer as determined by absorption spectrophotometry using an extinction coefficient e_{280} = 49,000 M^{-1} cm^{-1} for actin and e_{540} = 50,000 M^{-1} cm^{-1} for tetramethyl rhodamine (Molecular Probes).

Pure rhodamine actin filaments were made by polymerizing 5 μg of rhodamine-labeled actin in 20 μl of F-buffer (50 mM KCl, 50 mM Tris-HCl, pH 8.0, 1 mM ATP, 0.2 mM DTT) and 1 μl of a 20× stock solution of oxygen scavengers (2 mg/ml catalase, 0.6 mg/ml glucose oxidase, 200 mM glucose, 20 μm DTT, 40 mg/ml hemoglobin) to prevent photo-damage and bleaching. This actin could be depolymerized by mixing with a 1:1 vol of *Xenopus* egg extract. Rhodamine-labeled actin was polymerized in *Xenopus laevis* egg extracts by mixing 1 μl of 5 mg/ml rhodamine actin with 10 μl of the extract and 0.5 μl of a 20× stock solution of oxygen scavengers. These rhodamine actin filaments could be removed by centrifugation at 436,000 × *g* in a TLA 100 rotor for 15 min.

ATP- or ADP-bound Actin

The free ATP was removed from 60 μg of rabbit muscle actin in 80 μl of G-buffer by spinning 3000 g for 2 min through a 0.7 ml Biogel P-6 column pre-equilibrated in G-buffer without ATP. Either ATP or ADP was added to a final concentration of 0.2 mM and incubated for 15′ at 4°C followed by 15′ at 20°C. High-pressure liquid chromatography (HPLC) analysis revealed that the actin incubated in ATP was 100% ATP bound, and the actin incubated in ADP was 50% ADP bound and 50% ATP bound.

Isolation of the Actin-bound Nucleotide from Xenopus Egg Extracts

Actin-bound nucleotide was isolated as described in Table 1. High speed supernatants (h.s.s.) of either 100 μl rabbit muscle actin or *Xenopus* extracts were spun through a 0.7 ml Biogel P-6 column pre-equilibrated in G-buffer without ATP to remove the majority of unbound nucleotides. The flow through (~120 μl) was then added to 25 μl of DNase 1 beads, and incubated at 4°C for 1 h with frequent vortexing. Nonspecific proteins were removed with two 0.5 ml washes of wash buffer (0.4 M NH_4Cl_2, 10 mM Tris, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM DTT) for 1 min and the washed beads were recovered by centrifuging for 1 min at 10 000 × *g* in an Eppendorf microfuge. In a typical experiment, only ≈23 pmol of actin or 2–3% of the total actin was lost during the washes. The actin was denatured and eluted with 8 M urea, 10 mM Tris, pH 7.4, 0.2 mM $CaCl_2$ at 100°C for 5 min. Ten percent of the eluate was removed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and the remainder was diluted threefold with H_2O and filtered through a 10,000 kDa cut-off spin filter unit. The isolated nucleotides in the filtrate were then analyzed by HPLC. Total free nucleotides from *Xenopus* extracts were isolated for HPLC analysis by filtering crude extract through a 10-kDa cut-off spin filter unit.

Table 1. Isolation and analysis of nucleotide from actin monomer

Step	Method
Make concentrated *Xenopus* egg extract	Crush CSF-arrested X.1. eggs @ 21,000 × *g* 10′
Spin out F-actin	436,000 × *g* 15′ @ 4°C
Remove unbound nucleotides	Pass over a Bio-gel P-6 column
Purify actin and bound nucleotide	Bind to DNase 1 beads at 4°C 1 h, wash
Isolate nucleotides from actin	Elute 8 M urea, 100°C; filter through a 10-kDa cut-off filter
Analyze nucleotides	HPLC analysis using a MONO-Q column

HPLC Analysis of the Actin-bound Nucleotide

The filtrate from the last step in Table 1 was analyzed on a 1 ml Mono-Q column equilibrated in 100 mM NH_4HCO_3 and eluted in a 100–500 mM NH_4HCO_3 gradient over 30 min at a flow rate of 1 ml/min. Peak areas were analyzed and recorded at OD_{254} using Gilson software. The entire DNase 1 filtrate or 5 μl of crude extract filtrate was loaded for each HPLC run.

Quantitation of Actin by SDS-PAGE

The concentration of rabbit skeletal muscle actin, the actin standard, was determined by absorption spectrophotometry using an extinction coefficient e_{280} = 49,000 M^{-1} cm^{-1}. Because the absorbance of ATP in the G-buffer may interfere with the absorbance of actin at OD_{280}, the free and bound ATP were removed before spectrophotometry by denaturating an aliquot of muscle actin with 6 M guanidine HCl and passing over a 0.7-ml Biogel P-6 column pre-equilibrated in G-buffer without ATP. DNase 1 bead eluates and a series of the above actin standards were analyzed by 10% SDS-PAGE followed by Coomassie blue staining. Actin was quantified by cutting the the DNase 1-derived actin and actin standard bands out of the stained gel and eluting the dye in 0.4 ml of 50% methanol, 7% acetic acid at 100°C. The eluted dye was quantitated by spectophotometrically at OD_{595}.

Nucleotide Exchange Analysis

The percentage of nucleotide exchange was determined by dividing the specific activity of actin-bound nucleotide by the specific activity of free nucleotide. To determine the specific activity of bound ATP and ADP from actin, ^{32}P-α ATP was incubated with crude extracts for either 0′ or 30′ at room temperature, then the actin was isolated as described in Table 1 and the released nucleotides were analyzed by HPLC. Each 1-ml fraction was collected and counted with 5 ml of scintillate on a Beckman Scintillation Counter. From the cpm counted per fraction, we determined the number of moles of ^{32}P-α ATP per moles of ATP in each peak. The specific activity of free nucleotides were determined in a similar manner using total nucleotide that passed through a 10-kDa cut-off filter.

Native Gel Electrophoresis

Proteins or extract h.s.s were analyzed on 7.5% native polyacrylamide gels at 4°C as described (Safer *et al.*, 1990) except that 0.2 mM $MgCl_2$ was added to the gel buffer. Gels were transferred electrophoretically to nitrocellulose in 20 mM Tris, 25 mM glycine, 20% methanol, 0.1% SDS. After blocking in 5% milk in TBST (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20), the blot was incubated with a 1:1000 dilution of mouse anti-actin monoclonal antibody (Amersham) in TBST + 0.02% NaN_3. Immunoreactive bands were detected using an alkaline phosphatase-conjugated rabbit anti-mouse antibody.

RESULTS

The Use of Xenopus Egg Extracts

We first sought to determine whether active turnover of actin filaments occurs in *Xenopus* egg extracts by assaying their effect on preformed filaments of rabbit muscle actin. When rhodamine-labeled actin filaments

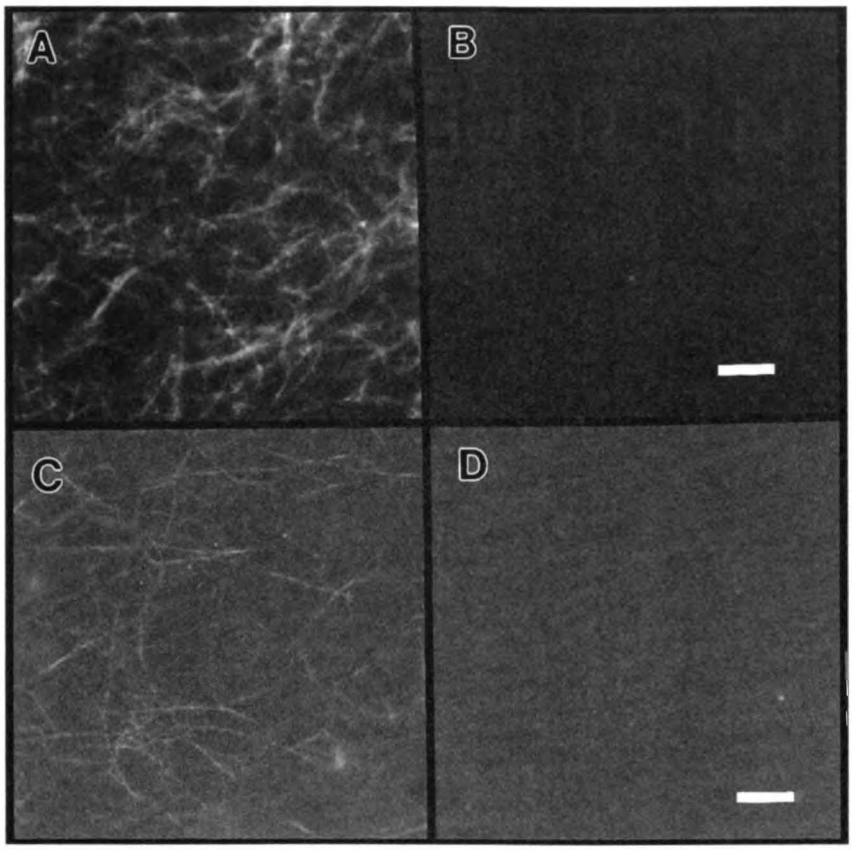

Figure 1. Micrographs of rhodamine-labeled actin filaments. Rhodamine-labeled rabbit muscle actin (0.25 mg/ml) polymerized in F-buffer alone (A) or mixed 1:1 with crude *Xenopus laevis* egg extracts (B). Rhodamine-labeled actin (5 μg) polymerized in 10 μl *Xenopus laevis* egg extracts before (C) and after (D) removal by high speed (436,000 × *g*) centrifugation. Bar represents 5 μm for (A) and (B) and 2.5 μm for (C) and (D).

(Figure 1A) were added to an equal vol of *Xenopus* extract they depolymerize within ≈1–2 min (Figure 1B). Dilution into buffer had no effect on these filaments. In fact, part of the released, labeled actin reincorporates into endogenous filaments in the extract, but these are not visible with the low amount of labeled actin in this experiment. In addition, higher concentrations of rhodamine-G-actin added to *Xenopus* egg extracts incorporated into endogenous filaments within ≈5 min (Figure 1C). By eye, these filaments could be seen to depolymerize and repolymerize over time suggesting that the actin in these extracts is not polymerized to equilibrium, but is fluxing rapidly between monomer and polymer to create a steady state, like actin in motile cells. Detailed imaging experiments are presently being done to more thoroughly analyze these actin dynamics. Although we could not see repolymerization of actin filaments in Figure 1B, we believe that the actin is being reassembled but that the rhodamine signal is too dilute to visualize these filaments, compared with that in Figure 1C where the actin is more concentrated. A possible caveat with these experiments is that the behavior of rhodamine-labeled rabbit muscle actin may not accurately report the behavior of endogenous *Xenopus* egg actin. Holliday *et al.* (1993) have reported that pyrene-labeled muscle actin is not an accurate reporter of endogenous actin behavior in *Acanthamoeba* extracts. Resolving this issue will require more work. However, we feel that the results of Figure 1 combined with the *Listeria* data (Theriot *et al.*, 1994) suggest that *Xenopus* egg extracts mimic cytoplasmic actin dynamics. Therefore, we used these extracts as a model for cellular actin dynamics from which we could isolate the unpolymerized actin and analyze its nucleotide content.

Isolation of Actin-Bound Nucleotide

To analyze the actin-bound nucleotide in *Xenopus* egg extract we developed the procedure shown in Table 1, which depends on the high affinity of DNase 1 for

actin, and the fact that DNase 1 binding blocks nucleotide exchange. Approximately half of the actin in the *Xenopus* egg extracts is sedimentable, presumably corresponding to F-actin. It was necessary to remove this F-actin from the extracts before isolating the G-actin. Although DNase 1 beads should preferentially bind G-actin, we found in test experiments that a significant amount of actin derived from filaments also bound to DNase 1 beads. This binding may result from DNase 1 binding to the pointed end of the actin filament or the induction of F-actin depolymerization by DNase 1 and subsequent binding of the G-actin (Hitchcock, 1980). Because the bulk of F-actin is ADP-bound, binding of either F-actin or recently depolymerized G-actin caused artificially high levels of ADP to appear in the DNase 1-derived actin in test experiments. To eliminate this potential artifact, high speed centrifugation was used to remove the F-actin. Comparison of extracts with added rhodamine-labeled actin before (Figure 1C) and after centrifugation (Figure 1D) by fluorescent microscopy confirmed that centrifugation was sufficient to remove detectable F-actin from the extracts. Quantitative Western blot analysis showed that the total actin concentration in *Xenopus* egg extracts varied between 15–25 μM and approximately 50% of the total actin remained soluble after centrifugation.

A P-6 desalting column was used to remove the majority of free nucleotide from the h.s.s. before actin isolation and to equilibrate the actin into a buffer in which it would not polymerize during the isolation step. Although DNase 1 binding inhibits actin nucleotide exchange, free nucleotide was removed to try to prevent exchange from occurring during the relatively slow binding step. By HPLC analysis of soluble components derived from the 10-kDa filtrate of a P-6 flow through, we estimate that >99.8% of the unbound nucleotide from *Xenopus* egg extracts is removed by the P-6 desalting column. However, after desalting, only 5% of the remaining ATP bound to DNase 1 beads under conditions where >90% of the actin bound (estimated by using radioactive ATP as a tracer). Thus, the desalted extracts contain an excess of ATP presumably bound to other nucleotide-binding proteins. This ATP can still potentially exchange onto actin during the DNase 1 binding step. In test experiments, we found that actin-bound nucleotide profiles were in fact similar with or without the desalting step. We retained this step to decrease the amount of exchange during nucleotide isolation and to ensure reproducibility.

DNase 1 beads proved effective in isolating actin and its bound nucleotide from *Xenopus* h.s.s (Figure 2, lane 3), and no detectable actin bound to the unconjugated beads (lane 4). By quantitating the supernatant and pellet of the *Xenopus* extract DNase 1 precipitation using Coomassie blue binding and Western blot analysis, we determined that 90–95% of the actin from

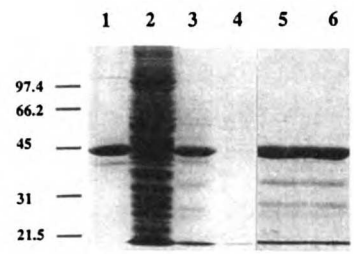

Figure 2. Coomassie blue stained SDS-PAGE of 5 μg rabbit skeletal muscle actin (lane 1), crude *Xenopus laevis* egg extract (lane 2), *Xenopus laevis* actin purified from DNase 1 beads (lane 3), and proteins bound to unconjugated Affigel-10 beads (lane 4). DNase 1 beads bind ATP-actin (lane 5) and ADP-actin (lane 6) equally well.

h.s.s. was recovered on the DNase 1 beads. In control experiments with pure actin we found that DNase 1 beads bind ATP- and ADP-actin equally well (Figure 2, lanes 5 and 6). Incubation of DNase 1 beads with ^{32}P-α ATP and excess cold ATP demonstrated that <2–4 pmol of the free nucleotide (<0.6% of the nucleotide bound to actin) bound to the DNase 1 beads in the absence of actin.

HPLC Analysis of Actin-bound Nucleotide

Actin and its bound nucleotide were released from the DNase 1 beads by denaturing with urea and heat treatment. The released nucleotides were analyzed on a Mono-Q HR 5–5 column (Figure 3, A-C). Two peaks were observed from nucleotide released from either pure actin or actin isolated from *Xenopus* egg extracts (Figure 3, A and B). These two peaks comigrate with ATP and ADP, and were quantitated using ATP and ADP standards. The total released nucleotide was compared with the amount of actin bound to the DNase beads quantitated by Coomassie blue binding. In a typical run, 0.95 nmol of nucleotide was released from 0.93 nmol of actin purified from 100 μl of crude *Xenopus* extract. The range over 22 experiments was between 0.7–1.1 nmol per 100 μl for both actin and released nucleotide. This corresponds to the soluble actin concentration of 7–11 μM, in agreement with our Western blot analysis. These values, together with our control experiments, make us confident that the majority of the nucleotide we were analyzing was derived from the bound actin.

As expected, greater than 99% of the nucleotide released from pure G-actin was ATP. Thus, no significant ATP hydrolysis occurs during the isolation steps. Nucleotide released from *Xenopus* extract actin (Figure 3B) had slightly more ADP, but the ratio of ATP to ADP was consistently high (≈9:1). This ratio was similar to that for free nucleotide in the same extracts (Figure 3C). The mean ATP:ADP ratio for actin-bound

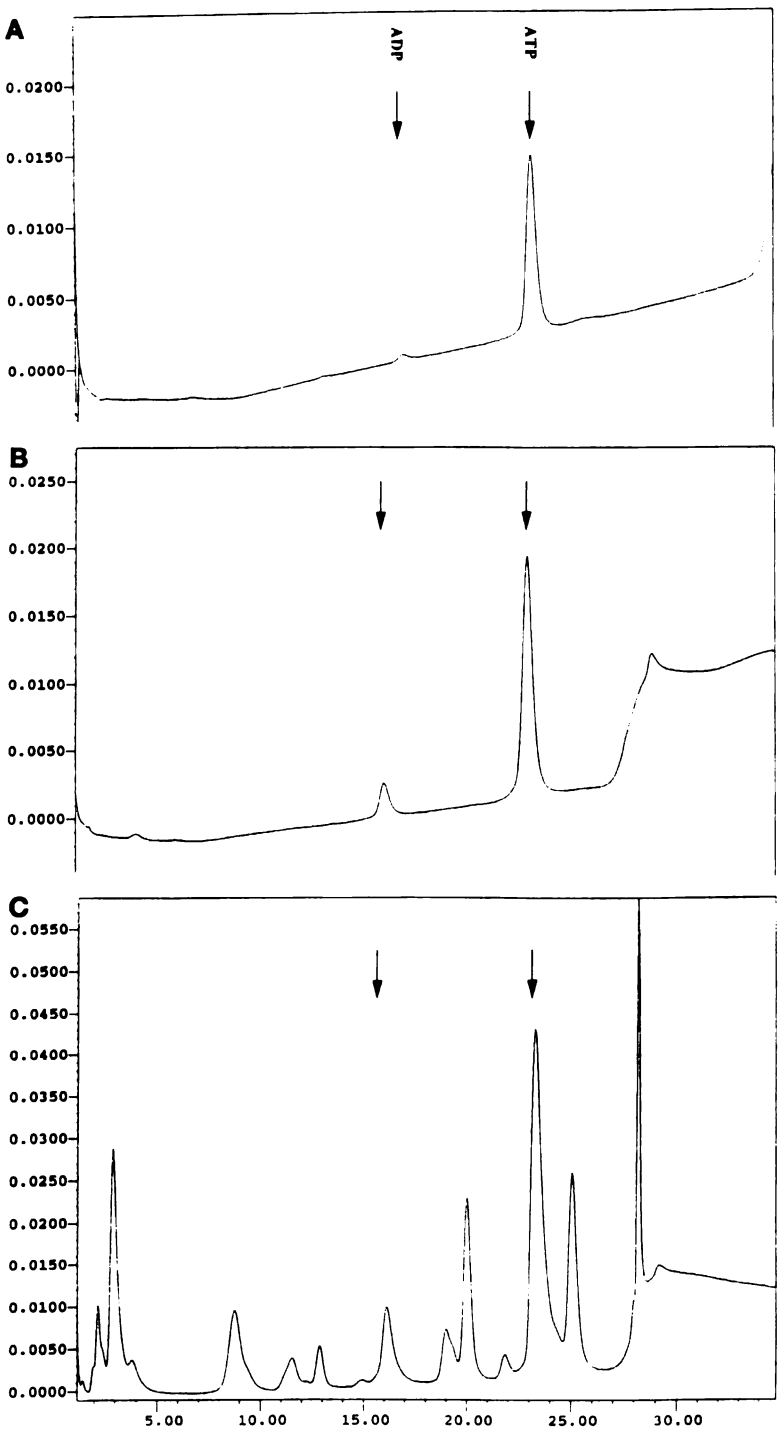

Figure 3. High pressure liquid chromatographs of nucleotides released from rabbit muscle actin DNase 1 bead eluate (A), *Xenopus* egg extract DNase 1 bead eluate (B), and nucleotides from 10-kDa cut-off filtrate of crude *Xenopus* egg extracts (C). The x-axis is elution time in minutes, and the *y*-axis is OD_{254}.

Table 2. Percentage of actin-bound nucleotide that exchanges with free nucleotide in *Xenopus* egg extracts

Incubation time before analysis[a]	% ATP exchanged	% ADP exchanged	Number of experiments
0' at 25°C	47 ± 3	30 ± 2.5	3
30' at 25°C	42 ± 7.5	30 ± 7	5

[a]Incubation time before analysis represents time that the ^{32}P-ATP is incubated with crude extracts before isolating the actin and its bound nucleotide. Therefore, 0' at 25°C represents the dead-time for nucleotide isolation.

nucleotide is 8.4 ± 4.2 over 22 experiments and the mean ratio for free nucleotides in *Xenopus* extracts is 7.6 ± 3.1 over nine experiments. Because >90% of the soluble actin was recovered in these experiments, and 1 mol of nucleotide was released per 1 mol of actin, these data indicate the absence of a large pool of ADP-actin.

Nucleotide Exchange in Soluble Actin

The similar ATP:ADP ratios for actin-bound and free nucleotide might reflect rapid nucleotide exchange in the soluble actin pool. If this were true, it would further suggest that control of nucleotide exchange is not the key regulator of actin polymerization. To test this possibility, we used ^{32}P-α ATP to estimate the rate and extent of nucleotide exchange occurring in the unpolymerized actin pool. ^{32}P-α ATP was added to crude extracts and allowed to incubate for either 0 or 30 min at 25°C. Actin was then isolated as outlined in Table 1, its nucleotide content analyzed by HPLC, and the specific activity determined. The specific activity of total unbound ATP and ADP was then determined by fractionating total unbound nucleotide, obtained as an ultrafiltrate, on HPLC. The percentage exchanged was obtained as the ratio of the specific activity of actin-bound nucleotide to the specific activity of free nucleotide in the same extract (Table 2). We found that the actin nucleotide exchange rate was rapid because the amount of exchange occurring within the dead-time of the isolation procedure (0 min at 25°C) was the same as that within 30 min at 25°C. We should note that this dead-time is difficult to accurately estimate but is potentially quite long. As discussed above, considerable nonactin bound ATP remains after the desalting step. Although this ATP is presumably bound to other proteins, it may still be able to exchange with actin-bound nucleotides during the DNase 1 binding step. Thus, the dead-time of the experiment could be as high as 60' at 4°C. Interestingly, only 30–50% of the actin exchanged with ^{32}P-α ATP at both time points. Thus, there appear to be two separate populations of actin in *Xenopus* extracts, one that exchanges nucleotide rapidly, and another which apparently does not exchange.

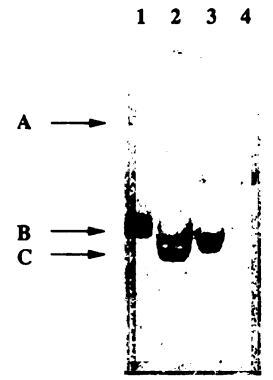

Figure 4. Anti-actin immunoblot of a nondenaturing gel of 0.9 μg rabbit muscle actin (lane 1), 85 μg *Xenopus laevis* extract h.s.s. (lane 2), 0.9 μg rabbit muscle actin mixed with ~1.6 μg *Xenopus laevis* extract boiled supernatant (lane 3), and ~1.6 μg boiled supernatant alone (lane 4). Arrow A indicates migration of unidentified actin complex and/or denatured actin, arrow B the migration of pure muscle actin, and arrow C the migration of actin complexed with a thymosin β_4-like protein.

The Bulk of Soluble Actin Is in a Protein Complex

Analysis of the soluble actin pool in a h.s.s. of *Xenopus* egg extract by the native gel electrophoresis method of Safer (Safer, 1989) demonstrated the existence of three distinct actin species (Figure 4, lane 2). The first is a faint streak of slowly migrating actin (band A) in the extract lane, which could represent denatured actin or large, poorly resolved actin complexes. The more striking species are the two bands that migrate near pure actin (bands B and C). The faster migrating band (C) could represent a complex of actin and thymosin β4 (T β4), or a T β4-like protein (Safer *et al.*, 1990). T β4 is heat stable, so we tested boiled extract supernatant for its ability to cause this same shift in actin mobility. When boiled extract supernatant was added to rabbit muscle actin it increased the mobility of rabbit muscle actin (lane 3) as expected from a T β4-like activity. This result suggests that *Xenopus* extracts contain a T β4-like activity that may account for band C in extracts. On average, native gels of *Xenopus* extracts showed that between 50% to 80% of the actin is in a complex with the T β4-like activity.

DISCUSSION

Actin in Xenopus Egg Extracts

We used *Xenopus* egg extracts to analyze the state of unpolymerized actin because they are made with little dilution of the cytoplasm. *Xenopus* egg extracts can support cycles of actin filament assembly/disassembly (Figure 1), although caution is required in interpreting the behavior of labeled muscle actin in extracts. A stronger argument that *Xenopus* extracts support normal actin dynamics comes from analysis of *Listeria* behavior. *Listeria* can rapidly induce polymerization from the soluble actin pool, and the fil-

aments in the resulting *Listeria* tail are rapidly depolymerized. Both events occur with kinetics similar to that seen in the cytoplasm of tissue culture cells (Theriot and Mitchison, 1992). It is formally possible that the actin in extracts is nondynamic, and *Listeria* induces dynamics, but we favor the simpler interpretation that the extracts mimic normal cytoplasmic regulation. Because *Listeria* motility does not depend on exogenously added actin, it reports on endogenous dynamics. Taken together, our data strongly suggest that actin polymerization in *Xenopus* egg extracts is subject to a similar regulation as that in somatic cells.

Analysis of the Actin-bound Nucleotide

In developing the protocol shown in Table 1, we sought to isolate the soluble actin pool using a rapid, nonperturbing method. However, some perturbations were inevitable for this isolation. We found it necessary to remove F-actin by high speed centrifugation before isolating the G-actin on DNase 1 beads because, in pilot experiments, a substantial amount of actin derived from filaments bound to DNase 1 beads. The centrifugation step could affect our nucleotide analysis because it disturbs the normal polymerization-depolymerization cycle that occurs within the extract by removing filaments. This perturbation could potentially alter the balance between nucleotide exchange and hydrolysis as compared with that in the cell. In addition, the centrifugation step could remove other unpolymerized actin populations in high molecular weight complexes that are important for polymerization (Hashimoto and Tatsumi, 1988). Thus, although we believe our results are relevant to the bulk pool of unpolymerized actin in cells, they may not be relevant to local regions such as the leading edge where rapid turnover of filaments is occurring.

Our analysis of actin-bound nucleotide in the soluble actin pool in *Xenopus* egg extracts showed that more than 80% of the unpolymerized actin contains bound ATP. Thus, when *Listeria* is recruiting soluble actin to rapidly assemble filaments in extracts, it must be recruiting actin that already contains ATP. This in turn means that the exchange of ADP for ATP is probably not a rate-limiting step for polymerization. The release of ATP-actin from the inhibitory effect of a sequestering protein is more likely to be a limiting step, or it may be that in fact filament elongation or nucleation are themselves limiting. Our results do not imply that the exchange reaction is not important because in the absence of profilin or some other exchange activity it is possible that ADP-actin would accumulate. For example, ADP-actin might accumulate as a kinetic intermediate in a region of the cell where the actin depolymerization rate is high. In such regions, profilin may be an important catalyst of nucleotide exchange (Goldschmidt *et al.*, 1992).

Nucleotide Exchange on Actin

Our experiments testing the rate of actin nucleotide exchange showed that the extent of exchange was independent of incubation time. In test experiments where we incubated extracts with ^{32}P-α ATP at 25°C over of a time course from 0 to 120 min before analysis, we found that the amount of actin nucleotide exchange had plateaued by 0 min, i.e., the dead-time of the assay. We had concerns with taking time points much longer than 2 h because the ATP becomes consumed and the extracts begin to die at such long time points. For these reasons, we performed careful analysis for just two time points, 0 and 30 min at 25°C. The time resolution of the experiment is severely limited by technical constraints discussed in the MATERIALS AND METHODS section. Given the long dead-time of our assay, it is clearly unsuited for determining accurate exchange kinetics in cytoplasm.

Although nucleotide exchange on actin appears to be fairly rapid, it occurs in less than half of the actin population. As yet, we have not been able to determine how the exchanging and nonexchanging actin populations relate to the different species resolved by native gel electrophoresis. T β_4 blocks actin nucleotide exchange, but because T β_4 itself exchanges with actin this molecule alone may not account for our nonexchangeable fraction. So far we have been unable to alter the extent of exchange by adding either *Listeria* or boiled supernatants to the extracts. The fact that nucleotide exchange on actin in these extracts is not complete may also be reflected by the fact that the ratio of ATP-actin:ADP-actin is similar to the ratio of free ATP:ADP within the extracts. Given that the affinity of actin for ATP is ≈fourfold greater than for ADP (Kinosian *et al.*, 1993), we might expect that the ratio of ATP-actin:ADP-actin would be higher that the free ATP:free ADP if all of the actin was freely exchanging.

Given our exchange data, how confidently can we assert that unpolymerized actin is mostly ATP-bound in vivo? For the nonexchanging ≈60% of the actin, the ATP we analyzed must be the same ATP that was bound in vivo. For the exchanging ≈40%, the ATP we analyzed was effectively introduced during isolation, and could potentially have been ADP in vivo. However, this fraction would tend to rapidly equilibrate with unbound nucleotide, and is thus presumably mostly ATP-bound in vivo. Thus we are confident that no large pool of ADP-actin exists in *Xenopus* egg extracts.

Monomer Binding Proteins

Band C from *Xenopus* egg extracts (Figure 4, lane 2) most likely represents an actin complex with T β_4 or a T β_4-like protein because boiled *Xenopus* extract supernatants (Figure 4, lane 3, band C) and 10-kDa filtrates of these supernatants (unpublished data), fractions that contain T β_4 in extracts of mammalian cells, can produce a similar band when bound to rabbit muscle actin. Although this band does not exactly comigrate with band C in the *Xenopus* extracts lane (lane 2), this difference in shift could be due to the use of a different species of actin. A similar slight difference in mobility has been seen with actin complexes found from supernatants from resting polymorphonuclear leukocyte extracts versus muscle actin complexed with a T β_4-containing fraction (Cassimeris *et al.*, 1992).

If band B in the *Xenopus* h.s.s. lane (lane 2) represents uncomplexed ATP-actin, its concentration ($\approx$2 μM-6 μM) appears to be quite high considering that it is unpolymerized and the critical concentration is 0.2 μM. When native gels were run without ATP in the buffer, pure rabbit muscle actin ran as a smear, presumably due to denaturation. However, omission of ATP did not affect the mobility of band B from *Xenopus* extracts. Thus, band B could represent an altered form of actin or uncharacterized actin complex that is sequestered from polymerization.

In summary, we have found that the majority of soluble actin from *Xenopus* extracts is ATP-bound. One population of the soluble actin exchanges with cytoplasmic nucleotide whereas the other does not. In addition, most of the soluble actin is in a complex with what appears to be the sequestering protein, thymosin β_4. Our results are not consistent with the existence of a large pool of ADP-actin in vivo, at least in resting cytoplasm. However, we could not rule out build up of significant levels of ADP-actin in areas of rapid filament depolymerization. Notwithstanding this possibility, our results on the bulk of cellular actin suggest that nucleotide exchange is probably not the key regulatory step for polymerization in vivo. More likely, actin assembly is regulated by release of ATP-actin from a complex with a sequestering protein. Our work shows considerable heterogeneity in the unpolymerized actin pool. Future work will need to examine how these different populations relate to each other, and which population of soluble actin is used when actin polymerization is induced.

