

Microinjection of Gelsolin into Living Cells

John A. Cooper,^{*‡} Joseph Bryan,[§] Bill Schwab III,^{*} Carl Frieden,^{*} David J. Loftus,^{*} and Elliot L. Elson^{*}

Departments of ^{*}Biological Chemistry and [‡]Pathology, Washington University School of Medicine, St. Louis, Missouri 63110, and [§]Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

Abstract. Gelsolins are actin-binding proteins that cap, nucleate, and sever actin filaments. Microinjection of cytoplasmic or plasma gelsolin into living fibroblasts and macrophages did not affect the shape, actin distribution, deformability, or ruffling activity of the cells. Gelsolin requires calcium for activity, but the NH₂-terminal half is active without calcium. Microinjection of this proteolytic fragment had marked effects: the cells rounded up, stopped ruffling, became soft, and stress fibers disappeared. These changes are similar to those seen with cytochalasin, which also caps barbed ends of actin filaments. Attempts to raise the cytoplasmic calcium concentration and thereby activate the injected gelsolin were unsuccessful, but the increases in calcium concentration were minimal or transient and may not have been sufficient. Our interpretation of these results is that at the low calcium concentrations normally found in cells, gelsolin does not express the activities observed in vitro at higher

calcium concentrations. We presume that gelsolin may be active at certain times or places if the calcium concentration is elevated to a sufficient level, but we cannot exclude the existence of another molecule that inhibits gelsolin.

Microinjection of a 1:1 gelsolin/actin complex had no effect on the cells. This complex is stable in the absence of calcium and has capping activity but no severing and less nucleation activity as compared with either gelsolin in calcium or the NH₂-terminal fragment. The NH₂-terminal fragment-actin complex also has capping and nucleating activity but no severing activity. On microinjection it had the same effects as the fragment alone. The basis for the difference between the two complexes is unknown.

The native molecular weight of rabbit plasma gelsolin is 82,500, and the extinction coefficient at 280 nm is 1.68 cm²/mg. A new simple procedure for purification of plasma gelsolin is described.

MANY proteins that bind to actin and influence its functional properties have been studied in recent years (for reviews see references 26 and 29). One group of these proteins bind to the barbed end of actin filaments and prevent the association and dissociation of monomers (called capping). Some proteins in this group nucleate the formation of filaments from monomers, and some can break one actin filament into two (called severing). Gelsolin is a well-studied example of this group. It possesses all these activities, requires calcium for activity, and is widely distributed among mammalian cell types (22, 32, 33, 36-38). Under nonpolymerizing conditions gelsolin and actin also form a 1:2 complex if calcium is present. On subsequent removal of calcium, one molecule of actin is removed, but the 1:1 gelsolin/actin complex persists (5, 20). This complex caps and nucleates but does not sever (6).

Given our knowledge of the mechanism of action of gelsolin in vitro, we asked whether it had similar activities and similar regulations in living cells and whether these activities could affect the actin-based cytoskeleton and cell mo-

These experiments were reported at the 1986 meeting of the American Society for Cell Biology (10).

tility. Whether gelsolin is active or not inside a cell is an important question because ionic and other conditions in cytoplasm are largely unknown and therefore cannot be duplicated in vitro. Especially, activation of gelsolin requires calcium ions, which are maintained at low concentrations in cells. We asked whether, for example, the sensitivity of gelsolin to calcium is enhanced in cells by an unknown factor so that it is active at ambient cytoplasmic calcium concentrations. The approach in these experiments was to microinject gelsolin directly into the cytoplasm of a living cell and monitor the shape, movement, and actin distribution of the cell. These features are reasonable assays for gelsolin activity because both treatment with cytochalasin and microinjection of other capping proteins (13) have dramatic effects on these features.

Gelsolin is found in slightly different forms in cytoplasm and plasma. The plasma form has been called brevin (12, 15) and actin-depolymerizing factor (7, 8, 16). The *M_r* of the plasma form is 3,000 larger than that of the cytoplasmic; this difference is due to an extra 25 amino acid residues at the NH₂-terminus (34). The two proteins are otherwise structurally similar (22, 31, 34) and are encoded by a single gene

(21). The functional properties of the cytoplasmic and plasma forms are similar (11, 12) except that cytoplasmic gelsolin may have a higher affinity for calcium (19, 37). The plasma form is easier to obtain in high purity, therefore we used plasma gelsolin in most of these experiments and then confirmed important results with cytoplasmic gelsolin.

Materials and Methods

Proteins and Chemicals

Ionomycin from Calbiochem-Behring Corp. (San Diego, CA) was stored in ethanol and diluted to its final concentration in aqueous medium before use. The aqueous solution was sonicated for several minutes. Fura-2, both pentapotassium and acetoxymethyl ester forms, was from Molecular Probes, Inc. (Eugene, OR). Other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Plasma gelsolin was prepared from frozen rabbit plasma (Pel-Freez Biologicals, Rogers, AR) by a combination of the methods of Soua et al. (28) and Chaponnier et al. (8). Briefly, the procedure includes ammonium sulfate fractionation, DEAE-Sepharose 6B chromatography, Sephacryl S-200 chromatography, and Blue-Sepharose chromatography. Our assay for plasma gelsolin in column fractions was a test tube inversion assay (25) with 1 mg/ml rabbit skeletal muscle actin, 0.1 M KCl, 2 mM MgCl₂, 1 mM CaCl₂, and 10% (vol/vol) column fraction. The purity of the final material varied, but only preparations >95% pure, as indicated by SDS polyacrylamide gels stained with Coomassie Blue, were used for experiments. The activity of the pure material was documented by its ability to nucleate actin polymerization, a quantitative assay described by Doi and Frieden (12). The protein was frozen in liquid nitrogen and stored at -70°C.

Human plasma and platelet gelsolins were prepared using a monoclonal antibody as described by Hwo and Bryan (18). Alternatively, human plasma gelsolin and porcine plasma gelsolin were purified using a simplified chromatographic procedure. Briefly, 500 ml of serum (Gibco, Grand Island, NY) was dialyzed against 25 mM Tris/HCl, 0.5 mM CaCl₂, pH 7.5, applied to a 1,000 ml DEAE-Sepharose column, and eluted with the same buffer. Fractions were analyzed by SDS-gel electrophoresis and the fractions containing gelsolin were pooled. EGTA was added to 5 mM, and the pH was adjusted to 7.5. This material was applied to a 2.5 × 20 cm column of DEAE-Sepharose equilibrated with 25 mM Tris/HCl, pH 7.5, 0.1 mM EGTA. Gelsolin binds to DEAE-Sepharose in EGTA, but not in calcium and was eluted from the second DEAE column with a 0–0.5 M NaCl gradient in 25 mM Tris/HCl, 0.1 mM EGTA, pH 7.5. We have used this method to purify human, bovine, porcine, chicken, and rabbit plasma gelsolins. The human protein shows the same activity as that purified by the immunoaffinity column method (18).

To determine the extinction coefficient of rabbit plasma gelsolin, we measured, with the assistance of Mr. Walter Nulty, protein concentration with the interference optical system on an analytical ultracentrifuge (model E; Beckman Instruments, Inc., Palo Alto, CA). For a 12 mm double sector cell, 4.1 fringes are equivalent to a 1 mg/ml solution (1). Our result was 1.68 cm²/mg for A₂₈₀ not corrected for light scattering, and 1.65 cm²/mg when corrected for light scattering (by subtracting two times A₃₃₃). Weeds determined a value of 1.24 cm²/mg for pig plasma gelsolin by a similar approach (14), and Kilhoffer and Gerard (19) determined a value of 1.63 cm²/mg for bovine plasma gelsolin by a different method, based on amino acid analysis. Knowing the extinction coefficient, we determined that the Bradford protein assay (3) with rabbit skeletal muscle actin as standard gives a value for rabbit plasma gelsolin concentration that is 95% of the true value. The Bradford protein assay was used for small volumes, when absorbance measurements were impractical.

The native molecular weight of rabbit plasma gelsolin was determined, with the assistance of Mr. Walter Nulty, by sedimentation equilibrium in an analytical ultracentrifuge (model E, Beckman Instruments, Inc.). 0.2 mg/ml plasma gelsolin in 0.1 M KCl, 10 mM imidazole/HCl, pH 7.0, was centrifuged in a 12 mm double sector cell at 16,000 rpm to equilibrium. A plot of ln concentration vs. radius squared was linear, and the slope yielded a molecular weight of 82,400 ± 1,000 (mean ± SD for 6 scans). The *M_r* calculated from the DNA sequence is 83,000 (21).

The NH₂-terminal half of human plasma gelsolin, called CT40, and the COOH-terminal half of human plasma gelsolin, called CT45, were prepared as described (4). Rabbit skeletal muscle actin was prepared and stored as previously described (30). BSA was labeled with lissamine rhodamine sul-

fonyl chloride (Molecular Probes, Inc.) by the procedure of Sanger et al. (27). The dye to protein ratio was 3.5:1.

