Ca^{2+} Regulation of Gelsolin Activity: Binding and Severing of F-actin

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ABSTRACT Regulation of the F-actin severing activity of gelsolin by Ca^{2+} has been investigated under physiologic ionic conditions. Tryptophan fluorescence intensity measurements indicate that gelsolin contains at least two Ca^{2+} binding sites with affinities of 2.5×10^7 M^{-1} and 1.5×10^5 M^{-1}. At F-actin and gelsolin concentrations in the range of those found intracellularly, gelsolin is able to bind F-actin with half-maximum binding at 0.14 μM free Ca^{2+} concentration. Steady-state measurements of gelsolin-induced actin depolymerization suggest that half-maximum depolymerization occurs at ~0.4 μM free Ca^{2+} concentration. Dynamic light scattering measurements of the translational diffusion coefficient for actin filaments and nucleated polymerization assays for number concentration of actin filaments both indicate that severing of F-actin occurs slowly at micromolar free Ca^{2+} concentrations. The data suggest that binding of Ca^{2+} to the gelsolin-F-actin complex is the rate-limiting step for F-actin severing by gelsolin; this Ca^{2+} binding event is a committed step that results in a Ca^{2+} ion bound at a high-affinity, EGTA-resistant site. The very high affinity of gelsolin for the barbed end of an actin filament drives the binding reaction equilibrium toward completion under conditions where the reaction rate is slow.

INTRODUCTION

Gelsolin is a Ca^{2+}-activated actin-binding protein consisting of six homologous domains (G1–6). A ternary complex of gelsolin and two actin monomers acts as a nucleus for actin polymerization, resulting in elongation at the pointed end of the actin filament (Wegner et al., 1994). Gelsolin binds to F-actin and severs the actin filament by breaking the noncovalent bonds between actin monomers in a polymer; the final products of the severing reaction are two actin filaments, one of which contains gelsolin bound to the barbed end with very high affinity (estimated to be $>10^{11}$ M^{-1}) (Janmey et al., 1985). High-affinity complexes of gelsolin with G-actin and F-actin (i.e., capped filaments), which form in the presence of Ca^{2+}, are not dissociated by chelation of the free Ca^{2+}, but require binding of phosphatidylinositol phosphates (phosphatidylinositol (4) phosphate (PIP) and phosphatidylinositol (4, 5) bisphosphate (PIP_2)) by gelsolin to dissociate the complexes (Janmey et al., 1987). In addition to the direct regulation of actin filaments, gelsolin may serve to modify a number of cellular activities through its interaction with membrane lipids, related enzymes, and cytoskeletal elements. The reported cellular roles of gelsolin include regulation of lipid metabolism (Baldassare et al., 1997; Singh et al., 1996; Steed et al., 1996), control of ion channels (Ismailov et al., 1997; Maximov et al., 1997), participation in apoptosis (Kothakota et al., 1997; Ohtsu et al., 1997), and activation of DNase I (Davoodian et al., 1997). Gelsolin is down-regulated in human neoplastic lesions, including bladder, breast, and colon cancers (Asch et al., 1996).

The Ca^{2+} regulation of gelsolin function has been described in a number of conflicting reports. Early studies indicated that ~1 μM Ca^{2+} was needed for activation of gelsolin binding and severing of F-actin (Yin and Stossel, 1979). More recent studies, however, suggest that 10 μM free Ca^{2+} is needed for half-maximum activation of gelsolin severing and nucleating activity (Lamb et al., 1993) and that F-actin binding by gelsolin is regulated by free Ca^{2+} in the 100-μM range (Allen and Janmey, 1994). It has been shown that a Ca^{2+}-induced gelsolin conformational change that is half-maximum at a free Ca^{2+} concentration of ~30 nM results in an increase in the hydrodynamic radius, which is thought to be due to the release of interactions between G6 and G2, which opens up the gelsolin molecule (Burtnick et al., 1997; Pope et al., 1997). The monomeric actin binding sites of gelsolin are reported to be exposed by Ca^{2+} binding to another site, resulting in half-maximum actin binding at 3 μM Ca^{2+} (Pope et al., 1997). Another study reported that monomeric actin binding was regulated by Ca^{2+} binding at two sites with dissociation constants of 25 μM and 200 μM (Ditsch and Wegner, 1995).