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REFERENCES

Balasubramanian, M.K., Hirani, B.R., Burke, J.D., and Gould, K.L. (1994). The *Schizosaccharomyces pombe* cdc3+ gene encodes a profilin essential for cytokinesis. J. Cell Biol. *125*, 1289–1301.

Buss, F., Temm, G.C., Henning, S., and Jockusch, B.M. (1992). Distribution of profilin in fibroblasts correlates with the presence of highly dynamic actin filaments. Cell Motil. Cytoskeleton *22*, 51–61.

Carlier, M.F., Jean, C., Rieger, K.J., Lenfant, M., and Pantaloni, D. (1993). Modulation of the interaction between G-actin and thymosin beta 4 by the ATP/ADP ratio: possible implication in the regulation of actin dynamics. Proc. Natl. Acad. Sci. USA *90*, 5034–5038.

Carlsson, L., Nystrom, L.E., Sundkvist, I., Markey, F., and Lindberg, U. (1977). Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells. J. Mol. Biol. *115*, 465–483.

Cassimeris, L., Safer, D., Nachmias, V.T., and Zigmond, S.H. (1992). Thymosin beta 4 sequesters the majority of G-actin in resting human polymorphonuclear leukocytes. J. Cell Biol. *119*, 1261–1270.

Cooley, L., Verheyen, E., and Ayers, K. (1992). chickadee encodes a profilin required for intercellular cytoplasm transport during *Drosophila* oogenesis. Cell *69*, 173–184.

Cooper, J.A. (1991). The role of actin polymerization in cell motility. Annu. Rev. Physiol. *53*, 585–605.

Finkel, T., Theriot, J.A., Dise, K.R., Tomaselli, G.F., and Goldschmidt, C.P. (1994). Dynamic actin structures stabilized by profilin. Proc. Natl. Acad. Sci. USA *91*, 1510–1514.

Goldschmidt, C.P., Furman, M.I., Wachsstock, D., Safer, D., Nachmias, V.T., and Pollard, T.D. (1992). The control of actin nucleotide exchange by thymosin beta 4 and profilin. A potential regulatory mechanism for actin polymerization in cells. Mol. Cell Biol. *3*, 1015–1024.

Haarer, B.K., Lillie, S.H., Adams, A.E., Magdolen, V., Bandlow, W., and Brown, S.S. (1990). Purification of profilin from *Saccharomyces cerevisiae* and analysis of profilin-deficient cells. J. Cell Biol. *110*, 105–114.

Hashimoto, K., and Tatsumi, N. (1988). Regulation of actin polymerization by membrane fraction of platelets. Biochem. Int. *16*, 267–278.

Haugwitz, M., Noegel, A.A., Karakesisolou, J., and Schleicher, M. (1994). *Dictyostelium* amoebae that lack G-actin-sequestering profilins show defects in F-actin content, cytokinesis, and development. Cell *79*, 303–314.

Hitchcock, S.E. (1980). Actin deoxyribonuclease I interaction. Depolymerization and nucleotide exchange. J. Biol. Chem. *255*, 5668–5673.

Holliday, L.S., Bubb, M.R., and Korn, E.D. (1993). Rabbit skeletal muscle actin behaves differently than *Acanthamoeba* actin when added to soluble extracts of *Acanthamoeba castellanii*. Biochem. Biophys. Res. Commun. *196*. 569–575.

Kellogg, D.R., Mitchison, T.J., and Alberts, B.M. (1988). Behavior of microtubules and actin filaments in living *Drosophila* embryos. Development *103*, 675–686.

Kinosian, H.J., Selden, L.A., Estes, J.E., and Gershman, L.C. (1993). Nucleotide binding to actin. Cation dependence of nucleotide dissociation and exchange rates. J. Biol. Chem. *268*, 8683–8691.

Korn, E.D. (1982). Actin polymerization and its regulation by proteins from nonmuscle cells. Physiol. Rev. *62*, 672–737.

Magdolen, V., Drubin, D.G., Mages, G., and Bandlow, W. (1993). High levels of profilin suppress the lethality caused by overproduction of actin in yeast cells. FEBS Lett. *316*, 41–47.

Mannherz, H.G., Goody, R.S., Konrad, M., and Nowak, E. (1980). The interaction of bovine pancreatic deoxyribonuclease I and skeletal muscle actin. Eur. J. Biochem. *104*, 367–379.

Murray, A.W. (1991). Cell cycle extracts. Methods Cell Biol. *36*, 581–605.

Ozaki, K., and Hatano, S. (1984). Mechanism of regulation of actin polymerization by *Physarum* profilin. J. Cell Biol. *98*, 1919–1925.

Pantaloni, D., and Carlier, M.F. (1993). How profilin promotes actin filament assembly in the presence of thymosin beta 4. Cell *75*, 1007–1014.

Pollard, T.D. (1986a). Assembly and dynamics of the actin filament system in nonmuscle cells. J. Cell. Biochem. *31*, 87–95.

Pollard, T.D. (1986b). Rate constants for the reactions of ATP- and ADP-actin with the ends of actin filaments. J. Cell Biol. *103*, 2747–2754.

Safer, D. (1989). An electrophoretic procedure for detecting proteins that bind actin monomers. Anal. Biochem. *178*, 32–37.

Safer, D., Golla, R., and Nachmias, V.T. (1990). Isolation of a 5-kilodalton actin-sequestering peptide from human blood platelets. Proc. Natl. Acad. Sci. USA *87*, 2536–2540.

Sanders, M.C., Goldstein, A.L., and Wang, Y.L. (1992). Thymosin beta 4 (Fx peptide) is a potent regulator of actin polymerization in living cells. Proc. Natl. Acad. Sci. USA *89*, 4678–4682.

Sohn, R.H., and Goldschmidt-Clermont, P.J. (1994). Profilin: at the crossroads of signal transduction and the actin cytoskeleton. BioEssays *16*, 465–472.

Spudich, J.A., and Watt, S. (1971). The regulation of rabbit skeletal muscle contraction: biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. J. Biol. Chem. *246*, 4866–4871.

Theriot, J.A., and Mitchison, T.J. (1992). The rate of actin-based motility of intracellular *Listeria monocytogenes* equals the rate of actin polymerization. Nature *357*, 257–260.

Theriot, J.A., Rosenblatt, J., Portnoy, D.A., Goldschmidt, C.P., and Mitchison, T.J. (1994). Involvement of profilin in the actin-based motility of *L. monocytogenes* in cells and in cell-free extracts. Cell *76*, 505–517.

Vojtek, A., Haarer, B., Field, J., Gerst, J., Pollard, T.D., Brown, S., and Wigler, M. (1991). Evidence for a functional link between profilin and CAP in the yeast *S. cerevisiae*. Cell *66*, 497–505.

Yu, F.X., Lin, S.C., Morrison, B.M., Atkinson, M.A., and Yin, H.L. (1993). Thymosin beta 10 and thymosin beta 4 are both actin monomer sequestering proteins. J. Biol. Chem. *268*, 502–509.

Yu, F.X., Lin, S.C., Morrison, B.M., and Yin, H.L. (1994). Effects of thymosin beta 4 and thymosin beta 10 on actin structures in living cells. Cell Motil. Cytoskeleton *27*, 13–25.

Chapter Three

Xenopus Actin Depolymerizing Factor/Cofilin (XAC) is responsible for the turnover of actin filaments in *Listeria monocytogenes* tails.

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Xenopus Actin Depolymerizing Factor/Cofilin (XAC) Is Responsible for the Turnover of Actin Filaments in *Listeria monocytogenes* Tails

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Abstract. In contrast to the slow rate of depolymerization of pure actin in vitro, populations of actin filaments in vivo turn over rapidly. Therefore, the rate of actin depolymerization must be accelerated by one or more factors in the cell. Since the actin dynamics in *Listeria monocytogenes* tails bear many similarities to those in the lamellipodia of moving cells, we have used *Listeria* as a model system to isolate factors required for regulating the rapid actin filament turnover involved in cell migration. Using a cell-free *Xenopus* egg extract system to reproduce the *Listeria* movement seen in a cell, we depleted candidate depolymerizing proteins and analyzed the effect that their removal had on the morphology of *Listeria* tails. Immunodepletion of *Xenopus* actin depolymerizing factor (ADF)/cofilin (XAC) from *Xenopus* egg extracts resulted in *Listeria* tails that were approximately five times longer than the tails from undepleted extracts. Depletion of XAC did not affect the tail assembly rate, suggesting that the increased tail length was caused by an inhibition of actin filament depolymerization. Immunodepletion of *Xenopus* gelsolin had no effect on either tail length or assembly rate. Addition of recombinant wild-type XAC or chick ADF protein to XAC-depleted extracts restored the tail length to that of control extracts, while addition of mutant ADF S3E that mimics the phosphorylated, inactive form of ADF did not reduce the tail length. Addition of excess wild-type XAC to *Xenopus* egg extracts reduced the length of *Listeria* tails to a limited extent. These observations show that XAC but not gelsolin is essential for depolymerizing actin filaments that rapidly turn over in *Xenopus* extracts. We also show that while the depolymerizing activities of XAC and *Xenopus* extract are effective at depolymerizing normal filaments containing ADP, they are unable to completely depolymerize actin filaments containing AMPPNP, a slowly hydrolyzible ATP analog. This observation suggests that the substrate for XAC is the ADP-bound subunit of actin and that the lifetime of a filament is controlled by its nucleotide content.

Actin polymerization is required for many cellular movements such as protrusion of the leading edge of the cell and intracellular movement of the pathogen, *Listeria monocytogenes* (Cooper, 1991; Bray and White, 1988; Sanger et al., 1992; Mitchison and Cramer, 1996). To maintain continuous polymerization during such movements, actin must be depolymerized and the subunits recycled. The intrinsic disassembly rates of pure filamentous actin (F-actin)[1] measured in vitro (0.044–1.14 μm/min) (Pollard, 1986) cannot account for the depolymerization rates found in the cell (up to 9 μm/min) (Theriot and Mitchison, 1991; Zigmond, 1993; Small et al., 1995). Therefore, one or more factors must catalyze actin depolymerization in vivo. Such factors could act by increasing the dissociation rate from existing ends, by severing to increase the number of ends, or by a combination of both mechanisms. To date, severing proteins have been best characterized.

Two classes of actin-severing proteins exist in most eukaryotic cells: the gelsolin family and a family of small severing proteins closely related in sequence and function that include actin depolymerizing factor (ADF) and cofilin. Structurally, these small proteins have a remarkable similarity to a single segment of the six repeated segments of gelsolin (Hatanaka et al., 1996). Both classes of severing proteins have been studied biochemically and much is known about their in vitro behavior and regulation. The gelsolin class of proteins includes tissue-specific isoforms such as villin (Pringault et al., 1986), scinderin (Rodriguez

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1. *Abbreviations used in this paper*: ADF, actin depolymerizing factor; ADP.Pi, ADP + inorganic phosphate; AMPPNP, 5'adenylylamido-diphosphate; F-actin, filamentous actin; XAC, *Xenopus* ADF/Cofilin.

et al., 1990), and adseverin (Sakurai et al., 1990) and species-specific forms such as fragmin (Ampe and Vandekerckhove, 1987) and severin (André et al., 1988). The molecular mass of gelsolin family members varies from 40–93 kD depending on the species or cell type. Gelsolin has strong actin-severing activity and can also cap the barbed end of actin filaments and nucleate filament formation. The activity of gelsolin is regulated positively by Ca^{2+} binding and inhibited by binding polyphosphoinositides (PIPs) (Janmey and Stossel, 1987).

The small actin-severing proteins include ADF (Bamburg et al., 1980) and cofilin (Nishida et al., 1984), as well as a number of species-specific isoforms (for review see Moon and Drubin, 1995). The ADFs have molecular masses ranging from 17–19 kD and the cofilins from 15–19 kD depending upon species type. The sequences of ADF and cofilin are ~70% identical to each other. Because of their similarities in sequence and function, members of either are often termed the ADF/cofilin family of proteins. While higher eukaryotes such as mammals and chicken contain both ADF and cofilin in their genomes, it is believed that all eukaryotes contain at least one copy of an ADF/cofilin protein (Moon and Drubin, 1995). Recently two proteins have been isolated from *Xenopus laevis* whose amino acid sequences are 77% identical to chick cofilin, 66% identical to chick ADF, and 93% identical to each other (Abe et al., 1996). These proteins have been named *Xenopus* ADF/cofilin 1 and 2 (XAC 1 and 2) since their sequence is intermediate between ADF and cofilin. Because of their high sequence homology and similar patterns of temporal and spatial expression, XAC 1 and 2 are thought to be allelic variants encoded by the pseudotetraploid *Xenopus laevis* genome.

Thus far, the XACs exhibit the same biochemical properties as other members of the ADF/cofilin family. ADF/cofilin family proteins can bind F-actin at pH 6.8 and depolymerize F-actin at pH 8.0 (Yonezawa et al., 1985; Hawkins et al., 1993; Hayden et al., 1993). ADF/cofilin proteins also bind monomeric actin (G-actin) (Hayden et al., 1993). However, their depolymerizing activity is thought to be derived from their ability to sever F-actin and not from their ability to bind and sequester G-actin (Maciver et al., 1991). The severing activity of ADF/cofilin proteins is much weaker than that of gelsolin in quantitative assays. The relative weakness of severing by ADF/cofilin may be explained by the fact that they preferentially sever at preexisting bends in filaments, whereas gelsolin induces bends and breaks at any point on the filament (Maciver et al., 1991). The activity of cofilin can be inhibited by tropomyosins (Bernstein and Bamburg, 1982; Bamburg and Berstein, 1991) and PIPs (Yonezawa et al., 1990). ADF/cofilin proteins in the cell are either unphosphorylated or phosphorylated on a serine near the NH_2 terminus (S3 in chick ADF) (Morgan et al., 1993; Agnew et al., 1995; Moriyama et al., 1996). The phosphorylated form has greatly reduced actin binding and depolymerizing activity. Several signal transduction pathways that cause reorganization of the actin cytoskeleton also cause rapid dephosphorylation of ADF and cofilin (for review see Moon and Drubin, 1995), suggesting that ADF/cofilin dephosphorylation may be important for regulating actin depolymerization in the cell.

Although biochemical studies show that gelsolin has stronger severing activity than the ADF/cofilin proteins, genetic studies of these two families have more strongly implicated ADF/cofilin proteins in the control of the actin cytoskeleton. Cofilin is an essential protein in *Saccharomyces cerevisiae* (Moon et al., 1993), *Drosophila melanogaster* (Gunsalus et al., 1995), and *Caenorhabditis elegans* (McKim et al., 1994). In addition, ADF and cofilin localize to the cleavage furrow of dividing cells and have been shown to be essential for cytokinesis (Gunsalus et al., 1995; Nagaoka et al., 1995; Abe et al., 1996). In contrast, the knockout of gelsolin in *Dictyostelium* (André et al., 1989) produced no obvious phenotype. Fibroblasts and neutrophils from gelsolin-deficient mice migrated more slowly than those from wild-type mice (Witke et al., 1995). However, the viability of animals lacking gelsolin in the above studies may be due to compensation by other proteins that are functionally redundant to gelsolin. Despite a combination of biochemical and genetic analyses, a clear role is lacking for either the ADF/cofilin or gelsolin classes of proteins in controlling the rapid depolymerization of actin filaments essential for lamellipodial protrusion and *Listeria* movement.

The half-life of actin polymer in *Listeria* tails is similar to that observed in the lamellipodia of moving cells (Theriot et al., 1992), suggesting that the depolymerization of actin filaments in *Listeria* tails may be a good model for turnover in other dynamic actin arrays. Concentrated *Xenopus* egg extracts can support the movement of *Listeria monocytogenes* at rates comparable to intact cell cytoplasm (Theriot et al., 1994) and can provide a system in which to dissect biochemically the components required for actin-based motility and actin dynamics. To determine if any of the known severing proteins are responsible for rapid turnover of actin filaments in the cell, we immunodepleted gelsolin and XAC from *Xenopus* egg extracts and tested whether the rapid polymerization and depolymerization seen in *Listeria* tails were perturbed.

Materials and Methods

Preparation of Recombinant Proteins and Xenopus Egg Extracts

The COOH-terminal 1,393 bp (464 amino acids) of X. l. gelsolin was cloned by PCR from a λ-YES (Stratagene, La Jolla, CA) *Xenopus* egg and embryo cDNA library (gift from Jeremy Minshull) (Kinoshita et al., 1995) into a pGEX-2T vector (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). XAC 2 was cloned into a pGEX expression vector as described by Abe et al. (1996). The pGEX expression plasmids were transformed into TG1, and recombinant proteins were expressed and purified on a glutathione column using standard procedures (Smith and Johnson, 1988). For use in the addback experiments or for purification of antibodies, the glutathione S-transferase (GST)-fusion proteins were cleaved with 0.4 mg/ml thrombin (Sigma Chemical Co., St. Louis, MO) in thrombin buffer (100 mM NaCl, 50 mM Tris HCl, pH 7.5, 2.5 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM DTT) at 37°C for 60 min. Thrombin was then removed by passing the cleaved protein over a *p*-aminobenzamidine Sepharose (Sigma Chemical Co.) column in thrombin buffer and concentrating the flow through a centriprep-10 concentrator (Amicon, Beverly, MA).

Xenopus egg extracts were made as described in Theriot et al. (1994). Briefly, after dejellying meiotically arrested *Xenopus laevis* eggs in 2% cysteine, pH 7.8, the eggs were washed 4× in 250 ml of XB (100 mM KCl, 10 mM Hepes, pH 7.7, 50 mM sucrose, 5 mM EGTA, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$), transferred to 5-ml tubes containing 50 μl 0.5 M EGTA, 5 μl 1 M $MgCl_2$, 5 μl 1,000× protease inhibitor mix (10 mg/ml leupeptin, pepstatin, and chymostatin in DMSO), and crushed at 10,000 rpm in an HB-4 rotor

(Sorvall Instruments, Newtown, CT) at 15°C. The cytoplasmic layer was removed with a 20-gauge needle and syringe and 1/20 volume of energy mix (150 mM creatine phosphate, 20 mM ATP, 2 mM EGTA, pH 7.7, and 20 mM $MgCl_2$) was added. Aliquots were frozen in liquid nitrogen and stored at −80°C for up to 6 mo.

Preparation of Anti-XAC and Antigelsolin Antibodies

GST fusion proteins with XAC 2 or gelsolin fragments expressed in *Escherichia coli* were used for rabbit antibody production (Berkeley Antibody Co., Berkeley, CA). The antibodies were affinity purified on pure XAC or gelsolin cleaved with thrombin from the corresponding GST-fusion proteins expressed in *E. coli*. Antibodies were affinity purified using published procedures (Harlow and Lane, 1988). Antibodies were eluted from the affinity column with 100 mM glycine, pH 2.5, 150 mM NaCl, neutralized, and dialyzed against 10 mM Hepes, pH 7.7, 100 mM KCl, concentrated using Aquacide II, and redialyzed against 10 mM Hepes, pH 7.7, 100 mM KCl.

Immunofluorescence

XL177 cells were grown on glass coverslips to ~60% confluency. Cells were infected with the *Listeria monocytogenes* strain 10403S as described (Theriot et al., 1994) except that ~10-fold more *Listeria* were used, and the cells were incubated for 8 h at 23°C (4 h before and 4 h after gentamycin addition) before processing. Coverslips were rinsed in TBS (20 mM Tris, pH 7.4, 150 mM NaCl) before fixation in 4% formaldehyde in TBS for 20 min. The coverslips were rinsed in TBS before cells were permeabilized in TBS + 0.5% Triton X-100 for 10 min. After blocking for 10 min in AbDil (TBS + 2% BSA and 0.5% Na azide), the coverslips were incubated for 30 min with either 2 μg/ml anti-XAC or 6 μg/ml antigelsolin antibodies in AbDil. Texas red–conjugated goat anti-rabbit secondary antibody was used to visualize gelsolin and XAC, and fluorescein-phalloidin (Molecular Probes, Eugene, OR) was used to visualize actin. Coverslips were mounted with FITC-guard (Testog Inc., Chicago, IL).

Immunodepletion

100 μg random rabbit IgG (Accurate Chemical and Scientific Corp., Westbury, NY), anti-XAC antibody, or antigelsolin antibody was bound to 30 μl Affiprep protein A (BioRad Labs, Hercules, CA) in TBST for 1 h at 4°C. The pellets were washed with 3× 1 ml XB and then incubated with 50 μl crude cytostatic factor–arrested *Xenopus* egg extracts for 1 h at 4°C on a rotator. The pellet was removed by centrifuging at 10,000 *g* in an Eppendorf centrifuge for 20 s. The supernatant was removed and treated as the immunodepleted extract. The pellets were washed 3× 1 ml in TBST, boiled in sample buffer, and analyzed by SDS-PAGE.

Western blots were performed by transferring SDS-PAGE gels electrophoretically to nitrocellulose in 20 mM Tris, 25 mM glycine, 20% methanol. Blots were incubated 1 h in AbDil followed by 1 h of incubation in 2.5 μg/ml antigelsolin or anti-XAC antibody in AbDil at room temperature. Alkaline phosphatase–conjugated anti-rabbit antibody was used as a secondary antibody (Promega Corp., Madison, WI). The amount of gelsolin or XAC depleted from extracts was determined using densitometry of immunoblots by comparing the band intensity of 1 μl of depleted extract to the band intensities of serially diluted undepleted extracts. Immunoblots were digitized using a scanner (model Power Look; UMAX Systems, Hsinchu, Taiwan) and analyzed using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA). Purified XAC and ADF as well as the immunoprecipitates were visualized by staining with 0.25% Coomassie blue R-250 in 45% methanol and 10% acetic acid followed by destaining in 25% methanol and 7% acetic acid.

Listeria Tail Assay

Listeria monocytogenes strain SLCC-5764 (Leimeister-Wachter and Chakraborty, 1989) was grown overnight at 37°C with constant shaking to stationary phase in brain–heart infusion broth (BHI; Difco Laboratories Inc., Detroit, MI). The *Listeria* were killed by adding 10 mM iodoacetic acid and incubating for 20 min at room temperature (Theriot et al., 1994). The bacteria were washed once in XB, resuspended in 1/5 original volume in 20% glycerol/XB, and stored at −80°C. Rabbit skeletal muscle actin covalently labeled with *N*-hydroxysuccinimidyl 5-carboxytetramethyl rhodamine (Molecular Probes Inc.) was made as previously described (Rosenblatt et al., 1995).

Listeria tail morphology and *Listeria* motility were assayed by mixing 5 μl of depleted or undepleted extract with 0.5 μl each of *Listeria* and 1 mg/ml rhodamine-labeled actin. In experiments where XAC or ADF proteins were added, XB or proteins were added in a volume of 0.5 μl. 1 μl of this mixture was removed and squashed between a microscope slide and a 22-mm^2 coverslip and allowed to incubate at room temperature for 25 min. Static images or movies of tails were collected using a CCD camera (Princeton Instruments, Trenton, NJ) and fluorescence movies of bacterial motility were acquired using a video camera (model SIT; Dage-MTI, Inc., Wabash, MI), respectively, during a period of 25–60 min after transferring reactions to room temperature. The lengths of tails and total tail fluorescence were quantitated using Winview software (Princeton Instruments, Trenton, NJ). Tail lengths were measured using the program Get Curve (Princeton Instruments), and the length in pixels was converted to microns using a micrometer standard. Total fluorescence in the *Listeria* tails was measured by multiplying the pixel area by average pixel intensity of a selected area minus the average pixel intensity of a background selected area. The CCD camera responds linearly to fluorescence intensity in the range of 10–3,000 counts/pixel, and we used illumination levels that avoided saturating the signal.

Production and Depolymerization of AMPPNP Actin Filaments

ATP or AMPPNP actin filaments were made by diluting rhodamine-labeled actin in G-buffer (5 mM Tris HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM DTT) containing either 0.2 mM ATP or 0.2 mM AMPPNP to a final concentration of 12.8 μM in 100 μl. The mixtures were either passed by gravity or spun through 1 ml G-25 (Pharmacia LKB Biotechnology, Inc.) columns preequilibrated in G-buffer plus the 0.2 mM of the appropriate nucleotide for 1 min in a clinical centrifuge at mid-speed into tubes containing 25 μl 0.25 M KCl, 0.25 M Tris, pH 8.0, and 1 μl 0.1 M of the appropriate nucleotide and incubated for 30 min at room temperature. Filamentous actin was recovered by centrifuging the actin for 15 min at 436,000 *g* in a centrifuge (model TLA100; Beckman Instrs., Palo Alto, CA) and resuspending the pellet in 100 μl F-buffer (50 mM KCl, 50 mM Tris, pH 8.0, 0.2 mM DTT, 0.5 mM ATP or AMPPNP).

Nucleotide incorporation was analyzed by centrifuging the various F-actin preparations through a 600 μl 40% glycerol F-buffer (50 mM KCl, 50 mM Tris, pH 8.0, 0.2 mM DTT) cushion at 436,000 *g* in a table top centrifuge (model TLA100; Beckman Instrs.) for 60 min. The pellet was resuspended in 50 μl 8 M urea for 15 min at room temperature and then diluted with 100 μl of H_2O and spun through a 10-kD cut-off filter (Millipore Corp., Bedford, MA). The nucleotides in the filtrate were then analyzed by HPLC on a 1 ml Mono Q column (Pharmacia LKB Biotechnology, Inc.) equilibrated in 100 mM NH_4HCO_3 and eluted in a 100–500 mM NH_4HCO_3 gradient over 30 min at a flow rate of 1 ml/min. Peak areas were analyzed and recorded at OD_{254} using Gilson software (Worthington, OH).

The ATP- or AMPPNP-containing F-actin was then mixed 1:1 with F-buffer, 0.1 mg/ml recombinant XAC, or crude cytostatic factor–arrested *Xenopus* egg extracts. Remaining filaments from the above mixtures were visualized on the microscope and quantitated by fluorimetry. Images of the above reactions were recorded by squashing 1 μl of each reaction between a microscope slide and a 22-mm^2 coverslip using a microscope (Nikon, Inc., Melville, NY), a CCD camera, and Winview software. After incubating the F-actins with buffer, XAC, or extract for 10 min at room temperature, the remaining F-actin in the mixture was pelleted at 436,000 *g* for 15 min at 4°C in a centrifuge (model TLA100; Beckman Instrs.). The pellets were resuspended in 0.1% SDS and the fluoresence was measured on a fluorimeter (model Aminco; SLM Instruments, Inc., Urbana, IL). Percent remaining F-actin was calculated as the fluorescence of XAC- or extract-treated pellet/fluorescence of buffer-treated pellet.

Results

Localization of XAC and Gelsolin in Listeria Actin Tails

Since we suspected that severing proteins might accelerate actin turnover in *Listeria* tails, we examined the localization of two candidate actin-severing proteins, gelsolin and XAC, in *Listeria*-infected *Xenopus* tissue culture cells (XL 177). Antibodies were raised to the COOH-terminal half

of *Xenopus laevis* gelsolin (Ankenbauer et al., 1988) and to the full-length XAC 2 protein (Abe et al., 1996) and affinity purified. Since XAC 1 and 2 differ by only four amino acids, we made only antibodies to XAC 2, which should also recognize XAC 1. Immunoblots of XL 177 lysate show that antibodies to gelsolin and XAC recognize a single band of the expected molecular mass in both cases (Fig. 1 *A*, lanes *2* and *3*). Fluorescein-labeled phalloidin and affinity-purified antibodies to XAC or gelsolin were used to visualize the intracellular distributions of F-actin (Fig. 1, *B* and *D*), gelsolin (Fig. 1 *C*), and XAC (Fig. 1 *E*) in XL 177 cells infected with *Listeria monocytogenes*. Both XAC and gelsolin colocalize with the F-actin staining in the *Listeria* tails. Both gelsolin and XAC antibodies also give punctate staining throughout the rest of the cell. In addition, gelsolin typically stained coincidentally with F-actin in the stress fibers of the cell, whereas XAC does not.

XAC Is Required for the Depolymerization of Actin in Listeria Tails

To determine the function of gelsolin and XAC in *Listeria* tails, we compared *Listeria* tails in mock-depleted *Xenopus* egg extracts to those in extracts depleted of XAC or gelsolin. Using our antibodies complexed to protein A beads, we were able to remove the bulk of either protein from *Xenopus* egg extracts. Quantitative immunoblots show that ~75% of XAC (Fig. 2 *B*, lane *2*) and >95% of gelsolin (Fig. 2 *A*, lane *3*) were depleted from extracts compared to random rabbit IgG–depleted extracts (Fig. 2, *A* and *B*, lane *1*). The XAC antibody precipitated XAC (19 kD) and an unknown band with an approximate molecular mass of 28 kD (Fig. 2 *C*, lane *2*). The gelsolin antibody precipitated only gelsolin (~93 kD) (Fig. 2 *C*, lane *3*) when compared to the rabbit IgG control pellet (lane *1*).

The IgG control depletion (Fig. 3 *A*) produced *Listeria* tails of the same length as untreated extract (15 ± 2.5 and 18 ± 1.5 μm, respectively). The tails in the gelsolin-depleted extracts (Fig. 3 *B*) were the same length and contained the same polymer mass as those of the IgG-depleted extracts (Fig. 3 *A*). However, the tails formed in the XAC-depleted extracts (Fig. 3 *C*) were on the average four to six times longer and displayed 13–21-fold more total fluorescence than those of the control extracts. Addback of pure recombinant XAC (Fig. 3 *D*) or chicken ADF to approximately endogenous concentrations (2.7 μM) rescued the long tail phenotype (12.3 ± 1.4 and 11.4 ± 1.6 μm, respectively). Addition of the same amount of a recombinant mutant version of ADF (S3E ADF) (Fig. 3 *E*), which behaves like constitutively phosphorylated ADF and has ~10% of wild-type ADF activity on pure actin (data not shown), does not rescue the long tail phenotype. The purity of the proteins used in the addback experiment (Fig. 2 *D*, lanes *1* and *2*) and the inability of the S3E ADF to rescue the XAC depletion phenotype strongly suggest that the observed long tail phenotype is due to the removal of XAC. The graph in Fig. 3 *F* shows the quantitation of the tail lengths and the amounts of tail fluorescence of the different phenotypes and provides quantitative support for our conclusion that increased tail length is due to XAC depletion, and not depletion of some other protein. These experiments demonstrate that XAC is required for the rapid depolymerization of actin filaments in *Listeria* tails in *Xenopus* extracts. While gelsolin is also concentrated within these tails, it does not appear to be essential for the depolymerization of *Listeria* tail actin.