Cells

3T3 cells (a mouse fibroblast line) were a gift of Dr. Luis Glaser (University of Miami) and were grown in DME with 10% FCS (KC Biological, Inc., Lenexa, KS). C3H cells (a mouse fibroblast line) were obtained from ATCC (American Type Culture Collection, Rockville, MD) and grown in Eagle's modified essential medium with 10% FCS. Chick embryo fibroblast cells were a gift of Dr. Milton Schlesinger (Washington University School of Medicine, St. Louis, MO). J774.2 cells (a mouse macrophage line) were a gift of Dr. Philip Stahl (Washington University School of Medicine) and were grown in alpha modified essential medium with 10% FCS. The medium was supplied by the Cancer Research Center at Washington University. Cells were grown on glass coverslips in a 5% CO₂ atmosphere and placed into a similar buffer with Hepes in place of bicarbonate before use.

Microinjection

Cells were injected at 37°C on a Zeiss IM-35 microscope with a Zeiss 40× Planapo phase contrast objective, NA 0.65, using a micromanipulator (Narishige Scientific Laboratory, Tokyo, Japan). The needles were pulled from 1 mm borosilicate Omega Dot glass capillaries (Frederick Haer & Co., Inc., Brunswick, ME) on a Brown-Flaming puller. The outer tip diameter was 0.2 μm by scanning electron microscopy. Proteins for injection were dialyzed into 150 mM KCl, 2 mM Hepes, pH 7.0, and centrifuged in a microfuge for 15 min to remove dust. Before centrifugation, proteins were often concentrated with Centricon-30 filter apparatuses (Amicon Corp., Danvers, MA). A fluorescent marker, either 40,000 *M_r* dextran labeled with FITC (FITC-dextran)¹ or BSA labeled with lissamine rhodamine (LRB-BSA), was added to the solution before centrifugation. The solution was back-loaded into the needle, the needle was mounted on the micromanipulator, and pressure was applied with a syringe. The solution was continuously flowing through the needle during the procedure.

In a control experiment rabbit plasma gelsolin labeled with fluorescein (prepared by Dr. Yukio Doi) was injected into J774.2 macrophages, and rabbit plasma gelsolin labeled with lissamine rhodamine B was injected into C3H fibroblasts. The labeled gelsolins were active in nucleation and capping assays with pyrene actin. The fluorescent gelsolin diffused rapidly (in 2 min) throughout the cytoplasm. After 2 h, the fluorescence still showed a diffuse distribution. These results show that injected plasma gelsolin has rapid access to the cytoplasm and that it remains in the cytoplasm for the duration of these experiments.

The injection volume was measured by a procedure suggested by Dr. Daniel P. Kiehart (Harvard University, Cambridge, MA). A fluorescent image of the injection needle containing FITC-dextran was digitized. The camera was in its linear range, and an appropriate background image was subtracted. The path length through the needle was assumed to be equal to the width of the needle. Bleaching was minimized by continuous flow. Fluorescent images of injected cells were also digitized. The fluorescence at a bright para-nuclear area was recorded and the height of the cell at that point was measured by focusing with differential interference contrast optics. The focus knob was calibrated previously. Percent volume injected was calculated as (cell fluorescence × needle width × 100)/(needle fluorescence × cell height). The mean was 10%, with values ranging from 4 to 15%. Cells injected with larger volumes generally died soon after injection.

Microscopy

Two approaches were used. In the first, one cell was injected and time-lapse video records were made with a Vidicon camera and a Panasonic NV 7030 video tape recorder. The 40× phase contrast objective was used as above. The shape and ruffling activity were assessed qualitatively by viewing the video tape. Light that would excite the injected fluorophore was excluded by appropriate colored-glass long-pass filters until the end of the recording period when fluorescence was checked to ensure that the cell had been injected and was still alive.

In the second approach, 20–100 cells on one coverslip were injected, returned to the incubator for some time, then fixed with formaldehyde and

1. *Abbreviations used in this paper:* FITC-dextran, 40,000 *M_r* dextran labeled with FITC; LRB-BSA, BSA labeled with lissamine rhodamine; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl.

stained with *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phalloidin (NBD-phalloidin; Molecular Probes, Inc.) as described (2). LRB-BSA was the fluorescent marker in these experiments because it remained in the cells after fixation. Photographs of phase contrast and fluorescence images were taken with a Zeiss Planapo 63 \times phase objective, NA 1.4, and Kodak Tri-X film. A 100-W mercury lamp was used as the light source for fluorescence.

Cell Deformability

The deformability of living cells was measured with a cell-poking device that indents cells with a glass microprobe. The instrument and its operation have been described (23, 24). Cells were injected on the IM-35 as described above and placed onto the cell-poking device. The temperature was 33°C. Maximum depth of indentation was <2.6 μ m. Velocity of indentation was 5.5 μ m/s, and the poker tip diameter was \sim 2 μ m. Flat, well-spread cells were indented in a para-nuclear area of cytoplasm, and small round cells were indented in their center. Cell appendages, such as ruffles and filopodia, are too small to have an effect on this measurement, which reflects properties of bulk cytoplasm. Stiffness was calculated as the linear slope of the initial portion of the force vs. displacement curve, corresponding to the initial phase of the indentation.

Permeabilized Cell Models

Fibroblasts on glass coverslips in culture were washed twice quickly with 150 mM NaCl, 5 mM Hepes, pH 7.2, and permeabilized for 120 s with HKM buffer (150 mM KCl, 2 mM MgCl₂, 2 mM Hepes, pH 7.0) plus 0.1% (wt/vol) Triton X-100. The cells were rinsed with HKM buffer twice quickly

and then incubated with various solutions for 30 min. They were rinsed with HKM buffer twice quickly, fixed with formaldehyde, and stained with NBD-phalloidin as above.

Calcium Measurements with Fura-2

Fibroblasts were either loaded with Fura-2/AM ester or microinjected with Fura-2/K⁺ salt. Cells were loaded with ester by incubating them in normal medium with 20 μ M Fura-2/AM for 45 min at 37°C or several hours at 4°C (to minimize uptake of dye by pinocytosis). The cells were washed briefly with Hepes-based medium and placed either on the Zeiss IM-35 microscope or in an Aminco fluorometer (SLM Instruments, Inc., Urbana, IL) at 37°C. On the microscope, fluorescent images were recorded with excitation band pass filters at 340 and 380 nm. A Zeiss 100 \times Ultrafluor objective was used. The Dage ISIT video camera was used at high sensitivity, and neutral density filters were placed in the excitation path to minimize bleaching. Background images, recorded from areas of the coverslip with no cells, were subtracted from the cell images, and the ratio of the 340 to the 380 image was calculated. A threshold value was applied during the ratio process, to avoid spuriously high ratio values at the cell edge, where the fluorescence values are low. Values were averaged over a 10 \times 10 pixel area. Standard solutions of EGTA-buffered calcium with Fura-2K⁺ were then placed on the microscope with the same settings as used for cell images. 340- and 380-nm images were recorded and backgrounds of solution without Fura were subtracted. The resulting ratios were used to calibrate the cell ratios. With the fluorometer, fluorescence was recorded with excitation at 340 and 380 nm, emission at 510 nm, excitation band width of 5 nm, and emission band width of 20 nm. Background fluorescence, from cells that were not loaded with

