

Modulation of Contraction by Gelation/Solation in a Reconstituted Motile Model

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Abstract. The actin-based cytoskeleton is a dynamic component of living cells with major structural and contractile properties involved in fundamental cellular processes. The action of actin-binding proteins can decrease or increase the gel structure. Changes in the actin-based cytoskeleton have long been thought to modulate the myosin II-based contractions involved in these cellular processes, but there has been some debate concerning whether maximal gelation increases or decreases contractile activity. To address this question, we have examined how contractile activity is modulated by the extent of actin gelation. The model system consists of physiologically relevant concentrations and molar ratios of actin filaments (whose lengths are controlled by gelsolin), the actin-cross-linking protein filamin, and smooth muscle myosin II. This system has been

studied at the macroscopic and light microscopic levels to relate the gel structure to the rate of contraction. We present results which show that while a minimal amount of structure is necessary to transmit the contractile force, increasing the gel structure inhibits the rate of contraction, despite an increase in the actin-activated Mg^{2+} -ATPase activity of myosin. Decreasing the total myosin concentration also inhibits the rate of contraction. Application of cytochalasin D to one side of the contractile network increases the rate of contraction and also induces movement comparable to flare streaming observed in isolated amoeba cytoplasm. These results are interpreted relative to current models of the relationship between the state of gelation and contraction and to the potential effects of such a relationship in the living cell.

FOR over 150 years biologists have studied the correlation between gel-sol transitions of the cytomatrix and cell movement. Recognition of the structure of cytoplasm, changes in the consistency of this structure, and the relationship between these changes and contractility in whole cells led to initial theories of amoeboid movement (for reviews see Allen, 1973; and Taylor and Condeelis, 1979). The study of nonmuscle cell extracts extended the understanding of the relationship between gelation and contraction and stimulated the identification of critical proteins for these processes (Taylor and Condeelis, 1979; Stossel, 1982; Stossel et al., 1985, Pollard and Cooper, 1986). The identification of the proteins involved in gelation, solation, and contraction enabled the reconstitution of these processes in vitro. Reconstitutions allowed selected and controlled conditions to be defined in order to explore the molecular basis of the phenomenology.

Two major hypotheses emerged from the early experiments exploring the relationship between the state of gelation of the actin-based cytoplasmic gel and contractility. Taylor and colleagues (see Taylor and Fehcheimer, 1982) proposed that a decrease in the gel structure (solation) decreased the resistance of sliding actin filaments, thus increasing the rate of contraction. The solation could result from a decrease in actin filament lengths and/or a decrease in the

extent of cross-linking. However a minimal structure was proposed to be necessary for transmission of tension. This solation-contraction coupling hypothesis evolved from studies of cell extracts (Condeelis and Taylor, 1977) and led to a detailed discussion of the hypothesis (Taylor and Fehcheimer, 1982). In contrast, Stossel and colleagues (see Stossel, 1982) suggested that maximal gelation was necessary for maximal contraction and that solation decreased the efficiency of contraction relative to more gelled domains (Stendahl and Stossel, 1980; Stossel, 1982). Both models recognized the importance of the relationship between gelation and force generation. The amplifying versus inhibiting effect of gelation on contraction was the major difference between the two models.

We have reexamined the relationship between gel structure and contractility in a reconstituted contractile system. This system allows a controlled study of gelation and contraction upon changing the extent of gelation by varying the relative number of actin cross-links, the average actin filament length, and the relative amount of myosin II. We have used mainly smooth muscle proteins, whose activities are analogous to their nonmuscle counterparts, as a paradigm for the nonmuscle, cytoplasmic machinery. Additionally, we used the fungal metabolite, cytochalasin D, to create a gradient of solation. From these studies, we conclude that, while

some minimal structure is necessary for contraction, increased resistance against the contractile force due to increased gelation, relative to the force of myosin, leads to a slower and less complete contraction. The *in vitro* and *in vivo* data here and in the accompanying paper (Kolega et al., 1991) are consistent with the solution-contraction coupling hypothesis.

Materials and Methods

Protein Isolation and Preparation

Actin. Actin was purified by the method of Spudich and Watt (1971), lyophilized, stored at -20°C , and resuspended as needed. The actin was further purified by G-150 Sephadex (Sigma Chemical Company, St. Louis, MO) gel filtration (MacLean-Fletcher and Pollard, 1980a). The column-purified actin was concentrated by polymerization and centrifugation, resuspended, and dialyzed exhaustively in buffer A (2 mM Tris, pH 8.0 at 25°C , 0.2 mM CaCl_2 , 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.005% Azide), clarified, and then dialyzed versus actin MOPS buffer (10 mM MOPS, pH 7.2 at 25°C , 0.2 mM CaCl_2 , 0.2 mM ATP, 0.1 mM DTT, 0.005% Azide). The concentration of the G-actin was determined using an extinction coefficient of 0.62 OD/mg/ml for the absorbance at 290 nm less the absorbance at 320 nm to correct for light scattering, i.e., $\text{mg/ml} = (\text{A}_{290} - \text{A}_{320})/0.62 \text{ OD/mg/ml}$.

Plasma Gelsolin/Fx-45 Fragment. Plasma gelsolin (brevin) was purified using a modified protocol of Cortese and Frieden (1988). In our hands the addition of 25% ammonium sulfate produced little or no precipitation and was, therefore, discontinued. In addition, for the second DEAE-Sepharose (Whatman Biosystems, Ltd., Maidstone, England) column we used two-step elutions. 75 mM NaCl was used to eliminate a major and several minor contaminating proteins. 300 mM NaCl was used to elute pure gelsolin. The clone for the calcium-independent fragment of gelsolin (Fx-45 fragment) was kindly provided by Dr. Helen Yin (University of Texas Southwestern Medical Center at Dallas, Dallas, TX) and was expressed and purified as instructed by Dr. Yin. Immediately before use the gelsolin/fragment was dialyzed versus gelsolin MOPS buffer (10 mM MOPS, pH 7.2 at 25°C , 100 mM NaCl, 0.1 mM EGTA, 0.1 mM DTT, 0.005% Azide, 1 mM PMSF) and the concentration and final activity determined. The concentration of plasma gelsolin and the Fx-45 fragment was determined either by the Bradford technique (Pierce Chemical Co., Rockford, IL) or by absorbance at 280 nm, using an extinction coefficient of 1.5 OD/mg/ml for the whole molecule (H. Yin, personal communication). The actin severing activity of gelsolin or the Fx-45 fragment was monitored throughout the purification and before final storage/use by the DNase I assay of Harris et al. (1982).

Filamin. Filamin was purified using a modified procedure of Wang (1977). In our hands, difficulties with the DEAE-Sepharose (Whatman Biosystems, Ltd., Maidstone, England) column step, necessitated a change to P11 phosphocellulose (Whatman Biosystems, Ltd., Maidstone, England) using a filamin phosphocellulose buffer (10 mM MOPS, pH 7.2 at 25°C , 20 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.005% Azide). When further concentration of the filamin was necessary, we used P11 phosphocellulose equilibrated in filamin phosphocellulose buffer, using 2 ml of P11 phosphocellulose per mg of filamin. After application and washing steps, the filamin was eluted with 600 mM NaCl and stored on ice until use, usually within two to four weeks. Immediately before use in the reconstitution studies, selected filamin fractions were dialyzed against filamin MOPS buffer (10 mM MOPS, pH 7.2 at 25°C , 200 mM NaCl, 1 mM EDTA, 0.1 mM DTT, 0.005% Azide). Filamin concentration was determined by absorbance at 280 nm using an extinction coefficient of 0.74 OD/mg/ml or by the Bradford technique (Pierce Chemical Co.). Throughout purification, storage, and before any contraction assay, the activity of the filamin was assayed using the technique of falling ball viscometry (MacLean-Fletcher and Pollard, 1980b; Rockwell et al., 1984) as follows: for each sample tested, filamin was mixed with 0.1 to 0.3 mg/ml Spudich & Watt G-actin at actin/filamin molar ratios of 50:1-300:1 and no filamin. The mixture was made 100 mM KCl and 2 mM MgCl_2 , mixed, and at least three 100 μl test samples were immediately drawn into glass capillary tubes. The capillaries were incubated at 28°C for 1 h and η_{app} determined as has been previously described (MacLean-Fletcher and Pollard, 1980b).