Reports indicating that high free Ca^{2+} concentrations are necessary for gelsolin activation bring into question the role gelsolin plays in vivo, where the resting intracellular Ca^{2+} levels are around 0.2 μM. However, evidence suggests that intracellular gelsolin does play a role in cell motility. Platelets, neutrophils, fibroblasts, and neurons from gelsolin-null mice exhibit impaired function (Lu et al., 1997; Witke et al., 1995), suggesting that gelsolin acts as a cytoskeletal regulator in certain cell types. It has been reported that in activated platelets, peripheral actin filaments are severed by gelsolin in response to a Ca^{2+} transient, and binding of PIP_2 or PIP by gelsolin uncaps the newly created actin filament

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barbed ends, allowing rapid actin polymerization (Hartwig, 1992; Hartwig et al., 1995; Lind et al., 1987). In activated neutrophils, pathways involving the GTP-binding protein Rac control gelsolin-actin dissociation and subsequent actin polymerization (Arcaro, 1998).

Evidence that describes the putative mechanism by which gelsolin can break the bonds between the tightly associated actin monomers within an actin filament has been derived mostly from structural data. Analysis of the actions of the domains of gelsolin has revealed three actin-binding sites: one F-actin binding site located on G2, and two G-actin binding sites, one each on G1 and G4 (Bryan and Kurth, 1984; Kwiatkowski et al., 1989; McLaughlin et al., 1993; Pope et al., 1995; Way et al., 1989). Recent reports of the crystal structure of gelsolin (without bound Ca^{2+}) (Burtnick et al., 1997) and the structure of the complex of F-actin with G2–6, based on electron microscopic reconstructions (McGough et al., 1998), support the view that initially G2 binds to F-actin and then G1 intercalates between two longitudinally adjacent actin subunits in the polymer, thereby breaking the bonds between them and severing the actin filament.

In previously published work we showed that, at high Ca^{2+} concentration (0.5 mM), gelsolin binds to F-actin with a diffusion limited second-order rate constant, $2 \times 10^7\ M^{-1}\ s^{-1}$, and severs F-actin in an apparent first-order process (Kinosian et al., 1996). In the present report, we investigate gelsolin binding and severing of F-actin over a range of Ca^{2+} concentrations and suggest a model for the Ca^{2+} regulation of gelsolin activity. Measurements of tryptophan fluorescence intensity indicate that gelsolin undergoes two conformational changes in response to Ca^{2+}, which are half-maximal at 0.04 μM and 7 μM Ca^{2+}. Based on measurements of gelsolin-induced actin depolymerization and cosedimentation experiments, we find that gelsolin acts in vitro to bind, sever, and cap F-actin at Ca^{2+} concentrations approximating those within resting cells ($\sim$0.2 μM). Analysis of the Ca^{2+} concentration dependence for the initial rate of gelsolin-induced actin depolymerization yields half-maximum activity at 40 μM; in contrast, measurements of the same samples at steady state show half-maximum depolymerization at 0.4 μM Ca^{2+}. These apparently conflicting results suggest an explanation for the inconsistent reports of the Ca^{2+} concentrations needed for regulation of gelsolin activity. We show here, using separate determinations of actin filament size and number, that the rate of F-actin severing by gelsolin is dependent on the free Ca^{2+} concentration, but the severing reaction proceeds to completion, even at micromolar free Ca^{2+} concentrations. The observed association rate of gelsolin with F-actin is linearly dependent on the free Ca^{2+} concentration and can be described as either a slow binding of Ca^{2+} to a gelsolin-F-actin complex with $k = 10^3\ M^{-1}\ s^{-1}$, or a third-order process with $k = 10^{10}\ M^{-2}\ s^{-1}$. The binding of Ca^{2+} by gelsolin in the presence of F-actin leads to a committed step that cannot be reversed by the chelation of Ca^{2+}. Our results suggest that an initial high-affinity (K_d = 40 nM) Ca^{2+} binding by gelsolin dissociates the G2-G6 connection, which exposes the F-actin binding site on gelsolin G2. Ca^{2+} binds to G1 in the gelsolin-F-actin complex and becomes trapped as a result of conformational changes during severing.