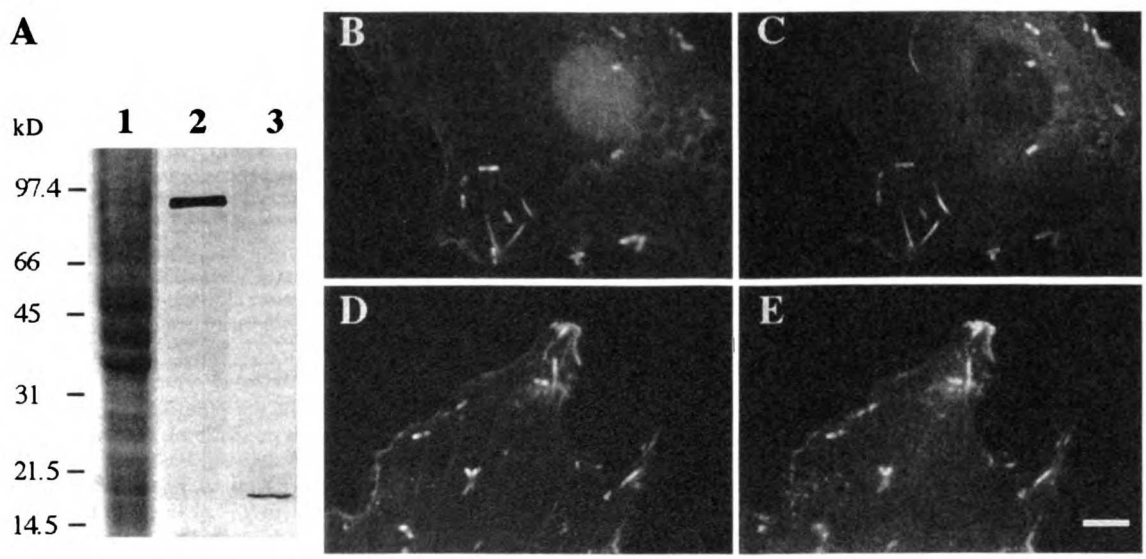


Figure 1. Localization of XAC and gelsolin in *Listeria*-infected XL 177 cells. (*A*) Western blot analysis. Lane *1*, Coomassie blue–stained SDS-PAGE gel of total XL 177 cell lysate; lane *2*, XL 177 lysate probed with anti-*Xenopus* gelsolin antibody; lane *3*, XL 177 lysate probed with anti-XAC antibody. Immunostaining of *Listeria*-infected XL 177 cells with gelsolin antibody (*C*) or XAC antibody (*E*). F-actin in *B* and *D* is visualized with fluorescein-phalloidin. Both gelsolin and XAC colocalize with F-actin in *Listeria* tails. Bar, 10 μm.

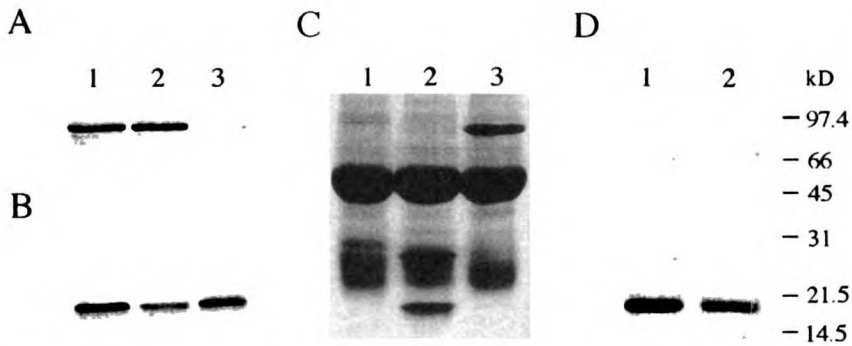


Figure 2. Gels of immunodepletion of XAC and gelsolin from *Xenopus laevis* egg extracts and purified XAC and ADF. (*A* and *B*) Immunoblots of immunodepleted extracts using antibodies to gelsolin (*A*) and XAC (*B*). For both *A* and *B*, lane *1* is the IgG-depleted control, lane *2* is the XAC-depleted extract, and lane *3* is the gelsolin-depleted extract. Quantitation of the depletion was performed by densitometry of the bands in *A* and *B* compared to a dilution series of pure extract. (*C*) Coomassie blue–stained SDS-PAGE gel of immunoprecipitated complexes with XAC and gelsolin antibodies. Lane *1*, the heavy and light chain of random rabbit IgG alone; lane *2*, XAC (19 kD) and another band at approximately 28 kD over the IgG heavy and light chain bands; lane *3*, gelsolin (~93 kD) over the IgG bands. (*D*) Coomassie blue–stained SDS-PAGE shows the purity of the recombinant XAC and chicken ADF mutant that were added back to the XAC immunodepletions. Lane *1*, wild-type XAC; lane *2*, S3E ADF. Apparent molecular mass markers for all gels are indicated on the right.

Addition of Excess XAC Decreases Listeria Tail Length

Quantitative immunoblots of *Xenopus* egg extracts using XAC antibodies and bacterially expressed XAC as a standard revealed that XAC is present at ~2.1 μM in extracts (data not shown). Addition of recombinant XAC to a final concentration of 7.1 μM in the extract (Fig. 4 *B*) produced tails that were ~0.33 times the length and had ninefold less total fluorescence than tails in a control extract (Fig. 4 *D*). Doubling the amount of XAC added to the *Listeria* assay to give a final concentration of 12.1 μM did not result in any further decrease in tail length (Fig. 4, *C* and *D*). When XAC was added to concentrations above 12.1 μM final concentration, few tails formed. Instead, rodlike structures containing rhodamine actin could be seen throughout the extract (data not shown). These rods appear analogous to those seen when actin and cofilin are concentrated in the nucleus upon heat shock or DMSO treatment to cells (Nishida et al., 1987; Ono et al., 1993) and probably represent a nonfilamentous coaggregate of XAC and actin. It is likely that few tails can form in such high concentrations of XAC since the actin required for *Listeria* tail formation may be sequestered in these XAC/actin rodlike aggregates. Thus, a *Listeria* tail segment of ~5 μm is resistant to XAC depolymerization even when XAC is added up to nearly saturating concentrations.

The Effect of XAC on Listeria Movement Rate

To determine whether the addition or depletion of XAC or gelsolin had an effect on the rate of actin polymerization, we measured the rate of *Listeria* movement in extracts either depleted of XAC or gelsolin, or containing additional XAC (Fig. 5). We used *Listeria* movement as an assay since the movement rate reflects the actin polymerization rate at the bacterial surface (Sanger et al., 1992; Theriot et al., 1992). The rate of *Listeria* movement in the XAC depleted extracts did not vary greatly from the mock-depleted extracts. The lack of effect that XAC-depletion had on actin assembly rates may indicate that XAC is not involved in actin polymerization. However, we cannot rule out such an involvement since only 75% of the XAC could be removed from the extracts with our reagents. Depletion of gelsolin increased the rate of *Listeria* movement by ~1 μm/min (~20%). The manipulations required for depletion slowed *Listeria* movement by ~1 μm/min (compare IgG-depleted to addition of buffer). This may be due to a decrease of ATP, dilution of actin, or other factors during the depletion procedure (2–3 h at 4°C). Addition of XAC to 5.0 μM seemed to increase the rate of polymerization by ~1 μm/min compared to when buffer alone is added, despite the shorter tails produced by this concentration (Fig. 4 *B*). These slight differences cannot account for the large changes in tail length and total fluorescence upon XAC depletion. Thus, the effects of XAC on actin depolymerization greatly outweigh those upon polymerization.

XAC Depolymerization Activity Depends upon the Nucleotide Content of the Filament

Upon polymerization, actin hydrolyzes its bound ATP and the terminal phosphate is slowly released. It has been suggested that the loss of the terminal phosphate could serve as a clock that regulates the lifetime of a filament by controlling the activity of ADF/cofilin proteins (Maciver et al., 1991; Moon and Drubin, 1995). To test this hypothesis, we made rhodamine-labeled actin filaments containing AMP-PNP, a slowly hydrolyzable ATP analog, and tested their resistance to the depolymerizing activities of *Xenopus* egg extracts and purified XAC. Rhodamine ATP-actin filaments depolymerize rapidly in the presence of XAC (Fig. 6 *C*) and *Xenopus* egg extract (Fig. 6 *E*) compared to buffer (Fig. 6 *A*). In contrast, rhodamine-labeled AMP-PNP filaments depolymerized to a limited extent in XAC (Fig. 6 *D*) or *Xenopus* egg extract (Fig. 6 *F*) compared to in buffer alone (Fig. 6 *B*). Thus, AMPPNP filaments were more stable to depolymerization in XAC or extract (Fig 6, *D* and *F*) compared to ATP filaments under the same conditions (Fig. 6, *C* and *E*). To quantitate actin filament de-

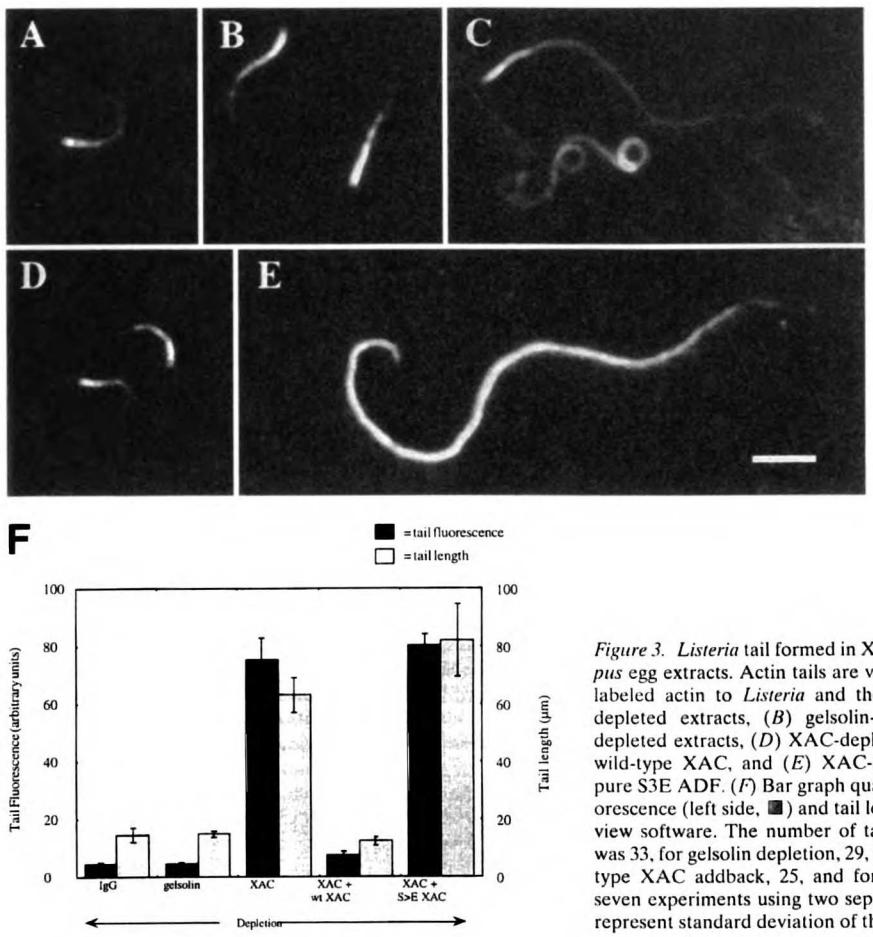


Figure 3. *Listeria* tail formed in XAC- or gelsolin-depleted *Xenopus* egg extracts. Actin tails are visualized by mixing rhodamine-labeled actin to *Listeria* and the following extracts: (*A*) IgG-depleted extracts, (*B*) gelsolin-depleted extracts, (*C*) XAC-depleted extracts, (*D*) XAC-depleted extracts plus 2.7 μM pure wild-type XAC, and (*E*) XAC-depleted extracts plus 2.7 μM pure S3E ADF. (*F*) Bar graph quantitating the amount of tail fluorescence (left side, ■) and tail length (right side, □) using Winview software. The number of tails analyzed for IgG depletion was 33, for gelsolin depletion, 29, for XAC depletion, 43, for wild-type XAC addback, 25, and for S3E ADF addback, 11, over seven experiments using two separate extracts preps. Error bars represent standard deviation of the mean. Bar, 10 μm.

polymerization, rhodamine-labeled filaments were added to *Xenopus* extracts or purified XAC and total polymerized actin was recovered by sedimentation. The fraction of the added actin left in polymer was determined by resuspending the pellets in SDS and quantitating the fluorescence with a fluorimeter (Fig. 6 *G*). AMPPNP caused twice as much of the added F-actin to be recovered in the pellet fraction. Comparing Fig. 6, *E* and *F*, with *G*, we should note that the visual and sedimentation assays are measuring different parameters. If the labeled actin repolymerized into new filaments in the presence of extract, the filaments will not be visible to the CCD camera because they are diluted with endogenous actin (Fig. 6, *E* and *F*). However the diluted actin will still sediment, giving rise to the high fraction of label in the pellet fraction in the presence of extract (Fig. 6 *G*, *Xenopus extract*). Both types of assay demonstrate that the AMPPNP filaments are relatively resistant to depolymerization by pure XAC and total extract. Two other slowly hydrolyzable ATP analogs, ATPγS and AMPPCP, showed similar stabilizing effects (data not shown), suggesting that the actin stability is due to the state of the bound nucleotide rather than nonspecific effects on actin structure. The results from both the microscopy and the fluorimetry assays suggest that the nucleotide content of actin filaments regulates XAC.

Incorporation of AMPPNP into actin filaments in these experiments was modest; ~12% of the ATP-binding sites in actin filaments incorporated AMPPNP, and the remainder contained ADP. By contrast, ATP actin filaments contained approximately only 2% ATP and 98% ADP. Despite low incorporation, ~1.5 and 2 times as much AMPPNP-containing actin filaments as ATP-containing actin filaments pelleted in the presence of *Xenopus* extract or pure XAC, respectively.

Discussion

We have used the ability to reconstitute *Listeria* motility in *Xenopus* egg extracts to test directly the role of the gelsolin and ADF/cofilin proteins in the promotion of actin filament depolymerization. We have raised specific antibodies to gelsolin and to XAC, the major ADF/cofilin protein known to be present in *Xenopus* egg extracts. Using immunodepletions and adding back purified proteins, our

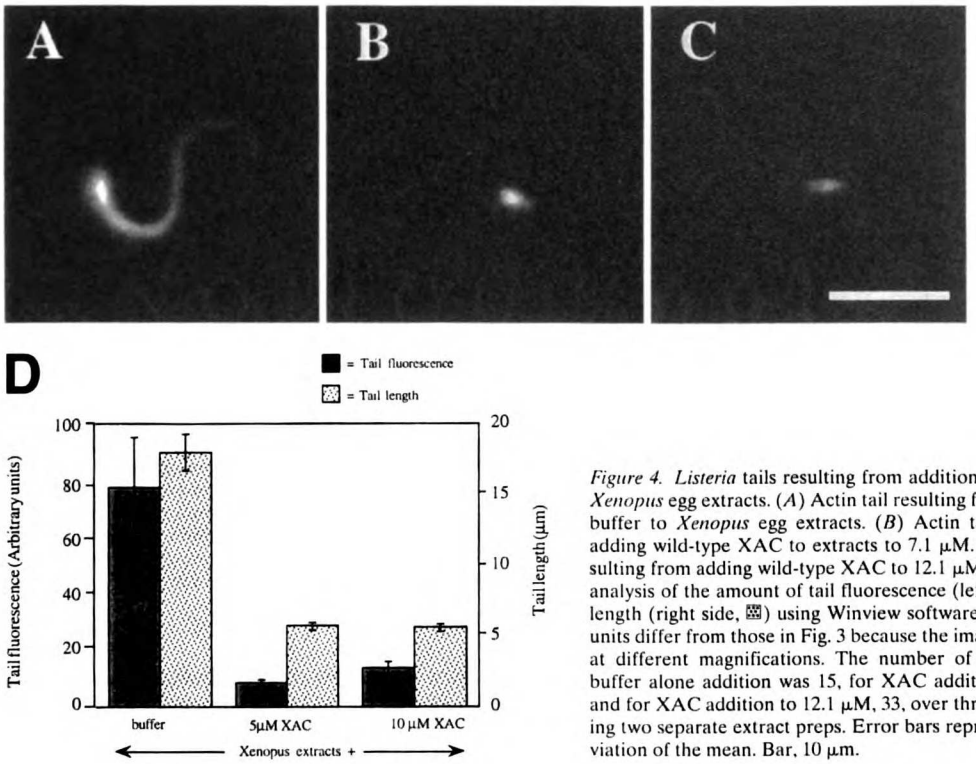


Figure 4. *Listeria* tails resulting from addition of excess XAC to *Xenopus* egg extracts. (*A*) Actin tail resulting from adding extract buffer to *Xenopus* egg extracts. (*B*) Actin tails resulting from adding wild-type XAC to extracts to 7.1 μM. (*C*) Actin tails resulting from adding wild-type XAC to 12.1 μM. (*D*) Quantitative analysis of the amount of tail fluorescence (left side, ■) and tail length (right side, ▩) using Winview software. The fluorescence units differ from those in Fig. 3 because the images were analyzed at different magnifications. The number of tails analyzed for buffer alone addition was 15, for XAC addition to 7.1 μM, 29, and for XAC addition to 12.1 μM, 33, over three experiments using two separate extract preps. Error bars represent standard deviation of the mean. Bar, 10 μm.

studies demonstrate that ADF/cofilin is the major factor responsible for the rapid turnover of actin filaments in *Listeria* tails. Removal of 75% of the ADF/cofilin from extracts considerably lengthened *Listeria* tails and greatly increased the total actin polymer mass present in the tails. Both tail length and actin polymer mass in tails were restored to control levels by adding back pure XAC at physiological concentrations to XAC-depleted extracts. In addition, adding excess XAC shortens *Listeria* tails and reduces the polymer mass in the tail, and AMPPNP actin is resistant to depolymerization by both XAC and whole extracts. Taken together, these data strongly implicate XAC as a central component of the machinery responsible for rapid actin filament turnover in extracts. Although we have no direct evidence, we predict that ADF/cofilin proteins are required for the dynamic organization of populations of actin filaments within living cells that turn over rapidly, such as the filaments within lamellipodia. Three arguments support our prediction: *Xenopus* egg extracts can support the actin polymerization and depolymerization in *Listeria* tails at rates comparable to those in intact cells and suggest they reflect the actin dynamics within cell cytoplasm. XAC is required to maintain the turnover of actin filaments in these tails in extracts. Because ADF/cofilin proteins are essential in every species in which they have been found, we may infer that they are required for essential processes like rapid actin turnover in the cell. Finally, since XAC is concentrated in *Listeria* tails and the leading edge of cells, we suspect it is also required for rapid filament turnover in vivo.

In contrast to XAC, gelsolin depletions had no significant effect on *Listeria* tail length or tail polymer mass, leaving the functional role of gelsolin in the regulation of actin dynamics an open question. Since gelsolin does not affect the depolymerization of *Listeria* tails, its concentration in these tails is curious. Perhaps gelsolin is concentrated in these tails strictly because of its actin binding activity, or by another of its activities such as actin capping. Although the *Xenopus* egg extracts used in our assays contain 5 mM EGTA, it is difficult to analyze any local concentrations of Ca^{2+} that may be due to vesicle release. Since Ca^{2+} is required for gelsolin activity, it is possible that the conditions in our cell-free egg extracts are not able to support the activity of gelsolin. Therefore, we cannot rule out a role for gelsolin in tail dynamics in vivo. However, the presence of gelsolin in *Listeria* tails does not greatly affect actin assembly or disassembly in *Listeria* tails in *Xenopus* extracts.

In our addition of varying amounts of excess XAC to extracts until all the actin was driven into abnormal rod structures, *Listeria* tails shrank to ~5 μm but no further. The persistence of a resistant tail segment in up to sixfold the normal concentrations of XAC suggests that an additional factor may control the extent of actin depolymerization by XAC. Several investigators have suggested that the energy of ATP hydrolysis could be used to regulate the lifetime of a filament (Pollard, 1986; Carlier, 1988; Maciver et al., 1991; Moon and Drubin, 1995). In vitro studies of actin filament assembly have shown that upon polymerization of ATP-actin, the bound ATP is hydrolyzed and

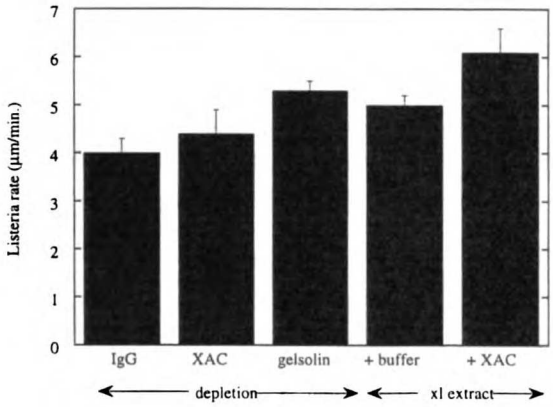
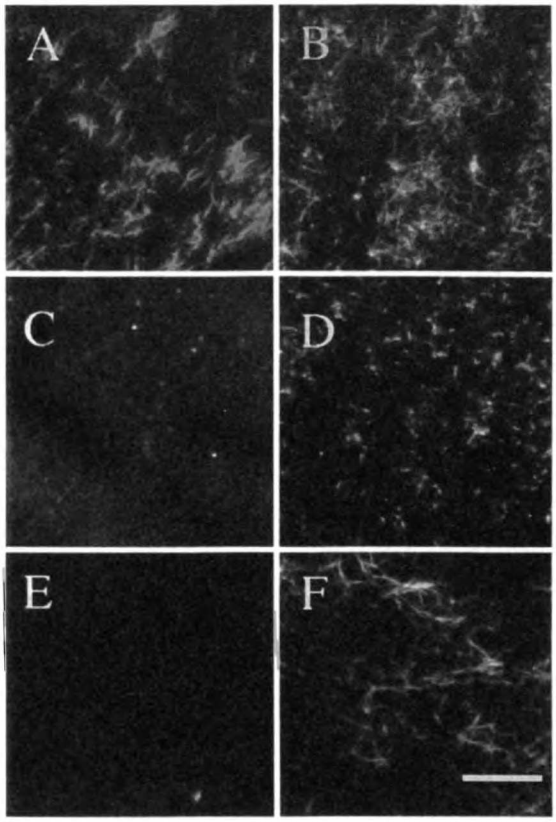
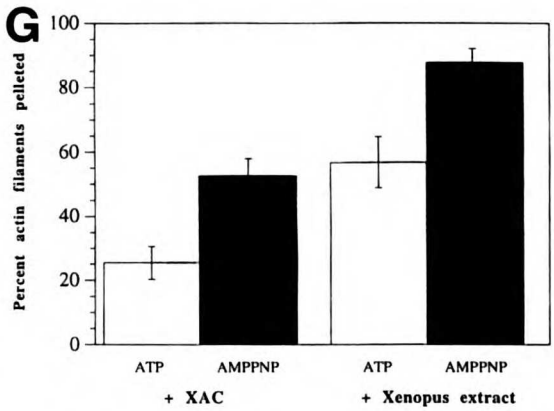


Figure 5. Bar graph representing analysis of *Listeria* movement rate during various extract treatments. The rates were measured using Image 1 software or Winview software. The number of tails measured for each treatment were: IgG depletion, 25; XAC depletion, 24; gelsolin depletion, 34; buffer addition, 27, and XAC addition (to 5.0 μM), 24, over four experiments using two separate extract preps. Error bars represent standard deviation of the mean.

the terminal phosphate is slowly released (Carlier, 1987). This slow rate of phosphate loss relative to the polymerization rate should then produce filaments that contain a segment of ADP + inorganic phosphate (ADP.Pi). Since ATP- and ADP.Pi-bound actin are known to make stronger intersubunit bonds than ADP-actin (Carlier, 1991; Carlier et al., 1985), phosphate release could play a role in regulating actin filament stability. However, since in vitro filaments depolymerize very slowly, phosphate release alone is not sufficient to direct rapid disassembly of actin filaments in the cell. In vivo, perhaps the role of slow phosphate release in actin is to regulate either the binding or activity of actin-severing proteins. Severing proteins such as ADF and cofilin may preferentially sever the ADP subunits of actin filaments while the ATP and ADP.Pi subunits are resistant to severing (Fig. 7). In support of this, the depolymerizing activity of actophorin, an *Acanthamoeba* member of the ADF/cofilin family, can be inhibited by addition of 25 mM phosphate, which presumably mimics ADP.Pi filaments (Maciver et al., 1991). In addition, actophorin has been shown to bind tightly to ADP-containing G-actin and weakly to ATP-actin (Maciver and Weeds, 1994). However, other studies show that chick ADF has a higher affinity for ATP-actin than for ADP-actin (Hayden et al., 1993) and have left the role of the bound nucleotide in regulating depolymerization in other species an open question. Our results showing the resistance of AMPPNP-containing actin filaments to the depolymerizing activities of both XAC and concentrated *Xenopus* egg extracts lend support to the idea that the nucleotide con-





ized with either ATP (*A*, *C*, and *E*) or AMPPNP (*B*, *D*, and *F*). Actin filaments mixed 1:1 with F-buffer (*A* and *B*), with 5.3 μM XAC (*C* and *D*), and with *Xenopus* extract (*E* and *F*). In both cases (*C–F*), XAC is in excess of rhodamine F-actin by approximately fivefold. (*G*) Quantitation of the amount of rhodamine-labeled ATP (□) versus AMPPNP (▨) F-actin pelleted in the presence of XAC or *Xenopus* egg extracts. Percent actin filaments remaining was calculated as the fluorescence of the rhodamine F-actin pelleted in XAC or extracts/the fluorescence of rhodamine F-actin pelleted in F-buffer. The bars represent an average of four experiments and the error bars represent standard deviation of the mean. Bar, 10 μm.

Figure 6. The resistance of AMPPNP–containing actin filaments to the depolymerizing activities of XAC and *Xenopus* egg extracts. Approximately 0.5 μM rhodamine-labeled actin polymer-

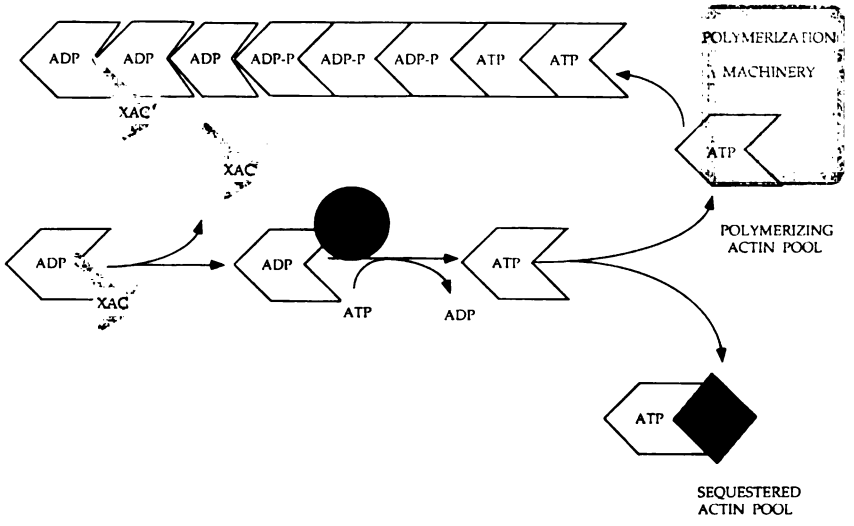


Figure 7. Model for recycling of actin by ADF/cofilin proteins. At areas of high filament turnover in the cell, ATP-bound actin is induced to polymerize by a complex of proteins. The ATP within the filament is hydrolyzed and the terminal phosphate is slowly released. The resulting ADP-containing subunits have weaker interactions with each other than those containing ATP. These subunits are now available for depolymerization by the ADF/cofilin family of proteins (*XAC*). Once XAC depolymerizes an actin subunit(s), it dissociates from actin. The released actin must exchange its ADP for ATP and the nucleotide exchange is probably catalyzed by profilin. The ATP-bound actin is then either repolymerized or sequestered for later use.

tent of an actin filament controls filament lifetime by regulating the activity of ADF/cofilin family members.

What is the mechanism of actin depolymerization by XAC? XAC could depolymerize by end-wise removal of subunits, by severing, or by both mechanisms. We interpret our results with AMPPNP actin as favoring the end-wise mechanism. We reason that a severing protein would not be greatly affected by an actin filament with only 12% of its subunits substituted with AMPPNP. By contrast, an end-wise depolymerizing protein would be blocked whenever AMPPNP subunits were present at the filament ends. Thus, the large inhibition of depolymerization by AMPPNP filaments may be accounted for if XAC primarily depolymerized using an end-wise mechanism. The most direct way of distinguishing severing and end-wise mechanisms will come from imaging depolymerization by ADF/cofilin proteins.

On the basis of our findings, we can postulate a model for how XAC recycles actin subunits in the *Listeria* tail (Fig. 7). A complex of proteins at the back of *Listeria* (Welch et al., 1997) induces polymerization of ATP-bound actin. Once actin polymerizes, the bound ATP is hydrolyzed and the terminal phosphate is slowly released. The resulting ADP-containing actin subunits interact more weakly within the filament than the ATP subunits and allow binding of XAC. XAC either depolymerizes single subunits or short fragments of filament. XAC is then released from the depolymerized actin subunit and recycled for another round of depolymerization. The ADP in the depolymerized actin is then exchanged for ATP by bulk mass or by catalysis from profilin, and this subunit is now available for another round of polymerization or remains unpolymerized by binding thymosin β_4 or other sequestering proteins.

This model provides a framework for understanding how an actin subunit is recycled from one round of polymerization to the next in regions of the cell that rapidly turn over actin filaments. Clearly, many questions remain regarding the details of this model. Future work will need to address whether XAC works primarily by severing or end-wise mechanisms in the cell. We are currently examining how XAC is recycled for another round of depolymerization after it is bound to the actin subunit and the role that XAC phosphorylation may play in its recycling. Other studies will need to focus on how the actin subunit is recycled for repolymerization. Analysis of the nucleotide content of actin has revealed that ~90% of unpolymerized actin in the cell is bound to ATP (Rosenblatt et al., 1995), suggesting that the actin nucleotide is exchanged early in the pathway. Information about whether the ATP- or ADP-bound actin is used for polymerization is still lacking. The 10% of actin that is ADP bound could be bound to XAC. This population of actin could undergo nucleotide exchange and be used directly for another round of polymerization, leaving the remaining 90% of actin sequestered from polymerization. However, the lack of an effect that XAC depletion has on the rate of polymerization at early time points would suggest that this is not the case. Future studies will need to address what population of actin is used for polymerization.

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References

Abe, H., T. Obinata, L.S. Minamide, and J.R. Bamburg. 1996. *Xenopus laevis* actin-depolymerizing factor/cofilin: a phosphorylation-regulated protein essential for development. *J. Cell Biol.* 132:871–885.

Agnew, B.J., L.S. Minamide, and J.R. Bamburg. 1995. Reactivation of phosphorylated actin depolymerizing factor and identification of the regulatory site. *J. Biol. Chem.* 270:17582–17587.

Ampe, C., and J. Vandekerckhove. 1987. The F-actin capping proteins of *Physarum polycephalum*: cap42(a) is very similar, if not identical, to fragmin and is structurally and functionally very homologous to gelsolin; cap42(b) is Physarum actin. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:4149–4157.

André, E., F. Lottspeich, M. Schleicher, and A. Noegel. 1988. Severin, gelsolin, and villin share a homologous sequence in regions presumed to contain F-actin severing domains. *J. Biol. Chem.* 263:722–727.