Myosin and Calcium-independent Myosin Light Chain Kinase. Myosin was purified using the protocol of Sellers et al. (1981) and stored in myosin MOPS buffer (10 mM MOPS, pH 7.2 at 25°C , 50 mM NaCl, 1 mM EGTA, 1 mM MgCl_2 , 0.1 mM DTT, 0.005% Azide). Myosin concentration was determined by absorbance at 280 nm using an extinction coefficient

of 0.53 OD/mg/ml. A calcium/calmodulin-independent myosin light chain kinase (IMLCK)¹ was kindly provided by Drs. Trudy Cornwell, Jim Sellers, and Robert Adelstein at National Institutes of Health (Bethesda, MD).

Other Materials. ATP was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Adenosine 5'-triphosphate, tetra(triethylammonium) salt, [γ -³²P] was purchased from New England Nuclear/DuPont (Wilmington, DE). All other chemicals and reagents were obtained from Sigma Chemical Company or Fisher Scientific (Pittsburgh, PA).

Production of Actin Filaments with Defined Lengths

Average actin filament length was controlled to create a homogeneous sample with a minimum of artifacts associated with a broad length distribution, including unknown cross-linking number, and regional inhomogeneities of gelation (Simon et al., 1988; Cortese and Frieden, 1988). Additionally, uncontrolled filament lengths could dramatically alter the viscosity and network structure of the system (reviewed in Stossel, 1982). Therefore, a relatively tight distribution of actin filaments with a defined average length was produced by addition of either the well-characterized, actin-severing protein, plasma gelsolin (brevin), or the amino-terminal, calcium-independent fragment of plasma gelsolin (Fx-45).

Theoretically, one gelsolin molecule severs one actin filament and remains bound to the "barbed" or "positive" end of the resulting filament fragment. Therefore, a molar ratio of one gelsolin to 366 actin subunits should result in filaments 1 μm in length. However, experimental studies of this effect do not agree exactly with calculated values based on this theoretical activity (Nunnally et al., 1981; Coleman and Mooseker, 1985; Janmey et al., 1986). Discrepancies may be due to a fraction of inactive gelsolin, the method of mixing, and/or changes in filament lengths during incubations and manipulations, including the method used for observation and measurement. We, therefore, defined the empirical relationship between different molar ratios of our gelsolin and actin in our hands, using the same addition and mixing protocols and similar observation and measurement methods as used in the complete reconstitution studies. The effect of severing on filament length was defined as follows: rhodamine-phalloidin in methanol (Molecular Probes, Eugene, OR) was air evaporated and then resuspended in buffer A. Column-purified actin was then added to give a final actin concentration of 0.5 mg/ml and a 2:1 rhodamine-phalloidin:actin ratio. The solution was made of 100 mM KCl and 2 mM MgCl_2 and allowed to polymerize overnight. Gelsolin and calcium were added at actin:gelsolin molar ratios of 50:1-400:1, vortexed lightly, and incubated at room temperature for 1 h. A gelsolin minus control was also prepared. Control experiments showed that gelsolin retained full function with actin filaments prelabeled with phalloidin (data not shown). The labeled actin was diluted to a final concentration of 10 nM to allow imaging of individual, rhodamine-phalloidin, actin filaments. The filaments were imaged with a universal microscope (Zeiss, Thornwood, NY) equipped with a 40 \times Planachromat lens (Carl Zeiss, Inc., Thornwood, NY) and an ISIT camera (Dage-MTI, Inc., Michigan City, IN) and recorded and processed with a Vicom Image Processor (Vicom Corporation, Inc., San Jose, CA). In addition, a micrometer was imaged under the same optical conditions.

Each filament in representative fields was measured and scored to determine the empirical filament length distribution at each specific actin:gelsolin ratio (Fig. 1). The number average length was plotted for each ratio to determine a mathematical relationship between actin:gelsolin molar amounts and length in micrometers (Fig. 2). We note that the values obtained in this study do not agree with theoretical values. However, the results are comparable to previous studies by other groups (Nunnally et al., 1981; Coleman and Mooseker, 1985; Janmey et al., 1986) and are consistent with the linear relationship characteristic of actin and gelsolin mixtures (Stossel, 1982; Janmey et al., 1986; Zaner and Hartwig, 1988). Most importantly, these results (a) verify that a narrow distribution of filament lengths is generated when compared to the broad distribution and aphysiological lengths of column purified actin (Hartwig and Stossel, 1979; Nunnally et al., 1981; Simon et al., 1988), and (b) define the empirical average length resulting from this mixing and incubation protocol.

In Vitro Reconstitution of Contractile Network

Strategy of Additions and Final Conditions. The molar ratios of the constituent proteins were an important consideration while developing the reconstituted system in order to prepare a model reflecting conditions *in vivo*. However, regional concentrations and ratios of the proteins have not

1. *Abbreviation used in this paper:* IMLCK, independent myosin light chain kinase.

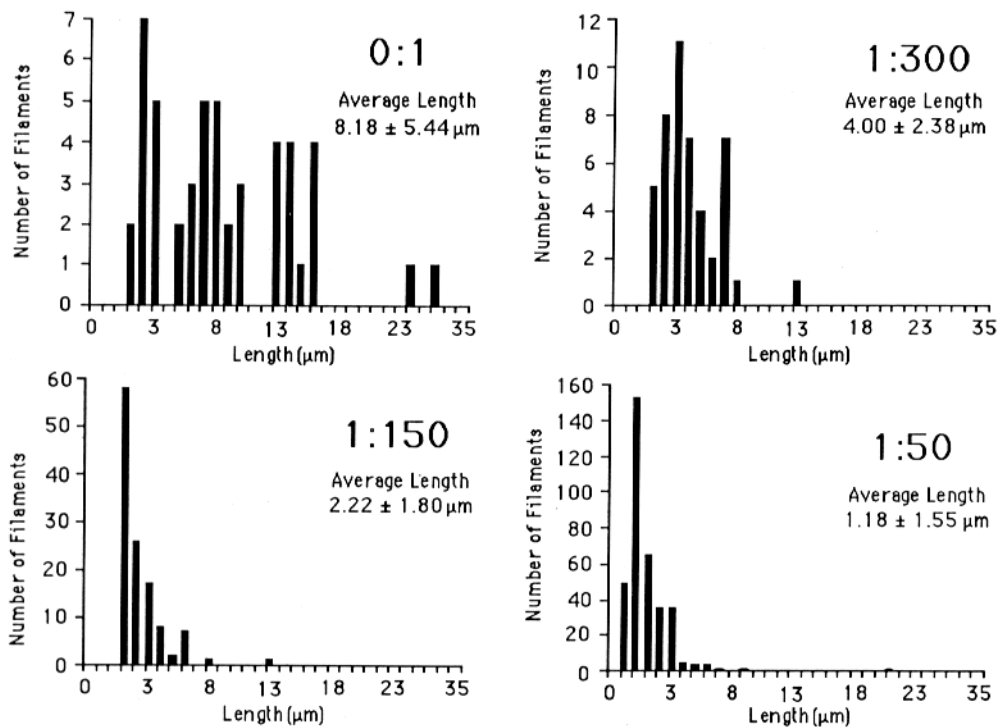


Figure 1. Actin filament length distribution in presence of varying actin/gelsolin molar ratio. Actin filament lengths were determined as described in Materials and Methods for actin/gelsolin molar ratios of 50:1, 150:1, 300:1, 400:1 (not shown), and no gelsolin. Number average length was used to determine relationship between gelsolin/actin ratio and measured actin filament length.

been strictly determined within nonmuscle cells. Additionally, biochemical considerations and stock concentrations were a limiting factor in achieving proper molar ratios of the constituent proteins. We, therefore, designed the system approximating average concentrations of these proteins determined from cell populations (Table I). The final actin concentration was 1.5 mg/ml, a value consistent with concentrations found in cell extracts and which technically permitted relevant concentrations of the other components. Gelsolin was added to obtain desired actin filament lengths, usually 1.5 μm . This length was based on reports which suggest a physiological range of ~ 0.2 –13 μm in nonmuscle cells (Stossel, 1982; Fath and Lasek, 1988; Hartwig and Yin, 1988; Podolski and Steck, 1990). We used a range of actin:filamin molar ratios of 50:1–400:1 that were consistent with measured values and covered the range of gel states that a motile cell might experience. Myosin has been reported to be present at molar ratios from approximately 24:1 to 79:1 actin/myosin; we normally used a ratio of 50:1. All experiments were performed with fully phosphorylated, 20-kD regulatory light chains of myosin so that the role of other regulatory or modulating factors could be tested.