MATERIALS AND METHODS

Protein preparation

Gelsolin was prepared from human plasma by the method of Kurokawa et al. (1990) and dialyzed against 10 mM HEPES, pH 7.0, 50 mM NaCl, 0.1 mM EGTA, 0.5 mM dithiotreitol (DTT), 0.01% NaN_3. When necessary, gelsolin was concentrated using Centriplus 30 concentrators (Amicon). Aliquots were quickly frozen in liquid nitrogen and stored at -80°C.

Actin was prepared from rabbit skeletal muscle acetone powder by a modification of previously published procedures (Estes et al., 1981) as follows. After acetone powder extraction, the slurry was centrifuged at 16,000 $\times$ *g* for 20 min, and the supernatant was further clarified by centrifugation at 200,000 $\times$ *g* for 30 min. The supernatant was converted to Mg-actin by the addition of 0.2 mM $MgCl_2$ and 0.5 mM EGTA. The Mg-actin was polymerized by the addition of 100 mM KCl, 2 mM $MgCl_2$, and 1 mM ATP. At this step, the actin is allowed to polymerize for at least 1 h and may be left overnight. After polymerization is complete, 0.8 M KCl (final concentration) was added and the solution was incubated for 2 h. The solution was then centrifuged at 200,000 $\times$ *g* for 2 h. The pellets were collected and homogenized in 150 ml of 2 mM Tris, pH 8.0, 100 mM KCl, 2 mM $MgCl_2$ 0.5 mM ATP, 0.5 mM EGTA, and 0.5 mM DTT and centrifuged at 200,000 $\times$ *g* for 2 h. The pellets were collected, homogenized in 2 mM Tris, pH 8.0, 0.2 mM ATP, 0.5 mM DTT, 0.2 mM $CaCl_2$, and 0.01% NaN_3, and brought to a concentration of $\sim$120 μM. The actin was then dialyzed against 2 liters of the same buffer with two changes. This monomeric actin was clarified by centrifugation at 200,000 $\times$ *g* for 3 h, and the supernatants were filtered with a 0.45-μm filter and stored at 4°C. Before use, a final chromatographic step was used in which 40–50 mg of actin was passed through a 2.5 $\times$ 100 cm Sephacryl S-300 column.

Actin was labeled with *N*-(1-pyrenyl) iodoacetamide (pyrene) (Kouyama and Mihashi, 1981) for fluorescence assays of gelsolin binding.

Free Ca^{2+} concentration determination

Free Ca^{2+} concentration up to 20 μM was buffered using $CaCl_2$ and EGTA, and the free Ca^{2+} concentration was calculated using a computer program (Perrin and Sayce, 1967). Free Ca^{2+} concentration was also measured using the fluorescent Ca^{2+} indicator Fura 2FF (Texas Fluorescence Labs), which is insensitive to Mg^{2+}. Good agreement was found between calculated and measured free Ca^{2+} concentrations.

Cosedimentation of gelsolin and F-actin

Mg-actin was polymerized with 100 mM KCl and 2 mM $MgCl_2$. Solutions of 80–100 μM F-actin were adjusted with $CaCl_2$ and EGTA to obtain various free Ca^{2+} concentrations, and gelsolin (0.2–0.3 μM) was added. The samples were typically incubated for half an hour, although overnight incubation gave similar results. Samples were centrifuged for 40 min at 30 psi in a Beckman Airfuge ($\sim$90,000 $\times$ *g*). Supernatants were analyzed by scanning densitometry (Molecular Dynamics model 300B) of Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels.

Gelsolin-induced actin depolymerization experiments

Pyrene-labeled Mg-actin was polymerized with 2 mM $MgCl_2$, 100 mM KCl and diluted to 0.2 μM in the same buffer; at steady state, the actin critical concentration (c_c) is 0.1 μM, and 0.1 μM polymer is present.

Severing and capping of the barbed actin filament end by gelsolin increases the actin c_c to that for the pointed end, 0.7 μM, and results in depolymerization of the F-actin. The free Ca^{2+} concentration in the F-actin samples was adjusted with EGTA. To minimize perturbations in the sample, rather than diluting F-actin to a concentration below the c_c, we added 0.01 μM gelsolin directly to 0.2 μM F-actin in the cuvette with stirring while the fluorescence intensity time course was being recorded; this procedure allowed accurate monitoring of the initial time course of depolymerization.