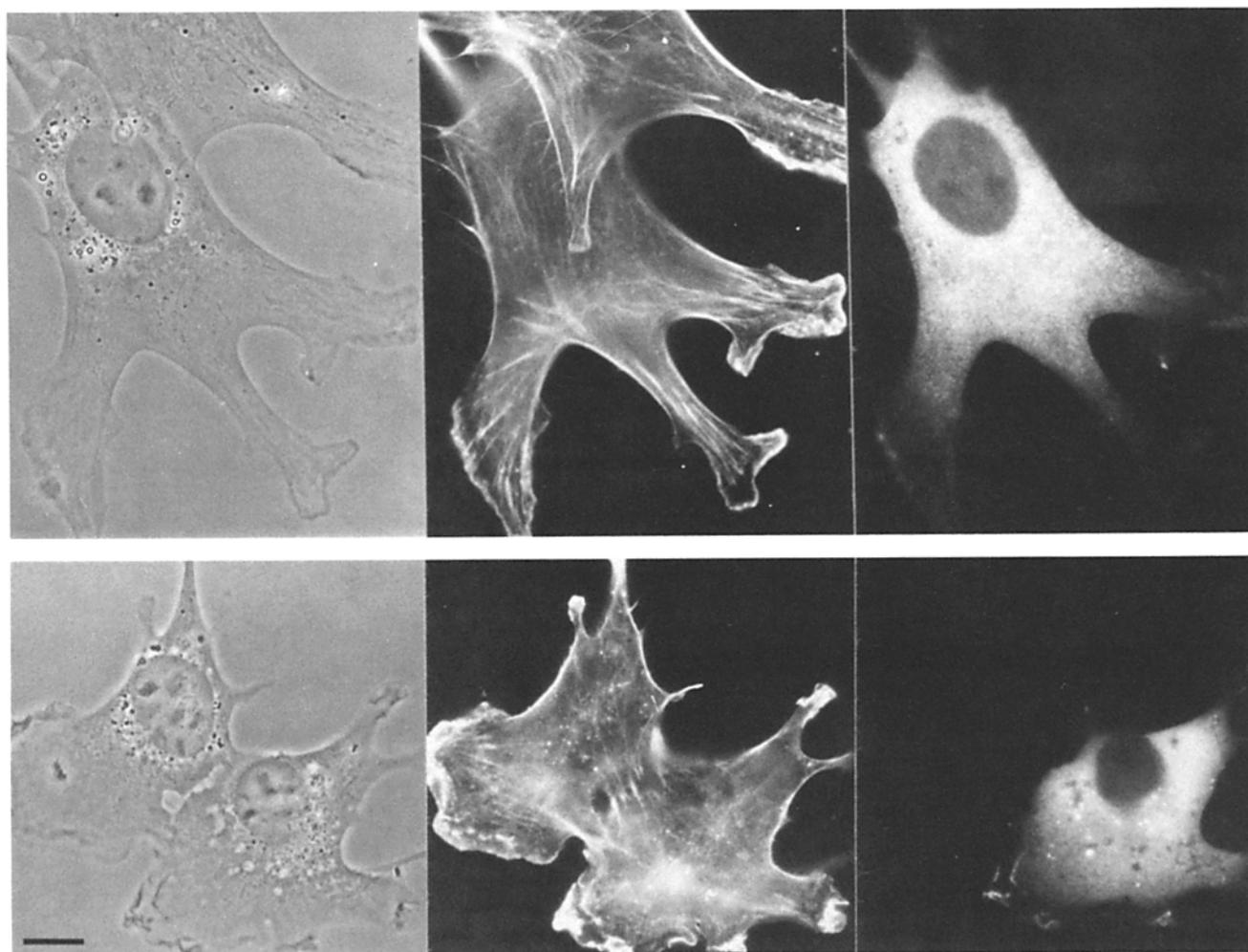
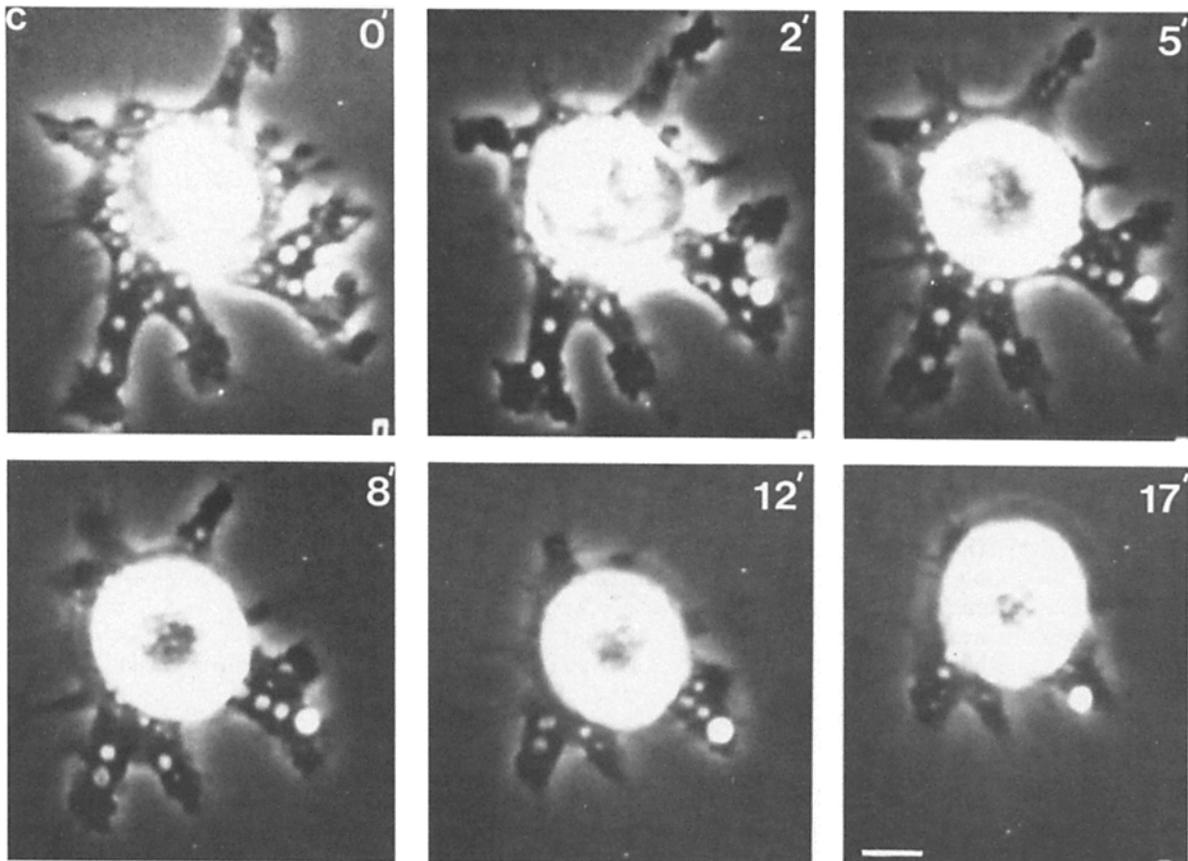
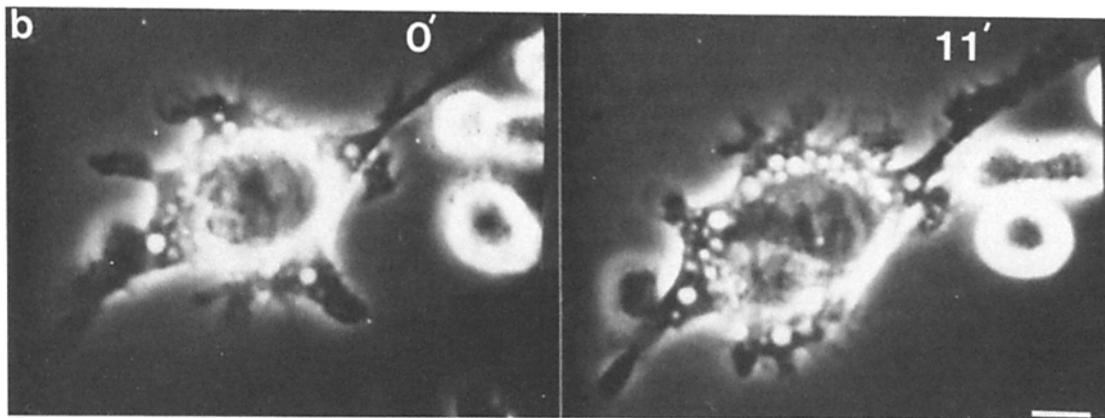
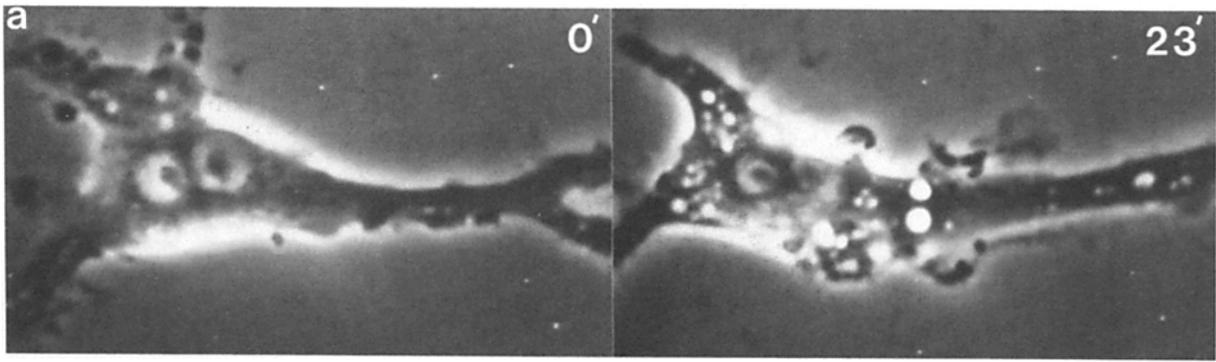


Figure 1. Microinjection of plasma gelsolin into fibroblasts. The micrographs are, left to right, phase contrast, NBD-phalloidin fluorescence, and LRB-BSA fluorescence. The LRB-BSA image shows which cell was injected. The cells have a normal spread shape, with actin-containing stress fibers (*top*) and membrane ruffles (*bottom*). Bar, 10 μ m.