Final buffer conditions of the reconstituted system were also chosen as a balance between the presumed environment of a mammalian cell and

known optimal biochemical environments for the constituent proteins. All components of the system were dialyzed into buffers containing 10 mM MOPS for reconstitution. The pKa of MOPS is 7.2 at room temperature, thereby offering optimal buffering capacity within the physiological range (7.0–7.4) (Bright et al., 1987). We chose a final KCl concentration of 50 mM in all assays, such that Mg^{2+} -ATPase activity was at or near its maximum value (Kron and Spudich, 1986; Janson, data not shown). Since MgCl_2 concentrations of 1–2 mM are optimal for actin polymerization, we maintained these concentrations throughout the reconstitution protocol. NaCl concentrations were maintained within 70–80 mM. CaCl_2 concentration was maintained below micromolar levels. 1 mM Mg^{2+} -ATP provided more than sufficient levels for the time of the assays based on the measured actin-activated Mg^{2+} -ATPase activity of the myosin.

Reconstitution Protocol. Our method of handling each protein, each combination of proteins, and the final network was developed to maintain the normal activity of each protein throughout the mixing process while resulting in the desired final ionic and chemical conditions which are suitable for the proteins and which approach the *in vivo* environment. Briefly, actin was polymerized overnight at room temperature at twice the desired final concentration with 100 mM KCl and 2 mM MgCl_2 . Gelsolin and calcium were added at one-tenth the actin volume to yield a predetermined, controlled average filament length. The amount of calcium added provided 2 mol of calcium per gelsolin, calcium to offset the EGTA in the gelsolin MOPS buffer, and additional calcium to provide a calcium concentration of 200 nM in the final reconstituted mixture. The activated gelsolin was incubated with the actin for 1 h at room temperature as described above and cooled on ice. Cooling inhibits gelation (Pollard and Ito, 1970; Stossel and Hartwig, 1976; Pollard, 1976; Kane, 1983) of cellular extracts or reconstituted mixtures and decreases myosin ATPase/contractile function (Thompson and Wolpert, 1963; Pollard and Ito, 1970; Stossel and Hartwig, 1976; Pollard, 1976; Condeelis and Taylor, 1977; Kane, 1983). Myosin and filamin solutions were also cooled immediately before addition. This cooling allowed addition and mixing of myosin and filamin with minimal premature gelation or contraction.

Unphosphorylated myosin filaments were solubilized with Mg^{2+} -ATP. Calcium-IMLCK was added at 50:1 or 100:1 myosin/IMLCK ratio and incubated at room temperature for 30–60 min. During this time, the mixture became somewhat turbid, indicating phosphorylation and the formation of filaments. Glycerol/polyacrylamide gels of this myosin showed full phosphorylation of the 20-kD regulatory light chains by this method (data not shown). At the end of the incubation, additional myosin MOPS buffer, MgCl_2 , and CaCl_2 were added to give the desired stock myosin concentration, calcium to bind EGTA from the added buffer, and 10 mM Mg^{2+} -ATP as well as additional MgCl_2 to maintain actin polymerization (see below).

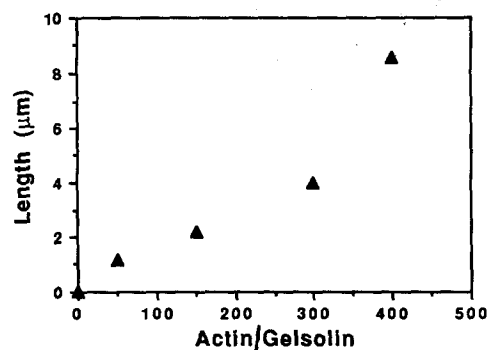


Figure 2. Average actin filament length versus gelsolin/actin molar ratio. Relationship between actin/gelsolin ratio and average filament length was used to generate a desired average filament length with a relatively narrow distribution. Control experiments showed that gelsolin retained full function with actin filaments prelabeled with phalloidin (data not shown). See text for further discussion.

Table I. Concentrations and Stoichiometric Ratios of Actin and Selected Actin-binding Proteins

Source	Actin	Filamin/ABP	Myosin	Gelsolin
Rabbit pulmonary macrophage (ingesting) (Stossel and Hartwig, 1976)	12.58 mg/ml	1.72 mg/ml (7:1)	1.91 mg/ml (7:1)	ND
Acanthamoeba (Pollard, 1976)	1.34 mg/ml	0.04 mg/ml (36:1)	0.13 mg/ml (11:1)	ND
Dictyostelium (S3 extract) (Condeelis and Taylor, 1977)	8.6 mg/ml	0.85 mg/ml (118:1)	2.8 mg/ml (34:1)	ND
Sea urchin (Kane, 1980)	~1.1 mg/ml	ND	6.5 mg/ml (50:1)	ND
Phagocytic leukocytes (Stossel, 1982)	ND	(120:1–143:1)	(79:1)	(50:1)
This study	1.5 mg/ml	(50:1–400:1)	(50:1)	(25:1–1000:1)

Concentration values are for whole tissue or extract. Molar ratios are presented as moles actin/moles selected actin binding protein. *ND*, not done.

Stock solutions of myosin and filamin were prepared as follows: myosin was diluted into myosin dilution buffer containing 10 mM MOPS, pH 7.2 at 25°C, 50 mM NaCl, 10 mM ATP, 15 mM MgCl₂, 1 mM EGTA, 1 mM CaCl₂, 0.1 mM DTT, 0.005% Azide. Filamin was diluted with filamin MOPS buffer. The diluted myosin and filamin were added in quick succession to give the desired actin to myosin and actin to filamin molar ratios. All ratios presented in text and figures are molar ratios of the noted components. The volume of myosin added was always 10% of the final volume; the volume of filamin represented 35% of the final volume. This was accomplished by diluting protein stocks but maintaining the same relative volumes of actin in actin MOPS buffer, gelsolin/calcium in Gelsolin MOPS buffer, myosin/IMLCK in myosin MOPS and myosin dilution buffer, and filamin in filamin MOPS buffer. Strict control of individual and combined protein environments assured that the assays were performed with the same defined ionic strength, concentration of components, and state of activity. The final reconstitution buffer conditions were 10 mM MOPS, pH 7.2 at 25°C, 50 mM KCl, 2 mM MgCl₂, 70–80 mM NaCl, 200 nM CaCl₂, 1 mM Mg²⁺-ATP, 0.1 mM DTT, 0.005% Azide.

Assays of Gelation and Contraction. Reconstitution mixtures in test tubes (0.5 ml final volume) allowed macroscopic tests of the relationship between gelation and contraction, thereby relating our results to previous experiments with cell extracts and *in vitro* reconstitutions (Rockwell et al., 1984). Final mixtures were vortexed lightly (three one second pulses at low power) and placed in a 28°C water bath, adapted so as to hold each test

tube firmly to eliminate any movement. At desired time points, two test tubes were carefully taken from the water bath, examined for contraction (see below) and assigned a qualitative score of gelation by the tube tipping assay as described in Rockwell et al. (1984) (Fig. 3 and Results). Gel state (+) is a slightly viscous fluid which flows readily, (++) is a cohesive mass, which flows slowly, (+++) is a solid, nonflowing mass which can support its own weight. Final gelation state was reached within 1 to 2 min of mixing and always preceded contraction by at least 5 min (except see Fig. 5, 200:1 sample—see figure legend). Test tubes were assayed only once and were not used further.