André, E., M. Brink, G. Gerisch, G. Isenberg, A. Noegel, M. Schleicher, J.E. Segall, and E. Wallraff. 1989. A *Dictyostelium* mutant deficient in severin, an F-actin fragmenting protein, shows normal motility and chemotaxis. *J. Cell Biol.* 108:985–995.

Ankenbauer, T., J.A. Kleinschmidt, J. Vandekerckhove, and W.W. Franke. 1988. Proteins regulating actin assembly in oogenesis and early embryogenesis of *Xenopus laevis*: gelsolin is the major cytoplasmic actin-binding protein. *J. Cell Biol.* 107:1489–1498.

Bamburg, J.R., and B.W. Berstein. 1991. Actin and actin-binding proteins in neurons. *In* The Neuronal Cytoskeleton. R.D. Burgeyne, editor. Wiley-Liss, New York. 121–160.

Bamburg, J.R., H.E. Harris, and A.G. Weeds. 1980. Partial purification and characterization of an actin depolymerizing factor from brain. *FEBS Lett.* 121:178–182.

Bernstein, B.W., and J.R. Bamburg. 1982. Tropomyosin binding to F-actin protects the F-actin from disassembly by brain actin-depolymerizing factor (ADF). *Cell Motil.* 2:1–8.

Bray, D., and J.G. White. 1988. Cortical flow in animal cells. *Science (Wash. DC).* 239:883–888.

Carlier, M.F. 1987. Measurement of Pi dissociation from actin filaments following ATP hydrolysis using a linked enzyme assay. *Biochem. Biophys. Res. Commun.* 143:1069–75.

Carlier, M.F. 1988. Role of nucleotide hydrolysis in the polymerization of actin and tubulin. *Cell Biophys.* 12:105–117.

Carlier, M.F. 1991. Nucleotide hydrolysis in cytoskeletal assembly. *Curr. Opin. Cell Biol.* 3:12–17.

Carlier, M.F., D. Pantaloni, and E.D. Korn. 1985. Polymerization of ADP-actin and ATP-actin under sonication and characteristics of the ATP-actin equilibrium polymer. *J. Biol. Chem.* 260:6565–6571.

Cooper, J.A. 1991. The role of actin polymerization in cell motility. *Annu. Rev. Physiol.* 53:585–605.

Gunsalus, K.C., S. Bonaccorsi, E. Williams, F. Verni, M. Gatti, and M.L. Goldberg. 1995. Mutations in *twinstar*, a *Drosophila* gene encoding a cofilin/ADF homologue, result in defects in centrosome migration and cytokinesis. *J. Cell Biol.* 131:1243–1259.

Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1–726.

Hatanaka, H., K. Ogura, K. Moriyama, S. Ichikawa, I. Yahara, and F. Inagaki. 1996. Tertiary structure of destrin and structural similarity between two actin-regulating protein families. *Cell.* 85:1047–1055.

Hawkins, M., B. Pope, S.K. Maciver, and A.G. Weeds. 1993. Human actin depolymerizing factor mediates a pH-sensitive destruction of actin filaments. *Biochemistry.* 32:9985–9993.

Hayden, S.M., P.S. Miller, A. Brauweiler, and J.R. Bamburg. 1993. Analysis of the interactions of actin depolymerizing factor with G- and F-actin. *Biochemistry.* 32:9994–10004.

Janmey, P.A., and T.P. Stossel. 1987. Modulation of gelsolin function by phosphatidylinositol 4,5-bisphosphate. *Nature (Lond.).* 325:362–364.

Kinoshita, N., J. Minshull, and M.W. Kirschner. 1995. The identification of two novel ligands of the FGF receptor by a yeast screening method and their activity in *Xenopus* development. *Cell.* 83:621–630.

Leimeister-Wachter, M., and T. Chakraborty. 1989. Detection of listeriolysin, the thiol-dependent hemolysin in *Listeria monocytogenes*, *Listeria ivanovii*, and *Listeria seeligeri*. *Infect Immun.* 57:2350–2357.

Maciver, S.K., and A.G. Weeds. 1994. Actophorin preferentially binds monomeric ADP-actin over ATP-bound actin: consequences for cell locomotion. *FEBS Lett.* 347:251–256.

Maciver, S.K., H.G. Zot, and T.D. Pollard. 1991. Characterization of actin filament severing by actophorin from *Acanthamoeba castellanii*. *J. Cell Biol.* 115:1611–1620.

McKim, K.S., C. Matheson, M.A. Marra, M.F. Wakarchuk, and D.L. Baillie. 1994. The *Caenorhabditis elegans* unc-60 gene encodes proteins homologous to a family of actin-binding proteins. *Mol. Gen. Genet.* 242:346–357.

Mitchison, T.J., and L.P. Cramer. 1996. Actin based cell motility and cell locomotion. *Cell.* 84:371–379.

Moon, A., and D.G. Drubin. 1995. The ADF/cofilin proteins: stimulus-responsive modulators of actin dynamics. *Mol. Biol. Cell.* 6:1423–1431.

Moon, A.L., P.A. Janmey, K.A. Louie, and D.G. Drubin. 1993. Cofilin is an essential component of the yeast cortical cytoskeleton. *J. Cell Biol.* 120:421–435.

Morgan, T.E., R.O. Lockerbie, L.S. Minamide, M.D. Browning, and J.R. Bamburg. 1993. Isolation and characterization of a regulated form of actin depolymerizing factor. *J. Cell Biol.* 122:623–633.

Moriyama, K., K. Iida, and I. Yahara. 1996. Phosphorylation of Ser-3 at cofilin regulates its essential function on actin. *Genes to Cells.* 1:73–86.

Nagaoka, R., H. Abe, K. Kusano, and T. Obinata. 1995. Concentration of cofilin, a small actin-binding protein, at the cleavage furrow during cytokinesis. *Cell Motil. Cytoskel.* 30:1–7.

Nishida, E., S. Maekawa, and H. Sakai. 1984. Cofilin, a protein in porcine brain that binds to actin filaments and inhibits their interactions with myosin and tropomyosin. *Biochemistry.* 23:5307–5313.

Nishida, E., K. Iida, N. Yonezawa, S. Koyasu, I. Yahara, and H. Sakai. 1987. Cofilin is a component of intranuclear and cytoplasmic actin rods induced in cultured cells. *Proc. Natl. Acad. Sci. USA.* 84:5262–5266.

Ono, S., H. Abe, R. Nagaoka, and T. Obinata. 1993. Colocalization of ADF and cofilin in intranuclear actin rods of cultured muscle cells. *J. Muscle Res. Cell Motil.* 14:195–204.

Pollard, T.D. 1986. Rate constants for the reactions of ATP- and ADP-actin with the ends of actin filaments. *J. Cell Biol.* 103:2747–2754.

Pringault, E., M. Arpin, A. Garcia, J. Finidori, and D. Louvard. 1986. A human villin cDNA clone to investigate the differentiation of intestinal and kidney cells in vivo and in culture. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:3119–3124.

Rodriguez, D., A. Castillo, S. Lemaire, L. Tchakarov, M. Jeyapragasan, J.P. Doucet, M.L. Vitale, and J.M. Trifaró. 1990. Chromaffin cell scinderin, a novel calcium-dependent actin filament-severing protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:43–52.

Rosenblatt, J., P. Peluso, and T.J. Mitchison. 1995. The bulk of unpolymerized actin in *Xenopus* egg extracts is ATP-bound. *Mol. Biol. Cell.* 6:227–236.

Sakurai, T., K. Ohmi, H. Kurokawa, and Y. Nonomura. 1990. Distribution of a gelsolin-like 74,000 mol. wt protein in neural and endocrine tissues. *Neuroscience.* 38:743–756.

Sanger, J.M., J.W. Sanger, and F.S. Southwick. 1992. Host cell actin assembly is necessary and likely to provide the propulsive force for intracellular movement of *Listeria monocytogenes*. *Infect. Immun.* 60:3609–3619.

Small, J.V., M. Herzog, and K. Anderson. 1995. Actin filament organization in the fish keratocyte lamellipodium. *J. Cell Biol.* 129:1275–1286.

Smith, D.B., and K.S. Johnson. 1988. Single-step purification of polypeptide expressed in *Escherichia coli* as fusions with glutathion S-transferase. *Gene.* 67:31–40.

Theriot, J.A., and T.J. Mitchison. 1991. Actin microfilament dynamics in locomoting cells. *Nature (Lond.).* 352:126–131.

Theriot, J.A., T.J. Mitchison, L.G. Tilney, and D.A. Portnoy. 1992. The rate of actin-based motility of intracellular *Listeria monocytogenes* equals the rate of actin polymerization. *Nature (Lond.).* 357:257–260.

Theriot, J.A., J. Rosenblatt, D.A. Portnoy, C.P. Goldschmidt, and T.J. Mitchison. 1994. Involvement of profilin in the actin-based motility of *L. monocytogenes* in cells and in cell-free extracts. *Cell.* 76:505–517.

Welch, M.D., A. Iwamatsu, and T.J. Mitchison. 1997. Actin polymerization is induced by the Arp 2/3 protein complex at the surface of *Listeria monocytogenes*. *Nature (Lond.).* 385:265–269.

Witke, W., A.H. Sharpe, J.H. Hartwig, T. Azuma, T.P. Stossel, and D.J. Kwiatkowski. 1995. Hemostatic, inflammatory, and fibroblast responses are blunted in mice lacking gelsolin. *Cell.* 81:41–51.

Yonezawa, N., E. Nishida, and H. Sakai. 1985. pH control of actin polymerization by cofilin. *J. Biol. Chem.* 260:14410–14412.

Yonezawa, N., E. Nishida, K. Iida, I. Yahara, and H. Sakai. 1990. Inhibition of the interactions of cofilin, destrin, and deoxyribonuclease I with actin by phosphoinositides. *J. Biol. Chem.* 265:8382–8386.

Zigmond, S.H. 1993. Recent quantitative studies of actin filament turnover during cell locomotion. *Cell Motil. Cytoskel.* 25:309–316.

Chapter Four

Recycling of depolymerized actin subunits into a polymerization competent-form.

Jody Rosenblatt, Matthew D. Welch, and Timothy J. Mitchison

Introduction:

Ameboid cell motility is important for many cellular processes such as chemotaxis of leukocytes, cell migration during wound closure and embyrogenesis. Protrusion of a membrane edge or lamellipodium is a crucial first step for this type of movement. Continuous movement of lamellipodia requires continuous cycles of actin polymerization and depolymerization. In order to biochemically dissect the mechanism and regulation of actin polymerization and depolymerization, we have used a simple model system which we believe represents the actin dynamics at the leading edge. The intracellular pathogen, *Listeria monocytogenes*, polymerizes the host cell actin at its surface to propel itself throughout the cytoplasm and into adjacent cells (Tilney and Portnoy, 1989; Tilney, 1990). Like lamellipodial protrusion, *Listeria* propulsion requires actin polymerization, and actin polymerization and depolymerization dynamics within the *Listeria* tail occur at rates similar to those in the lamellipodium (Tilney et al., 1992; Theriot et al., 1994). Because *Listeria* expresses ActA at its surface, a protein that acts as a constitutive signal for inducing actin polymerization, and can move in cellular extracts as well as intact cells, *Listeria* movement provides an excellent system for biochemically purifying the host factors required for actin dynamics (Theriot et al., 1994; Appendix A).

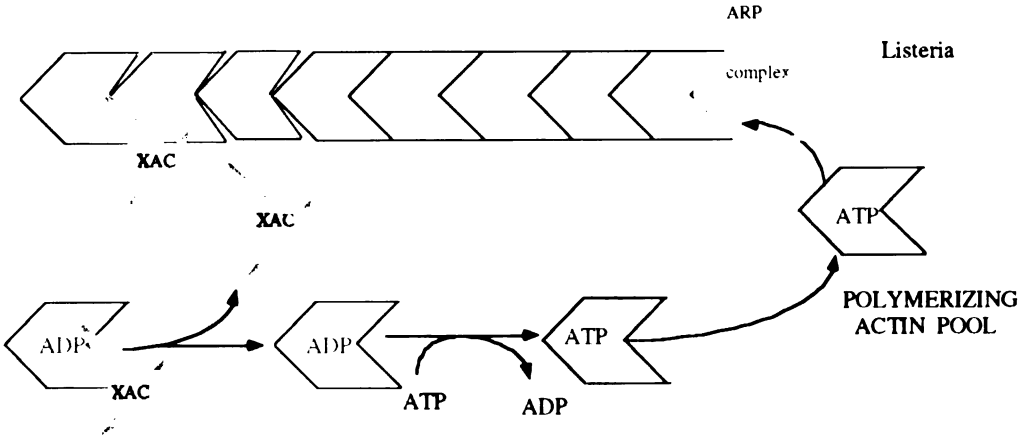
In an effort to better understand the mechanism of actin polymerization/depolymerization cycles within the cell, we would like to reconstitute Listeria movement using pure components in an *in vitro* system. To this end, we have purified the host cell factors required for catalyzing actin polymerization and depolymerization. Using the *Listeria*

system, Welch et al. have purified a complex of proteins, the Arp 2/3 complex, which in conjunction with ActA nucleate actin polymerization at the *Listeria* surface (Welch et al., 1997; Welch et al., 1998). I have found that the ADF/cofilin family of proteins are responsible for actin depolymerization in the *Listeria* tail and, by inference, we predict for actin disassembly in the lamellipodium (Rosenblatt et al., 1997). Although actin polymerization is crucial for *Listeria* and lamellipodial movement, actin depolymerization is equally important. If actin depolymerization is blocked, as in the case where extracts are depleted for the Xenopus ADF cofilin protein (XAC), eventually actin monomer is depleted and actin polymerization at the *Listeria* surface ceases (Rosenblatt et al., 1997). Therefore, we can assume that actin depolymerization is an early requirement for actin polymer recycling.

What other events must occur in order to complete recycling of an actin subunit into a polymerizable form? The model in Fig. 1 shows the events that might contribute to actin subunit recycling. When ATP bound actin polymerizes, the ATP within the polymer hydrolyzes and the terminal phosphate is slowly released to form ADP-containing subunits. From experiments using non-hydrolyzible ATP analog containing filaments, the subunits containing ADP appear to be more susceptible to depolymerization by XAC (Rosenblatt et al., 1997). Thus, recently depolymerized actin subunits are expected to contain ADP and, because the ADF/cofilin family of proteins bind with an approximate 100-fold greater affinity to ADP-actin than ATP actin (Moon and Drubin, 1995; Theriot, 1997), may continue to bind XAC after depolymerization has occurred. Therefore a step that is likely to be important for recycling is release of XAC

Figure 4-1. Recycling of actin subunits. Actin filaments are nucleated at the back of *Listeria monocytogenes* by the Arp 2/3 complex. After polymerization the ATP within the actin filament is hydrolyzed and ADF/cofilin proteins (here, XAC) depolymerize the ADP containing subunits. Once a subunit is depolymerized, XAC must be released and the bound ADP must be exchanged for ATP so that the subunit may be repolymerized.

Fig. 1


ARP
complex
Listeria
XAC
XAC
ATP
POLYMERIZING
ACTIN POOL
ADP
ADP
ATP
XAC
ATP
ADP

from the depolymerized subunit. Since ATP-actin has a 10-fold lower critical concentration for polymerization than does ADP-actin (Pollard, 1986), another predicted step for completing actin subunit recycling may be nucleotide exchange of ADP for ATP on actin. I have developed an assay to biochemically test candidate proteins and fractionate for factors required for recycling a subunit from polymer back into polymer.

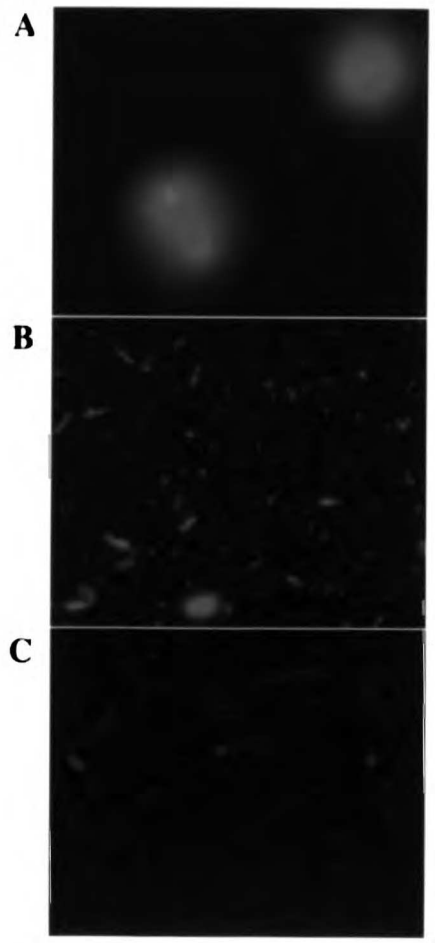
Results:

Depolymerized F-actin must be further recycled to assemble into *Listeria* tails.

As a first step in assessing how actin is recycled I wanted to determine what forms of actin were capable of polymerization. To do this, I developed an assay using pure reagents. Since we knew that pure rhodamine-labeled G-actin can polymerize onto the back of *Listeria* monocytogenes when the Arp2/3 complex is present ((Welch et al., 1997) and Fig. 2A), I used the *Listeria*/Arp2/3 complex polymerization machine to test what other forms of actin were capable of assembly. Addition of rhodamine actin polymer to this mix demonstrated that *Listeria* actin clouds could only be produced by de novo polymerization of actin monomer and not by binding previously formed actin polymers (Fig. 2B and also (Welch et al., 1998)). Given that actin must be in a monomer form to polymerize onto *Listeria* with the Arp complex, I tested whether actin monomer produced from depolymerization of rhodamine actin polymer could also work in this assay. Fig. 2C shows that actin polymer depolymerized with XAC can not assemble onto *Listeria* in the presence of the Arp complex. Thus, although the Arp/*Listeria* system requires actin monomer to assemble actin clouds

Figure 4-2. Depolymerized F-actin must be recycled to assemble into *Listeria monocytogenes* tails. Micrographs showing the 0.4 μM Arp 2/3 complex and DAPI-labeled *L. monocytogenes* mixed with (A) 0.5 μM unpolymerized TMR-actin, (B) 0.5 μM TMR-actin filaments, and (C) 0.5 μM TMR-actin filaments plus 0.8 μM XAC.

Fig. 2



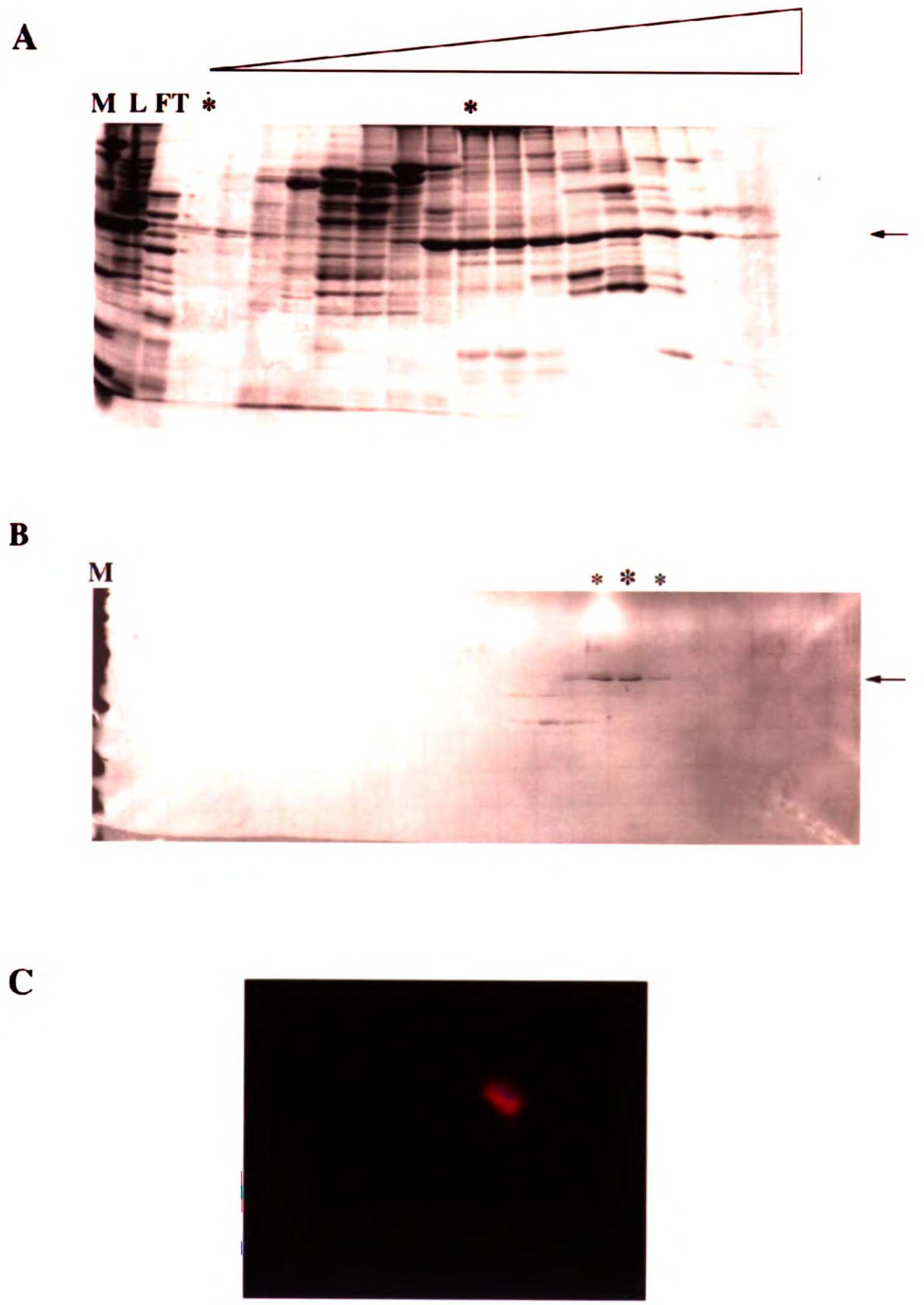
and tails, this actin monomer must first be regenerated into a form capable of polymerization.

Fractionation for an actin-recycling factor from platelet extracts.

The results from Fig. 2C provided a useful assay to fractionate for factors that could recycle depolymerized actin filaments (F-actin) into a form that could repolymerize onto *Listeria* tails. Platelet extracts were used as a starting material for fractionation because they contain high concentrations of actin-binding proteins and had proven successful when used for purifying the Arp 2/3 complex (Welch et al., 1997). As a first step in purifying the recycling factor, platelet extracts were loaded onto a Hi Trap Q column and eluted with a 40-500 mM KCl gradient by FPLC. The fractions were run on gels and assayed for recycling activity by mixing them with a cocktail containing *Listeria*, the Arp 2/3 complex, rhodamine-labeled actin polymer, and XAC (Fig. 3 A). The asterisks above the gel denote the two active fractions. While the fraction that eluted later in the gradient had recycling activity, its protein profile was more complex and was also capable of forming *Listeria* tails rather than simply actin clouds. The ability of this fraction to form tails may indicate that it contained actin cross-linking and bundling proteins in addition to recycling proteins. Because I was fractionating only for proteins that could recycle actin into clouds (as seen with Fig. 2A, *Listeria* + G-actin), I used only the early eluting, cloud forming fraction for further purification. Since this fraction contained few proteins, complete purification was achieved by gel filtration. Fig. 3 B shows the fractions from a Superose 12 gel filtration column with asterisks above the active fractions. An example of the actin clouds that formed

Figure 4-3. Purification of F-actin recycling factor. (A) Coomassie Blue stained SDS-PAGE of elution profile of platelet extracts run on a Hi-Trap Q column. M, marker, L, load, and FT, flow through. (B) Coomassie Blue stained SDS-PAGE of fractions 4 and 5 run on gel filtration column. M, marker. For (A) and (B), asterisk indicates lanes containing recycling activity and arrow indicates where actin migrates on 12% SDS-PAGE. (C) Fluorescence micrograph of actin halo formed around *Listeria monocytogenes* when actin fraction from (B) is mixed with DAPI-labeled *Listeria*, the Arp 2/3 complex, XAC, and TMR-actin filaments.

Fig. 3



around *Listeria* when gel filtration fraction 18 (the largest asterisk) was added to the recycling assay is shown in Fig. 3 C. The only protein in the most active fraction migrated at approximately 45 kD and was therefore predicted to be actin.

To test whether actin alone could recycle depolymerized F-actin into polymer, pure platelet or rabbit skeletal muscle actin was added to the recycling assay. Both types of actin were capable of recycling when present at 6 μM in the assay and platelet actin was active at concentrations as low as 0.3 μM (rabbit skeletal muscle actin was not tried at lower dilutions). The less pure actin-containing fractions eluting off the HiTrap Q column in Fig. 3A (arrow shows migration of actin), suggest that actin in complex with other proteins is not able to recycle actin. The fact that these actin-containing fractions are incapable of recycling actin in *Listeria*/Arp/F-actin/XAC assay suggests that actin in cells, much of which is in complex with other proteins, may not be the true recycling protein in a cell.

Attempts to deplete actin from extracts prior to fractionation for recycling activity.

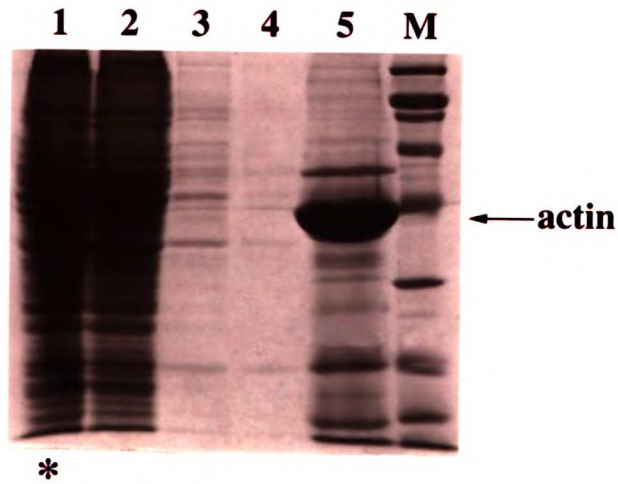
Because pure actin may not be a physiological recycling protein in the context of a cell but could score in the recycling assay, I tried to deplete extracts of actin before fractionating for the recycling factor. To do this, I tried to either inactivate the cellular actin or to deplete actin directly. Actin requires ATP for its stability and activity and because depletion of ATP from actin by desalting or Dowex treatment inactivates actin, this might provide a simple way of treating the extracts prior to fractionation. However, platelet extracts treated with Dowex beads or desalted over a P-4 column were rendered inactive.

Treatment of actin with 8 M urea can also destroy its activity. Thus, extracts were treated with 8 M urea, desalted and assayed for their activity in the recycling assay. Urea-treated extracts were capable of recycling depolymerized F-actin into actin halos around *Listeria*, but, when further fractionated over a Q column, all activity was lost. The loss of activity after fractionation may be due to removal of chaperones or other proteins important for refolding actin or putative recycling factor into a stable, active form. Thus, neither treatment with urea or ATP-depletion provided good methods of blocking actin activity prior to fractionation.

Because these general treatments were insufficient to remove actin without inactivating all recycling activity, direct depletion of actin from extracts was tried. DNase 1 binds tightly and specifically to actin and therefore provides a good reagent for depleting actin from extracts. Fig. 4 shows a gel of Xenopus egg extracts before (lane 1) and after (lane 2) DNase 1 depletion of actin (asterisk indicates lanes with recycling activity). While undepleted extracts are active, those treated with DNase 1 beads no longer contain recycling activity. DNase 1 depletion of recycling activity may be due to depletion of actin binding proteins in addition to actin. Because it is likely that an actin recycling factor would bind actin, the DNase 1 beads were eluted with 250 mM NaCl (Fig. 4, lane 3) and 600 mM NaCl (Fig. 4, lane 4) to try to recover actin binding proteins bound to the DNase 1 beads. The salt elutions were not efficient at eluting many proteins off of the DNase 1 beads and, after desalting, these elutions did not contain any recycling activity. Fig. 4, lane 5 shows the proteins remaining on the DNase 1 beads after the NaCl elutions. However, the actin and actin-binding

Figure 4-4. Elutions off of DNase 1 beads. Coomassie-Blue stained 12% SDS-PAGE showing lane 1, concentrated *Xenopus* egg extracts (load), lane 2, DNase 1 bead flow through, lane 3, 250 mM NaCl elution off of DNase 1 beads, lane 4, 600 mM NaCl elution off of DNase 1 beads, lane 5, DNase 1 beads boiled in SDS-PAGE loading buffer. M, marker. Asterisk indicates fraction active for F-actin recycling activity.

Fig. 4



protein contained in this fraction could not be assayed for recycling activity because their elution required boiling in SDS sample buffer.

Candidate actin-binding proteins tried in recycling assay.

In addition to random isolation of recycling proteins by fractionation, I also decided to assay whether candidate actin-binding proteins might recycle actin. From the model in Fig. 1, one might assume that for actin to be recycled into a form that can repolymerize, at least two events must take place: the actin must be released from XAC, and the ADP from the depolymerized actin subunit must be exchanged for ATP. With these assumptions, proteins that promote either release of XAC from actin or nucleotide exchange on actin might serve as good candidates for recycling proteins. The ADF/cofilin family of proteins have a much lower binding affinity for ATP-actin than ADP-actin monomer (Moon and Drubin, 1995; Theriot, 1997) and do not bind actin when they are phosphorylated (Morgan et al., 1993; Agnew et al., 1995; Moriyama et al., 1996). Therefore, proteins that either act as nucleotide exchange factors on actin or that can phosphorylate ADF/cofilins may help in releasing actin from XAC as well as recycle XAC for further rounds of depolymerization. Since nucleotide exchange on actin could recycle actin by both releasing it from XAC and recharging the nucleotide, I addressed whether profilin, a known nucleotide exchange factor for actin (Goldschmidt et al., 1992), was capable of recycling actin.

To test whether profilin may act as an actin recycling protein, I assayed whether pure profilin was active when added directly to the recycling assay and also whether active gel filtration fractions contained profilin. Profilin purified from human platelets did not appear to have any

recycling activity when added to the *Listeria*/Arp/F-actin/XAC assay at molar ratios of 1:4 to 2:1 profilin:rhodamine-actin. Thymosin ß4, an actin sequestering protein which may contribute to profilin's actin polymerization enhancing activities (Goldschmidt et al., 1992; Pantaloni and Carlier, 1993), did not have any recycling activity when it was added alone or in combination with profilin to the assay. The native gel blotted with anti-actin antibodies in Fig. 5 A shows that both profilin and thymosin ß4 were both active in their abilities to bind actin. The migration of actin alone (lane 1, *) is retarded when mixed with profilin (lane 2, shift to **) and enhanced when mixed with thymosin ß4 (lane 3, shift to ***).

Fig. 5 B shows a Western blot of fractions from a Superose 12 gel filtration column blotted with antibodies to VASP and profilin (migration of each is indicated by arrows on right side of gel). The size of asterisk below each lane correlates with the degree of recycling activity. Note that the fractions containing only profilin or only VASP have only slight recycling activity. Fraction 17 has the most robust recycling activity and also contains both profilin and VASP. Since VASP is thought to be an accessory protein for profilin to bind to the ActA protein of *Listeria* (Reinhard et al., 1995; Pollard, 1995), it is possible that both VASP and profilin are required for efficient recycling of actin at the *Listeria* surface.