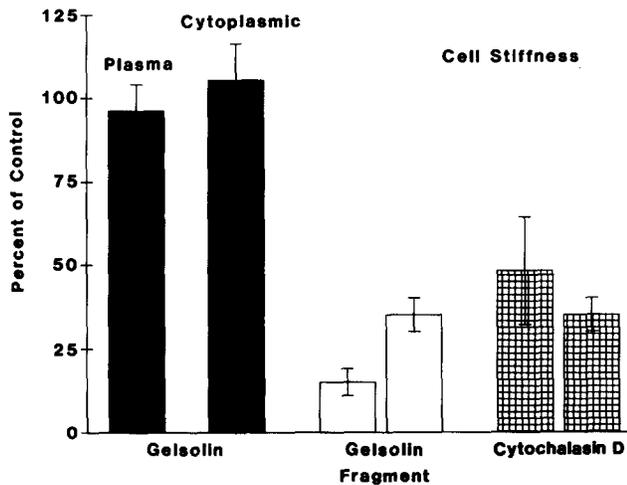


Figure 3. Deformability of cells. The percent of cell stiffness for injected vs. uninjected cells is plotted for six different experiments. These data are also listed in Table I. Cells were injected with plasma or cytoplasmic gelsolin, NH₂-terminal fragment of gelsolin (CT40), or treated with cytochalasin D. Each bar represents one experiment, performed on a day separate from the other experiments. The error marks are 1 SEM.

Fura₂ was subtracted from each value and ratios were calculated. Calcium standard solutions, described above, were used to calibrate the ratios.

For microinjection, fibroblasts were placed on the microscope with the optics described above and injected with 500 μ M Fura-2/K⁺ in 150 mM KCl. In some experiments 1 mM CaCl₂ was included in the injection buffer. Many injected cells lost their fluorescence over several minutes, whether calcium was in the solution or not. This phenomenon does not occur with injections of Lucifer Yellow or FITC-dextran in our hands and we have no good explanation for it. We prefer microinjection of salt to loading with ester because less dye associates with vesicles and it avoids the potential problem of partially hydrolyzed ester that is trapped but not responsive to calcium.

Results

Microinjection of plasma gelsolin into living cells had no effect on their shape, actin distribution, deformability, or ruffling. Fibroblasts were injected with 30–38 μ M plasma gelsolin and incubated for 15 min to 2 h. The concentrations given for this and other microinjection experiments represent the concentration of protein in the needle, not the injected cell. 30–80 cells were injected on each coverslip and each experiment was repeated at least once. The injected cells were well-spread with a normal complement of stress fibers and ruffles (Fig. 1). This experiment was repeated with gelsolin prepared from rabbit, human, and pig plasma. Macrophages were injected with 30 μ M rabbit plasma gelsolin and the motility of single cells was followed with time-lapse video microscopy (Fig. 2 a). The shape and extensive ruffling activity of these cells was unaffected by the injection compared with injection of similar concentrations of BSA (Fig. 2 b). These observations are qualitative and subjective. However, cell deformability, which is a quantitative and objective measurement of the state of the cytoskeleton, also showed no difference between fibroblasts injected with rabbit

Table I. Deformability of Cells

Treatment	Deformability (mean \pm SEM)		
	Injected	Uninjected	Percent
	<i>mdyn/μm</i>	<i>mdyn/μm</i>	
Plasma gelsolin	0.25 \pm 0.02	0.26 \pm 0.04	96
Cytoplasmic gelsolin	0.20 \pm 0.02	0.19 \pm 0.02	105
Fragment of plasma gelsolin	0.04 \pm 0.01	0.26 \pm 0.03	15
	0.07 \pm 0.01	0.20 \pm 0.02	35
Low dose	0.21 \pm 0.03	0.20 \pm 0.02	105
Cytochalasin D	0.10 \pm 0.03	0.21 \pm 0.07	48
	0.07 \pm 0.01	0.20 \pm 0.02	35
Plasma gelsolin/actin complex	0.22 \pm 0.02	0.24 \pm 0.03	92
Controls			
FITC-dextran	0.24 \pm 0.06	0.21 \pm 0.07	114
LRB-BSA	0.22 \pm 0.02	0.24 \pm 0.03	92
Ionomycin/calcium treatment			
Plasma gelsolin (30 min)	0.28 \pm 0.04	0.31 \pm 0.06	90
Plasma gelsolin (110 min)	0.41 \pm 0.08	0.40 \pm 0.08	103
LRB-BSA (60 min)	0.23 \pm 0.02	0.24 \pm 0.03	95
Injections with calcium			
Plasma gelsolin	0.20 \pm 0.01	0.19 \pm 0.02	105
	0.20 \pm 0.02	0.17 \pm 0.01	118
Cytoplasmic gelsolin	0.18 \pm 0.02	0.19 \pm 0.02	95
BSA	0.57 \pm 0.06	0.55 \pm 0.07	104

The values represent the stiffness of cells during the initial portion of the indentation, where force vs. displacement is relatively linear. Living cells were microinjected with 38 μ M plasma or cytoplasmic gelsolin. NH₂-terminal fragment of plasma gelsolin, or plasma gelsolin/actin complex. Control injections were with 2 mg/ml FITC-dextran or 0.3 mg/ml LRB-BSA. Cytochalasin D treatment consisted of 2 μ M extracellular cytochalasin D for 30 min. The "uninjected" values for the cytochalasin treatments represent cells from other coverslips on the same day that were not treated with cytochalasin. For ionomycin/calcium treatment, the ionomycin concentration was 1.3 μ M, extracellular calcium concentration was 2 mM, and extracellular pH was 8.1. When calcium was included in the injection buffer, its concentration was 500 μ M.

The number of cells in each group was 15–50. This value is included in calculation of SEM. In these data, the conclusion that two populations are the same (i.e., not different) is not justified for the many cases where the values appear to be the same, because of beta (type II) error. For example, with 30 data points in each sample, one has a 90% chance of detecting the difference between means of 0.25 and 0.16 (a 35% difference) given our typical SD of 0.10.

plasma gelsolin and cells not injected (Fig. 3; Table I). Microinjection of similar concentrations of cytoplasmic gelsolin from human platelets into fibroblasts also had no effect on cell shape, actin distribution, or cell deformability (Figs. 3 and 4; Table I).

Because gelsolin requires calcium for activity, we injected a fragment of gelsolin that does not need calcium to be active. The NH₂-terminal half of plasma gelsolin (CT40), produced by chymotryptic proteolysis, has the full functional activity of the intact molecule but is active without calcium. Microinjection of this fragment at 38 μ M had dramatic effects on all injected cells. Fibroblasts rounded up and stopped ruffling. Their stress fibers were lost, and perinuclear punctate structures were seen with the NBD-phalloidin stain (Fig. 5). The cells became very soft in the deformability measurements (Fig. 3; Table I). These effects resemble those

Figure 2. Microinjection of macrophages. Selected phase contrast images from a time-lapse video record are shown. The times indicated are minutes after injection. The injected protein was (a) plasma gelsolin, (b) BSA, or (c) NH₂-terminal fragment of gelsolin (CT40). In a and b the injected cells maintain their spread shape and ruffling activity, but in c the cell rounds up and stops ruffling. Bar, 10 μ m.