Reconstitution mixtures used for microscopic examination of contraction (<0.1 ml final volume) were identical to test tube assay preparations, except for the inclusion of 1.4 μm polystyrene latex beads which allowed easier monitoring of the contraction using darkfield microscopy. Control experiments using solutions without beads showed no difference in time to contraction. All buffers used in the microscopic system were degassed to reduce the formation of air bubbles in the mixture during warmup. The final mixture was quickly but gently pipetted into a microscopic chamber (0.9 × 0.7 × 0.053 cm) made of a glass slide, 0.53-mm silastic spacers (Dow Corning Corp., Midland, MI), and a glass coverslip. The sample was sealed in the chamber using vacuum grease and quickly placed on the microscope stage. Total time between sample mixing and the initial image acquisition was usually 45 s or less.

Experiments using a gradient of cytochalasin D used the same micro-

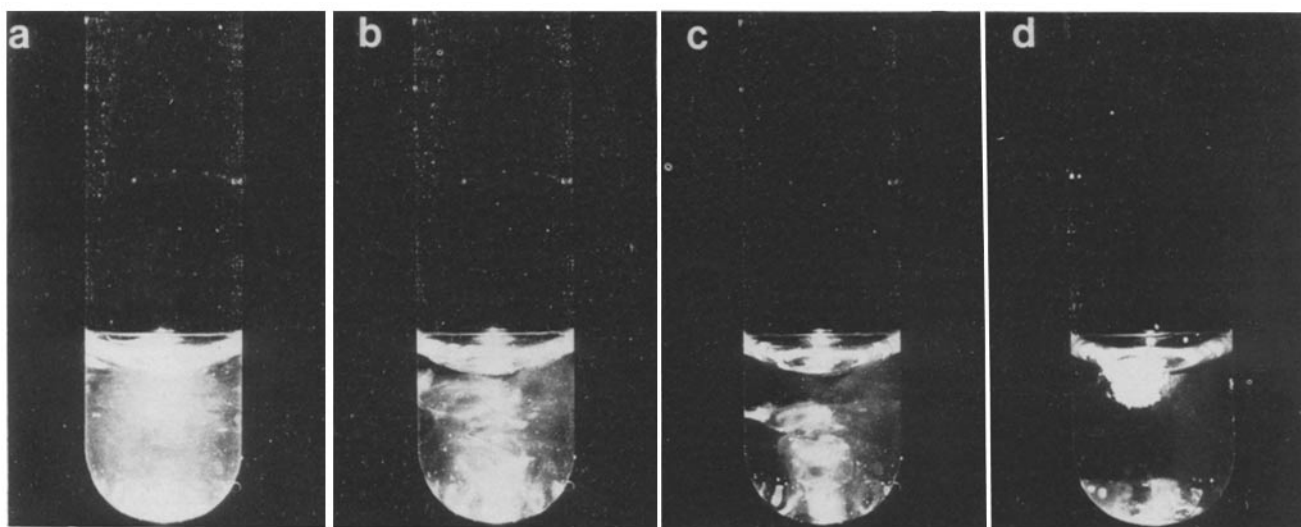


Figure 3. Macroscopic contraction of the reconstituted system. Homogeneous mixture (a) gradually pulls away from bottom and sides of test tube (b and c) until reaching the fully contracted state (d). Contracted masses were almost always found at the top of the mixture and were surrounded by a (+) fluid (see Materials and Methods). Total time for the above sequence was ~15 min.

scopic assay system as previously described with only minor changes. The freshly mixed reconstitution system was gently pipetted into a microscopic chamber $0.9 \times 0.7 \times 0.1$ cm in dimension until $\sim 90\%$ of the chamber was filled. The chamber was then sealed on one end with vacuum grease and allowed to incubate at room temperature for 2.5 min to allow full gelation. After incubation $50 \mu\text{M}$ cytochalasin D (Sigma Chemical Company) in the final reconstitution buffer (10 mM MOPS, pH 7.2 at 25°C , 50 mM KCl, 2 mM MgCl_2 , 70–80 mM NaCl, 200 nM CaCl_2 , 1 mM Mg^{2+} -ATP, 0.1 mM DTT, 0.005% Azide) at 28°C was added to the open end of the chamber with a Hamilton syringe. Total volume of cytochalasin D added was $\sim 10\%$ of the final volume in all cases. The remaining open end of the chamber was then sealed with vacuum grease and the sample monitored at 15-s intervals for 30 min as described below. Control samples had final reconstitution buffer (at 28°C) without cytochalasin D added to the open end of the chamber. Great care was taken throughout the preparation of all microscopic assay samples to not jar the assay chamber or disturb the assay sample.

Microscopic samples were monitored by darkfield microscopy using a microscope (SZH-ZB Zoom Stereo; Olympus Corporation, Overland Park, KS) equipped with a IX DF Plan objective and an NFK $2.5\times$ photo eyepiece. Final magnification with adjustments to the zoom magnification knob was $\sim 37.5\times$. Images were acquired with a camera (C-2400 CCD; Hamamatsu Photonics K.K., Hamamatsu City, Japan) and stored on optical discs. Images included at least 90% of the chamber area and were acquired at 15-, 30-, or 60-s intervals for 30 to 40 min. During acquisition time, samples were maintained at 28°C using an air curtain with a temperature feedback control as described in Bright et al. (1987). Image sequences were analyzed either using the Biovision Image Processor or a MacIntosh-based AOTools time lapse program (Perceptics Corp., Knoxville, TN) with our own modifications. After image acquisition, the sequence of images was examined to determine the time to contraction of the mixture (see below). This microscopic system allowed a closer examination of the structure of the reconstituted mixtures during all phases of the contraction and a more continual temporal examination of the contraction.

General Description of Contractions/Time to Contraction. The *in vitro* reconstituted system enabled the study of reproducible contractions with the ability to modulate protein concentrations to change motor activity, gel structure, and filament lengths. These contractions could be monitored visually at the macroscopic level in 10×75 mm test tubes or microscopically using darkfield optics. Test tube experiments usually started as homogeneous samples. However, samples with high filamin concentration often showed some macroscopic bundling which could be clearly and easily distinguished by appearance from contractions. As contraction proceeded, the gelled sample pulled away from the sides and bottom of the test tube, forming a central opaque mass (Fig. 3). This mass continued to condense to a final, unchanging, contracted state which was usually found at the top of the mixture. Time to contraction for bulk assays indicates elapsed time to reach this fully contracted mass (Fig. 3 *d*). Qualitative assessment of the size of contracted masses showed that samples with more filamin (i.e., increased gel state) ended in larger masses than samples with less filamin.

When viewed in the microscopic chamber, reconstituted gels usually began as a homogeneous network which condensed into one or more masses. Microscopic examination also showed bundling at high filamin concentration and with long filament lengths. Reconstituted mixtures condensed isotropically to a contracted state, representing 90% or more of the final mass, sometimes followed by nonisotropic contractions. Time to contraction of microscopic images was determined as the elapsed time from the initial mixture to the cessation of all isotropic contractions. This contracted state closely resembled the contracted mass seen in the bulk assay (see Fig. 3 *d*). Like the test tube contractions, samples with higher cross-linking or longer average filament lengths did not contract into as tight a mass as samples with lower cross-linking or shorter filaments.

Assay of Mg^{2+} ATPase Activity. Mg^{2+} -ATPase activity was determined under the same conditions as the reconstitution assays in test tubes using radioactive ATP by the method of Sellers et al. (1981). Assays were done in duplicate. Activity is reported relative to the 200:1 actin/filamin for each assay series to correct for changing filamin and myosin activity.