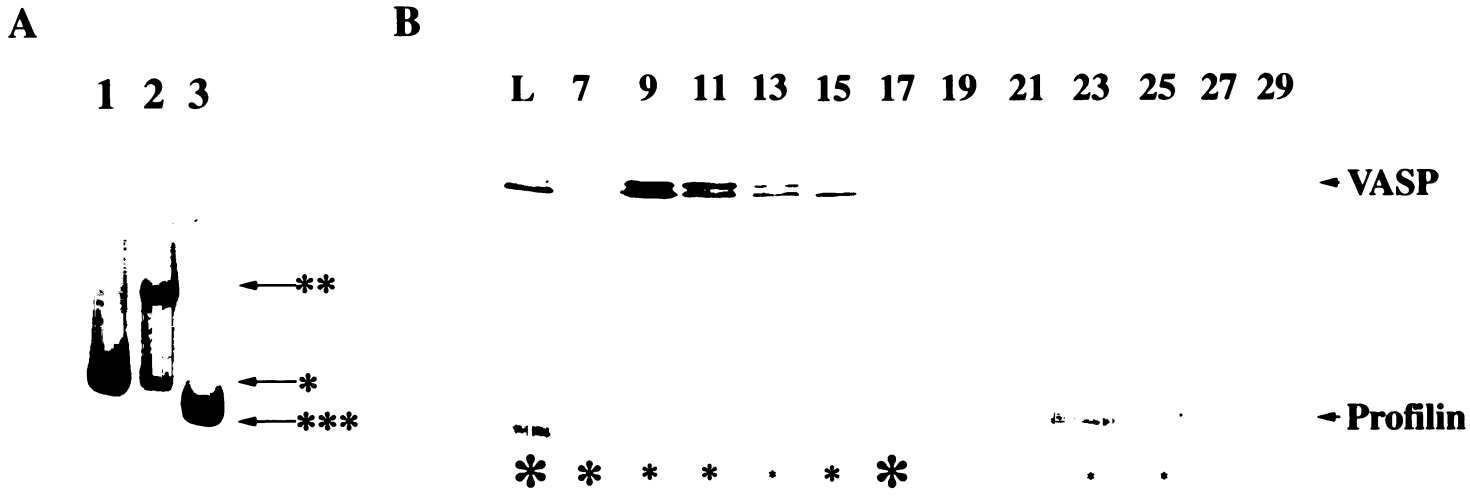
Discussion:

Is actin the real recycling protein?

Using conventional chromatographic methods to isolate a protein that could recycle depolymerized actin into a form which could be repolymerized, the only active protein I found was actin. Ordinarily when activities are purified by standard methods, the activity is present at

Figure 4-5. Candidate actin binding proteins tried in the F-actin recycling assay. (A) Anti-actin immunoblot of native gel showing migration of actin when mixed with: lane 1, G-buffer, lane 2, profilin, and lane 3, thymosin ß4. Single asterisk indicates the migration of pure actin, double asterisk, actin plus profilin, and the triple asterisk, actin plus thymosin ß4. (B) Anti-profilin and anti-VASP immunoblot of 12% SDS-PAGE of platelet extracts run on a gel filtration column. L, load. Asterisks indicate active fractions.

Fig. 5



moderate levels in the starting material and becomes greater as the activity is purified. However, here, where actin was purified as a recycling protein, many fractions that contained actin originally did not have any recycling activity. This suggests that actin may only act as a recycling protein when it is pure and not in a complex. In my assay, pure actin can recycle actin only when present at concentrations of 0.3 μM and higher. However, the critical concentration for actin polymerization at the barbed end of a filament has been measured at 0.1-0.2 μM (Pollard, 1986). Therefore, it is not likely that uncomplexed actin can remain unpolymerized at concentrations as high as 0.3 μM in the cell. Thus, one can not conclude whether actin can act as a recycling protein in a cell if unpolymerized, uncomplexed actin does not exist at concentrations required for recycling activity.

How pure actin could act as a recycling protein is also unclear. Addition of pure ATP-bound actin should not enhance the off rate of the rhodamine ADP-bound actin from XAC. Furthermore, given that ADF/cofilin proteins bind ADP-actin with approximately two orders of magnitude higher affinity than ATP-actin, any rhodamine ADP-actin that is released would be more likely to rebind XAC than would the added ATP-actin. How addition of pure unpolymerized actin could enhance nucleotide exchange on rhodamine ADP-actin is also hard to imagine. Therefore, the most likely possibility is that any trace amounts of unpolymerized rhodamine actin within the mix incorporate into the actin tails when unlabeled, ATP-actin is added. These trace amounts of rhodamine actin may be too far below the critical concentration for polymerization to polymerize on their own until extra actin is added. This mechanism of "recycling" would explain how the actin clouds formed in

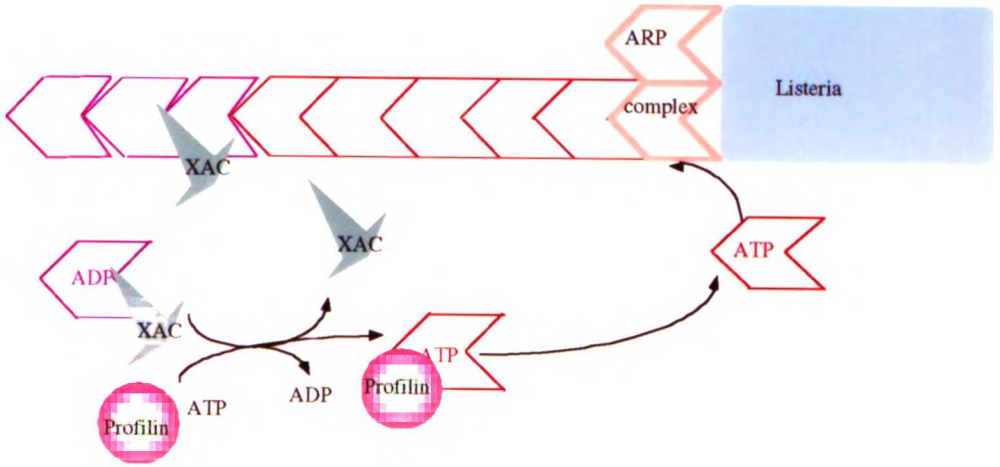
Fig. 3 C from addition of the purified actin fraction are small and faint when compared to those formed in Fig. 2 A. Both figures should contain the same total amount of rhodamine actin but the clouds in Fig. 2A (*Listeria*/Arp/TMR-G-actin) are capable of incorporating all of the rhodamine actin whereas the clouds in Fig. 3 C can only incorporate a fraction of the total rhodamine-actin. If actin were a true recycling protein, one would expect addition of actin to transfer nearly all of the depolymerized rhodamine actin back into clouds similar to those seen in Fig. 2 A. A similar looking halo of actin could also be seen when pure platelet actin was added to the recycling assay in the absence of XAC (data not shown). Again, this would argue that trace amounts of unpolymerized rhodamine actin, perhaps depolymerized following actin polymer isolation, were incorporating into the clouds containing mostly unlabeled actin. Although we can not definitively rule out whether actin is a recycling protein, the arguments above suggest that it is not.

If actin is not the real recycling factor, further work in search of recycling factor(s) will require efficient removal of actin prior to fractionation. My attempts to remove actin without removing potentially interesting actin-binding proteins were unsuccessful but by no means exhaustive. A better approach for removing actin would be to use DNase 1 beads to deplete actin specifically. The use of DNase 1 beads has the advantage of concentrating for actin binding proteins which are likely to contain the putative recycling factor since recycling activity is absent in extracts which have bound DNase 1 beads (see Fig. 4). Gradient elutions of salt, urea, or formamide may provide better methods for eluting actin

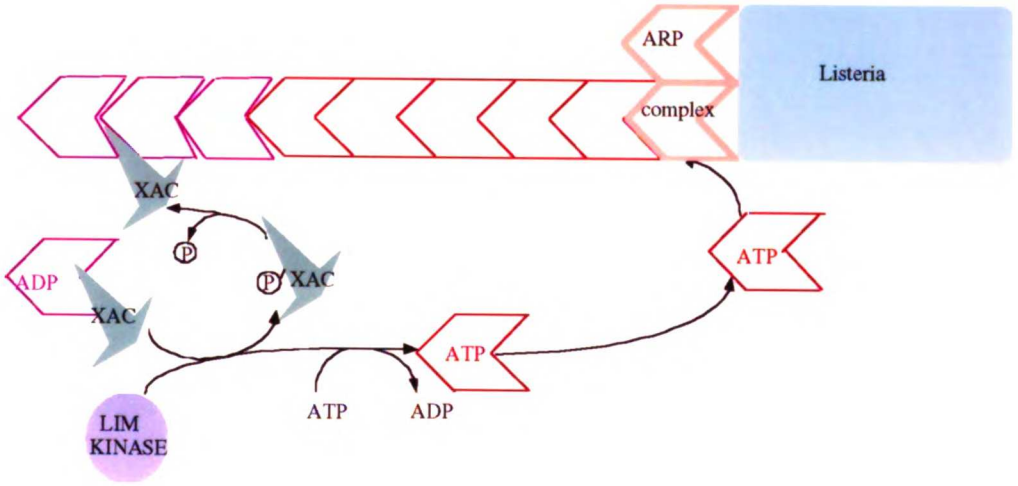
Figure 4-6. Models for recycling actin subunits. (A) Model for how profilin may recycle actin subunits for repolymerization. Profilin may cause nucleotide exchange on actin while XAC is still bound. XAC would be removed through the process of nucleotide exchange because it has a lower affinity for ATP-actin than for ADP-actin. (B) Model for how LIM kinase could recycle actin subunits for repolymerization. LIM kinase phosphorylation of XAC would cause XAC to release from actin because phosphorylated XAC can not bind actin. To complete recycling, the ADP on the released subunit could be exchanged for ATP with or without profilin.

Fig. 6

A


ARP
complex
Listeria
XAC
XAC
ADP
XAC
ATP
ATP
Profilin
ATP
ADP
Profilin

B


ARP
complex
Listeria
XAC
P
P
XAC
ADP
XAC
ATP
ATP
ATP
ADP
LIM
KINASE

binding proteins off the actin/DNase 1 beads complex without simultaneously eluting actin as well.

Are profilin and LIM kinase good candidates for recycling factors?

Because several known actin binding proteins stand out as prime candidates for recycling factors, directly assaying these proteins for recycling activity may be the most powerful approach for isolating the recycling factor. By the model in Fig. 6 A, profilin as a nucleotide exchange protein for actin could recycle a depolymerized actin subunit by exchanging the actin-bound ADP for ATP and/or releasing it from XAC. The role of profilin in the cell is not known. Profilin has been shown to inhibit actin polymerization when present at high concentrations such as during the acrosome reaction where it acts as an actin sequestering protein (Carlsson et al., 1977; Tilney et al., 1983; Ozaki and Hatano, 1984). At concentrations sub-stoichiometric to actin, profilin may also promote actin polymerization. This may be accomplished by two activities: by enhancing nucleotide exchange on actin and increasing the pool of ATP-bound actin (as mentioned above) (Korn, 1982; Goldschmidt et al., 1992) and/or by decreasing the critical concentration for actin polymerization at the barbed end (Pantaloni and Carlier, 1993). Because profilin's activity is dependent on its relative concentration to actin, it may be hard to reconstitute profilin's activity *in vitro* compared to in a cell. In a cell, although profilin may exist at low concentrations compared to actin, it is localized to sites where actin polymerization in initiated (Carlsson et al., 1977; Buss et al., 1992; Theriot et al., 1994) and, therefore, the local concentration of profilin may be quite high. Although the cellular function of VASP is uncertain, its ability to bind both profilin and ActA suggests that it serves to localize

profilin at *Listeria* (and the cellular equivalent sites of actin assembly) (Pollard, 1995; Pistor et al., 1995). Therefore, it may be significant that the only gel filtration fraction that contained profilin and VASP was active for recycling. Adding profilin alone at low concentrations that may be at the right stoichiometry for enhancing actin polymerization may be at concentrations too low to localize at the *Listeria* surface without the aid of VASP. Addition of profilin at higher concentrations might circumvent the localization problem but would also act to sequester actin monomer and prevent polymerization. Thus, to properly test whether profilin may recycle actin subunits, VASP should be purified and included in the assay.

Another model for how actin subunits could be recycled by release of XAC from actin is shown in Fig. 6 B. Since phosphorylated XAC can not bind actin (Morgan et al., 1993; Agnew et al., 1995; Moriyama et al., 1996), XAC could release its bound actin subunit by becoming phosphorylated. Then the actin could undergo nucleotide exchange and the XAC could become dephosphorylated and recycled for further rounds of depolymerization. In this model, nucleotide exchange on actin may occur with or without the help of profilin since actin intrinsically has a ~four-fold higher affinity for ATP than for ADP (Kinosian et al., 1993).

While I was reaching the end of this project, the kinase that phosphorylates cofilin, LIM Kinase 1, had just been discovered (Arber et al., 1998; Yang et al., 1998). I tried to purify LIM kinase in attempt to see whether it had any recycling activity in my assay. I obtained a mouse clone of wild type and dominant negative LIM kinase which I subcloned into a vector to make N-terminal fusions with glutathione S transferase (GST). I was able to purify a GST-dominant negative LIM kinase protein as a

control, but unfortunately the wild type LIM kinase was degraded upon expression. I also tried to immunoprecipitate Myc-tagged versions of these proteins which I had transfected into HeLa cells, but the transfection rates were too low. Without knowing whether pure recombinant LIM kinase is active on its own or needs other proteins to activate it, I feel the best approach for isolating active LIM kinase would be to either immunoprecipitate it from transfected cells or to reisolate recombinant LIM kinase after mixing with "activating" extracts. Since previous work has shown that N-terminally tagging LIM kinase does not interfere with its activity (Arber et al., 1998), the N-terminal Myc or GST tags should be active. If one is to reisolate LIM kinase after incubation with extracts, however, it would be advantageous to use a tag which would not compete for glutathione binding in the cell as the GST tag might.

Recycling of actin subunits for further rounds of polymerization is crucial for the movements that depend on actin polymerization. If depolymerization as a first step for actin polymer recycling is blocked using the drug Jasplakinolide, protrusion of lamellipodia is quickly abrogated (Louise Cramer, personal communication). Therefore, it is important to know how actin can complete its recycling and what factors are responsible for this recycling. Due to time limitations, I was not able to complete this project. However, had I more time, I think that the best approach to isolating the putative recycling factor(s) may come from directly analyzing LIM kinase, profilin and VASP. Should any of these proteins serve as recycling proteins in the actin recycling assay, they may also give an indication of the mechanism by which actin is recycled.

Methods:

Recycling assay

The Arp 2/3 complex was purified as describe in (Welch et al., 1997). XAC was purified as described in (Rosenblatt et al., 1997). Actin was purified as described in (Spudich, 1971), labeled with N-hydroxysuccinimidyl 5-carboxytetramethylrhodamine (TMR) using the method described in (Kellogg et al., 1988), and stored at -80°C in G-buffer (5 mM Tris HCl (pH 8.0), 0.2 mM $CaCl_2$, 0.2 mM adenosine triphosphate (ATP), 0.2 mM dithiothreitol (DTT)). TMR-labeled actin filaments were polymerized by adding Tris HCl (pH 8.0) to 50 mM and NaCl to 50 mM and isolated by centrifugation through an F-buffer cushion (50 mM NaCl, 50 mM Tris HCl, 0.2 mM DTT, 0.5 mM ATP, 40% v/v glycerol) at 228,000 g for 15 min. at 20° C in a TLA 120 rotor. The TMR-F-actin pellet was then resuspended in F-buffer (50 mM NaCl, 50 mM Tris HCl, 0.2 mM DTT, 0.5 mM ATP) by pipetting and its concentration, relative to unpolymerized TMR-actin, was determined by spotting 1 µl of each on a piece of nitrocellulose and staining with Ponceau S stain.

Equal concentrations of G- and F-TMR actin (0.5 µM) were incubated with Arp 2/3 complex (0.3-0.5µM), 0.05% Triton X 100, Energy Mix (0.15 mg/ml creatine kinase, 7.5 mM creatine phosphate, 1 mM ATP, 1 mM $MgCl_2$, 0.1 mM EGTA (pH 7.7)), antifade mix (2 mM glucose, 0.02 mg/ml glucose oxidase, 0.04 mg/ml catalase) and 4', 6-diamidino-2-phenylindole (DAPI)-labeled L. monocytogenes strain SLCC-5764 as described in (Welch et al., 1997). In the case where depolymerized TMR-F-actin was used, XAC was added to 0.08 µM. Chromatographic fractions or purified proteins tested for recycling activity were assayed by adding the sample or

comparable volume of control buffer to the above mixes containing either TMR-F-actin + XAC or TMR-G-actin (as a control). The mix was squashed between a glass slide and coverslip and incubated at 22° C for 20 min before it was viewed by fluorescence microscopy.

Preparation of platelet extracts

Outdated human platelet phoresis or platelet concentrates stored at 22° C were obtained from Irwin Memorial Blood Bank. Platelets were centrifuged in a GSA rotor at 1000 rpm for 15 min. at 22° C to remove red blood cells. The pellets were discarded and the supernatants were recentrifuged in the GSA rotor at 4500 rpm for 15 min. at 22° C. The platelet pellet was washed twice by gently resuspending in 200 mls platelet buffer (50 mM Tris (pH 8.0), 50 mM sucrose, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA (pH 8.0), and 1 mM EDTA (pH 8.0)) and recovering by centrifugation in a GSA rotor at 4,500 rpm for 15 min. at 22° C. The pellets were resuspended in 10 mls cold platelet buffer + 1 mM PMSF, 2 mM benzamidine, and 10µg/ml each leupeptin, pepstatin, and chymostatin. The platelet mixture was then lysed by sonicating 12 times for 10 secs. on ice, or until platelets appeared to be lysed by microscopy and Bradford analysis. The platelet sonicate was centrifuged in a 70.1 Ti Beckman ultracentrifuge rotor at 50,000 rpm for 1.5 hr at 4 ° C. The supernatant was removed and aliquots were frozen in liquid nitrogen and stored at -80°C. Note: extreme care was taken while handling human platelets. All liquid waste was bleached and dry waste was autoclaved.

Column Chromatography

A Pharmacia 1 ml HiTrap Q column was washed in 5 mls elution buffer (1 M KCl, 20 mM MOPS (pH 7.0), 5 mM EGTA (pH 8.0), 2 mM $MgCl_2$,

1mM EDTA (pH 8.0), 0.2 mM DTT, 0.2 mM ATP) and pre-equilibrated in 5 mls loading buffer (40 mM KCl, 20 mM MOPS (pH 7.0), 5 mM EGTA (pH 8.0), 2 mM $MgCl_2$, 1mM EDTA (pH 8.0), 0.2 mM DTT, 0.2 mM ATP). One ml platelet extract was filtered through a 0.2 µm filter and loaded onto the pre-equilibrated HiTrap Q column, washed with 10 mls loading buffer and eluted in a 40 mM to 500 mM KCl gradient while collecting 1 ml fractions. Each fraction was analyzed on a 12% SDS-PAGE gel followed by Coomassie Blue staining and assayed for recycling activity by addition to the recycling assay described above. Active fractions #4 and #5 were concentrated to 0.6 mls using a Centricon-10 and run on a Pharmacia Superose 6 column in loading buffer and 0.5 ml fractions were collected. Each fraction was assayed for recycling activity using the recycling assay and analyzed for protein content by SDS-PAGE analysis, as above.

Gel filtration of platelet extracts used in the profilin/VASP western was done using a Pharmacia Superose 12 column. Platelet extract was centrifuged at 436,000 g for 15 min in a TLA 120.1 rotor at 4° C and the supernatant was filtered through a 0.2 µm filter. 0.6 mls filtered supernatant was loaded onto a Superose 12 column pre-equilibrated in Superose buffer (100 mM KCl, 20 mM MOPS (pH 7.0), 5 mM EGTA (pH 8.0), 2 mM $MgCl_2$, 1mM EDTA (pH 8.0), 0.2 mM DTT, 0.2 mM ATP, and 10% v/v glycerol) and 0.5 ml fractions were collected. Each fraction was assayed for recycling activity using the recycling assay and for protein content by SDS-PAGE followed by Coomassie Blue staining and western blotting using antibodies to human profilin and VASP.

Preparation of human thymosin ß4

Platelet phoresis was centrifuged in GSA rotor at 1000 rpm for 15 min. at 22° C to remove red blood cells. The supernatant was recentrifuged in a GSA rotor at 3400 rpm for 15 min. at 22° C and the platelet pellet was washed three times by resuspending gently in 200 ml lysis buffer (20 mM KPipes (pH 6.8), 40 mM KCl, 5 mM EGTA (pH 8.0), and 1mM EDTA (pH 8.0)) and centrifuging at 3400 rpm for 15 min. at 22° C in a GSA rotor. The pellet was resuspended in 25 mls lysis buffer and chilled on ice for 10 min. 25 mls lysis buffer + 0.05 mM ATP, 1% Triton X 100, 2 mM PMSF, 4 mM Benzamidine, and 20 µg/ml each of leupeptin, pepstatin, and chymostatin was added and incubated for 5 min. on ice. The lysate was centrifuged at 4300 rpm in a GSA rotor for 2 min. at 4° C and the supernatant was quick frozen in liquid nitrogen and stored at -80° C as 'triton-soluble platelet extract'. The triton-soluble extract was boiled for 5 min. and centrifuged in an Sorvall SS34 rotor at 10,000 g for 30 min. The supernatant was transferred to 3,500 MW dialysis tubing and concentrated to 10 mls using Aquacide II, filtered through a 0.2 µm filter, and then filtered through a Centriprep-10 filter overnight at 3000 rpm in a GSA rotor. The filtrate was loaded over a 64 ml Biogel P-10 column pre-equilibrated in H_2O at 0.5 ml/min. 2 ml fractions were collected and analyzed on a 10-20% gradient SDS-PAGE gel followed by Coomassie Blue staining. Peak fractions were pooled, aliquoted, frozen in liquid nitrogen, and stored at -80° C.

Profilin was isolated as described in (Janmey, 1991). Profilin and thymosinß4 were analyzed for actin-binding activity by native gel analysis. 3 µg of actin was mixed with either G-buffer, 1 µg profilin, or 0.5 µg thymosinß4 for 5 min. at 22° C and run on native gels and western blotted with actin antibodies as described in (Rosenblatt et al., 1995).

Actin depletion using DNase 1 beads

DNase 1 beads and Xenopus egg extracts were made as described in (Rosenblatt et al., 1995). Xenopus egg extracts were centrifuged at 228,000g in a TLA 100 rotor for 15 min at 4° C and the supernatant was removed to a fresh tube. 50 µl DNase 1 beads were washed with 1 ml high salt buffer (0.6 M NaCl, 20 mM Tris HCl (pH 8.0), 2 mM EGTA (pH 8.0), 2 mM $MgCl_2$, 1mM EDTA (pH 8.0), 1 mM ßME), and twice with 1 ml low salt buffer (25 mM NaCl, 20 mM Tris HCl (pH 8.0), 2 mM EGTA (pH 8.0), 2 mM $MgCl_2$, 1mM EDTA (pH 8.0), 1 mM ßME) and incubated with 200 µl Xenopus egg extracts supernatant for 60 min. at 4° C. The slurry was centrifuged at 14,000 rpm in an Eppendorf microfuge for 2 min. and the depleted supernatant was removed to a fresh tube. The pellets were washed 3 X with 1 ml with low salt buffer and eluted with 100 µl medium salt buffer (250 mM NaCl, 20 mM Tris HCl (pH 8.0), 2 mM EGTA (pH 8.0), 2 mM $MgCl_2$, 1mM EDTA (pH 8.0), 1 mM ßME) for 10 min. at 22° C, and consecutively with 100 µl high salt buffer for 10 min. at 22° C. The salt eluted pellets were SDS-eluted by boiling the pellets with 50 µl of SDS gel loading buffer for 5 min. The medium and high salt elutions were desalted by spinning each through a 1 ml Biogel P-4 column in low salt buffer. The Xenopus extract supernatant, DNase 1 supernatant, and desalted medium and high salt elutions were analyzed for protein content by running on a 12% SDS-PAGE gel followed by Coomassie Blue staining and for recycling activity by adding to the recycling assay described above.

Depletion of ATP in an attempt to inactivate actin was accomplished by treating 50 µl of platelet extracts or 0.3 mg/ml platelet actin twice with 20 µl Dowex beads in platelet buffer for 60 min. on ice, or by passing each over

1 ml Biogel P-4 spin columns in platelet buffer. Inactivation of actin by urea treatment was done by mixing 10 µl of 5-fold concentrated platelet extracts (using a Microcon-10 filter) or 10 µl 1.5 mg/ml platelet actin with 40 µl freshly made 10 M urea for 10 min at 22° C. The urea-treated extracts or actin were then passed over 1 ml Biogel P-4 spin columns in platelet buffer to remove urea and ATP was added to 5 mM.

References:

Agnew, B. J., Minamide, L. S., and Bamburg, J. R. (1995). Reactivation of phosphorylated actin depolymerizing factor and identification of the regulatory site. J Biol Chem *270*, 17582-7.

Arber, S., Barbayannis, F., Hanser, H., Schneider, C., Stanyon, C., Bernard, O., and Caroni, P. (1998). Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature *393*, 805-809.

Buss, F., Temm, G. C., Henning, S., and Jockusch, B. M. (1992). Distribution of profilin in fibroblasts correlates with the presence of highly dynamic actin filaments. Cell Motil Cytoskeleton *22*, 51-61.

Carlsson, L., Nystrom, L. E., Sundkvist, I., Markey, F., and Lindberg, U. (1977). Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells. J Mol Biol *115*, 465-83.

Goldschmidt, C. P., Furman, M. I., Wachsstock, D., Safer, D., Nachmias, V. T., and Pollard, T. D. (1992). The control of actin nucleotide exchange by thymosin beta 4 and profilin. A potential regulatory mechanism for actin polymerization in cells. Mol Biol Cell *3*, 1015-24.

Janmey, P. A. (1991). Polyproline affinity method for purification of platelet profilin and modification with pyrene-maleimide. Methods Enzymol *196*, 92-9.

Kellogg, D. R., Mitchison, T. J., and Alberts, B. M. (1988). Behaviour of microtubules and actin filaments in living Drosophila embryos. Development *103*, 675-86.

Kinosian, H. J., Selden, L. A., Estes, J. E., and Gershman, L. C. (1993). Nucleotide binding to actin. Cation dependence of nucleotide dissociation and exchange rates. J Biol Chem *268*, 8683-91.

Korn, E. D. (1982). Actin polymerization and its regulation by proteins from nonmuscle cells. Physiol Rev *62*, 672-737.

Moon, A., and Drubin, D. G. (1995). The ADF/cofilin proteins: stimulus-responsive modulators of actin dynamics. Mol Biol Cell *6*, 1423-31.

Morgan, T. E., Lockerbie, R. O., Minamide, L. S., Browning, M. D., and Bamburg, J. R. (1993). Isolation and characterization of a regulated form of actin depolymerizing factor. J Cell Biol *122*, 623-33.

Moriyama, K., Iida, K., and Yahara, I. (1996). Phosphorylation of Ser-3 at cofilin regulates its essential function on actin. Genes to Cells *1*, 73-86.

Ozaki, K., and Hatano, S. (1984). Mechanism of regulation of actin polymerization by Physarum profilin. J Cell Biol *98*, 1919-25.

Pantaloni, D., and Carlier, M. F. (1993). How profilin promotes actin filament assembly in the presence of thymosin beta 4. Cell *75*, 1007-14.

Pistor, S., Chakraborty, T., Walter, U., and Wehland, J. (1995). The bacterial actin nucleator protein ActA of Listeria monocytogenes contains multiple binding sites for host microfilament proteins. Curr Biol *5*, 517-25.

Pollard, T. D. (1995). Actin cytoskeleton. Missing link for intracellular bacterial motility? Curr Biol *5*, 837-40.

Pollard, T. D. (1986). Rate constants for the reactions of ATP- and ADP-actin with the ends of actin filaments. J Cell Biol, 2747-54.

Reinhard, M., Giehl, K., Abel, K., Haffner, C., Jarchau, T., Hoppe, V., Jockusch, B. M., and Walter, U. (1995). The proline-rich focal adhesion and microfilament protein VASP is a ligand for profilins. Embo J *14*, 1583-9.

Rosenblatt, J., Agnew, B. J., Abe, H., Bamburg, J. R., and Mitchison, T. J. (1997). Xenopus actin depolymerizing factor/cofilin (XAC) is responsible for

the turnover of actin filaments in Listeria monocytogenes tails [see comments]. Journal of Cell Biology *136*, 1323-32.

Rosenblatt, J., Peluso, P., and Mitchison, T. J. (1995). The Bulk of Unpolymerized Actin in *Xenopus* Egg Extracts Is ATP-bound. Mol. Biol. Cell *6*, 227-236.

Spudich, J. A., and Watt, S (1971). The regulation of rabbit skeletal muscle contraction: biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. Journal of Biological Chemistry *246*, 4866-4871.

Theriot, J. A. (1997). Accelerating on a treadmill: ADF/cofilin promotes rapid actin filament turnover in the dynamic cytoskeleton [comment]. J Cell Biol *136*, 1165-8.

Theriot, J. A., Rosenblatt, J., Portnoy, D. A., Goldschmidt, C. P., and Mitchison, T. J. (1994). Involvement of profilin in the actin-based motility of L. monocytogenes in cells and in cell-free extracts. Cell *76*, 505-17.

Tilney, L. G., Connelly, P.S., and Portnoy, D.A. (1990). Actin Filament Nucleation by the Bacterial pathogen, Listeria monocytogenes. J. Cell Biology *111*, 2979-2988.

Tilney, L. G., Bonder, E. M., Coluccio, L. M., and Mooseker, M. S. (1983). Actin from Thyone sperm assembles on only one end of an actin filament: a behavior regulated by profilin. J Cell Biol *97*, 112-24.

Tilney, L. G., DeRosier, D. J., Weber, A., and Tilney, M. S. (1992). How Listeria exploits host cell actin to form its own cytoskeleton. II. Nucleation, actin filament polarity, filament assembly, and evidence for a pointed end capper. J Cell Biol *118*, 83-93.

Tilney, L. G., and Portnoy, D. A. (1989). Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, listeria monocytogenes. J. Cell Biol. *109*, 1597-1608.

Welch, M. D., Iwamatsu, A., and Mitchison, T. J. (1997). Actin polymerization is induced by Arp2/3 protein complex at the surface of Listeria monocytogenes. Nature *385*, 265-9.

Welch, M. D., Rosenblatt, J., Skoble, J., Portnoy, D. A., and Mitchison, T. J. (1998). The human Arp2/3 complex is composed of evolutionarily conserved subunits and is localized to cellular regions of dynamic actin filament assembly. Science *281*, 105-8.

Yang, N., Higuchi, O., Ohashi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E., and Mizuno, K. (1998). Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. Nature.*393*, 809-813.

