Landmarks in the discovery of a role for actin in cell locomotion

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ABSTRACT **During the late 1960s four independent lines of research implicated actin in cellular motility. This Retrospective recounts how biochemistry, light and electron microscopy, and inhibitory natural products all contributed to this breakthrough.**

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INTRODUCTION

Remarkably little was known about the molecular basis of cell locomotion at the time of a fascinating symposium held in 1963. The proceedings, published in a 1964 book entitled *Primitive Motile Systems in Cell Biology* (Allen and Kamiya, 1964), provides the historic context for the breakthrough work establishing the role of actin in cellular movements later in the decade. Twenty years earlier, Banga and Straub purified actin and myosin from skeletal muscle in the laboratory of Albert Szent-Gyorgyi in Szeged, Hungary, which Szent-Gyorgyi summarized in a long report at the end of World War II (Szent-Gyorgyi, 1945). Subsequently, Straub and Feuer (1950) discovered that actin hydrolyzes its bound adenosine triphosphate (ATP) during polymerization, beginning a decade of basic discoveries about actin polymerization led by Fumio Oosawa and his colleagues in Japan (see, for example, Kasai *et al.*, 1962). Nevertheless, "actin" and "myosin" were rarely mentioned at the 1963 symposium on motile systems, and a few of the participants even held onto vitalistic beliefs that cell movements were a magical property of live cells rather than the result of chemical reactions of cellular macromolecules.

The new work that opened up the field used biochemical purification of the participating proteins, electron microscopy to identify actin filaments in motile cells, and observations of cytoplasmic streaming associated with actin filaments in cell extracts and treatment of cells with cytochalasin, a natural product that inhibits actin polymerization and cellular motility. Collectively, this work in the late 1960s established the existence of actin in cells other than muscle and linked it to cellular movements.

PURIFICATION AND CHARACTERIZATION OF ACTIN FROM NONMUSCLE CELLS

The pioneering work that identified actin in nonmuscle cells was done independently by Sadashi Hatano working with Professor Fumio Oosawa in Nagoya, Japan, and Mark Adelman working with Professor Edwin Taylor at the University of Chicago. Both groups used the acellular slime mold *Physarum* as the source of actin. "Acellular" refers to the fact that these organisms are giant syncytia with millions of nuclei. *Physarum* was a logical choice, given its spectacular cytoplasmic streaming (Kamiya and Kuroda, 1973) and the ease of growing massive amounts of material to start the biochemical purification with more than 100 g of material. Their work in the 1960s was built on the isolation from *Physarum* of crude mixtures of proteins with some properties of striated muscle actomyosin by Ariel Loewy (1952) and others (Ts'o *et al.*, 1957; Nakajima, 1964) and from human platelets by M. Bettex-Galland and E. F. Luscher (1965). I will describe both purification methods to give readers a feeling for how challenging this work was 60 years ago without the benefit of SDS–polyacrylamide gel electrophoresis to monitor purity.

Hatano's work appeared first in 1966 and 1968 (Hatano and Oosawa, 1966; Hatano and Tazawa, 1968). He converted ~1 kg of slime mold into a dry powder by treatment with acetone and air drying followed by extraction with a low-salt buffer with the reducing agent cysteine—similar to the standard initial steps used to purify actin from skeletal muscle. He then added purified muscle myosin to precipitate actin filaments from the extract. A second cycle of acetone treatment and drying denatured the myosin, allowing