Results

Changing Myosin Concentration

The effect of changing myosin concentration on the rate of contraction was explored. Throughout these tests we main-

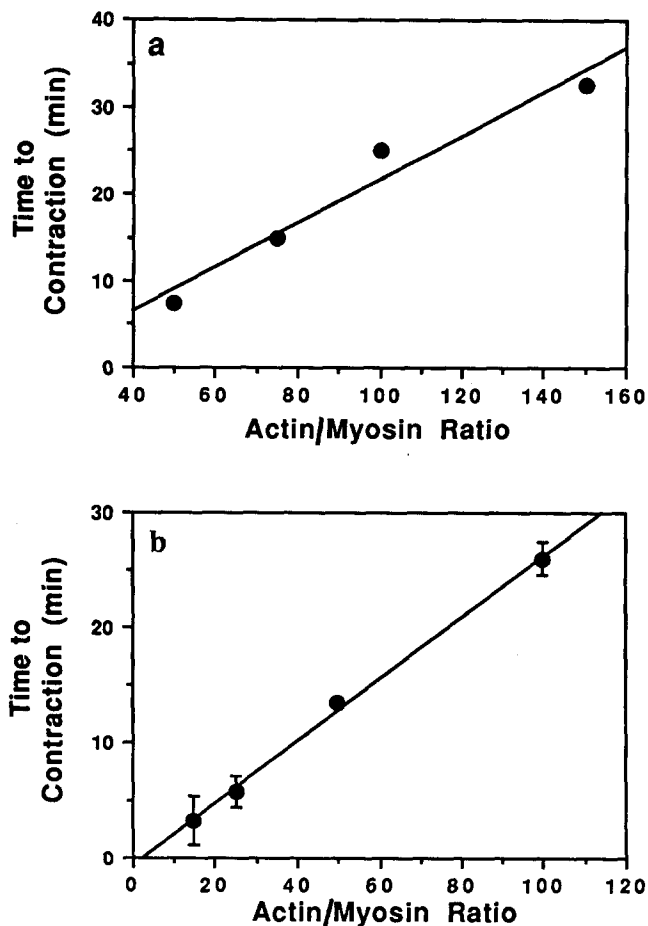


Figure 4. Effect of changing actin/myosin molar ratio on rate of contraction. (*Top*) Macroscopic assay. Samples with actin/myosin molar ratios of 50:1, 75:1, 100:1, and 150:1 were examined at 28°C using the macroscopic assay as described in Materials and Methods. All samples had a 100:1 actin/filamin ratio and an actin/gelsolin ratio of 91:1 to yield an average actin filament length of $1.5 \mu\text{m}$. Samples with no myosin showed no signs of contraction up to 90 min (not indicated). (*Bottom*) Microscopic assay. Microscopic samples with actin/myosin ratios of 15:1, 25:1, 50:1, and 100:1 were examined at 28°C for up to 35 min using the microscopic assay as described in Materials and Methods. All samples had a 100:1 actin/filamin ratio and an actin/gelsolin ratio of 91:1 to yield an average actin filament length of $1.5 \mu\text{m}$. Time to final contracted state for multiple samples was determined. Error bars represent ± 1 SD.

tained constant actin filament number and length, using 1.5 mg/ml actin with an average filament length of $1.5 \mu\text{m}$, and an unchanged cross-linking number, using an actin/filamin molar ratio of 100:1 in all samples. Our results showed a direct relationship between the amount of myosin and the time to complete contraction, since samples with higher concentrations of myosin contracted faster than those samples with less myosin (Fig. 4 *a*). The addition of unphosphorylated myosin produced no contraction. Additionally, control experiments with no myosin also yielded no contraction and gels with weaker gel structure as measured by the tube tipping assay (Rockwell et al., 1984; and data not shown). We re-examined this effect with the microscopic system using the same conditions as the test tube assay. We found that increas-

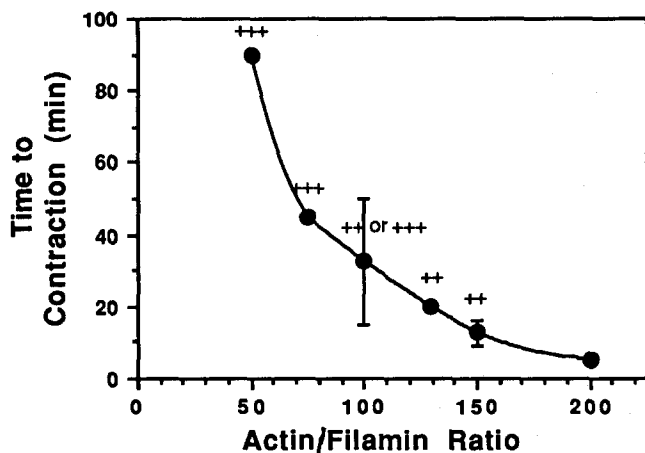


Figure 5. Effect of changing actin/filamin molar ratio on rate of contraction—macroscopic assay. Samples with actin/filamin ratios of 50:1, 100:1, 129:1, 150:1, 200:1, and no filamin were examined at 28°C for contraction and gel state at 5, 10, 20, 30, 45, 60, and 90 min after mixing using the macroscopic assay as described in Materials and Methods. All samples had a 50:1 actin/myosin ratio and an actin/gelsolin ratio of 91:1 to yield an average actin filament length of 1.5 μm . Times indicated are from one assay or the average of multiple assays. Gel state above sample point indicates the state of the mixture before contraction. All samples had reached their final gel state before contraction. However, the 200:1 sample was contracted before the first time point and was, therefore, not scored for gel state. 200:1 control samples without myosin or with unphosphorylated myosin were always (+). Samples without filamin were not contracted as of 90 min (not indicated). Error bars represent ± 1 SD.

ing the concentration of fully phosphorylated myosin II, with a fixed amount of actin and filamin, resulted in higher rates of contraction of the reconstituted system (Fig. 4 *b*). Under the conditions of this assay, an actin/myosin ratio of 200:1 did not cause complete contraction by 40 min. Microscopic assay samples with no myosin or with unphosphorylated myosin also showed no signs of contraction for up to 40 min (data not shown). These results indicated that some minimal level of myosin II force generation is necessary to overcome the resistance of the gelled network.

Changing the State of Gelation

We used the *in vitro* reconstitution system to examine how changing the gel structure of the network affected the rate of contraction. We varied gelation by changing cross-linking and filament length, two parameters known to modulate gel structure (see Discussion).

Changing Cross-linking Number. When we increased the strength of the gel in the reconstituted network by increasing the total filamin concentration, a longer time was required to complete contraction (Fig. 5). Time to contraction for macroscopic assays varied from <5 min (200:1 actin/filamin ratio) to 90 min (50:1 actin/filamin ratio). Samples with no filamin and, therefore, no gel structure did not contract even 90 min from the time of addition of myosin. All samples contained a 50:1 actin/myosin molar ratio and 91:1 actin/gelsolin molar ratio, yielding an average filament length of 1.5 μm (see Materials and Methods above). Measures of gelation, scored as described in Materials and Methods, showed expected in-

creases with increasing filamin. Therefore, there is a minimal level of gel structure that is required for a macroscopic contraction of the reconstituted system. We also measured the actin-activated, myosin Mg^{2+} -ATPase activity of identical reconstituted mixtures in order to compare the enzymatic activity with the shortening of the sample. These measurements showed that the Mg^{2+} -ATPase activity of the myosin increased with increased cross-linked structure (Fig. 6), despite the fact that the more highly gelled sample took longer to contract.

Microscopic assays also showed a direct relationship between increasing the gel structure and increased time for contraction (Fig. 7). As seen in the macroscopic assays, higher concentrations of filamin led to slower contractions than samples with less filamin. At high filamin concentrations (actin/filamin molar ratio of 75:1, 60:1, and 50:1) the contractions were not completed within the 35-min assay time. We also noted a transformation to a partially anisotropic or bundled network at molar ratios of 100:1–50:1 actin/filamin (Rockwell et al., 1984; Simon et al., 1988; Hou et al., 1990). The microscopic assay also allowed a closer examination of the lower limit of gel structure which still supported contractile activity in the test tube assays (200:1 actin/filamin down to no filamin). In this range, contractions often did not involve the entire sample, but consisted of smaller more localized areas of the sample. These smaller contractions were still noted in samples with as little as a 400:1 actin/filamin molar ratio but the samples with no filamin (1:0 actin/filamin) showed no signs of contraction up to the time limit of observation. These results clearly showed that some threshold amount of gel structure was necessary to connect the entire sample in order to propagate a full contraction across the network. However, above this threshold level, increasing filamin concentrations progressively slowed the time to contraction.