Chapter Five

Conclusions

Actin dynamics are important for protrusion of the leading edge membrane as well as other cellular movements. The aim of my thesis was to determine how actin dynamics are regulated in the cell. From the work of this dissertation as well as other publications, we can now sketch out roughly what happens to an actin subunit throughout polymer dynamics in a cell. Actin polymerization at the back of *Listeria monocytogenes* and, presumably, within lamellipodia is nucleated by activation of the Arp 2/3 complex. Actin polymer reaches a discreet length dictated by ATP hydrolysis within the filament. Once production of ADP actin within the filament weakens actin subunit interactions, the ADF/cofilin proteins depolymerize the filament. After an actin subunit is depolymerized, it must be recycled for further rounds of polymerization. Recycling of actin subunits is likely to require removal of ADF/cofilin protein from the depolymerized subunit and exchange of actin-bound ADP for ATP. Possible candidates for subunit recycling include profilin and VASP and/or the LIM kinase protein.

Aside from determining how actin subunits are recycled, a number of unanswered questions remain: How do the signals that activate lamellipodial protrusion activate the Arp 2/3 complex and the ADF/cofilin proteins? How is the Arp 2/3 complex localized and activated to nucleate actin filaments at the right time and place? What population of actin monomers are used for polymerization, the thymosin ß4-sequestered actin or the recently depolymerized actin subunits? How are the ADF/cofilin family of proteins regulated to depolymerize actin filaments only within specific regions of the cell? Do the ADF/cofilin proteins depolymerize actin by end-wise removal of subunits or by severing pieces of filaments?

Appendix A

Involvement of profilin in the actin-based motility of *L. monocytogenes* in cells and in cell-free extracts.

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I contributed to this publication by purifying and labeling profilin and demonstrating that it localizes to the rear of *Listeria monocytogenes* in the prescence but not the absence of *Xenopus laevis* egg extracts. I also show that the localization of profilin is dependent on expression of the *Listeria monocytogenes* gene ActA.

Involvement of Profilin in the Actin-Based Motility of L. monocytogenes in Cells and in Cell-Free Extracts

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Summary

Within hours of Listeria monocytogenes infection, host cell actin filaments form a dense cloud around the intracytoplasmic bacteria and then rearrange to form a polarized comet tail that is associated with moving bacteria. We have devised a cell-free extract system capable of faithfully reconstituting L. monocytogenes motility, and we have used this system to demonstrate that profilin, a host actin monomer-binding protein, is necessary for bacterial actin-based motility. We find that extracts from which profilin has been depleted do not support comet tail formation or bacterial motility. In extracts and host cells, profilin is localized to the back half of the surface of motile L. monocytogenes, the site of actin filament assembly in the tail. This association is not observed with L. monocytogenes mutants that do not express the ActA protein, a bacterial gene product necessary for motility and virulence. Profilin also fails to bind L. monocytogenes grown outside of host cytoplasm, suggesting that at least one other host cell factor is required for this association.

Introduction

Listeria monocytogenes is a ubiquitous gram-positive bacterium that can cause serious food-borne infections in pregnant women, newborns, and immunocompromised adults (Gellin and Broome, 1989). It grows directly in the cytoplasm of infected host cells and moves rapidly throughout the infected cell at rates up to 1.4 μm/s using a remarkable form of actin-based motility (Tilney and Portnoy, 1989; Mounier et al., 1990; Dabiri et al., 1990). Within a few hours after infection, host cell actin filaments initially form a dense cloud around the intracytoplasmic bacteria and then rearrange to form a polarized comet tail, which is associated with all moving bacteria. The comet tail is made up of short (0.3 μm) actin filaments cross-linked into a meshwork in which the majority of filaments have their barbed (rapidly growing) ends oriented toward the bacterium (Tilney and Portnoy, 1989; Tilney et al., 1992). New actin filament polymerization occurs only at the front of the tail, adjacent to the surface of the bacterium; polymerization occurs at the same rate as bacterial propulsion (Theriot et al., 1992; Sanger et al., 1992).

Although the behavior of pure actin has been well-studied in vitro, the regulation of the dynamic behavior of actin inside intact cells is still poorly understood. In particular, the mechanisms by which actin filament polymerization is harnessed by cells to produce movement have proven difficult to elucidate (reviewed by Stossel, 1993; Theriot and Mitchison, 1992; Heath and Holifield, 1991; Smith 1988). The actin dynamics involved in the intracellular movement of L. monocytogenes bear some resemblance to actin dynamics in the lamellipodia of motile cells (Theriot et al., 1992; Theriot and Mitchison, 1992), suggesting that L. monocytogenes motility may provide a tractable model system for dissecting the molecular basis of actin-based cell motility.

Several host actin-binding proteins have been localized throughout the comet tails of moving intracellular L. monocytogenes, including α-actinin, tropomyosin (Dabiri et al., 1990), and fimbrin (C. Kocks, M. Arpin, and P. Cossart, personal communication). It is not yet known whether any of these proteins plays a functionally important role in bacterial propulsion. Since actin filament polymerization occurs only at the very front of the tail, immediately adjacent to the rear surface of the bacterium, any protein responsible for promoting filament polymerization in this system should be restricted in its activity or location to the bacterial surface and should not be active throughout the tail.

The L. monocytogenes surface protein ActA is necessary for the bacterium to direct the polymerization of host actin filaments; transposon insertion into or deletion of the *actA* gene prevents the infecting bacteria from promoting assembly of actin filament clouds or comet tails inside host cells, although the bacteria invade cells and multiply within the cytoplasm normally (Kocks et al., 1992; Domann et al., 1992). However, bacteria expressing ActA are not able to efficiently nucleate actin filament polymerization in vitro (Tilney et al., 1990; 1992). Thus, it seems plausible that one or more host cell cytoskeletal protein responsible for promoting actin filament nucleation and elongation during L. monocytogenes propulsion may associate with the intracellular bacteria by binding to ActA.

In this report, we describe a cell-free extract system capable of supporting L. monocytogenes motility that can be used to biochemically identify host cytoskeletal proteins necessary for comet tail formation and bacterial movement. Furthermore, we show that the actin monomer–binding protein profilin is localized to the surface of moving intracellular L. monocytogenes, that this localization is dependent on the presence of the ActA protein, and that profilin is functionally required for L. monocytogenes movement in the extract system.

Results

L. Monocytogenes Motility Can Be Reconstituted in Xenopus Egg Cytoplasmic Extracts

Concentrated cytoplasmic extracts of Xenopus laevis eggs (Murray et al., 1989) have proven useful for the reconstitution of various complex cytoskeletal processes, including mitotic spindle assembly (Sawin and Mitchison, 1991) and cell cycle–dependent regulation of microtubule dynamics (Belmont et al., 1990). These extracts are typically >60 mg/ml in protein concentration and are rich in the cytoskeletal components necessary for early embryonic development.

Meiotic (cytostatic factor) extracts were prepared as described (Murray et al., 1989), except that cytochalasin D was omitted. These extracts showed a tendency to gel and contract after several hours at room temperature, but they could be kept on ice for at least 8 hr without noticeable actomyosin aggregation or contraction. Temperature-dependent actomyosin gelation and contraction have

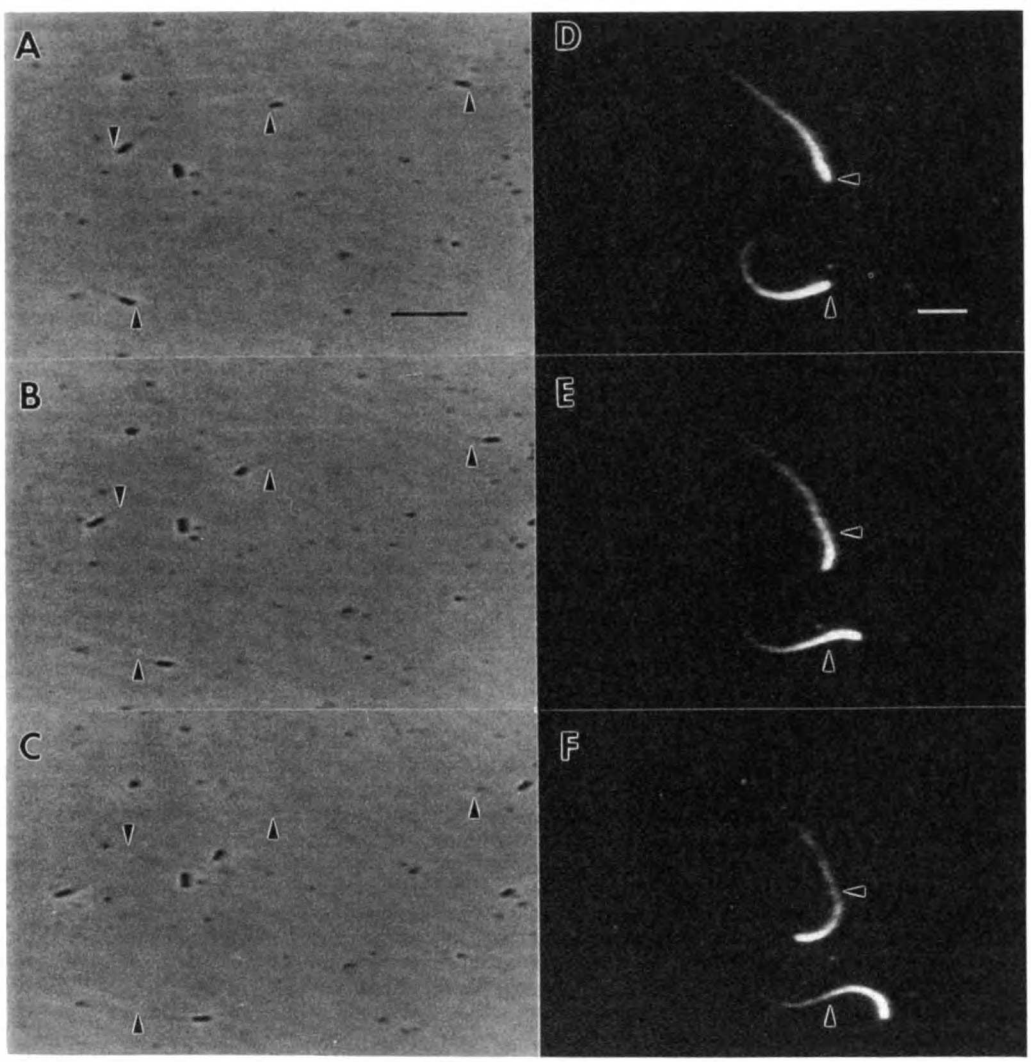

Figure 1. Movement of Living L. Monocytogenes (Strain SLCC-5764) in Xenopus Egg Extracts

(A–C) Movement observed by phase-contrast microscopy. The same field is shown at 30 s intervals. Arrowheads mark fixed positions in the field. Bar is 10 μm.

(D–F) Fluorescent signal arising from trace TMR–actin mixed in extracts with L. monocytogenes. Actin-rich comet tails are clearly visible. The same field is shown at 1 min intervals. Arrowheads mark fixed positions in the field. Bar is 10 μm.

been previously observed in concentrated cytoplasmic extracts of other cells, including sea urchin eggs (Kane, 1980), Acanthamoeba (Pollard, 1976), and Dictyostelium (Condeelis and Taylor, 1977). L. monocytogenes strain SLCC-5764 added to a Xenopus egg extract and incubated at room temperature started moving within 20 min, and typically 70–80% of the bacteria in an extract were moving within 60–90 min. Strain SLCC-5764 differs from some other wild-type isolates of L. monocytogenes in that it expresses unusally high levels of virulence factors after in vitro growth (Leimeister-Wächter and Chakraborty, 1989). Strain 10403S, which does not exhibit such high levels of virulence gene expression outside of host cytoplasm, did not reproducibly move in extracts. Movement of L. monocytogenes strain SLCC-5764 usually continued for at least 6 hr, and the bacteria continued to grow and divide throughout the incubation period. The bacteria tended to move in curved trajectories reminiscent of their movement inside infected host cells (Figures 1A–1C). When the bacteria divided, the two daughters always moved in opposite directions with the newly formed ends leading; this behavior is also invariably observed inside infected cells and probably reflects inherent polarity of the bacteria (Kocks et al., 1993). The average rate of movement of L. monocytogenes in the extracts varied somewhat among different extract preparations. Generally, it was comparable to but slower than the rate of movement inside infected tissue culture cells (6.3 μm/min [SD = 2.0, n = 58] in extracts versus 11.4 μm/min [SD = 4.8, n = 41] in PtK2 potoroo kidney epithelial cells). An average of 73% of the bacteria in an extract were moving (Table I). This frequency is almost identical to the average of 77% moving bacteria observed in PtK2 cells (J. A. T. and D. A. P., unpublished data). The slower movement rate in Xenopus egg extracts compared with that in tissue culture cells may have been partially due to the temperature difference in the two systems; PtK2 cells were kept at 37°C, whereas extracts were kept at room temperature (20–22°C). The slower rate may also have been a consequence of nonoptimal concentrations of cytoskeletal factors in the Xenopus egg extracts. L. monocytogenes in infected tissue culture cells move at a wide range of rates depending on the cell line (Dabiri et al., 1990).

Extracts diluted 2-fold or more with Xenopus extract buffer (XB) did not support motility of L. monocytogenes (data not shown). Initial attempts to reconstitute L. monocytogenes motility in cytoplasmic extracts made from a variety of mammalian tissue culture cells did not succeed, perhaps because the extracts made by homogenizing the cells in several volumes of buffer were too dilute in cytoskeletal factors.

To show that this movement involved actin, tetramethylrhodamine-labeled (TMR-labeled) actin was added as a fluorescent tracer. Fluorescent clouds of actin were associated with L. monocytogenes in the extract within 20 min, and moving bacteria invariably displayed fluorescent actin-rich tails (Figures 1D–1F). The fluorescence intensity (reflecting the filament density) of actin in the tails decreased exponentially with increasing distance from the bacteria (Figure 2), similar to the distribution of actin filament density previously seen in the tails of L. monocytogenes moving inside infected tissue culture cells (Theriot et al., 1992). Average filament half-life could be indirectly measured by calculating the rate of decrease of fluorescence intensity over time in a region of the tail fixed in space. The average half-life of actin filaments in the tails of bacteria moving in extracts measured by this method was 42 s (SD = 8, n = 11), similar to the half-life of 33 s (SD = 16, n = 22) measured in infected cells using fluorescence photoactivation (Theriot et al., 1992). By the criteria of tail morphology, movement rate, actin filament density distribution in the tails, and actin filament half-life, the actin-based L. monocytogenes motility in Xenopus egg extracts appears to be a faithful reconstitution of motility in infected cells.

Remarkably, L. monocytogenes did not need to be alive to move in the extracts. Bacteria killed with iodoacetic acid (10 mM for 10 min in XB) or formaldehyde (3.2% for 10 min in XB) could form actin filament clouds and tails and move normally, although they could not grow and divide in the extracts (data not shown). This indicates that the bacterially produced proteins necessary for interaction with the host cytoskeleton and motility must be stably present on the surface of the bacteria, rather than continuously secreted or renewed. Furthermore, these bacterial protein factors are not inactivated by treatment with iodoacetic

Table 1. Rate and Frequency of Movement of L. Monocytogenes in Xenopus Egg Extract

Condition	Rate[a]	Number Measured	Moving (%)
Untreated	5.76 (0.82)	12	73
Mock-depleted extract (Bovine serum albumin–sepharose)	5.37 (0.88)	10	74
Depleted extract (Poly-L-proline–sepharose)	0.00 (0.00)[b]	49	0
Depleted extract plus 1.5 mg/ml actin	0.00 (0.00)[b]	23	0
Depleted extract plus 1.5 mg/ml actin plus 0.3 mg/ml profilin	1.02 (0.18)	20	71

[a]The rate is shown in μm/min. Standard deviation is shown in parentheses.
[b]No directed movement was observed.

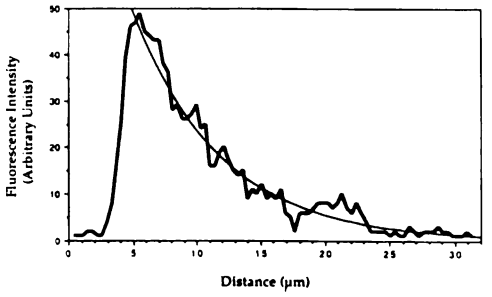

Figure 2. Fluorescence Intensity Profile through the Tail of an L. Monocytogenes Moving in a TMR-Actin-Doped Xenopus Egg Extract
The thin line shows an exponential decay curve fitted to the intensity profile.

acid or formaldehyde and therefore probably do not contain any sulfhydryl or primary amine groups necessary for their function. The bacterial protein ActA has these features: the sequence contains no cysteines (Kocks et al., 1992; Domann et al., 1992), the protein is not secreted nor found in the tail, but is found only on the surface of the bacteria (Niebuhr et al., 1993; Kocks et al., 1993), and the protein is stable for hours in the cytoplasm (D. A. P., unpublished data).

Host Profilin Is Localized to the Surface of Motile Intracellular L. Monocytogenes

A host protein that promotes actin filament polymerization at the front of the comet tail during bacterial propulsion must have its function or position restricted to the surface of the motile bacteria. Such a host factor might be localized by directly binding to the ActA bacterial surface protein. Examination of the predicted ActA amino acid sequence reveals a striking motif of 11 amino acids repeated four times with slight variations on the sequence DFPPPPTDEEL (Kocks et al., 1992; Domann et al., 1992). We conjectured that this motif, found in the middle of the predicted large external domain of the ActA protein, might represent a binding site for a host factor involved in actin filament polymerization. The actin monomer-binding protein profilin is known to bind strongly and specifically to poly-L-proline (Tanaka and Shibata, 1985). Four prolines by themselves are probably not sufficient to bind profilin, since profilin will not bind in solution to a proline oligomer 6 residues long, though it will bind an oligomer with 10 residues (L. Machesky, personal communication).

In HeLa human cervical carcinoma tissue culture cells infected with L. monocytogenes strain 10403S, profilin could be seen to specifically associate with the surface of motile intracellular bacteria by indirect immunofluorescence (Figures 3A–3C). At high magnifications, it was apparent that the distribution of profilin over the bacterial surface is not uniform, but is more concentrated on the rear half of the bacterium where the actin comet tail originates. On dividing bateria, profilin shows a bilobed distribution in which the signal is stronger at both ends than in the middle (Figures 3D–3F). Association of profilin with the bacteria was dependent on the presence of the ActA protein. *actA*$^-$ bacteria infected and grew inside of host cells normally, but did not associate with profilin or with actin at all (Figures 3G–3I). The ActA protein itself appears also to be asymmetrically distributed on the surface of infecting L. monocytogenes, again with a higher apparent protein density on the rear halves of moving bacteria and at the two ends of dividing bacteria (Kocks et al., 1993). Similar profilin distributions were seen in infected BSC1 African green monkey kidney cells (data not shown).

Profilin localization to the surface of infecting bacteria was readily evident within 3 hr after the initiation of infection in HeLa cells, at about the same time comet tail formation and movement begin. However, profilin could not always be seen on the surfaces of intracellular bacteria at earlier times, even when rudimentary actin clouds were present (data not shown). Profilin was associated with L. monocytogenes when the infected cells had been incubated in 0.2–0.6 μg/ml cytochalasin D for 4 hr (Figure 4). Under these conditions, actin tails were not observed, but some clouds of actin filaments could still be seen associating with the bacteria by labeling with fluorescent phalloidin.

Profilin was also observed to bind to moving intracellular bacteria when fluorescently labeled profilin was microinjected into live infected cells. Calf thymus profilin was covalently labeled with TMR-N-hydroxy-succinimide ester. TMR-profilin at a concentration of 0.12 mg/ml was injected into PtK2 potoroo kidney epithelial cells infected with L. monocytogenes. The injected profilin clearly associated with moving intracellular bacteria, and the bound TMR-profilin appeared to move with the bacteria (Figure 5). This is in contrast with microinjected labeled α-actinin, which is incorporated throughout the entire tail (Dabiri et al., 1990).

To examine whether profilin concentration inside an infected host cell had any effect on L. monocytogenes motility, we compared the rates of bacterial movement before and after microinjection of 3 mg/ml purified calf thymus profilin into infected PtK2 potoroo kidney epithelial cells. The bacteria moved more rapidly immediately after microinjection. For example, in one infected cell, the bacteria in the cytoplasm were moving at an average rate of 9.5 μm/min (SD = 3.7, n = 11) 1 min prior to microinjection of profilin and sped up to an average rate of 12.8 μm/min (SD = 2.9, n = 11) at 3 min after injection. This difference was statistically significant (unpaired t test, $p = 0.03$). However, profilin microinjection under similar conditions has been shown to disrupt stress fibers and other filamentous actin structures in tissue culture cells (Cao et al., 1992). Thus, it is possible that the increase in the rate of L. monocytogenes motility after profilin microinjection was simply a result of a decrease in the viscous drag on the moving bacteria due to general solation of the cytoplasm.

Profilin Is Required for L. Monocytogenes Motility In Extracts

To determine whether profilin is functionally required for L. monocytogenes motility, we examined whether L. monocytogenes could move in Xenopus egg extracts from

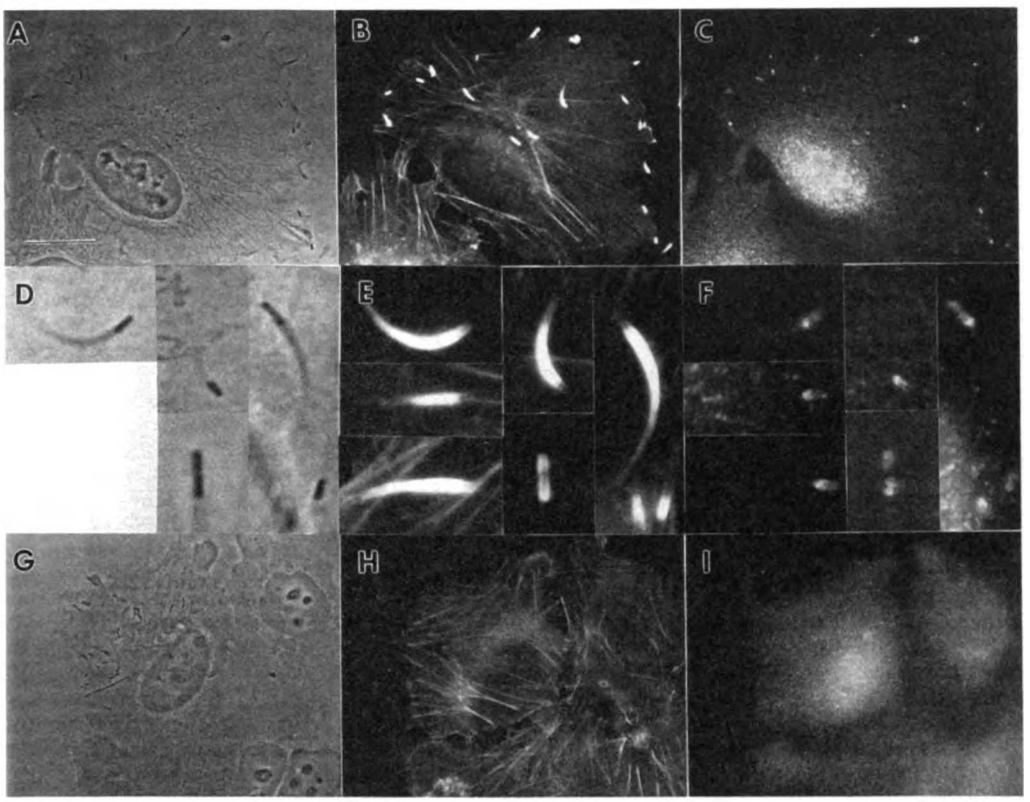

Figure 3. Immunolocalization of Profilin to the Surface of L. Monocytogenes in Infected HeLa Cells

(A) Phase-contrast image of wild-type (strain 10403S) L. monocytogenes in an infected cell. (B) Distribution of filamentous actin in the same cell (labeled with fluorescein–phalloidin). (C) Distribution of profilin in the same cell (by indirect immunofluorescence). (D–F) Collage of higher-magnification images of wild-type bacteria, seen with phase-contrast (D), fluorescein–phalloidin (E), or indirect immunofluorescence of profilin (F). Note the asymmetric distribution of profilin on the bacterial surface. Dividing bacteria exhibit a bilobed profilin distribution, in which profilin is depleted from the septation zone. Phase-contrast images were not available for two of the bacteria shown in this collage. (G) Phase-contrast, (H) fluorescein–phalloidin, and (I) profilin indirect immunofluorescence of *actA*$^-$ (strain DP-L1942) L. monocytogenes in an infected cell. *actA*$^-$ L. monocytogenes do not spread throughout the cell as efficiently as wild-type and do not associate with actin or profilin. Bar in (A) is 20 μm.

which the profilin had been depleted. Polyproline–sepharose beads (or as a control, bovine serum albumin–sepharose beads) were added to extracts and mixed at 4°C for 4–8 hr. Profilin and the profilin–actin complex were expected to bind to the polyproline beads. Examination of extract proteins by polyacrylamide gel electrophoresis before and after polyproline depletion revealed that a protein of approximately 12–14 kd became bound to the beads and was removed from the depleted extracts (Figure 6A). After the beads were removed by centrifugation, depleted extracts did not support L. monocytogenes motility. Mock-depleted extracts, which had been incubated in parallel with an equal volume of bovine serum albumin–sepharose beads, produced normal motility (Table I). Bacteria in profilin-depeleted extracts were not entirely unable to promote actin filament polymerization. Actin clouds of various shapes were often seen associated with L. monocytogenes in depleted extracts, although normal comet tails were never observed (data not shown).

Since some actin had been removed by the incubation with polyproline–sepharose beads, it seemed plausible that the reason the extracts could not support motility might be that the actin concentration was no longer high enough. Addition of purified rabbit skeletal muscle actin up to a final concentration of approximately 1.5 mg/ml did not restore motility in the profilin-depeleted extracts. However, addition of 1.5 mg/ml actin plus 0.3 mg/ml purified calf thymus profilin did partially restore movement in the depleted extract, and the bacteria formed comet tails and moved normally at reduced rates (Table 1). The calf thymus profilin preparation used in these experiments was >95% pure (Figure 6B), but we cannot rule out the possibility that a trace contaminant in the preparation contributed to the restoration of motility. Addition of 0.3 mg/ml profilin

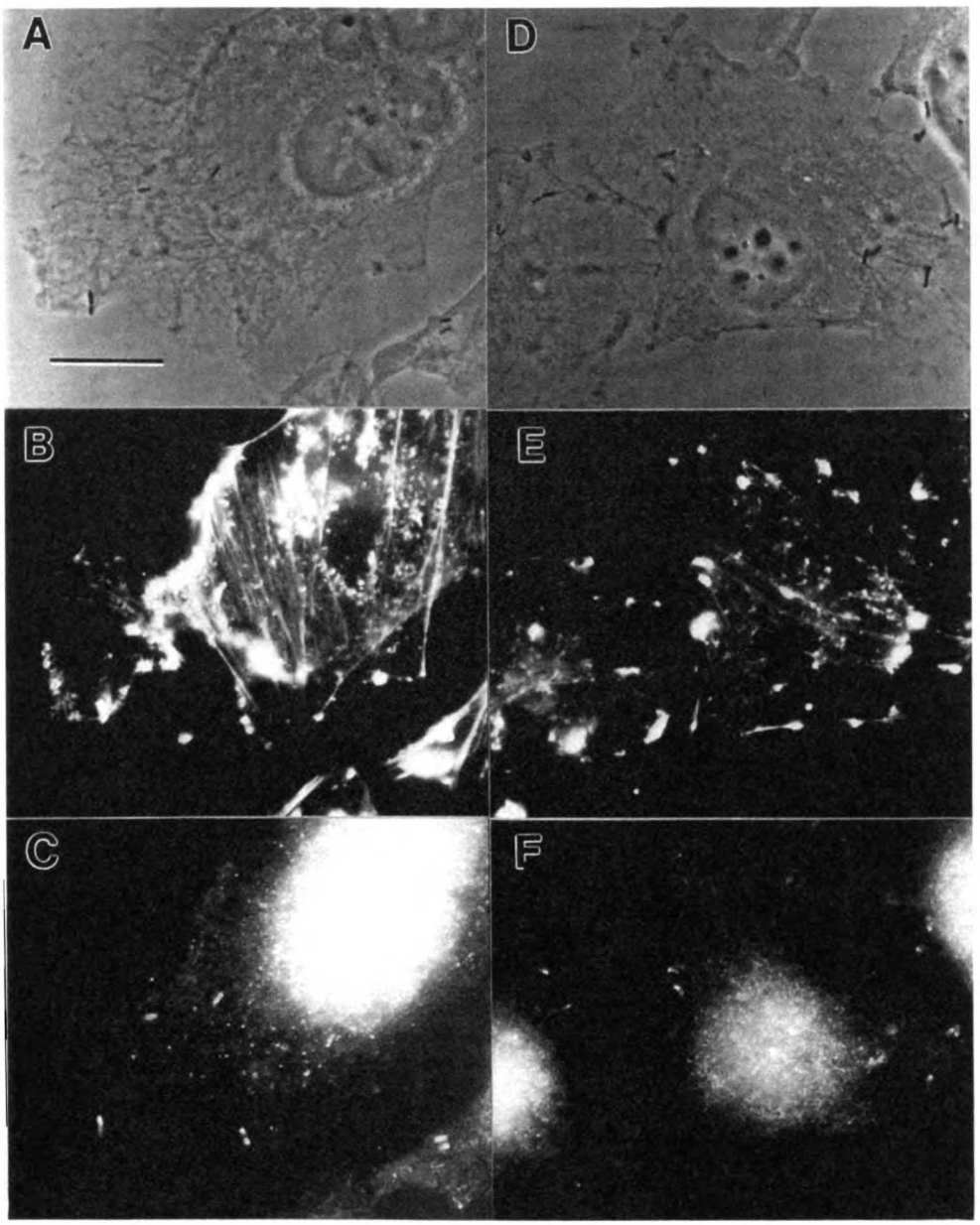

Figure 4. Immunolocalization of Profilin to the Surface of L. Monocytogenes (Strain 10403S) in Infected HeLa Cells Treated with Cytochalasin

(A and D) Phase-contrast image of L. monocytogenes in infected cells.

(B and E) Distribution of filamentous actin in the same cells (labeled with fluorescein–phalloidin).

(C and F) Distribution of profilin in the same cells (by indirect immunofluorescence). Infected cells had been treated with 0.2 μg/ml (A–C) or 0.6 μg/ml (D–F) cytochalasin D to disrupt the actin cytoskeleton for 4 hr prior to fixation.

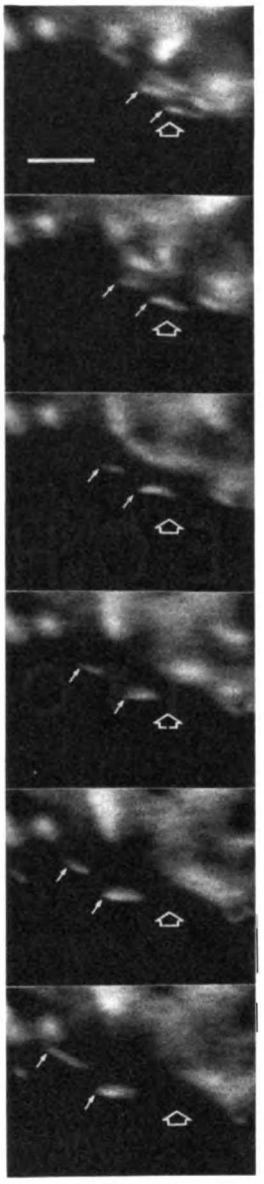

Figure 5. Association of TMR–Profilin with Moving L. Monocytogenes (Strain 10403S) in an Infected PtK2 Cell

The same field is shown at 30 s intervals. The open arrow marks a fixed position in the field. Two bacteria (indicated by small white arrows) just above the arrow in the first frame are moving to the left at different rates. Bar is 10 μm.