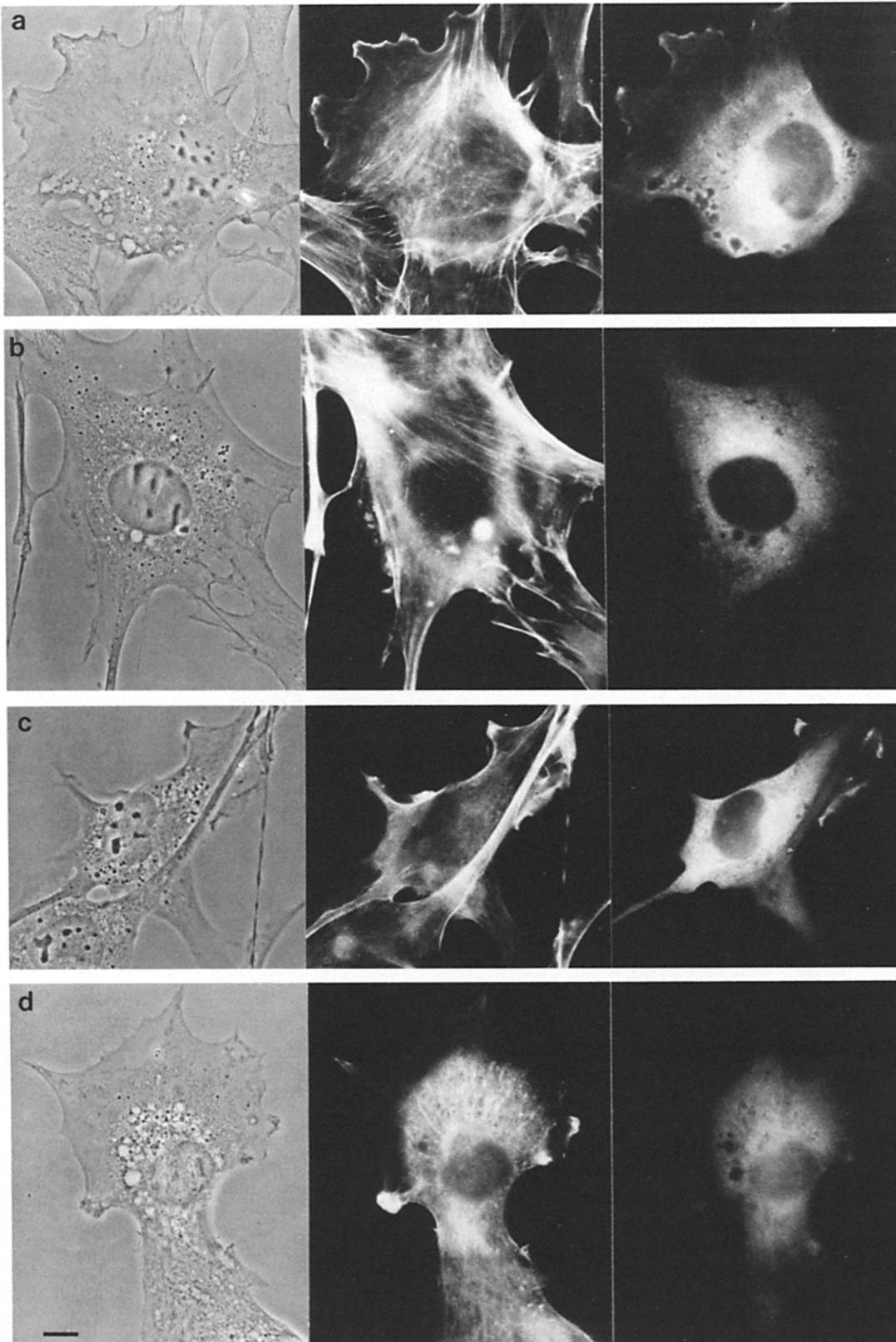


Figure 4. Microinjection of fibroblasts with cytoplasmic gelsolin. The micrographs are, left to right, phase contrast, NBD-phalloidin fluorescence, and LRB-BSA fluorescence. (*Top to bottom*) (*a*) no calcium treatment; (*b*) 500 μM CaCl_2 in injection solution; (*c*) ionomycin/calcium treatment after injection; (*d*) 500 μM CaCl_2 in injection solution and ionomycin/calcium treatment after injection. The injected cells in *a* and *b* are normal. Cells treated with ionomycin (*c* and *d*) are slightly retracted with partial loss of stress fibers, but the injected cells still resemble the uninjected ones. Bar, 10 μm .

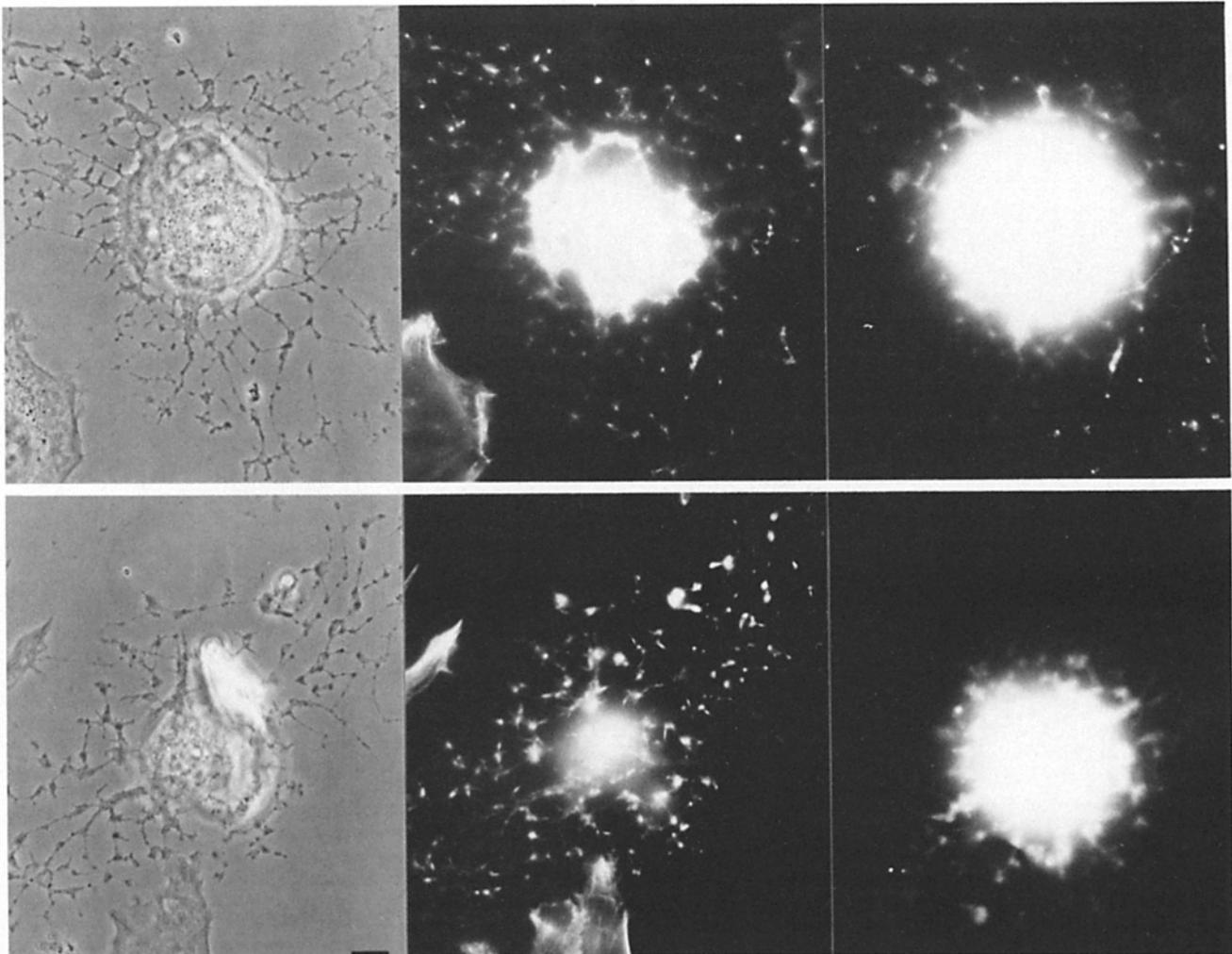


Figure 5. Microinjection of NH₂-terminal fragment of plasma gelsolin (CT40) into fibroblasts. The micrographs are (left to right) phase contrast, NBD-phalloidin fluorescence, and LRB-BSA fluorescence. Injected cells are round, having left a thin network of cytoplasm attached to the coverslip. Fluorescence shows the loss of stress fibers and appearance of perinuclear punctate stain. Bar, 10 μ m.

of cytochalasin D (Figs. 3 and 6; Table I). Macrophages also rounded up and stopped ruffling (Fig. 2 c).

The injection added 3–4 μ m of gelsolin to the cells based on our measured injection volume of 10%. The concentration of endogenous gelsolin in cells is not known precisely, but Soua et al. (28) measured 1–4 μ g gelsolin/mg protein in a variety of tissues. If cells contain 75 mg protein/ml, then the gelsolin concentration is 1–4 μ M. Therefore the injection introduces a quantity similar to that already present. The actin polymer concentration is \sim 100 μ M, so a molar ratio of about 1 gelsolin fragment to 30 actin protomers is present when the dramatic effects are seen.

In both types of cells the effects began immediately upon injection and persisted without reversal for up to 6 h (the longest time point examined). Microinjection of lower concentrations, 18 and 9 μ M, of NH₂-terminal fragment into fibroblasts caused similar effects on shape and actin distribution, but the effects were variable and less pronounced. Some cells showed loss of stress fibers, either partial or total, without rounding up. Microinjection of the COOH-terminal half of human plasma gelsolin (CT45) at 38 μ M had no effect on the shape or actin distribution of fibroblasts (no micrographs are shown).

We attempted to activate plasma gelsolin in the injected cells by increasing intracellular calcium with ionophores and calcium injections. Treatment of gelsolin-injected cells with 1.3 μ M ionomycin or 0.5 μ M A23187 in the presence of 2 mM extracellular calcium had no effect (micrographs not shown; Table I). Raising the extracellular calcium to 12 mM in such an experiment caused many injected and uninjected cells to die as indicated by the loss of LRB-BSA (the fluorescent marker), but the surviving cells showed none of the changes described above with the NH₂-terminal fragment (Fig. 7). Raising the pH of the medium to 8.1 increases the effectiveness of ionomycin and ultimately caused all the cells to die. With shorter treatment times, cells were slightly retracted with partial loss of stress fibers, but again there was no difference between injected and uninjected cells (Fig. 4, c and d). In other experiments 0.5 mM calcium was included with gelsolin in the injection solution. A larger fraction of the injected cells died but the survivors were not different from uninjected cells (Fig. 4, b and d; Table I).