Changing Filament Length. Stossel and colleagues have

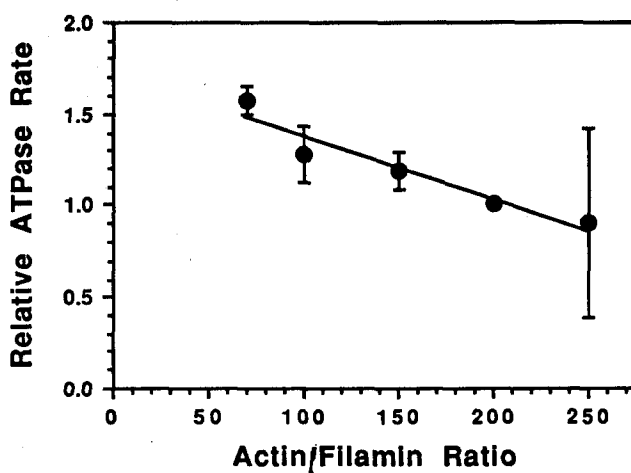


Figure 6. Effect of changing actin/filamin molar ratio on actin-activated Mg^{2+} -ATPase. Actin-activated Mg^{2+} -ATPase activity was determined as described in Materials and Methods for duplicate macroscopic samples with actin/filamin ratios of 75:1, 100:1, 150:1, 200:1, and 250:1. Rates were normalized to 200:1 sample for each assay series. All samples had a 50:1 actin/myosin ratio and an actin/gelsolin ratio of 91:1 to yield an average actin filament length of 1.5 μm .

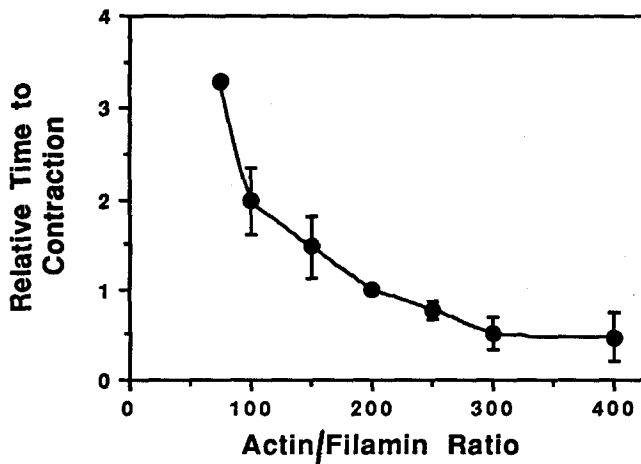


Figure 7. Effect of changing actin/filamin molar ratio on rate of contraction (microscopic assay). Samples with actin/filamin ratios of 50:1, 60:1, 75:1, 100:1, 150:1, 200:1, 250:1, 300:1, 400:1, and no filamin were examined at 28°C for up to 35 min using the microscopic assay as described in Materials and Methods. All samples had a 50:1 actin/myosin ratio and an actin/gelsolin ratio of 91:1 to yield an average actin filament length of 1.5 μm . Time to contraction was determined for complete condensation of the mixture into a contracted mass. These data are from several assays on multiple days. They were normalized to the time to contraction for the 200:1 sample for that day to account for varying filamin and/or myosin activities. 50:1 and 60:1 samples were not completely contracted after 35 min (not indicated). The samples with no filamin showed no signs of contraction up to 35 min, and are not indicated. Error bars represent ± 1 SD.

shown that increasing the average actin filament length with a fixed number of cross-links leads to an increase in the gel structure (reviewed in Stossel, 1982). We used the microscopic system to examine the effect of changing the gel structure, by changing the average actin filament length. The number of cross-links remained constant. Actin/Fx-45 molar ratios of 25:1, 100:1, 1000:1, and no Fx-45 were chosen. Actin/myosin and actin/filamin ratios of 50:1 and 150:1, respectively, were maintained in all samples. Increased gelation, generated by increasing the length of actin filaments, but maintaining a constant amount of cross-linking, inhibited the contraction (Fig. 8). Therefore, increased gel structure by increased filament length also slowed time to contraction.

Gradient of Cytochalasin D. Cytochalasin D was used as a tool for inducing a gradient of solation of gelled actin (Stossel and Hartwig, 1976; Pollard, 1976; Condeelis and Taylor, 1977). The mechanism of solation has been discussed in detail (Cooper, 1987). The addition of cytochalasin D to one end of the microscopic assay chamber created a gradient of solation and allowed simultaneous observation of the sample at different stages of solation. The gradient of solation led to two noted effects on the contraction of the system. The end of the sample with the highest cytochalasin concentration and initial solation showed increased rates of contraction in comparison to the opposite or low cytochalasin end (Fig. 9). Therefore, the contracting mass moved from the site of solation towards the more gelled side. As the rate of contraction at the end of the chamber with high cytochalasin concentration increased, a phenomenon similar in ap-

pearance to flare streaming of isolated amoeba cytoplasm was created (Allen, 1973; Taylor et al., 1973) (Fig. 9). The contracting mass formed a central structure near the high cytochalasin end, reminiscent of the plasmagel sheet seen in amoeboid movement (Taylor et al., 1980a), which moved towards the low cytochalasin end of the chamber. As the contraction proceeded across the chamber it caused a displacement of material to the sides and back towards the high cytochalasin end of the chamber. This displacement was reminiscent of the flare streaming in single cell models (Taylor et al., 1973). Control samples with only buffer contracted but showed no effect on either rate or direction of contraction and did not display flare streaming. Therefore, the solation of the gel by shortening actin filaments with cytochalasin D accelerated the contraction and created a less structured phase that could be displaced by the contracting mass and which exhibited flare streaming.

Discussion

Design of a Model to Study the Structural and Contractile Properties of Cytoplasm

The cytomatrix is the poorly characterized material that contains and surrounds the organelles of a living cell. Studies of cytoplasm from single cells and cell extract models have established the concept that the cytomatrix is not a simple, dilute, aqueous solution, but a concentrated, three-dimensional protein matrix that fills the cytoplasmic volume (Allen, 1973; Taylor and Condeelis, 1979; Luby-Phelps et al., 1988). This gel network has both solid and fluid properties, undergoing reversible changes in cytoplasmic structure classically termed gelation and solation (Taylor and Condeelis, 1979; Luby-Phelps et al., 1988). It has been proposed that gel-sol transitions and contractions of the gel network are intimately involved in fundamental cellular processes, such as

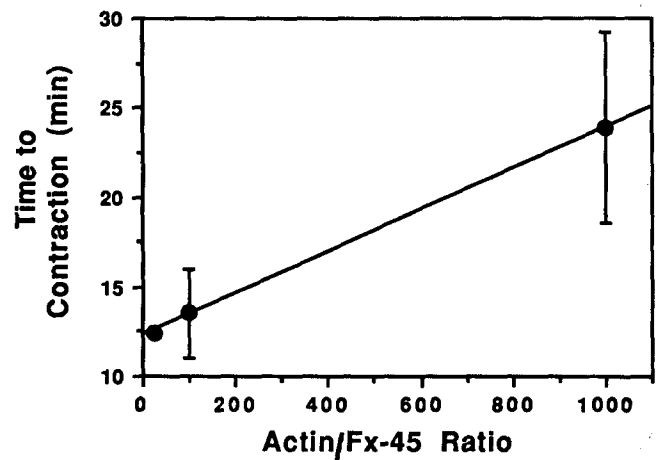


Figure 8. Effect of changing actin/Fx-45 molar ratio on rate of contraction. Samples with actin/Fx-45 ratios of 25:1, 100:1, 1000:1, and no gelsolin were examined at 28°C for up to 35 min using the microscopic assay as described in Materials and Methods. All samples had a 50:1 actin/myosin ratio and an actin/filamin ratio of 100:1. Time to contraction was determined for complete condensation of the mixture into a contracted mass. The samples with no gelsolin showed no signs of contraction up to 35 min, and are not shown. Error bars represent ± 1 SD.