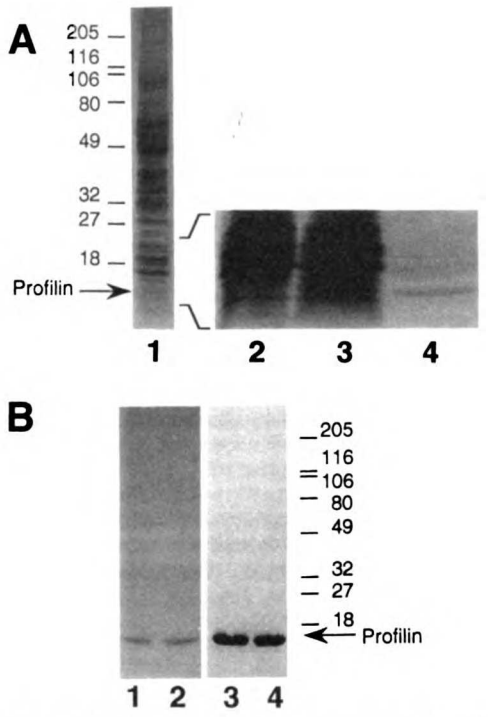

Figure 6. Removal of Profilin from Extracts with Polyproline–Sepharose Beads

(A) Depletion of profilin from Xenopus egg extracts: lane 1, 30 μg total extract protein; lane 2, extract supernatant after incubation with polyproline–sepharose beads; lane 3, mixture of extract and beads before depletion; and lane 4, protein remaining bound to beads, 4–20% gradient polyacrylamide gel stained with Coomassie brilliant blue. Lanes 2–4 are 200% enlargements of the region indicated in lane 1 and represent 90 μg of total extract protein. Similar results were obtained with two different extract preparations.

(B) Calf thymus profilin preparations used in these experiments: lane 1, 1.9 μg of profilin preparation used for addback experiments (Table 1); lane 2, 2 μg of profilin preparation used for TMR labeling (see Figures 5 and 7), 4–20% gradient polyacrylamide gel stained with Coomassie brilliant blue; and lanes 3 and 4, Western blot of lanes 1 and 2 using affinity-purified anti-human profilin rabbit polyclonal antibody. Positions of molecular weight markers are indicated in each panel.

alone restored motility in one experiment, but was generally not sufficient. Addition of both actin and profilin succeeded in restoring motility in five separate experiments on three different extract preparations. Addition of <0.2 mg/ml profilin never restored motility, and addition of >0.8 mg/ml profilin usually resulted in complete disappearance of all filamentous actin in the extracts. Addition of 1.5 mg/ml actin, 0.3 mg/ml profilin, or both to mock-depleted or undepleted extracts had no reproducible effects on L. monocytogenes motility. Thus, profilin is functionally required for L. monocytogenes motility in the Xenopus egg extracts, and purified calf thymus profilin can at least partially substitute for Xenopus profilin in this system. We do not yet know whether the reduced rate of motility after addition of purified calf thymus profilin to depleted extracts is due to differences between calf thymus and Xenopus egg profilins, differences between rabbit skeletal muscle and Xenopus egg actins, or nonoptimal concentrations of these two proteins in the reconstituted system. It is also

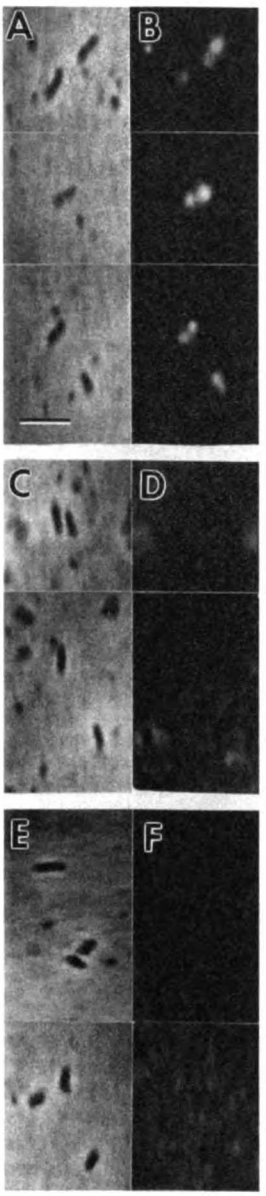

Figure 7. Association of TMR–Profilin with L. Monocytogenes in a High-Speed Supernatant of a Xenopus Egg Extract

(A and B) Phase-contrast and fluorescence images of wild-type (strain SLCC-5764) L. monocytogenes mixed with TMR–profilin in Xenopus egg extract high-speed supernatant. Note asymmetric or polar distribution of profilin. Bacterium in center panel and upper bacterium in bottom panel are dividing and show characteristic depletion of TMR–profilin from the septation zone. The high-speed supernatant does not support actin binding, tail formation, or motility. Bar is 5 μm.

(C and D) Phase and fluorescence images of *prfA*⁻ (strain DP-L1376) L. monocytogenes mixed with TMR–profilin in Xenopus egg extract high-speed supernatant.

(E and F) Phase and fluorescence images of wild-type (strain SLCC-5764) L. monocytogenes mixed with TMR–profilin in the absence of extract.

likely that at least part of the reduction in rate is due to polyproline-specific depletion of other unidentified host factors, perhaps proteins containing Src homology 3 (SH3) domains, which are known to bind to proline-rich sequences (Ren et al., 1993).

Profilin Binds to L. Monocytogenes Only in Infected Cells or in Extracts

Since calf thymus profilin could functionally substitute for Xenopus profilin in the extracts, we wished to test whether we could develop a simple visual assay for the interaction between calf thymus profilin and the bacterial surface in the extracts. TMR–profilin added to Xenopus egg extracts containing L. monocytogenes became bound to the bacteria. This association was easily observed in Xenopus egg extracts diluted 3-fold with XB and in high-speed supernatants (after centrifugation at 100,000 × g for 1 hr) of extracts (Figure 7), although diluted extracts and high-speed supernatants could not support motility or actin filament cloud or tail formation by the bacteria. The distribution of TMR–profilin on the surface of the bacteria often reflected the polarized or bilobed distribution seen by immunofluorescence in infected cells (Figures 7A and 7B). TMR–profilin did not associate with isogenic bacteria carrying a transposon insertion in the *prfA* gene, the gene coding for the positive transcriptional regulator necessary for expression of the ActA protein and other virulence factors (Figures 7C and 7D). TMR–profilin also failed to associate with L. monocytogenes in the absence of Xenopus egg extract (Figures 7E and 7F). Thus, the association between TMR–profilin and L. monocytogenes requires the presence of ActA and also of another as yet uncharacterized factor or factors supplied by the host cell and by the Xenopus egg extract.

Discussion

Reconstitution of Actin-Based Motility in a Cell-Free System

Genetic and reverse-genetic approaches to dissecting the control of cell motility have begun to provide useful insight into the role of particular actin-binding proteins in whole-cell motility (see DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Cox et al., 1992). However, biochemical dissection of the mechanisms of lamellipodial protrusion is particularly difficult because of the intimate role of the plasma membrane in localizing actin filament nucleation (Symons and Mitchison, 1991; Shariff and Luna, 1992) and perhaps also in generating force (Oster and Perelson, 1987). The motility of intracellular L. monocytogenes appears to provide a simplified model system that mimics the actin filament dynamics involved in lamellipodial protrusion in the absence of the plasma membrane and host cell signal transduction.

Several types of actin-based motility involving myosin motors have been successfully reconstituted in vitro, including cytoplasmic gel contraction (Pollard, 1976; Condeelis and Taylor, 1977; Kane, 1980) and gliding of actin filaments over myosin (Kron and Spudich, 1986). However, forms of actin-based motility requiring concomitant

actin polymerization (such as lamellipodial protrusion and L. monocytogenes propulsion) have proven less tractable. In this report, we have shown faithful reconstitution of actin-based L. monocytogenes motility and associated actin dynamics in a cytoplasmic extract. To our knowledge, this is the first report of the reconstitution of any type of polymerization-associated actin-based motility in a cell-free extract system. This system is now accessible to traditional biochemical techniques for identifying host cell proteins that are functionally required for motility.

Association of Profilin with L. Monocytogenes Motility

Athough several cytoskeletal proteins, including α-actinin, tropomyosin (Dabiri et al., 1990), and fimbrin (C. Kocks, M. Arpin, and P. Cossart, personal communication), but interestingly enough not myosin (Dabiri et al., 1990), have been localized to the comet tail of moving intracellular L. monocytogenes, no functional role for any of them has yet been established. Examinations of actin filament dynamics in the tail have revealed that new filaments polymerize only at the front of the tail, immediately adjacent to the back half of the bacterium, and depolymerize uniformly throughout the tail (Theriot et al., 1992; Sanger et al., 1992). Thus, host cytoskeletal proteins responsible for promoting actin filament nucleation and elongation should be localized only to the front of the tail, perhaps binding to the surface of the bacterium.

By indirect immunofluoresence, the localization of profilin on intracytoplasmic L. monocytogenes is consistent with its having a role in promoting actin filament growth associated with motility. Furthermore, extracts from which profilin has been depleted are not capable of supporting L. monocytogenes movement, and readdition of purified profilin and actin to depleted extracts partially restores movement. Thus, profilin must be functionally required for motility. The concentration of purified profilin added back to restore motility in depleted extracts was critical; too little profilin had no effect and too much profilin caused the disappearance of all filamentous actin in the extracts. The sensitivity of actin-based motility to the exact concentration of some actin-associated proteins has also been observed in intact cells; the migratory ability of human malignant melanoma cells transfected with the actin-binding protein filamin is dependent on the filamin concentration; cells with too much filamin move as poorly as cells with too little filamin (Cunningham et al., 1992).

Profilin does not appear to be absolutely required for the association of actin filaments with infecting bacteria. Profilin is not reproducibly observed on bacteria after less than 2 hr of infection, before motility has begun but after actin clouds are associated with intracytoplasmic bacteria. Likewise, L. monocytogenes in profilin-depleted extracts do often exhibit actin clouds, but these clouds do not rearrange into comet tails and they are not associated with movement (though it is possible that trace amounts of profilin remain in the depleted extracts). Thus, infecting bacteria appear to be able to nucleate or trap actin filaments in the absence of associated profilin, but profilin is necessary for the rapid actin dynamics involved in motility. Since L. monocytogenes grown outside of host cells do not efficiently nucleate actin polymerization (Tilney et al., 1990; 1992), and nucleation can occur without profilin, a second host protein must be responsible for nucleation. This unidentified factor might be a cytoskeletal nucleating protein that binds to the surface of the bacterium, or it might be an enzyme that processes a bacterial protein or protein complex to enable the bacterial proteins to directly nucleate actin filament polymerization.

Only one bacterial surface protein necessary for L. monocytogenes association with actin, the product of the *actA* gene, has been identified genetically (Kocks et al., 1992; Domann et al., 1992). Disruption of this gene results in bacteria that can infect host cells and grow normally, but ones that are markedly reduced in virulence because of inefficient intra- and intercellular spread. The ActA protein is not uniformly distributed on the surface of the bacteria, but is concentrated at the back end near the tail or at both poles of dividing bacteria (Kocks et al., 1993). This distribution is strikingly similar to the distribution of profilin on the surface of bacteria in infected cells and in extracts (Figure 3F, inset; Figure 7B). Since the ActA protein is required for localization of profilin to the surface of intracellular L. monocytogenes, contains four proline-rich repeats, and is polarized in the same way as profilin, it is tempting to speculate that profilin binds directly to ActA. However, we have as yet no direct evidence that this is the case. Indeed, since TMR–profilin will not associate with bacteria expressing ActA when grown in broth, but will associate with bacteria in extracts or inside infected cells, another host factor (or factors) must be necessary for this association. This unidentified factor might bind with profilin in a complex, stabilizing the association, or it might process ActA to allow profilin binding. It has recently been shown that the ActA protein is phosphorylated by the host cell after infection (Brundage et al., 1993). It is possible that this phosphorylation is necessary for ActA to bind profilin, to bind the unidentified host nucleating factor, or both.

Possible Role of Profilin in L. Monocytogenes Motility

The interaction between profilin and actin is complex and may yet be only partially understood. Profilin was originally identified as an actin monomer–binding protein that inhibits actin filament nucleation in vitro (Carlsson et al., 1977). Profilin strongly inhibits elongation of actin filaments at the pointed (slowly growing) end, but does not substantially inhibit elongation at the barbed end (Tilney et al., 1983). In fact, profilin–actin complexes bind directly to the barbed end of preexisting filaments, and then the profilin is released, leaving the actin monomer behind (Pollard and Cooper, 1984; Pring et al., 1992). It has recently been shown that elongation of actin filaments by the addition of the profilin–actin complex is thermodynamically more favorable than elongation through the addition of monomer alone. Because of this, profilin can lower the critical concentration for ATP–actin elongation at the barbed end. This effect is most pronounced in the presence of the ubiq-

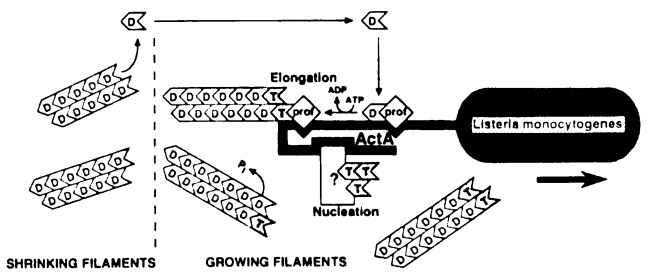

Figure 8. Model for Role of Profilin in Actin Filament Elongation at the Surface of Moving Intracellular L. Monocytogenes

Actin monomers (chevrons) bind to profilin associated with the bacterial surface, probably through the ActA protein. Profilin (diamonds) promotes the exchange of ATP for ADP on actin monomers. The profilin–actin complexes can then rapidly elongate nearby filaments promoting growth only at the barbed ends of the filament. ATP hydrolysis is associated with polymerization. A second host factor (?, as yet unknown) may also associate with ActA and promote filament nucleation. As the bacterium is pushed away, older filaments are no longer in a profilin-rich environment and cease elongation. These filaments then depolymerize to regenerate the actin monomer pool of the host cell.

uitous actin monomer–binding and –sequestering protein, thymosin β4 (Pantaloni and Carlier, 1993).

Profilin also acts as a nucleotide exchange factor for actin. When bound, profilin increases the rate constant for dissociation of adenine nucleotide from actin by three orders of magnitude (Goldschmidt-Clermont et al., 1991). Relatively low concentrations of profilin can catalytically effect nucleotide exchange on actin monomers, even in the presence of thymosin β4 (Goldschmidt-Clermont et al., 1992). Thus, spatial variations in profilin concentration could control local ratios of ATP–actin to ADP–actin. Since ATP–actin polymerizes somewhat more rapidly and with a substantially lower critical concentration than does ADP–actin, the nucleotide exchange activity of profilin might substantially promote filament elongation in a local region of a cell.

The action of profilin in promoting filament formation at the L. monocytogenes surface might involve its ability to lower the critical concentration for elongation at the barbed end of existing filaments, its nucleotide exchange function, or both (Figure 8). If there is an independent factor promoting filament nucleation at the surface of L. monocytogenes, profilin–actin complex loosely bound (perhaps) to ActA will provide a concentrated local monomer pool to rapidly elongate the filaments. If there is a pool of ADP–actin monomers present in the cell, this elongation will proceed even more efficiently owing to nucleotide exchange. Acceleration of filament elongation by profilin could contribute to increasing the force for L. monocytogenes propulsion by a Brownian ratchet mechanism (Peskin et al., 1993).

If profilin is indeed responsible for rapid elongation at the surface of the bacterium, we would predict that after the bacterium moves away from newly polymerized filaments, those filaments that are now no longer in an environment promoting elongation would stop growing and then depolymerize. Consistent with this idea, it has been observed that the average rate of filament loss in the tail away from the bacterial surface is uniform and rapid (Theriot et al., 1992). However, the actin filaments in the tail appear to be about the same length regardless of distance from the bacterium (Tilney and Portnoy, 1989). If all the filaments were shrinking at the same rate, it would be expected that the filaments further away from the bacterial surface would be shorter than those nearby. It is possible that filament depolymerization in the tail is a random and catastrophic event, such that filaments transit from a growing or stationary state to a shrinking state in a stochastic fashion, and once they begin shrinking they disappear very rapidly. This might imply that actin filaments in cells undergo a type of dynamic instability (Theriot and Mitchison, 1992).

These observations are consistent with a growing body of evidence that profilin, although it is an actin monomer–binding protein, acts inside cells to promote actin filament growth in at least some dynamic actin structures. When profilin is deleted in yeast (Saccharomyces cerevisiae), actin cables fail to form and the cells are unable to divide normally (Haarer et al., 1990). In Drosophila, elimination of an ovary-specific transcript of profilin (a product of the *chickadee* gene) causes female sterility because a cytoplasmic actin network critical for proper oocyte development fails to form in the egg chambers of mutant flies (Cooley et al., 1992). Conversely, stable overexpression of profilin in CHO cells stabilizes actin bundles to a degree directly proportional to the amount of overexpression (Finkel et al., 1994). Here we have shown that profilin is necessary for L. monocytogenes motility and appears to be involved in promoting rapid filament elongation at the surface of the bacteria. It is interesting to note in this context that profilin is also concentrated throughout the lamellipodia of motile fibroblasts (Buß et al., 1992) and in the actin-rich cleavage furrow of dividing Tetrahymena (Edamatsu et al., 1992).

The faithful reconstitution of actin-based L. monocytogenes motility in a cell-free extract system has facilitated a direct biochemical approach to identify the roles of cytoskeletal proteins in bacterial propulsion. We have established a functional requirement for profilin in this motility, and we intend to use this system to further probe its mechanism of action. The biochemical dissection of the molecular mechanisms of L. monocytogenes motility may shed light on the study of lamellipodial protrusion in motile cells as well.

Experimental Procedures

Preparation of Extracts

Meiotically arrested cytoplasmic extracts (cytostatic factor) of Xenopus laevis eggs were prepared as described (Murray et al., 1989) with the modifications that the spin through Versilube oil was omitted and cytochalasin D was omitted at all steps. Sucrose was added to 150 mM and the extracts were used immediately or frozen in liquid nitrogen and stored at −80°C.

Bacterial Strains and Culture Conditions

L. monocytogenes strain 10403S and derivatives thereof were used for all experiments involving infection of tissue culture cells. Strain DP-L1942 is a derivative of 10403S from which the *actA* gene has been deleted (Brundage et al., 1993). Bacteria were grown to stationary phase overnight in brain–heart infusion broth (BHI; Difco) at room temperature without aeration and were washed once in phosphate-buffered saline before being added to cell cultures.

L. monocytogenes strain SLCC-5764 (Leimeister-Wächter and Chakraborty, 1989) and derivatives thereof were used for all experiments in Xenopus egg extracts. This isolate expresses unusually high levels of ActA and other products of virulence genes after in vitro growth. Strain DP-L1376 is a derivative of SLCC-5764 with an insertion of transposon Tn917 in the *prfA* gene, which encodes a transcription factor that positively regulates the expression of all known L. monocytogenes virulence genes (Mengaud et al., 1991; Chakraborty et al., 1992), including *actA* (Vazquez-Boland et al., 1992). Bacteria were grown to stationary phase overnight at 37°C with constant shaking and were washed once in XB (Murray and Kirschner, 1989) before being added to extracts.

Observation of Movement in Extracts

L. monocytogenes were grown overnight to stationary phase in BHI, pelleted for 1 min in an Eppendorf centrifuge, and resuspended at their original density in XB. Resuspended bacteria (1 μl) was mixed with 6 μl of Xenopus egg extract by vortexing, and the mixture was incubated at room temperature for 15 min or more. The mixture (1 μl) was then removed and squashed between a microscope slide and a 22 mm square coverslip. For observations with fluorescent actin as a tracer, 1 μl of 0.2 mg/ml actin covalently labeled with TMR–iodoacetamide (Molecular Probes) (Theriot and Mitchison, 1991) in XB was added to the 6 μl of extract prior to the addition of bacteria. Any further additions (of actin, profilin, etc.) were made in a volume of 1–2 μl.

Video Microscopy and Image Analysis

All recording was performed on a Zeiss IM35 inverted microscope. Phase images of bacteria moving in extracts were recorded using a Hamamatsu Newvicon camera. Fluorescent images of TMR–actin in extracts or TMR–profilin in extracts or infected cells were recorded using a standard rhodamine filter set and an intensified silicon-intensified tube camera (Cohu). Video frames (8) were averaged for each recorded frame by a Maxvision AT1 image processor (Datacube), and frames were recorded on optical disc (Panasonic).

All image analysis was performed using Image-1 (Universal Imaging). Fluorescence intensity profiles were measured by averaging three adjacent pixel lines. Actin filament half-lives were determined by measuring the total fluorescence intensity in an 8 × 8 pixel box over the tails of moving bacteria visualized in extracts doped with TMR–actin for at least 10 video frames at 10 s intervals and by fitting an exponential decay curve to each plot of fluorescence intensity versus time. Rates of L. monocytogenes movement were determined by averaging the distance moved in 10 s for five adjacent time intervals.

Tissue Culture and Immunofluorescence

HeLa cells were a gift from Drs. Rebecca Bernat and Linda Wordeman. This line of HeLa cells was chosen for its relatively well-spread morphology. HeLa cells were grown in RPMI 1640 medium with 25 mM HEPES and 10% fetal calf serum on acid-washed glass coverslips. Cells were infected with L. monocytogenes at a density of approximately 10^7/ml for 1 hr and then were rinsed 3 × in phosphate-buffered saline and returned to culture medium. After 30 min, gentamicin was added to 5 μg/ml. Infected cells were fixed with 3.2% formaldehyde in phosphate-buffered saline after a total of 5 hr from initiation of infection. Indirect immunofluorescence was performed using affinity-purified rabbit anti-human profilin serum (Finkel et al., 1994) as the primary antibody and Texas red-conjugated goat anti-rabbit immunoglobulin as the secondary antibody, with fluorescein–phalloidin (Molecular Probes) added to the second incubation. Coverslips were mounted in FITC-guard (Testog).

PtK2 potoroo kidney epithelial cells were grown as described (Mitchison, 1988). Infection (Dabiri et al., 1990) and microinjection (Theriot and Mitchison, 1991) were performed as described.

Profilin Purification and Labeling

Profilin was purified from calf thymus by chromatography on poly-L-proline sepharose (Kaiser et al., 1989). Profilin at 4.5 mg/ml in low salt buffer (5 mM Tris [pH 8.0], 0.2 mM $CaCl_2$) was mixed with a 1.5-fold molar excess of TMR-N-hydroxysuccinimide ester (Molecular Probes) from a 100 mM DMSO stock at 4°C overnight. Unreacted dye was removed by gel filtration. TMR–profilin at 3 mg/ml was frozen in liquid nitrogen and stored at −80°C. Protein concentration was determined by Bradford assay (Bio-Rad) and the amount of TMR was measured by absorption assuming ε = 80,000 at 560 nm. TMR–profilin retained most of its ability to bind to polyproline; under conditions where 94% of unlabeled profilin could be precipitated by polyproline–sepharose beads, 64% of TMR–profilin could be so precipitated. TMR–profilin also mostly retained its ability to interact with monomeric actin and inhibit polymerization. Addition of physiological salt to 0.05 mM monomeric rabbit skeletal muscle actin resulted in 89% of the actin polymerizing into filaments, with 11% remaining monomeric. The addition of unlabeled calf thymus profilin in a 1:1 molar ratio with the actin resulted in 83% of the actin failing to polymerize. The addition of TMR–profilin in a 1:1 molar ratio with the actin resulted in 60% of the actin failing to polymerize. Thus, TMR–profilin retains about 68% of the polyproline-binding ability and about 72% of the actin polymerization-inhibiting ability of unlabeled calf thymus profilin.

Polyacrylamide Gel Electrophoresis and Western Blotting

Profilin and Xenopus egg extract samples were run on 4–20% gradient reducing and denaturing SDS–polyacrylamide gels and stained with Coomassie brilliant blue. Profilin was transferred onto nitrocellulose, probed with affinity-purified rabbit polyclonal immunoglobulin Gs against human profilin (dilution 1:1000), and detected using chemiluminescence (Finkel et al., 1993). This antibody did not recognize Xenopus profilin on Western blots. For depletions, 35 μl of pelleted polyproline–sepharose beads were mixed with 85 μl of Xenopus egg extract for 8 hr at 4°C. Samples were compared of the extract supernatant after depletion, the mixture of beads and extract, and the protein remaining bound to the beads after 3 washes in 0.1 M NaCl, 0.1 M glycine, 0.01 mM DTT, 10 mM Tris (pH 7.8), and 1 M urea.

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References

Belmont, L. D., Hyman, A. A., Sawin, K. E., and Mitchison, T. J. (1990). Real-time visualization of cell cycle–dependent changes in microtubule dynamics in cytoplasmic extracts. Cell *62*, 579–589.

Brundage, R. A., Smith, G. A., Camilli, A., Theriot, J. A., and Portnoy, D. A. (1993). Expression and phosphorylation of the *Listeria monocytogenes* ActA protein in mammalian cells. Proc. Natl. Acad. Sci. USA *90*, 11890–11894.

Buß, F., Temm-Grove, C., Henning, S., and Jockusch, B. M. (1992). Distribution of profilin in fibroblasts correlates with the presence of highly dynamic actin filaments. Cell Motil. Cytoskel. *22*, 51–61.

Cao, L.-g., Babcock, G. G., Rubenstein, P. A., and Wang, Y.-l. (1992). Effects of profilin and profilactin on actin structure and function in living cells. J. Cell Biol. *117*, 1023–1029.

Carlsson, L., Nystrom, L.-E., Sundkvist, I., Markey, F., and Lindberg, U. (1977). Actin polymerizability is influenced by profilin, a low molecular weight protein in nonmuscle cells. J. Mol. Biol. *115*, 465–483.

Chakraborty, T., Leimeister-Wächter, M., Domann, E., Hartl, M., Goebel, W., Nichterlein, T., and Notermans, S. (1992). Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the *prfA* gene. J. Bacteriol. *174*, 568–574.

Condeelis, J. S., and Taylor, D. L. (1977). The contractile basis of amoeboid movment. V. The control of gelation, solation, and contraction in extracts from *Dictyostelium discoideum*. J. Cell Biol. *74*, 901–927.

Cooley, L., Verheyen, E., and Ayers, K. (1992). *chickadee* encodes a profilin required for intercellular cytoplasm transport during Drosophila oogenesis. Cell *69*, 173–184.

Cox, D., Condeelis, J., Wessels, D., Soll, D., Kern, H., and Knecht, D. A. (1992). Targeted disruption of the ABP-120 gene leads to cells with altered motility. J. Cell Biol. *116*, 943–955.

Cunningham, C. C., Gorlin, J. B., Kwiatkowski, D. J., Hartwig, J. H., Janmey, P. A., Byers, H. R., and Stossel, T. P. (1992). Actin-binding protein requirement for cortical stability and efficient locomotion. Science *255*, 325–327.

Dabiri, G. A., Sanger, J. M., Portnoy, D. A., and Southwick, F. S. (1990). *Listeria monocytogenes* moves rapidly through the host-cell cytoplasm by inducing directional actin assembly. Proc. Natl. Acad. Sci. USA *87*, 6068–6072.

DeLozanne, A., and Spudich, J. A. (1987). Disruption of the *Dictyostelium* myosin heavy chain gene by homologous recombination. Science *236*, 1086–1091.

Domann, E., Wehland, J., Rohde, M., Pistor, S., Hartl, M., Goebel, W., Leimeister-Wächter, M. Wuenscher, M., and Chakraborty, T. (1992). A novel bacterial virulence gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to the proline-rich region of vinculin. EMBO J. *11*, 1981–1990.

Edamatsu, M., Hirono, M., and Watanabe, Y. (1992). *Tetrahymena* profilin is localized in the division furrow. J. Biochem. *112*, 637–642.

Finkel, T., Theriot, J. A., Dise, K. R., Tomaselli, G. F., and Goldschmidt-Clermont, P. J. (1994). Dynamic actin structures stabilized by profilin. Proc. Natl. Acad. Sci. USA, in press.

Gellin, B. G., and Broome, C. V. (1989). Listeriosis. J. Amer. Med. Assoc. *261*, 1313–1320.

Goldschmidt-Clermont, P. J., Furman, M. I., Wachsstock, D., Safer, D., Nachmias, V. T., and Pollard, T. D. (1992). The control of actin nucleotide exchange by thymosin β4 and profilin. A potential regulatory mechanism for actin polymerization in cells. Mol. Biol. Cell *3*, 1015–1024.

Goldschmidt-Clermont, P. J., Machesky, L. M., Doberstein, S. K., and Pollard, T. D. (1991). Mechanism of the interaction of platelet profilin with actin. J. Cell Biol. *113*, 1081–1089.

Haarer, B. K., Lillie, S. H., Adams, A. E. M., Magdolen, V., Bandlow, W., and Brown, S. S. (1990). Purification of profilin from *Saccharomyces cerevisiae* and analysis of profilin-deficient cells. J. Cell Biol. *110*, 105–114.

Heath, J. P., and Holifield, B. F. (1991). Cell locomotion: new research tests old ideas on membrane and cytoskeletal flow. Cell Motil. Cytoskel. *18*, 245–257.

Kaiser, D. A., Goldschmidt-Clermont, P. J., Levine, B. A., and Pollard, T. D. (1989). Characterization of renatured profilin purified by urea elution from poly-L-proline agarose columns. Cell Motil. Cytoskel. *14*, 251–262.

Kane, R. E. (1980). Induction of either contractile or structural actin-based gels in sea urchin egg cytoplasmic extract. J. Cell Biol. *86*, 803–809.

Knecht, D. A., and Loomis, W. F. (1987). Antisense RNA inactivation of myosin heavy chain gene expression in *Dictyostelium discoideum*. Science *236*, 1081–1085.

Kocks, C., Gouin, E., Tabouret, M., Berche, P., Ohayon, H., and Cossart, P. (1992). *L. monocytogenes*-induced actin assembly requires the *actA* gene product, a surface protein. Cell *68*, 521–531.

Kocks, C., Hellio, R., Gounon, P., Ohayon, H., and Cossart, P. (1993). Polarized distribution of *Listeria monocytogenes* surface protein ActA at the site of directional actin assembly. J. Cell Sci. *105*, 699–710.

Kron, S. J., and Spudich, J. A. (1986). Fluorescent actin filaments move on myosin fixed to a glass surface. Nature *83*, 6272–6276.

Leimeister-Wächter, M., and Chakraborty, T. (1989). Detection of listeriolysin, the thiol-dependent hemolysin in *Listeria monocytogenes*, *Listeria ivanovii* and *Listeria seeligeri*. Infect. Immun. *57*, 2350–2357.