The intracellular calcium concentration was measured in fibroblasts either injected with Fura-2 salt or loaded with Fura-2 ester. Fluorescence measurements were made both by digital microscopy on single cells and by fluorometry on

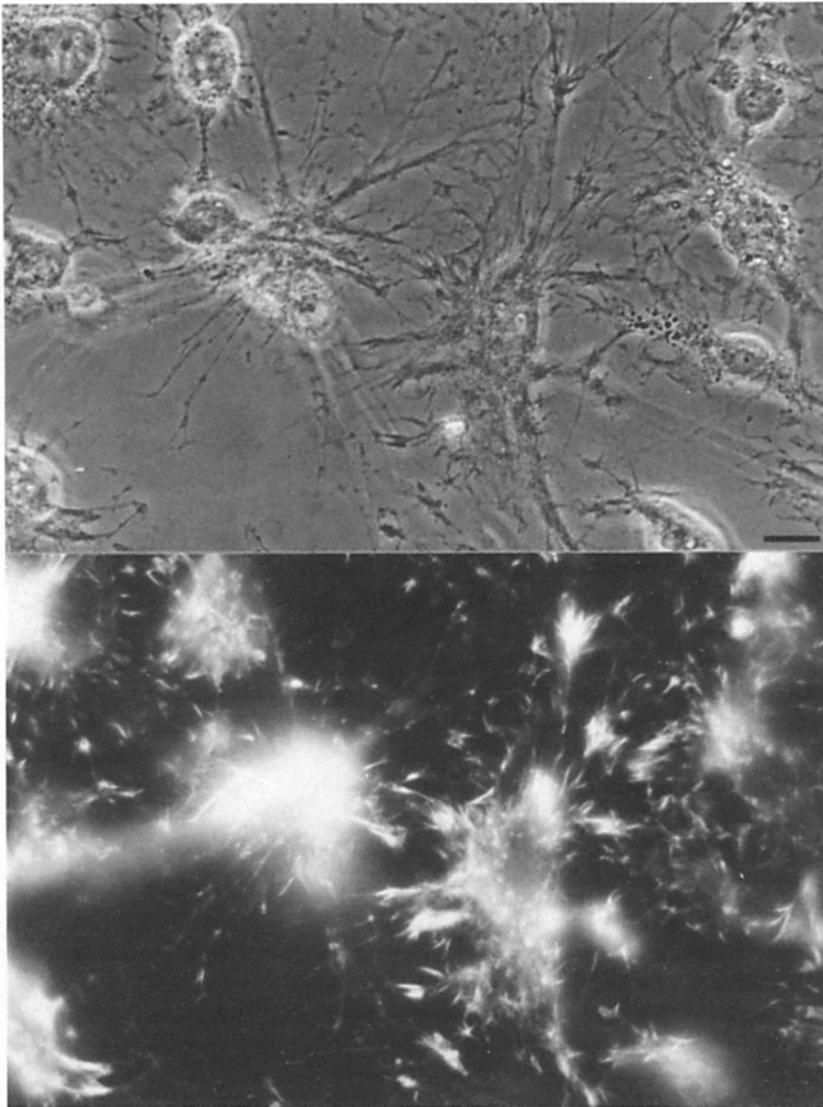


Figure 6. Treatment of fibroblasts with cytochalasin D. Phase contrast and NBD-phalloidin fluorescence micrographs are shown. The shape and actin distribution of the cells are similar to those of fragment-injected cells. Bar, 10 μm .

populations of cells. The resting calcium was 70 nM. Treatment of cells with 1.3 μM ionomycin and 2 mM extracellular calcium at pH 7.4 only raised the calcium to 270 nM. When 1 mM calcium was included with the Fura-2 salt in the injection buffer, the initial intracellular calcium measurement was 100 μM , but by 12 min it fell to 200 nM. These results show that the cells control their intracellular calcium concentration so well that the ionophores and calcium treatments cannot raise the calcium high enough to activate microinjected plasma or cytoplasmic gelsolin. The cells probably die before their calcium concentration rises to a sufficient level.

As another positive control, we examined whether plasma gelsolin could remove stress fibers from permeabilized cell models in which the calcium was high enough to activate the gelsolin. Triton-permeabilized fibroblasts were treated with 0.3 μM plasma gelsolin and 100 μM CaCl_2 for 30 min. Stress fibers were totally removed (Fig. 8).

To ask whether the capping, nucleating, or severing activity of the NH_2 -terminal fragment (CT40) was responsible for the effects on cells upon microinjection, we injected two gelsolin preparations that do not sever. Gelsolin can be made

to form a 1:1 complex with actin, which is active in the absence of calcium. Calcium is required for the formation of the complex, but on subsequent chelation of calcium the complex dissociates very slowly, with a half-time of 30 d. The complex has capping and partial nucleation activity but no severing activity (6). We confirmed this for our preparation of rabbit plasma gelsolin by measuring the effect of the complex on the kinetics of actin polymerization from monomers and the final steady state level of polymerization with pyrene actin fluorescence. For microinjection, plasma gelsolin was mixed with actin in a 1:2 molar ratio in the presence of 100 μM CaCl_2 . After 10 min, 100 μM EGTA was added and the complex was concentrated to 36 μM . Injection of the complex into fibroblasts had no effect on cell shape and actin distribution (micrographs not shown) and cell deformability (Table I). A complex of CT40 with actin was made by mixing 2.5 mol of actin per mol of CT40, and it was dialyzed into injection buffer. The absence of severing activity was confirmed by a depolymerization assay with pyrene actin and by a cytoskeleton assay like the one in Fig. 8. Injection of this complex at 39 μM into fibroblasts had dramatic

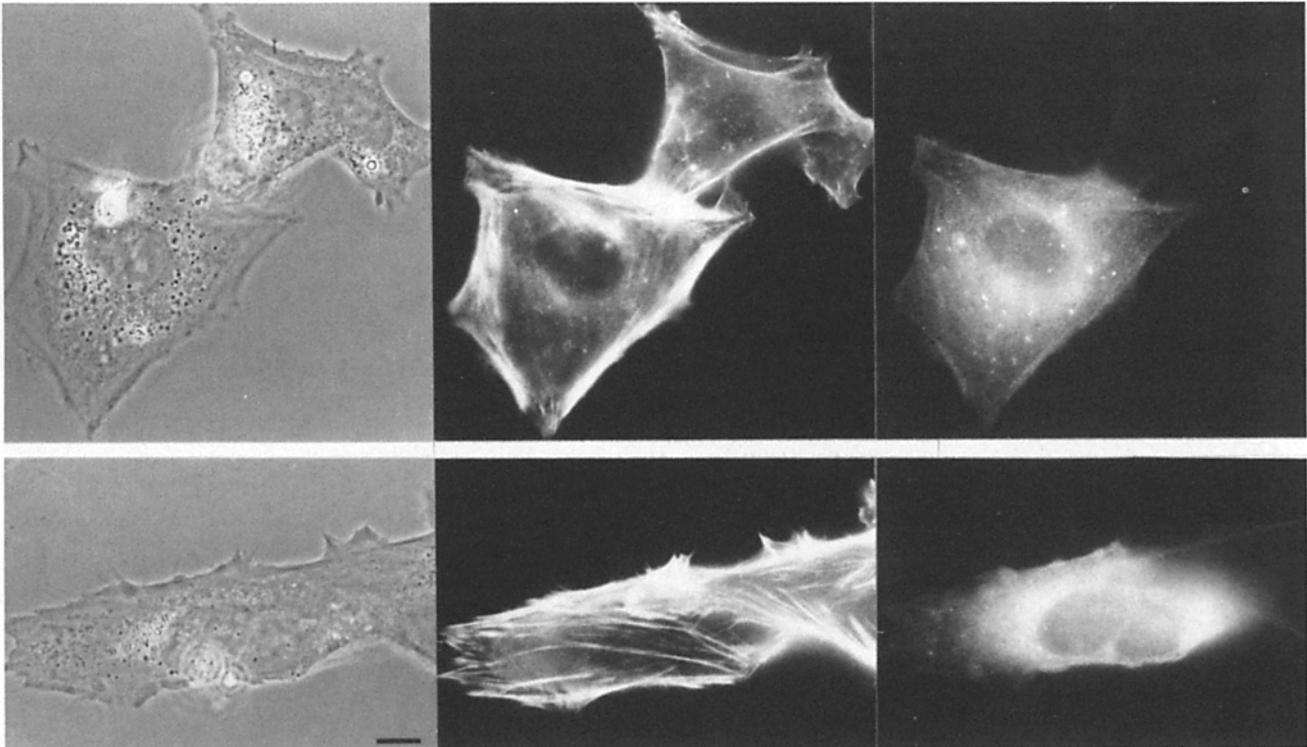


Figure 7. Ionomycin/calcium treatment of fibroblasts injected with plasma gelsolin. After injection with 38 μM rabbit plasma gelsolin, cells were incubated for 60 min with 1.3 μM ionomycin and 12 mM CaCl_2 at pH 7.4. All the cells are slightly retracted with partial loss of stress fibers, but there is no difference between injected and uninjected cells. Bar, 10 μm .