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Abbreviation used: ATP, adenosine triphosphate.
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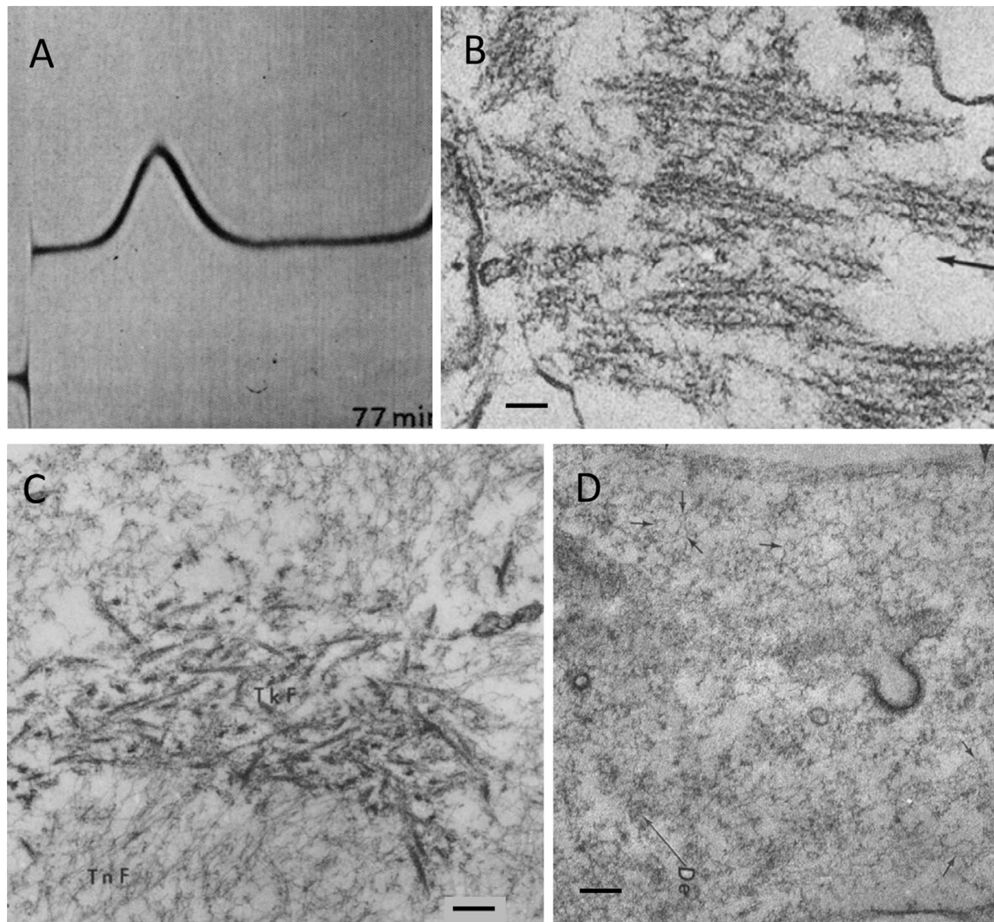

FIGURE 1: Data from the historic papers on the discovery of actin in nonmuscle cells. (A) Sedimentation velocity profile of purified slime mold actin (Hatano and Oosawa, 1966). (B, C) Transmission electron micrographs of thin sections. These images are from pdf files on journal websites, so some of the quality of the original micrographs was lost during digitization. (B) Actin filaments in an epithelial cell decorated with heavy meromyosin (Ishikawa *et al.*, 1969). (C) Actin filaments (TnF) and thick filaments (TkF) (likely myosin-II in a motile extract of *A. proteus* (Pollard and Ito, 1970). (D) Meshwork of filaments (arrows), likely actin filaments, in a cultured neuron (Yamada *et al.*, 1971). Scale bars are 100 nm.

~100 mg of actin monomers to be extracted with low-salt buffer with cysteine and to be concentrated by isoelectric precipitation at pH 4.7. The final step in the purification was careful precipitation with 15–35% ammonium sulfate.

Adelman and Taylor (1969a,b) devised a different method to purify *Physarum* actin. They concentrated crude actomyosin from a cell extract by precipitation with 45% saturated ammonium sulfate followed by gel filtration in a low-salt buffer with ATP to separate myosin in the void volume from actin monomers. One cycle of polymerization, pelleting, and depolymerization yielded ~50 mg of purified actin monomers from 100 g of slime mold.

Both Hatano and Adelman used sedimentation velocity analytical ultracentrifugation to assess purity (Figure 1A). *Physarum* actin monomers sedimented as a single peak at 3.3 S, like muscle actin monomers. Like muscle actin, *Physarum* actin polymerized in physiological salt solutions and depolymerized in dilute buffers as assessed by capillary viscometry. The Adelman preparation had a higher specific viscosity (viscosity/protein concentration), so it may have been somewhat purer. Adding muscle myosin to the actin filaments in a high-salt buffer made the solution very viscous, and addition of ATP lowered the viscosity by dissociating myosin from the actin filaments. *Physarum* actin filaments bound muscle myosin and activated its Mg-ATPase activity. Direct measurements showed that *Physarum* actin monomers bound ~1 molecule of ATP and hydrolyzed the γ-phosphate during polymerization into filaments ~5 nm in diameter. The amino acid composition of *Physarum* actin was very similar to that of muscle actin.