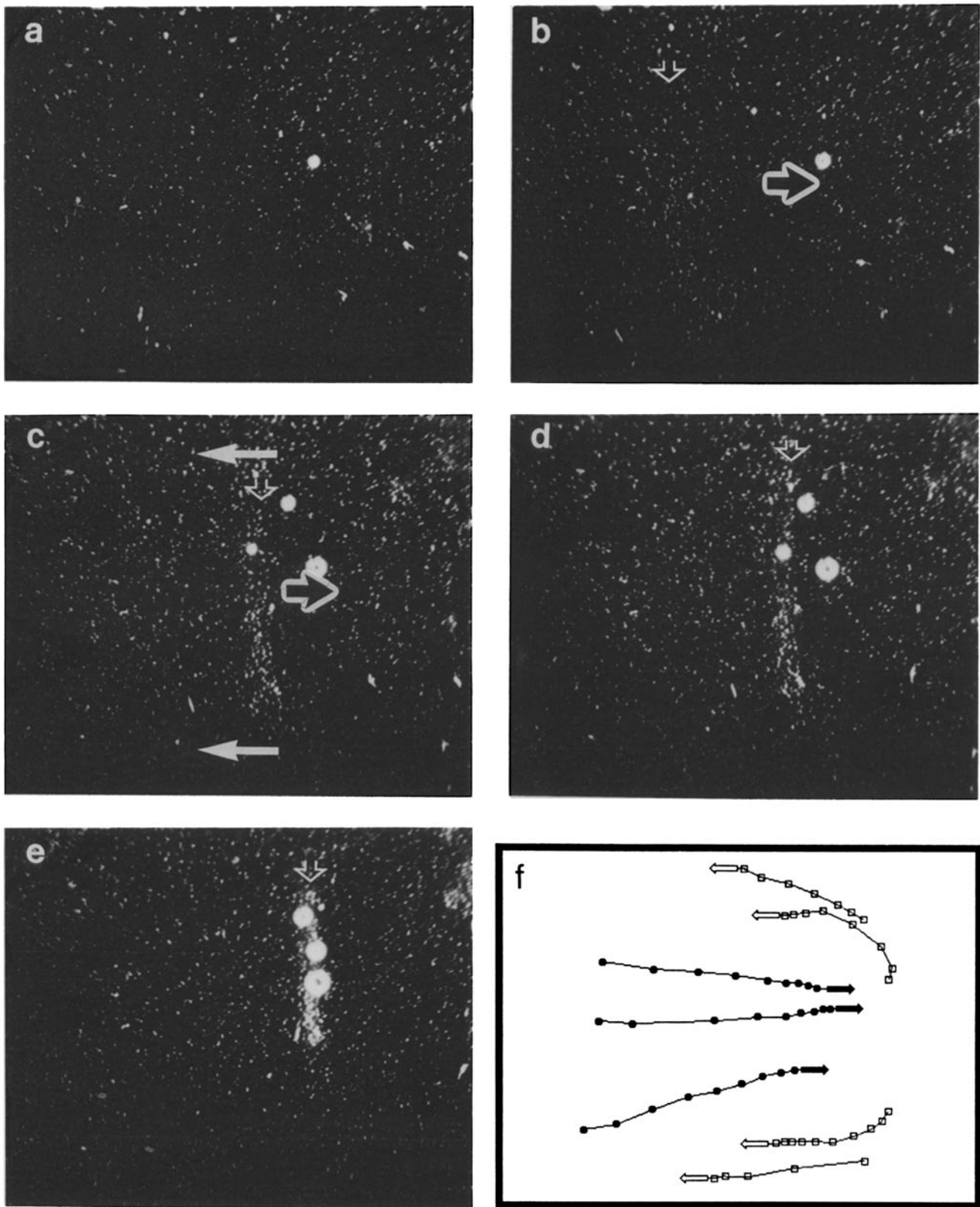


Figure 9 Example of microscopic contraction with cytochalasin D gradient. Reconstituted proteins with polystyrene latex beads were placed in the microscopic chamber and monitored with time-lapse dark field imaging (see Materials and Methods). Cytochalasin D was added to the left side of the chamber (*left side of image*) as described in Materials and Methods. (*a*) The cytochalasin D initiated solation and contraction on the left side of the chamber. The contraction caused the formation of a fiber, resembling the plasmagel sheet in amoeba (*open arrowheads*), which contracted from the high cytochalasin D side across the whole chamber (*b, c, d, and e*) (*large arrows*). As cytochalasin D diffused across the chamber, the contracting mass moved towards the low cytochalasin side of the chamber, displacing material in the opposite direction (*c, d, and e*; *small arrows*) reminiscent of the flare streaming of isolated amoeba cytoplasm (Allen, 1973; Taylor et al., 1973). *f* shows representative tracks of polystyrene latex beads, illustrating the contraction (●) and flare streaming (□).

the metabolism of cells, determination and modulation of cell shape and polarity, cellular locomotion, and cell division (Taylor and Fehcheimer, 1982; Stossel, 1982; Pollard, 1984). As a result, force generation must be considered in light of the gel structure of cytoplasm. One important approach to understanding the significance of the gel-sol transition is the reconstitution of a model system capable of exhibiting both cytoskeletal structure and contractile activity. This model system should consist of physiologically relevant concentrations and molar ratios of proteins, as well as ions and metabolites. The complementary approach of investigating the dynamics in living cells is discussed in the accompanying paper (Kolega et al., 1991).

We have developed a reconstituted system which allows a critical examination of the relationship between the gel state of the actin-based cytomatrix and contraction generated by a myosin II motor. The reconstitution is an extension of previous *in vitro* models (e.g., Stendahl and Stossel, 1980; Nunnally et al., 1981; Sobue et al., 1982; Simon et al., 1988), and uses components and buffer conditions which are designed to model the actin-based cytomatrix of nonmuscle cells (Table I). The method of reconstitution allows each protein and component a suitable environment throughout the mixing protocol so that native function is not impaired or changed (Kane, 1983). The results from test tube, microscopic, and microscopic gradient assays appear very similar to phenomena seen in single cell cytoplasmic models (Taylor et al., 1973), cell extracts (Thompson and Wolpert, 1963; Pollard and Ito, 1970; Stossel and Hartwig, 1976; Pollard, 1976; Condeelis and Taylor, 1977), and in live cells (Allen, 1973), thereby attesting to the potential physiological significance of this system. The reconstitution model presented here complements existing motility assays, designed to explore single molecule activities (Sheetz and Spudich, 1983; Kron and Spudich, 1986) and adds the complexity of gel structure. It should ultimately be possible to fully reconstitute the actin-based cytomatrix and to use this combined solid phase and fluid phase milieu in exploring many cellular processes.

Significance of Solation-Contraction Coupling for Cell Movement

Relationship between Extent of Gelation and Contraction.

The results with our reconstitution model system are consistent with the major tenants of the solation-contraction coupling hypothesis (Taylor and Fehcheimer, 1982) which are listed in the accompanying paper (Kolega et al., 1991). Our results support the hypothesis in three major areas.

(a) A minimal level of structure is necessary to transmit tension across the network. The data illustrate that a threshold amount of gel structure is necessary for contraction (Figs. 5 and 7). Macroscopic and microscopic samples without filamin (1:0 molar ratio actin/filamin) and, therefore, without gel structure showed no signs of contraction within the assay time (40–90 min). Microscopic samples with a small amount of filamin (400:1 molar ratio actin/filamin) contracted quickly, although the contraction was not propagated throughout the entire sample. In support of these results, a calculation of the critical cross-linking value (Hartwig and Stossel, 1981) for our reconstitution system indicates that an actin/filamin molar ratio of at least 999:1 is necessary to produce a gel network capable of transmitting

contractile tension across the network. Absence of contraction in samples with no cross-linker molecule (no gelation) has been noted in previous model systems by Stossel and Hartwig (1976), Stendahl and Stossel (1980), and Kane (1983). An examination of the results of Stendahl and Stossel (1980), which served as a basis for the gelation-contraction or rigidity-shear hypothesis, showed that the authors most likely examined the area of minimal gel structure required by the solation-contraction coupling hypothesis. Therefore, these authors also demonstrated that when starting below this threshold value, increased gel structure is necessary for any kind of contractile event.