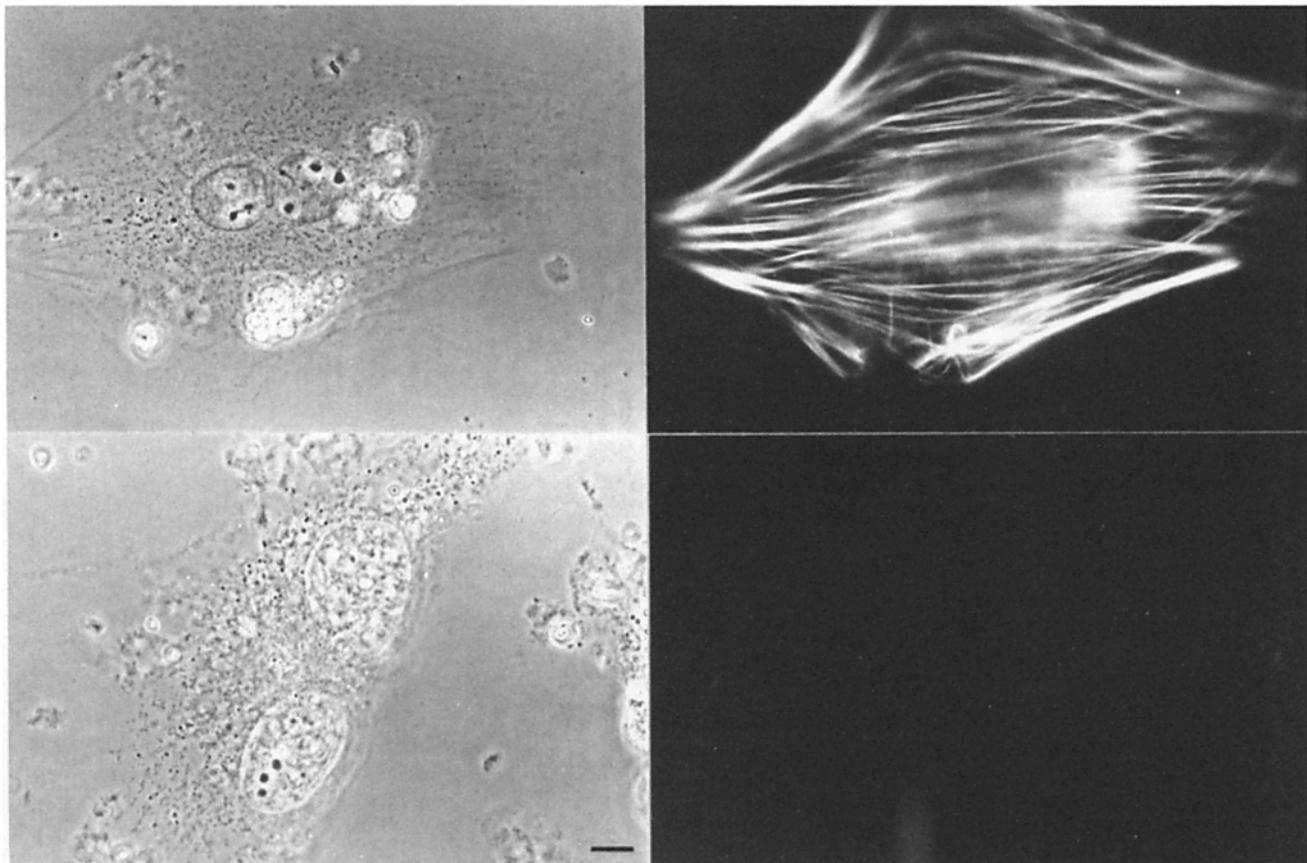


Figure 8. Effect of plasma gelsolin and calcium on stress fibers in permeabilized fibroblasts. Fibroblasts were permeabilized with Triton and then treated with a solution of 0.3 μM plasma gelsolin and 100 μM CaCl_2 . Control cells, treated with calcium but not gelsolin, are shown in the upper panel and gelsolin-treated cells are below. Bar, 10 μm .

effects on cell shape and actin distribution, fully resembling those of the fragment alone shown in Fig. 5. The micrographs from this experiment are not shown.

Discussion

The first step toward understanding the physiologic role of gelsolin is to determine whether it can be active in the environment of cytoplasm. In these experiments we injected gelsolin into living cells and observed no effect on various parameters of cell motility and the actin-based cytoskeleton. Gelsolin-injected cells had normal shape and membrane ruffling. The distribution of actin filaments, observed by fluorescence microscopy, was unchanged. Cell deformability, a quantitative measurement that mainly reflects the state of actin (24), was also unchanged. We conclude that the majority of the injected gelsolin is not active in living cells, and therefore infer that the majority of the endogenous gelsolin is also not active. The conclusion is limited by the cell types, fibroblasts and macrophages, the functional state of the cells, and the parameters of cell motility examined in these experiments.

Is gelsolin not active because of a lack of calcium? To answer this question we injected a proteolytic fragment of gelsolin that has all the activities, capping, nucleation, and severing, of gelsolin but does not require calcium (4). Injection of this fragment at concentrations similar to those used for the ineffective gelsolin injections markedly affected all of the observed cell parameters: shape, ruffling, actin distribution, and deformability. This positive result serves as a control to show that the negative result with intact gelsolin is valid. These results also suggest that the injection of gelsolin is ineffective because the calcium concentration in the cells is too low for gelsolin to be active.

We attempted to confirm this conclusion by raising the calcium concentration in cells injected with gelsolin. Neither treatment with calcium ionophores and high extracellular calcium nor injection of calcium activated the injected gelsolin. The most severe treatments simply killed the cells. The intracellular calcium concentration, measured by fluorescence of Fura-2, increased only from 70 to 270 nM with these treatments. Should gelsolin have been active at these concentrations of calcium? Direct binding studies show that both macrophage and platelet cytoplasmic gelsolins bind 2 mol of calcium with a dissociation constant of 1 μ M (5, 37), and titration of fluorescence polarization shows that bovine plasma gelsolin binds more than 1 mol of calcium with a dissociation constant of 20 μ M (19). Therefore our treatments probably did not increase the calcium concentration enough to activate most of the injected gelsolin. The injections of lower concentrations of fragment show that most of the injected gelsolin would have to be active to produce a detectable effect on the cells. The absence of an effect when calcium was injected with gelsolin is more difficult to understand. The calcium concentration fell rapidly, perhaps before gelsolin could act.

Our interpretation of these results is that gelsolin, which is present in cytoplasm at a substantial concentration, is normally inactive due to the low concentration of calcium, \sim 0.1 μ M. These molecules may be activated by an increase of the calcium concentration to the range of 1 μ M. Another possibility, although less likely in our view, is that gelsolin is in-

hibited by another protein, which is currently undescribed, and is activated by relief of this inhibition. Cytoplasmic gelsolin can be isolated uncomplexed with actin (27), which supports the view that it is an unactivated state in cells. If gelsolin is activated and interacts with actin before isolation, it is obtained as a complex with actin, which is irreversible to the removal of calcium ions (5, 20). It is also possible, however, that there is a constituent of cytoplasm that can dissociate gelsolin/actin complexes (9). Why do cells apparently retain substantial quantities of cytoplasmic gelsolin in an inactive state? One possible explanation is that cell activities that require active gelsolin involve only a localized portion of the cell and are activated by a local and transient signal such as an increase in calcium concentration. Possible examples of such functions include phagocytosis, exocytosis, ruffling, and locomotion. As our attempts to increase intracellular calcium concentration suggest, a local increase would dissipate very rapidly. Hence, a high concentration of gelsolin at the site of the transient rise in ion concentration might be necessary to yield a sufficient quantity of the activated protein. A direct test of this idea would require a correlation of local increases in calcium ion concentration with local activation of gelsolin.

Other capping proteins do not require calcium for activity, and previous microinjection experiments showed that brain capping protein and *Physarum* 42(a + b) capping protein can disrupt the stress fibers of fibroblasts (13). Gelsolin may be involved in calcium-mediated processes, whereas these other proteins may be constitutively active or regulated in other ways.

The results obtained with the gelsolin fragment also indicate what types of effects gelsolin might have on cells. The most novel finding, based on a quantitative assay on living cells, is that the mechanical properties of cytoplasm can be changed by gelsolin. Another finding is that stress fibers can be disrupted. Such a disruption is associated with mitosis and translocation (17). Also, ruffling can be inhibited, and cells can round up. These findings are predicted from the mechanism of action of gelsolin on actin *in vitro*, and show that capping proteins, when active, can have powerful effects on several aspects of cell motility.

Gelsolin has capping, nucleating, and severing activities. To test which of these activities causes the effects seen on injection of the NH₂-terminal fragment, we injected gelsolin/actin and fragment/actin complexes. Neither complex has severing activity, but both cap and nucleate. Surprisingly, the two complexes have different effects on cells. The gelsolin/actin complex has no effect and the fragment/actin complex has the full effect of the fragment alone. This result suggests three possible interpretations, which can be tested in future experiments. First, cells may possess a mechanism to dissociate the complexes, which are very stable *in vitro*. The free gelsolin would be inactive, and the free fragment would be active. Second, another molecule may interact with gelsolin, but not the fragment, to inactivate the complex. Third, the capping and nucleating activities of the two complexes may not be quantitatively identical.

We are grateful to Kalliopi Patane for preparing rabbit plasma gelsolin and actin, to Margaret McHugh for photography, to Jei-In Young for culturing cells, to Bill McConnaughey for building and maintaining the cell poker, to Walter Nulty for operation of the analytical ultracentrifuge, to Steve Tur-

ney and Jim Voyvodic for computer programming, and to Susan Cooper for editing the manuscript. Dan Kiehart provided valuable advice and assistance in setting up the microinjection technique.