Both groups also purified from *Physarum* a myosin with properties similar to those of muscle myosins (Hatano and Tazawa, 1968; Adelman and Taylor, 1969b). Together, these seminal papers established the existence of actin and myosin in cells other than muscle. The properties of these cytoplasmic contractile proteins strongly suggested that they participate in cellular movements like their counterparts in muscle.

IDENTIFICATION OF ACTIN FILAMENTS IN NONMUSCLE CELLS BY ELECTRON MICROSCOPY

In parallel with these biochemical studies, Harunori (Hal) Ishikawa, a postdoc with Professors Lee Peachey and Howard Holtzer at the University of Pennsylvania, devised a simple method to identify actin filaments in electron micrographs of thin sections of muscle and nonmuscle cells (Ishikawa *et al.*, 1969). Ishikawa used a soluble proteolytic fragment of skeletal muscle myosin called heavy meromyosin to "decorate" actin filaments with arrowhead-shaped complexes first described by Hugh Huxley (1963). These arrowheads repeat with the turn of the actin helix and reveal the polarity of

each filament. Ishikawa treated cells and tissues with concentrated glycerol solutions to lyse the plasma membrane while preserving the filaments, an established method to isolate myofibrils from striated muscles.

Iskikawa's heavy meromyosin decoration method showed that nonmuscle cells have abundant actin filaments (Figure 1B) and opened up the study of cytoplasmic actin by the cell biology community. Many labs subsequently applied his method to identify actin filaments in a variety of cells and tissues. His method also revealed that muscle and nonmuscle cells have filaments ~10 nm in diameter that do not bind heavy meromyosin. He named them "intermediate filaments," because their diameters are intermediate between those of actin filaments and striated muscle thick filaments composed of myosin-II. Thus, this seminal paper defined two of the three cytoskeletal polymers

ACTIN FILAMENTS IN MOTILE CELL EXTRACTS

Robert Allen pioneered an orthogonal approach when he discovered that cytoplasm released from a giant amoeba into a confined space can continue to stream for an hour (Allen *et al.*, 1960). Lewis Wolpert and colleagues in London (Thompson and Wolpert, 1963; Wolpert *et al.*, 1964) extended this work. They chilled live *Amoeba proteus* cells and prepared crude cytoplasm by lysis and low-speed centrifugation. After adding ATP and warming to room temperature, the extract seemed to come to life with rapid streaming of organelles and some bulk contractions. Electron micrographs of thin sections of these extracts revealed short filaments ~12 nm wide and 500 nm long associated with some "spongy material" that they suggested might aggregate into the thicker filaments (Wolpert *et al.*, 1964).

As a medical student in 1968 I did experiments with Harvard Medical School cell biologist Susumu Ito that confirmed the movements in extracts of the giant amoeba *A. proteus* and implicated two types of filaments in these movements (Pollard and Ito, 1970). Fortunately, we were unaware that others had difficulties reproducing the Wolpert experiments, and after considerable trial and error, I observed dramatic movements of bulk cytoplasm outside the living cell. Our electron micrographs of thin sections of the amoeba extracts before and during the streaming movements showed that the spongy material reported by the Wolpert group consists of 5 nm thin filaments that assemble from soluble precursors, fill the entire extract, and interact with the thicker filaments (Figure 1C). Some thin filaments formed bundles, visible in real time by phase contrast microscopy, which interacted with clusters of the thicker filaments to produce movements in the extracts. Concurrent with assembly of the thin filaments, the extracts formed a gel. The idea that interactions between thin filaments of actin and thicker myosin filaments might produce cellular movements was so new that our department chair was initially skeptical that the filaments might be actin and myosin. (All of us were unaware of the pioneering biochemistry papers on actin, illustrating how siloed the biological research community was at the time.) Subsequently, decoration with heavy meromyosin confirmed that the thin filaments in the *A. proteus* extracts are actin (Pollard and Korn, 1971).