(b) The gel resists the contractile force of myosin II, but the solating gel cannot resist this force. Previous work by others and our own results suggest that structure and organization of the gel network may have some effect on myosin's ability to cause a contraction. We assumed that at least some motor activity would be necessary to cause a contraction of the system. However, myosin II can also serve a structural role by "cross-linking" actin filaments (Brotschi et al., 1978; Stossel et al., 1985; Coleman and Mooseker, 1985). Myosin II can also be involved in a nonproductive interaction with actin, which may cause a viscouslike frictional drag (Sellers et al., 1985; Tawada and Sekimoto, 1991). Additionally, the myosin ATPase results suggested that changing the gel structure may affect myosin II-actin interactions. We determined that there is an approximately linear relationship between myosin concentration and time to contraction and that a threshold level of force generation is necessary to perform work on a gelled network (Fig. 4 *a* and *b*). Stendahl and Stossel (1980) also noted that contraction of their *in vitro* system was dependent on myosin concentration with a threshold value of myosin below which no contraction occurred. A similar result was obtained in HeLa cell extracts (Weihsing, 1977) and in a sea urchin egg system (Kane, 1983).

(c) Contraction can be induced by decreasing the gel structure (solation). Cross-linking of actin filaments represents a major modulator of actin-based gel structure (Stossel et al., 1985). The cross-linking of actin filaments by filamin and the closely related actin-binding protein of macrophages has been well characterized by several laboratories (Brotschi et al., 1978; Hartwig and Stossel, 1981; Stossel, 1982; Rockwell et al., 1984; Weihsing, 1985; Hou et al., 1990). It has also been shown that increasing the average filament length in the presence of a fixed number of cross-linkers leads to a direct rise in viscosity and gel state (Flory, 1940; Stossel, 1982). We observed that an increase in gel state by either increased cross-linking or increased filament length leads to a slower and less complete contraction (Figs. 5, 7, and 8). These results cannot be explained by inhibition of the Mg^{2+} -ATPase activity of the myosin since enzymatic activity actually increases with increasing filamin concentrations within the range of molar ratios that we investigated (Fig. 6). We believe that this increased ATPase activity may be due to ordering of the actin by filamin cross-linking, thereby allowing more crossbridge cycling, but that this additional force produced by the myosin filaments cannot overcome the increased strength of the highly cross-linked networks (see also Coleman and Mooseker, 1985). Similar effects on ATPase activity have been noted by others at these filamin/actin ratios (Dabrowska et al., 1985) and for similar ratios of actin and other actin-cross-linking proteins (Seraydarian et al.,

1967; Shimo-Oka and Watanabe, 1981; Wagner, 1984; Coleman and Mooseker, 1985). Our data also show that solation of a rigid gel by decreasing the mean length of the actin filaments with gelsolin or cytochalasin D, induced contractions that were propagated across the decreased gel network (Figs. 5, 7, 8, and 9). Solation-induced contraction also releases a portion of the components of the contracting gel into a more fluid fraction as part of a self-destruct mechanism. When a gradient of cytochalasin D was used, the transition between the gel and fully solated states also created a less structured medium that could be induced to stream by the contractile activity (Fig. 9). The streaming activity was comparable to that observed in single cell models (Allen, 1973; Taylor et al., 1973). This streaming resulted from the displacement of the less structured material as the sample contracted.

Regulation of Motile Processes. The solation-contraction coupling hypothesis suggests another level of regulation of cell movement beyond the regulation of myosin heavy and light chain phosphorylation (Sellers and Adelstein, 1987) and other potential actin- or myosin-linked regulation, including caldesmon (Last et al., 1986; Sutherland and Walsh, 1989). The present results demonstrate at least a modulating role for the gel-sol transformation on contraction. The accompanying paper (Kolega et al., 1991) demonstrates that decreasing the gel structure alone can induce contraction. The results from these studies taken together are also suggestive of a regulatory role for the gel-sol transformation. The value of coupling a force generation step with the creation of a more fluid cytoplasm by a self-destruct mechanism is readily apparent in amoeboid movement (see below). However, the solation-contraction coupling mechanism could play a significant role in other motile events including cytokinesis and smooth muscle contraction. Cytokinesis involves the transient assembly of an actin, actin-binding protein (multiple types), and myosin II machine. The contractile event must involve a self-destruct mechanism since the end of cytokinesis results in the loss of the cytokinetic structure. Therefore, the self-destruct aspect of the solation-contraction coupling hypothesis could explain the dynamics. A combination of myosin II phosphorylation and other regulatory mechanisms including solation-contraction coupling could initiate the contractile process. It is also possible that the cross-linking of actin by filamin in smooth muscle cells is part of the latch mechanism (Hai and Murphy, 1989) and/or part of the regulatory fine tuning combined with myosin light chain phosphorylation (Sellers and Adelstein, 1987) and caldesmon (Lash et al., 1986; Sutherland and Walsh, 1989). The physiological solation in all of the motile events could be induced by one or a combination of decreasing the number of crosslinks (Simon et al., 1988) and/or shortening the actin filaments with gelsolin or related actin modulating proteins.

Amoeboid Movement and the Solation-Contraction Coupling Hypothesis. It is clear that the cytoplasm of amoeboid cells, including mammalian fibroblasts in culture, is a complex medium exhibiting both gel and sol properties (Luby-Phelps et al., 1988). The most dramatic example is the relationship between gel-sol transitions and motility in free-living amoeba (Allen, 1973; Taylor and Condeelis, 1979; Taylor and Fehcheimer, 1982). The cytoplasm cycles through a less gelled state (sol) as the central endoplasm streams forward, to a more gelled state as the endoplasm in-

verts at the leading edge to form the ectoplasmic tube (cell cortex). The ectoplasm continues transporting to the rear of the cell where solation-contraction coupling generates force and produces new endoplasm that is driven forward.

The site or sites of force generation, relative to the changes in gel structure, have been debated for many years (Allen, 1973; Taylor and Condeelis, 1979; Taylor and Fehcheimer, 1982). The solation-contraction coupling hypothesis has been used to describe at least part of the relationship between cytoplasmic structure and force generation in amoeboid movement (Taylor et al., 1980a,b; Taylor and Fehcheimer, 1982). In short, solation-contraction coupling of the ectoplasmic tube was proposed as a self-destruct process that not only maximizes the contraction in the tail, but also creates the solated endoplasm that is driven forward by positive hydrostatic pressure. The solated endoplasm re-gelled by the time it inverted to form the anterior region of the ectoplasmic tube. Pseudopod extension was proposed to involve the localized weakening of the actin gel by the separation of the actin-based cortex from the membrane. This proposal was supported by the distribution of actin structures and fluctuations in the free calcium concentration (Taylor et al., 1980a,b). Recent evidence of a second class of myosin motor (myosin I) located at the anterior of the amoeboid stage of *Dictyostelium discoideum*, while myosin II is concentrated in the tail (Fukui et al., 1989) indicated that the myosin II may be involved in the tail contraction, but that a second mechanism of force generation may involve the leading edge. This idea is further supported by the ability of myosin II-minus mutants to exhibit a nonwild type movement (De Lozane and Spudich, 1987; Knecht and Loomis, 1987) and the loss of cell shape in locomoting fibroblasts injected with anti-myosin II antibodies (Honer et al., 1988). However, the integrated role of gel structure and contractility must be considered for any actin-based motor, whether in the front or rear of the cell. It is believed that similar, but less dramatic changes occur during the locomotion of other amoeboid cells (Taylor and Condeelis, 1979). Similar cyclical formation and breakdown of actin-based structures occurs in stress fibers in serum-deprived fibroblasts (Giuliano and Taylor, 1990).

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