J. A. Cooper is a postdoctoral fellow of the Damon Runyon-Walter Winchell Cancer Fund. The research was supported by National Institutes of Health grants GM 13332 to C. Frieden, GM 27160 to E. Elson, and GM 26091 and HL 26973 to J. Bryan.

Received for publication 4 September 1986, and in revised form 11 November 1986.

Note Added in Proof: We refer the reader to two recent publications that describe some experiments similar to these: Füchtbauer, A., B. M. Jockusch, E. Leberer, and D. Pette, 1986, *Proc. Natl. Acad. Sci. USA.*, 83:9502-9506; and Jockusch, B. M., A. Füchtbauer, C. Wiegand, and B. Höner, 1986, *In Cell and Molecular Biology of the Cytoskeleton*, J. W. Shay, editor, Plenum Publishing Corp., New York, 7:1-40.

References

1. Babul, J., and E. Stellwagen. 1969. Measurement of protein concentration with interference optics. *Anal. Biochem.* 28:216-221.
2. Barak, L. S., R. R. Yocum, E. A. Nothnagel, and W. W. Webb. 1981. Fluorescence staining of the actin cytoskeleton in living cells with 7-nitrobenz-2-oxa-1,3-diazole-phalloidin. *Proc. Natl. Acad. Sci. USA.* 77:980-984.
3. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
4. Bryan, J., and S. Hwo. 1986. Definition of an N-terminal actin-binding domain and a C-terminal Ca^{2+} regulatory domain in human brevin. *J. Cell Biol.* 102:1439-1446.
5. Bryan, J., and M. C. Kurth. 1984. Actin-gelsolin interactions. Evidence for two actin-binding sites. *J. Biol. Chem.* 259:7480-7487.
6. Bryan, J., and L. M. Coluccio. 1985. Kinetic analysis of F-actin depolymerization in the presence of platelet gelsolin and gelsolin-actin complexes. *J. Cell Biol.* 101:1236-1244.
7. Chaponnier, C., R. Borgia, E. Rungger-Brandle, R. Weil, and G. Gabbiani. 1979. An actin-destabilizing factor is present in human plasma. *Experientia (Basel).* 35:1039-1040.
8. Chaponnier, C., P. Patebex, and G. Gabbiani. 1985a. Human plasma actin-depolymerizing factor. Purification, biological activity and localization in leukocytes and platelets. *Eur. J. Biochem.* 146:267-276.
9. Chaponnier, C., H. L. Yin, and T. P. Stossel. 1985b. Reversible actin-gelsolin complex formation in macrophages. *J. Cell Biol.* 101:136a. (Abstr.)
10. Cooper, J. A., J. Bryan, C. Frieden, D. J. Loftus, B. Schwab, III, and E. L. Elson. 1986. Microinjection of gelsolin into living cells. *J. Cell Biol.* 103:286a. (Abstr.)
11. Coue, M., and E. D. Korn. 1985. Interaction of plasma gelsolin with G-actin and F-actin in the presence and absence of calcium ions. *J. Biol. Chem.* 260:15033-15041.
12. Doi, Y., and C. Frieden. 1984. Actin polymerization. The effect of brevin on filament size and rate of polymerization. *J. Biol. Chem.* 259:11868-11875.
13. Fuchtbauer, A., B. M. Jockusch, H. Maruta, M. W. Kiliamann, and G. Isenberg. 1983. Disruption of microfilament organization after injection of F-actin capping proteins into living tissue culture cells. *Nature (Lond.).* 304:361-364.
14. Harris, H. E. 1985. Covalent complexes formed between plasma gelsolin and actin with a zero-length cross-linking compound. *Biochemistry.* 24:6613-6618.
15. Harris, D. A., and J. H. Schwartz. 1981. Characterization of brevin, a serum protein that shortens actin filaments. *Proc. Natl. Acad. Sci. USA.* 78:6798-6802.
16. Harris, H. E., and A. G. Weeds. 1983. Plasma actin depolymerizing fac-

tor has both calcium-dependent and calcium-independent effects on actin. *Biochemistry.* 22: 2728-2741.

17. Herman, I. M., N. J. Crisona, and T. D. Pollard. 1981. Relation between cell activity and the distribution of cytoplasmic actin and myosin. *J. Cell Biol.* 90:84-91.

18. Hwo, S., and J. Bryan. 1986. Immuno-identification of Ca^{2+} -induced conformational changes in human gelsolin and brevin. *J. Cell Biol.* 102:227-236.

19. Kilhoffer, M.-C., and D. Gerard. 1985. Fluorescence study of brevin, the M_r 92,000 actin-capping and -fragmenting protein isolated from serum. Effect of Ca^{2+} on protein conformation. *Biochemistry.* 24:5653-5660.

20. Kurth, M. C., and J. Bryan. 1984. Platelet activation induces the formation of a stable gelsolin-actin complex from monomeric gelsolin. *J. Biol. Chem.* 259:7473-7479.

21. Kwiatkowski, D. J., T. P. Stossel, S. H. Orkin, J. E. Mole, H. R. Colten, and H. L. Yin. 1986. Plasma and cytoplasmic gelsolins are encoded by a single gene and contain a duplicated actin-binding domain. *Nature (Lond.).* 323:455-458.

22. Markey, F., T. Persson, and U. Lindberg. 1982. A 90,000-dalton actin-binding protein from platelets. Comparison with villin and plasma brevin. *Biochim. Biophys. Acta.* 709:122-133.

23. Pasternak, C., and E. L. Elson. 1985. Lymphocyte mechanical response triggered by cross-linking surface receptors. *J. Cell Biol.* 100:860-872.

24. Petersen, N. O., W. B. McConaughy, and E. L. Elson. 1982. Dependence of locally measured cellular deformability on position on the cell, temperature, and cytochalasin D. *Proc. Natl. Acad. Sci. USA.* 79:5327-5331.

25. Pollard, T. D., and J. A. Cooper. 1982. Methods to characterize actin filament networks. *Methods Enzymol.* 85:211-233.

26. Pollard, T. D., and J. A. Cooper. 1986. Actin and actin-binding proteins. A critical evaluation of mechanisms and functions. *Annu. Rev. Biochem.* 55:987-1035.

27. Sanger, J. W., B. Mittal, and J. M. Sanger. 1984. Interaction of fluorescently-labeled contractile proteins with the cytoskeleton in cell models. *J. Cell Biol.* 99:918-928.

28. Soua, Z., F. Porte, M.-C. Harricane, J. Feinberg, and J.-P. Capony. 1985. Bovine serum brevin. Purification by hydrophobic chromatography and properties. *Eur. J. Biochem.* 153:275-287.

29. Stossel, T. P., C. Chaponnier, R. M. Ezzell, J. H. Hartwig, P. A. Janmey, D. J. Kwiatkowski, S. E. Lind, D. B. Smith, F. S. Southwick, H. L. Yin, and K. S. Zaner. 1985. Nonmuscle actin-binding proteins. *Annu. Rev. Cell Biol.* 1:353-402.

30. Tellam, R., and C. Frieden. 1982. Cytochalasin D and platelet gelsolin accelerate actin polymer formation. A model for regulation of the extent of actin polymer formation in vivo. *Biochemistry.* 21:3207-3214.

31. Thorstensson, F., G. Utter, and R. Norberg. 1982. Further characterization of the calcium-dependent F-actin-depolymerizing protein of human serum. *Eur. J. Biochem.* 126:11-16.

32. Wang, L.-L., and J. Bryan. 1981. Isolation of calcium-dependent platelet proteins that interact with actin. *Cell.* 25:637-649.

33. Yin, H. L., J. H. Hartwig, K. Maruyama, and T. P. Stossel. 1981. Calcium control of actin filament length. Effects of macrophage gelsolin on actin polymerization. *J. Biol. Chem.* 256:9693-9697.

34. Yin, H. L., D. J. Kwiatkowski, J. E. Mole, and F. S. Cole. 1984. Structure and biosynthesis of cytoplasmic and secreted variants of gelsolin. *J. Biol. Chem.* 259:5271-5276.

35. Yin, H. L., and T. P. Stossel. 1979. Control of cytoplasmic actin gel-sol transformation by gelsolin, a calcium dependent regulatory protein. *Nature (Lond.).* 281:583-586.

36. Yin, H. L., J. H. Albrecht, and A. Fattoum. 1981. Identification of gelsolin, a calcium dependent regulatory protein of actin gel-sol transformation, and its intracellular distribution in a variety of cells and tissues. *J. Cell Biol.* 91:901-906.

37. Yin, H. L., and T. P. Stossel. 1980. Purification and structural properties of gelsolin, a calcium activated regulatory protein of macrophages. *J. Biol. Chem.* 255:9490-9493.

38. Yin, H. L., K. S. Zaner, and T. P. Stossel. 1980. Calcium control of actin gelation. Interaction of gelsolin with actin filaments and regulation of actin gelation. *J. Biol. Chem.* 255:9494-9500.