INHIBITION OF CELL MOVEMENTS WITH CYTOCHALASIN

The foregoing studies established the presence of actin filaments in motile cells and extracts but did not associate them with actual cellular movements. At the time neither genetic nor molecular genetic approaches were in use, so it was fortunate that Stephen Carter (1967) discovered a pharmacological approach. He reported in *Nature* that his colleague W. B. Turner at Imperial Chemical Industries Limited had isolated four related compounds from two species of fungi. Carter showed that these natural products inhibit cytokinesis by causing regression of the cleavage furrow and inhibit the motility of cultured L cells. He named these compounds cytochalasins for their "cell relaxing" activities.

Tom Schroeder, then a starting faculty member at Stanford University, linked the effects of cytochalasin to the disruption of thin filaments in the cytokinetic contractile ring. In electron micrographs of thin sections, he observed 5 nm thin filaments forming the contractile rings of jellyfish eggs undergoing their first cleavage (Schroeder, 1968) and reported in an abstract that cytochalasin B disrupts similar filaments in sea urchin eggs (Schroeder, 1969). Schroeder shared his findings with his Stanford colleague Norman Wessells, whose laboratory then set out to investigate how cytochalasins inhibit other cell movements.

Postdocs Ken Yamada and Brian Spooner in the Wessells lab found that cytochalasin B inhibited the movements of sensory nerve growth cones and glial cells in cultures of chick dorsal root ganglia (Yamada *et al.*, 1970, 1971; Spooner *et al.*, 1971). Motility resumed within hours of removing cytochalasin from the medium.

To examine how cytochalasin B alters these cells, they used state-of-the-art electron microscopy methods, fixing the cells with glutaraldehyde followed by osmium tetroxide before dehydration, embedding in plastic, and cutting thin sections (Figure 1D). Their method preserved microtubules and intermediate filaments beautifully (see Figures 3 and 11 in Yamada *et al.*, 1971). Their micrographs also showed polygonal latticeworks of short, 5 nm filaments in growth cones and at the leading edges of glial cells. These filaments associated with the plasma membrane and extended into cell protrusions called "microspikes," where the filaments tended to align along the long axis. Later work showed that osmium tetroxide, a strong oxidizing agent, fragments actin filaments even after they are cross-linked with glutaraldehyde (Maupin-Szamier and Pollard, 1978), so the short 5 nm filaments in Yamada and Spooner's thin sections were surely what was left after fixation of the cortical networks of branched actin filaments (Svitkina *et al.*, 1997). Yamada and Spooner also reported bundles of longer 5 nm filaments along the basal surfaces of the glial cells, which likely corresponded to stress fibers (Buckley and Porter, 1967), where associated proteins can protect against osmium tetroxide (Maupin-Szamier and Pollard, 1978).

Cytochalasin B caused the microspikes to collapse and the tips to axons to round up without compromising the appearance of microtubules, intermediate filaments, or bundles of 5 nm filaments in the thin sections. However, the latticeworks of 5 nm filaments collapsed into denser networks in which it was difficult to distinguish individual filaments. Thus, the effect of cytochalasin on the filaments was not completely clear.

Yamada *et al.* (1971) interpreted the latticework of 5 nm filaments as a contractile network and speculated that "contractility pulling the lateral sides of polygons toward one another could generate the elongated polygons observed in microspikes." The papers do not mention actin or cite the pioneering work by Hatano, Adelman, or Ishikawa.

In 1970 *Science* magazine published a long review of published and unpublished work from the Wessells lab on the effects of cytochalasin on microfilaments and cell movements in a wide range of cells (Wessells *et al.*, 1970). The review rightly credits Schroeder with discovering that cytochalasin disrupts the 5 nm filaments of the cytokinetic contractile ring (Schroeder, 1969). The review cites Hatano for "extraction of actin-like or actomyosin-like proteins from cells other than muscle" but seems not to have appreciated the powerful implications of the biochemical characterization of actin

and myosin by Hatano and Oosawa as well as Adelman and Taylor. The review cites Ishikawa's decoration of cellular actin filaments with heavy meromyosin but associates the decorated filaments with the bundles of 5 nm filaments in stress fibers that are resistant to cytochalasin. The review concludes that "little of substance can be said about the molecular bases of primitive contractile systems." From our current perspective, we now appreciate that the four lines of research covered here had already provided convincing evidence that actin filaments are responsible for cellular movements.

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