Mengaud, J., Dramsi, S., Gouin, E., Vazquez-Boland, J.-A., Milon, G., and Cossart, P. (1991). Pleiotropic control of *Listeria monocytogenes* virulence factors by a gene which is autoregulated. Mol. Microbiol. *5*, 2273–2283.

Mitchison, T. J. (1988). Polewards microtubule flux in the mitotic spindle: evidence from photoactivation of fluorescence. J. Cell Biol. *109*, 637–652.

Mounier, J., Ryter, M., Coquis-Rondon, M., and Sansonetti, P. J. (1990). Intracellular and cell-to-cell spread of *Listeria monocytogenes* involves interaction with F-actin in the enterocytelike cell line Caco-2. Infect. Immun. *58*, 1048–1058.

Murray, A., and Kirschner, M. (1989). Cyclin synthesis drives the early embryonic cell cycle. Nature *339*, 275–280.

Murray, A., Solomon, M., and Kirschner, M. (1989). The role of cyclin synthesis in the control of maturation-promoting factor activity. Nature *339*, 280–296.

Niebuhr, K., Chakraborty, T., Rohde, M., Gazlig, T., Jansen, B., Köllner, P., and Wehland, J. (1993). Localization of the ActA polypeptide of *Listeria monocytogenes* in infected tissue culture cell lines: ActA is not associated with actin comets. Infect. Immun. *61*, 2793–2802.

Oster, G. F., and Perelson, A. S. (1987). The physics of cell motility. J. Cell Sci. (Suppl.) *8*, 35–54.

Pantaloni, D., and Carlier, M.-F. (1993). How profilin promotes actin filament assembly in the presence of thymosin β4. Cell *75*, 1007–1014.

Peskin, C. S., Odell, G. M., and Oster, G. F. (1993). Cellular motions and thermal fluctuations-the Brownian ratchet. Biophys. J. *65*, 316–324.

Pollard, T. D. (1976). The role of actin in the temperature-dependent gelation and contraction of extracts of *Acanthamoeba*. J. Cell Biol. *68*, 579–601.

Pollard, T. D., and Cooper, J. A. (1984). Quantitative analysis of the effect of *Acanthamoeba* profilin in actin filament nucleation and elongation. Biochemistry *23*, 6631–6641.

Pring, M., Weber, A., and Bubb, M. R. (1992). Profilin–actin complexes directly elongate actin filaments at the barbed end. Biochemistry *31*, 1827–1836.

Ren, R., Mayer, B. J., Cicchetti, P., and Baltimore, D. (1993). Identification of a ten-amino acid proline-rich SH3 binding site. Science *259*, 1157–1161.

Sanger, J. M., Sanger, J. W., and Southwick, F. S. (1992). Host cell actin assembly is necessary and likely to provide the propulsive force for intracellular movement of *Listeria monocytogenes*. Infect. Immun. *60*, 3609–3619.

Sawin, K. E., and Mitchison, T. J. (1991). Poleward microtubule flux in mitotic spindles assembled *in vitro*. J. Cell Biol. *112*, 941–954.

Shariff, A., and Luna, E. J. (1992). Diacylglycerol-stimulated formation of actin nucleation sites at plasma membranes. Science *256*, 245–247.

Smith, S. J. (1988). Neuronal cytomechanics: the actin-based motility of growth cones. Science *242*, 708–715.

Stossel, T. P. (1993). On the crawling of animal cells. Science *260*, 1086–1094.

Symons, M. H., and Mitchison, T. J. (1991). Control of actin polymerization in live and permeabilized fibroblasts. J. Cell Biol. *114*, 503–513.

Tanaka, M., and Shibata, H. (1985). Poly (L-proline)-binding proteins from chick embryos are profilin and profilactin. Eur. J. Biochem. *151*, 291–297.

Theriot, J. A., and Mitchison, T. J. (1991). Actin microfilament dynamics in locomoting cells. Nature *352*, 126–131.

Theriot, J. A., and Mitchison, T. J. (1992). The nucleation-release model of actin filament dynamics in cell motility. Trends Cell Biol. *2*, 219–222.

Theriot, J. A., Mitchison, T. J., Tilney, L. G., and Portnoy, D. A. (1992). The rate of actin-based motility of intracellular *Listeria monocytogenes* equals the rate of actin polymerization. Nature *357*, 257–260.

Tilney, L. G., and Portnoy, D. A. (1989). Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. J. Cell Biol. *109*, 1597–1608.

Tilney, L. G., Bonder, E. M., Coluccio, L. M., and Mooseker, M. S. (1983). Actin from Thyone sperm assembles on only one end of an actin filament: a behavior regulated by profilin. J. Cell Biol. *97*, 112–124.

Tilney, L. G., Connelly, P. S., and Portnoy, D. A. (1990). Actin filament nucleation by the bacterial pathogen, *Listeria monocytogenes*. J. Cell Biol. *111*, 2979–2988.

Tilney, L. G., DeRosier, D. J., Weber, A., and Tilney, M. S. (1992). How *Listeria* exploits host cell actin to form its own cytoskeleton. II. Nucleation, actin filament polarity, filament assembly, and evidence for a pointed end capper. J. Cell Biol. *118*, 83–93.

Vazquez-Boland, J.-A., Kocks, C., Dramsi, S., Ohayon, H., Geoffroy, C., Mengaud, J., and Cossart, P. (1992). Nucleotide sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread. Infect. Immun. *60*, 219–230.

Note Added in Proof

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Appendix B

Interaction of human Arp 2/3 complex and the *Listeria monocytogenes* ActA Protein in Actin Filament Nucleation.

Matthew D. Welch, Jody Rosenblatt, Justin Skoble, Daniel A Portnoy and Timothy J. Mitchison

(Published in Science, Vol. 281, 105-108, 1998)

I contributed to this publication by demonstrating that the Arp 2/3 complex produces actin clouds around *Listeria monocytogenes* by incorporating actin monomer, not by recruiting actin polymer. I also show by electron microscopy that the Arp 2/3 complex and ActA nucleate actin filaments.

Interaction of Human Arp2/3 Complex and the *Listeria monocytogenes* ActA Protein in Actin Filament Nucleation

Matthew D. Welch,* Jody Rosenblatt, Justin Skoble, Daniel A. Portnoy, Timothy J. Mitchison

Actin filament assembly at the cell surface of the pathogenic bacterium *Listeria monocytogenes* requires the bacterial ActA surface protein and the host cell Arp2/3 complex. Purified Arp2/3 complex accelerated the nucleation of actin polymerization in vitro, but pure ActA had no effect. However, when combined, the Arp2/3 complex and ActA synergistically stimulated the nucleation of actin filaments. This mechanism of activating the host Arp2/3 complex at the *L. monocytogenes* surface may be similar to the strategy used by cells to control Arp2/3 complex activity and hence the spatial and temporal distribution of actin polymerization.

The pathogenic bacterium *Listeria monocytogenes* initiates actin filament polymerization at its cell surface after it gains access to the cytosol of infected host cells (*1*). Actin polymerization is tightly coupled to intracellular bacterial motility (*2*) and may provide the motile force (*3*). Thus the *L. monocytogenes* cell surface is functionally similar to the leading edge of lamellipodia in locomoting cells, where actin polymerization is linked with membrane protrusion (*4*). Understanding the mechanism by which polymerization is instigated by *L. monocytogenes* should shed light both on an essential aspect of bacterial pathogenesis and on the general mechanisms by which actin filament assembly is modulated in cells.

Actin polymerization at the *L. monocytogenes* surface is mediated by bacterial and

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host cell factors. The only essential bacterial component is ActA (*5, 6*), a cell surface protein that recruits host cell factors that promote actin assembly. A critical host factor is the Arp2/3 complex (*7*), an evolutionarily conserved protein complex that contains actin-related proteins (Arp) in the Arp2 and Arp3 subfamilies as well as five additional proteins (*8–10*). This protein complex promotes actin assembly at the bacterial surface, mediates bacterial motility in vitro (*7*), and is localized throughout actin "comet tails" assembled by moving *L. monocytogenes* in vivo (*7, 10*). Moreover, the Arp2/3 complex is concentrated in the lamellipodia of mammalian cells (*10, 11*) and in pseudopodia of *Acanthamoeba castellanii* (*8, 12, 13*), which suggests that it is important for membrane protrusion. Genetic analysis in yeast has demonstrated that the Arp2/3 complex is essential for actin function and cell viability (*9, 14*).

To further understand the biochemical function of the Arp2/3 complex in cells, we sought to determine how it promotes actin polymerization at the *L. monocytogenes* surface. Structural models of Arp2 and Arp3 (*12*) suggest that the complex may serve as a nucleating site for the assembly of actin monomer (G-actin). Nucleation is the rate-limiting step in spontaneous actin polymerization and thus represents a kinetic barrier to actin assembly. Alternatively, the Arp2/3 complex may recruit actin filaments (F-actin) (*13*), which themselves serve as a template for polymerization. To distinguish between these mechanisms, bacteria were incubated with Arp2/3 complex and equal concentrations of rhodamine-labeled G-actin or F-actin (*15*). Actin clouds were observed surrounding bacteria incubated with Arp2/3 complex and G-actin (Fig. 1A). In contrast, no actin was associated with bacteria in the presence of Arp2/3 complex and F-actin (Fig. 1B). This strongly favors the nucleation model for Arp2/3 complex function on *L. monocytogenes*.

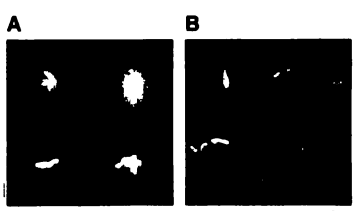

Fig. 1. Function of the Arp2/3 complex at the *L. monocytogenes* cell surface. (**A**) Composite image of DAPI-labeled *L. monocytogenes* (blue) that were incubated with 0.5 μM TMR-labeled G-actin (red) and 0.07 μM Arp2/3 complex (*15*). Between 30 and 50% of bacteria assembled actin clouds. (**B**) Composite image *L. monocytogenes* (blue) that were incubated with 0.5 μM TMR-labeled F-actin (red) and 0.07 μM Arp2/3 complex (*15*). No actin was associated with bacteria. These data represent a compilation of 17 individual experiments. Bar = 10 μm.

To determine whether the Arp2/3 complex nucleates actin polymerization in the absence of *L. monocytogenes*, we observed the effect of pure complex on the kinetics of actin assembly. Polymerization kinetics were monitored in vitro by an assay that employs pyrene-actin, a fluorescent derivative of actin that exhibits much higher intensity of fluorescence when present as F-actin than as G-actin (*16*). In this assay (*17*), actin alone exhibited typical assembly kinetics marked by an initial lag phase, indicative of the kinetic barrier to nucleation, followed by a period of rapid assembly that represents filament elongation (Fig. 2A). In the presence of the Arp2/3 complex, the kinetics of polymerization were accelerated relative to actin alone (Fig. 2A), but the initial lag phase of assembly was not significantly shortened, even at higher ratios of Arp2/3:actin. This effect on polymerization is consistent with an ability to accelerate actin filament generation by either facilitating nucleation or severing newly formed filaments. However, pure Arp2/3 complex did not affect the rate of filament depolymerization (*17*), indicating that it does not sever filaments and suggesting that it facilitates nucleation.

Actin nucleation by the Arp2/3 complex at the *L. monocytogenes* surface also requires the bacterial ActA surface protein (*7*), and synthetic peptides derived from ActA bind to G- and F-actin (*18*), which suggests that ActA may itself possess nucleating activity. To determine how ActA affects polymerization kinetics, we constructed and purified a variant of ActA called ActA-His (*19*) (Fig. 3, A and B). The kinetics of actin polymerization in the presence of ActA-His and in the absence of added protein were identical (Fig. 2C), indicating that full-length ActA does not affect actin assembly (*18*).

In addition to testing the effects of Arp2/3 complex and ActA on actin assembly individually, we monitored polymerization kinetics in the presence of both pure proteins. In the presence of Arp2/3 complex and ActA-His, the initial rate of actin assembly was accelerated up to 50-fold relative to the reactions in the presence of Arp2/3 or ActA alone (Fig. 2E). Moreover, with both factors present, the lag phase of polymerization was eliminated (Fig. 2G), indicating that Arp2/3 complex and ActA function together as a highly efficient nucleating site, which is kinetically comparable to the end of an actin filament. Addition of increasing amounts of ActA to a fixed concentration of Arp2/3 complex caused a dose-dependent acceleration of the kinetics of actin polymerization (Fig. 2H). This effect was specific to ActA-His because

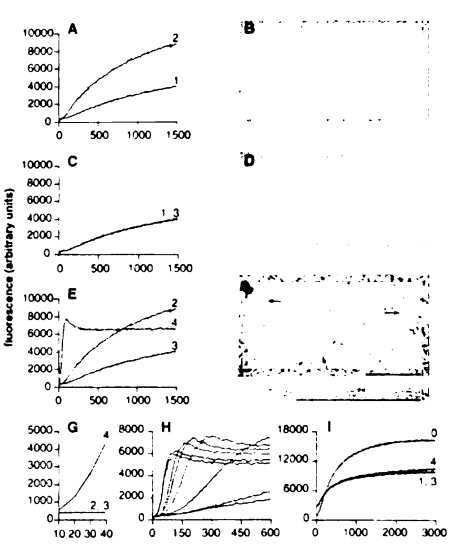

Fig. 2. Effects of the Arp2/3 complex and ActA on actin polymerization. (**A**, **C**, and **E**) Graphs of fluorescence intensity versus time after initiating polymerization in the pyrene-actin assay (*17*). Curve 1, 2 μM actin; curve 2, 2 μM actin with 30 nM Arp2/3 complex (1:65 ratio Arp2/3:actin); curve 3, 2μM actin with 30 nM ActA-His (1:65 ratio ActA:actin); curve 4, 2μM actin with 30 nM Arp2/3 complex and 30 nM ActA (1:1:65 ratio Arp2/3:ActA:actin). (**B**, **D**, and **F**) Electron micrographs of grids spotted with polymerization reaction mixtures 30 s after initiating polymerization (*21*). (B) Actin (2 μM) with 70 nM Arp2/3 complex [corresponding to (A) curve 2]. (D) Actin (2μM) with 70 nM ActA-His [corresponding to (C) curve 3]. (F) (Top) Actin (2 μM) with 70 nM ActA and 70 nM Arp2/3 complex [corresponding to (E) curve 4]. Arrows indicate actin filaments. (Bottom) Higher magnification view of an actin filament from the same reaction. Bars = 500 nm. (**G**) Expanded view of the initial 40 s of the graph in (E). (**H**) Graphs from the pyrene-actin assay. Actin (2 μM) with 20 nM Arp2/3 and, from right to left, 0, 0.04, 0.40, 4, 8, 20, 40, and 60 nM ActA-His. (**I**) Graphs from the pyrene-actin assay in the absence and presence of CD. Curve 0, 4 μM actin; curve 1, 4 μM actin with 1 μM CD, curve 3, 4 μM actin with 18 nM Arp2/3 and 1 μM CD; curve 4, 4μM actin with 18 nM Arp2/3, 10 nM ActA-His, and 1 μM CD.

addition of an unrelated protein (His-XCTK2 tail, the COOH-terminal domain of a kinesin family protein) (*20*) to the Arp2/3 complex did not accelerate polymerization kinetics relative to the Arp2/3 complex alone.

To confirm that the assembly kinetics measured by the pyrene-actin assay represented the kinetics of filament formation, we visualized filaments in polymerization reactions by electron microscopy during the lag phase of spontaneous polymerization (30 s after initiating assembly) (*21*). Filaments were observed in the reaction mixtures containing both ActA and Arp2/3 complex (Fig. 2F) but not those with Arp2/3 complex or ActA alone (Fig. 2, B and D) (*21*). These results, together with those obtained by the pyrene-actin assay, demonstrate that the Arp2/3 complex and ActA act synergistically to nucleate actin assembly. We suggest that the nucleation activity of the Arp2/3 complex is stimulated by a physical interaction with ActA because the Arp2/3 complex on its own accelerates actin polymerization, whereas ActA does not. However, we cannot rule out the possibility that ActA participates directly in nucleation.

Actin filaments in the *L. monocytogenes* comet tail are oriented with their barbed (fast growing) ends toward the bacterial surface, and barbed-end elongation is thought to drive motility (*22*). To determine which end is elongating in filaments nucleated by Arp2/3 complex and ActA, we performed the pyrene-actin assay in the presence of cytochalasin D (CD). This compound prevents

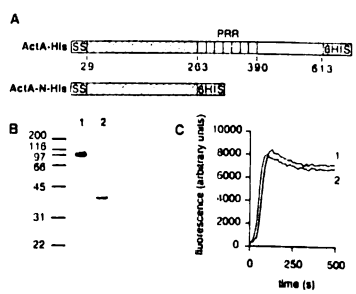

Fig. 3. ActA derivatives and their effect on actin nucleation. (**A**) Schematic representation of ActA-His and ActA-N-His (*19*) showing the signal sequence (SS), the NH_2-terminal region that is essential for actin polymerization (hatched box; amino acids 29 to 263) (*18, 24*), the central region containing four proline-rich repeats (gray boxes, PRR; amino acids 263 to 390), and the COOH-terminal tag of six histidine residues (6HIS; replaces the ActA transmembrane domain in ActA-His). (**B**) Purified ActA-His (lane 1) and ActA-N-His (lane 2) (*19*) visualized on a SDS–12% polyacrylamide gel stained with Coomassie blue. (**C**) Graphs from the pyrene-actin assay (*17*). Curve 1, 2 μM actin with 20 nM ActA-His and 20 nM Arp2/3 complex; curve 2, 2 μM actin with 20 nM ActA-N-His and 20 nM Arp2/3 complex.

assembly at barbed ends (*23*) and hence limits actin polymerization to pointed ends. When CD was included in the reaction mixture, the kinetics of Arp2/3 and ActA nucleated actin assembly were nearly identical to the kinetics in the presence of the Arp2/3 complex or actin alone (Fig. 2I). This indicates that filaments nucleated by the Arp2/3 complex and ActA elongate predominantly at their barbed ends.

We next sought to determine which domain of ActA is responsible for its activity. The NH_2-terminal region (Fig. 3A) is essential for ActA to induce actin assembly (*18, 24*). In contrast, the four proline-rich repeats in the central region (Fig. 3A) are not essential but enhance the efficiency of polymerization and motility (*24, 25*). We generated and purified ActA-N-His, which consists only of the NH_2-terminal domain of ActA (*19*) (Fig. 3, A and B). Equal amounts of ActA-N-His and ActA-His were equivalent in their ability to activate Arp2/3 complex nucleation activity in the pyrene-actin assay (*17*) (Fig. 3C). ActA-N-His alone had no effect on actin polymerization (*17*). Thus, the Arp2/3 complex interacts with the NH_2-terminal region of ActA to form a nucleating activity, and the proline-rich repeats do not contribute to nucleation in this assay. These repeats may enhance actin polymerization and bacterial motility by recruiting the Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family of proteins (*26*) and profilin (*27*), which may promote the elongation of filaments nucleated by the Arp2/3 complex and ActA.

Our findings indicate that the Arp2/3 complex and ActA function together to nucleate actin assembly at the *L. monocytogenes* cell surface. We propose the following model for the potential role of these two proteins in actin polymerization and *L. monocytogenes* motility. Before encountering ActA, the Arp2/3 complex only weakly enhances the kinetics of actin polymerization. Upon interacting with the NH_2-terminal domain of ActA, the activity of the complex is stimulated and it nucleates actin assembly, generating actin filaments whose elongation propels the bacterium forward (*3*). Activation of the Arp2/3 complex may occur by two mechanisms. Interaction with ActA may induce a conformational change in the complex. Alternatively, the complex may be activated by self-association facilitated by ActA, which is a dimer on the bacterial surface (*28*). In either case, the ability of ActA to activate the Arp2/3 complex explains how the complex can generate actin filaments only at the bacterial surface, as is observed in vivo (*2*), although it is present throughout the actin tails assembled by moving bacteria (*7, 10*).

Activation of the Arp2/3 complex with a spatially localized factor such as ActA may represent a general strategy used to regulate the distribution of actin polymerization in cells. Cellular proteins with functions similar to ActA may recruit the complex to lamellipodia and activate its nucleating activity, leading to the generation of filaments that elongate to drive membrane protrusion. Although ActA is the only known regulator of the Arp2/3 complex, other factors such as posttranslational modification (*7, 10*) may also modulate its function. Thus, multiple pathways may operate in concert to regulate Arp2/3 complex activity. A more complete understanding of the cellular mechanisms that control actin polymerization awaits further determination of how Arp2/3-mediated nucleation is regulated and how it is integrated with other processes such as filament uncapping, elongation, cross-linking, and depolymerization.

References and Notes

1. L. G. Tilney and D. A. Portnoy, *J. Cell Biol.* **109**, 1597 (1989).
2. J. A. Theriot, T. J. Mitchison, L. G. Tilney, D. A. Portnoy, *Nature* **357**, 257 (1992); J. M. Sanger, J. W. Sanger, F. S. Southwick, *Infect. Immun.* **60**, 3609 (1992).
3. A. Mogilner and G. Oster, *Biophys. J.* **71**, 3030 (1996).
4. T. J. Mitchison and L. P. Cramer, *Cell* **84**, 371 (1996).
5. C. Kocks *et al.*, *ibid.* **68**, 521 (1992); E. Domann *et al.*, *EMBO J.* **11**, 1981 (1992).
6. G. A. Smith, D. A. Portnoy, J. A. Theriot, *Mol. Microbiol.* **17**, 945 (1995).
7. M. D. Welch, A. Iwamatsu, T. J. Mitchison, *Nature* **385**, 265 (1997).
8. L. M. Machesky, S. J. Atkinson, C. Ampe, J. Vandekerckhove, T. D. Pollard, *J. Cell Biol.* **127**, 107 (1994).
9. D. Winter, A. V. Podtelejnikov, M. Mann, R. Li, *Curr. Biol.* **7**, 519 (1997).
10. M. D. Welch, A. H. DePace, S. Verma, A. Iwamatsu, T. J. Mitchison, *J. Cell Biol.* **138**, 375 (1997).
11. L. M. Machesky *et al.*, *Biochem. J.* **328**, 105 (1997).
12. J. F. Kelleher, S. J. Atkinson, T. D. Pollard, *J. Cell Biol.* **131**, 385 (1995).
13. R. D. Mullins, W. F. Stafford, T. D. Pollard, *ibid.* **136**, 331 (1997); R. D. Mullins, J. F. Kelleher, J. Xu, T. D. Pollard, *Mol. Biol. Cell* **9**, 841 (1998).
14. E. Schwob and R. P. Martin, *Nature* **355**, 179 (1992); J. P. Lees-Miller, G. Henry, D. M. Helfman, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 80 (1992); D. McCollum, A. Feoktistova, M. Morphew, M. Balasubramanian, K. L. Gould, *EMBO J.* **15**, 6438 (1996); M. K. Balasubramanian, A. Feoktistova, D. McCollum, K. L. Gould, *ibid.*, p. 6426; V. Moreau, A. Madania, R. P. Martin, B. Winsor, *J. Cell Biol.* **134**, 117 (1996); M. E. Huang, J. L. Souciet, J. C. Chuat, F. Galibert, *Yeast* **12**, 839 (1996).
15. The Arp2/3 complex was purified as described in [(*7*); M. D. Welch and T. J. Mitchison, *Methods Enzymol.* **298** (1998)]. Actin was purified [J. A. Spudich and S. Watt, *J. Biol. Chem.* **246**, 4866 (1971)], labeled with *N*-hydroxysuccinimidyl 5-carboxytetramethylrhodamine (TMR) [D. R. Kellogg, T. J. Mitchison, B. M. Alberts, *Development* **103**, 675 (1988)], and stored in G-buffer [5 mM tris-HCl (pH 8.0), 0.2 mM $CaCl_2$, 0.2 mM adenosine triphosphate (ATP), 0.2 mM dithiothreitol (DTT)]. Monomeric TMR-labeled actin was isolated by centrifuging actin in G-buffer at 228,000*g* for 15 min to remove filaments. TMR-labeled actin filaments were polymerized by adding tris-HCl (pH 8.0) to 50 mM and NaCl to 50 mM and isolated by centrifugation through an F-buffer cushion [50 mM NaCl, 50 mM tris-HCl (pH 8.0), 0.2 mM DTT, 0.5 mM ATP] at 228,000*g* for 15 min. Equal concentrations of G- and F-actin (0.5 μM) were incubated with Arp2/3 complex (0.3 to 0.5 μM) and 4′,6-diamidino-2-phenylindole (DAPI)–labeled *L. monocytogenes* strain SLCC-5764 as described [(*7*); M. D. Welch and T. J. Mitchison, *Methods Enzymol.* **298** (1998)]. The mix was squashed between a glass slide and coverslip and

incubated at 22°C for 20 min before it was viewed by fluorescence microscopy.
16. T. Kouyama and K. Mihashi, *Eur. J. Biochem.* **114**, 33 (1981); J. A. Cooper, S. B. Walker, T. D. Pollard, *J. Muscle Res. Cell Motil.* **4**, 253 (1983).
17. The pyrene-actin assay measures the weight concentration of polymer over time and is not sensitive to the distribution of filament lengths (*16*). Pyrene-actin was prepared as described in (*16*). Pyrene-labeled G-actin and unlabeled G-actin were isolated by centrifuging actin in G-buffer at 228,000*g* for 15 min to remove F-actin. For polymerization reactions, the Arp2/3 complex, ActA-His, and/or ActA-N-His were mixed and brought to 5 μl in control buffer [20 mM Mops (pH 7.0), 100 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM EDTA, 0.2 mM ATP, 0.5 mM DTT, 10% (v/v) glycerol]. This solution was immediately mixed with 5 μl of 10× initiation buffer (20 mM $MgCl_2$, 10 mM EGTA, 5 mM ATP) and immediately added to 40 μl of G-actin solution (90% unlabeled actin and 10% pyrene-actin in G-buffer) to initiate polymerization. To monitor actin depolymerization, 7 μM pyrene G-actin was polymerized for 4 to 5 hours at 20°C by adding 10× initiation buffer (20 mM $MgCl_2$, 10 mM EGTA, 5 mM ATP). Depolymerization was initiated by diluting 2 μl of F-actin into 48 μl of G-buffer containing 1 μl of control buffer or 1 μl of 6.4 μM Arp2/3 complex in control buffer (0.27 μM actin, 0.13 μM Arp2/3). Pyrene fluorescence was monitored with a fluorometer (excitation wavelength, 365 nm; emission wavelength, 407 nm).
18. I. Lasa *et al.*, *EMBO J.* **16**, 1531 (1997).
19. To generate ActA-His, a truncated *actA* gene encoding amino acids 1 to 613 but lacking the transmembrane domain was amplified by polymerase chain reaction (PCR). Primers used were GGAAGCTTGTGGATGCTTCTGAAAGTGAC (upstream of the *actA* promoter) and GGGGATCCCGTCGTATGGTTCCCTGG (bases 1812 to 1839 of *actA*; Bam HI site is underlined). The PCR product was digested with Bam HI and Hin dIII (site upstream of promoter) and was subcloned into pAM401, yielding pDP2683. To introduce the His_6 tag, the Bam HI–Xba I fragment of pQE-60 (Qiagen) encoding amino acids GSRSHHHHHH (G, Gly; S, Ser; R, Arg; H, His) was subcloned into pDP2683 to yield pDP2717. This was transformed into *L. monocytogenes* strain DPL1545 (6) to yield strain DPL2723. To generate ActA-N-His, a fragment of *actA* encoding amino acids 1 to 263 was amplified by PCR. Primers used were CGGGATCCTGAAGCTTGGGAACCAG (upstream of *actA* promoter; Bam HI site underlined) and GCTCTAGATTAGTGGTGGTGGTGGTGGTGCGAAGCATTTACCTCTTCACT (bases 769 to 789 of *actA*; Xba I site underlined), which encodes the His_6 tag. The PCR product was digested with Bam HI and Xba I and ligated into pAM401 to yield pDP3624. This was transformed into *L. monocytogenes* strain DPL1545 to yield strain DPL3625. Secreted ActA-His and ActA-N-His were purified from culture media; 475 ml of brain heart infusion media containing chloramphenicol (10 μg/ml) (BHI cm10) was inoculated with 25-ml overnight cultures of strain DPL2723 or DPL3625 in BHI cm10 and was grown at 37°C for 6 to 8 hours. After bacteria were pelleted, protein was precipitated from the supernatant by adding ammonium sulfate to 40% (ActA-His) or 60% (ActA-N-His) saturation. Precipitated protein was resuspended in binding buffer [20 mM Mops (pH 7.0), 100 mM KCl, 20 mM imidazole (pH 7.0)] and bound to 0.5 ml of nickel nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen). The resin was washed with binding buffer and proteins were eluted with elution buffer [20 mM Mops (pH 7.0), 100 mM KCl, 250 mM imidazole (pH 7.0)]. Eluted proteins were further purified by Superose-6 (Pharmacia) gel-filtration chromatography in control buffer for the pyrene-actin experiments (*17*) and were frozen in N_2(l) and stored at −80°C.
20. C. E. Walczak, S. Verma, T. J. Mitchison, *J. Cell Biol.* **136**, 859 (1997). His-XCTK2 tail alone (20 nM) had no effect on the polymerization of 2 μM actin in the pyrene-actin assay (*17*).
21. For electron microscopy, ActA-His (70 nM) and/or Arp2/3 complex (70 nM) were mixed with 10 μl of 2× polymerization buffer [100 mM KCl, 20 mM imidazole (pH 7.0), 2 mM EGTA, 2 mM $MgCl_2$] and immediately added to 10 μl of 5 μM G-actin (in G-buffer containing 0.5 mM ATP). After polymerizing for 30 s, 2 μl of actin mix was spotted onto Formvar-coated glow-discharged grids (Pelco), washed with 1× polymerization buffer and distilled H_2O, and negatively stained with 1% uranyl acetate in 50% methanol. At higher actin concentrations or longer polymerization times, some filaments were observed in all reaction mixtures, although more filaments were consistently observed in reaction mixtures containing both the Arp2/3 complex and ActA.
22. L. G. Tilney, D. J. DeRosier, M. S. Tilney, *J. Cell Biol.* **118**, 71 (1992); L. G. Tilney, D. J. DeRosier, A. Weber, M. S. Tilney, *ibid.* p. 83.
23. J. A. Cooper, *ibid.* **105**, 1473 (1987).
24. S. Pistor, T. Chakraborty, U. Walter, J. Wehland, *Curr. Biol.* **5**, 517 (1995); I. Lasa, V. David, E. Gouin, J. B. Marchand, P. Cossart, *Mol. Microbiol.* **18**, 425 (1995).
25. G. A. Smith, J. A. Theriot, D. A. Portnoy, *J. Cell Biol.* **135**, 647 (1996).
26. T. Chakraborty *et al.*, *EMBO J.* **14**, 1314 (1995); F. B. Gertler, K. Niebuhr, M. Reinhard, J. Wehland, P. Soriano, *Cell* **87**, 227 (1996); M. Reinhard *et al.*, *EMBO J.* **14**, 1583 (1995).
27. J. A. Theriot, J. Rosenblatt, D. A. Portnoy, C. P. Goldschmidt, T. J. Mitchison, *Cell* **76**, 505 (1994); J. B. Marchand *et al.*, *J. Cell Biol.* **130**, 331 (1995).
28. P. Mourrain *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 10034 (1997).
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