Dynamic light scattering

Samples were prepared by diluting actin to the desired concentration and filtering (0.08 μm Nuclepore) directly into optical cuvettes (sample volume 0.4–1 ml). Small volumes of polymerizing salts (0.1 M KCl ± 2 mM $MgCl_2$) were added to the actin, and after monitoring polymerization by dynamic light scattering (DLS), EGTA and/or $CaCl_2$ was added to bring the sample to the desired free calcium concentration. At time 0, gelsolin was added to a molar ratio of 20:1 actin:gelsolin, and DLS was used to monitor the severing of actin filaments. DLS data were obtained at room temperature at a laser wavelength of 514.5 nm and a scattering angle of 90°, as previously described (Newman et al., 1982). Consecutive 20-s experiments were performed to monitor the changes in actin diffusion coefficient, *D*, determined by cumulant analysis. The time course of *D* was fit by a single-exponential function to obtain a characteristic severing rate for gelsolin at the given solvent conditions.

Monomer add-on assay for actin filament number concentration

The actin filament number concentration was assayed by monitoring the rate of addition of monomeric pyrenyl-actin to the pointed ends of gelsolin-severed actin filaments. In the presence of various free Ca^{2+} concentrations, 50 nM gelsolin was added to 1 μM unlabeled F-actin. At timed intervals EGTA (in excess of $CaCl_2$) was added to stop severing, 1 μM pyrenyl-G-actin was added, and the resultant fluorescence intensity increase recorded. The observed rate constant for polymerization is proportional to the number concentration of actin filaments present. An exponential function was fit to the time course of polymerization:

$$F(t) = F_0 + \Delta F\{1 - \exp(-k_{obs}t)\} \quad (1)$$

where F_0 is the initial fluorescence intensity value, ΔF is the change in fluorescence intensity, and k_{obs} is the observed first order rate constant for polymerization. The observed rate constant $k_{obs} = mk_p$, where m is the number concentration of actin filaments and k_p is the second order rate constant for monomer addition onto the pointed ends of filaments (the barbed ends are gelsolin-capped). The number concentration of filaments, m, produced by the severing of F-actin by gelsolin, was calculated:

$$m = G \times (k_{obs} - k_{min})/(k_{max} - k_{min}) \quad (2)$$

where *G* is the gelsolin concentration, k_{min} is the value obtained for k_{obs} in the absence of Ca^{2+}, and k_{max} is the value obtained for k_{obs} in the presence of 500 μM Ca^{2+}. Thus k_{min} represents k_{obs} in the presence of no additional filaments produced by severing, and k_{max} represents k_{obs} in the presence of the maximum number of filaments produced by severing.

Gelsolin binding to F-actin in the presence of phalloidin

Actin was polymerized with 100 mM KCl, 2 mM $MgCl_2$, 0.2 mM ATP, and either equimolar phalloidin or tetramethylrhodamine isothiocyanate-phalloidin (TRITC-phalloidin). Experiments were carried out by rapidly mixing gelsolin with F-actin in a 1 × 1 cm cuvette with constant stirring. Fluorescence intensity was monitored with an Aminco Bowman AB2 spectrofluorometer. F-actin was diluted to either 10 nM or 100 nM concentrations, and the desired free Ca^{2+} concentrations were obtained with mixtures of $CaCl_2$ and EGTA. The fluorescence intensity time course was recorded for pyrene-F-actin as either 10 nM gelsolin was added to 100 nM pyrene-F-actin or 100 nM gelsolin was added to 10 nM pyrene-F-actin. The fluorescence intensity for TRITC-phalloidin was recorded as 100 nM gelsolin was added to 10 nM F-actin containing equimolar TRITC phalloidin. The time course data were fit by an equation containing two exponential rate constants:

$$F(t) = \Delta F_1 \cdot \exp(-k_1 t) + \Delta F_2 \cdot \exp(-k_2 t) + F(\infty) \quad (3)$$

where ΔF_1 and ΔF_2 are the fluorescence intensity changes for each phase, k_1 and k_2 are the rate constants for each phase, and $F(\infty)$ is the final fluorescence intensity value. All experiments are considered to be pseudo-first-order because the concentration of one reactant is 10 times greater than the other; thus the value for k_1 is equal to either k_+[gelsolin] for experiments containing 100 nM gelsolin or k_+[F-actin] for experiments containing 100 nM F-actin, where k_+ is the second-order association rate constant.

RESULTS

Ca^{2+} binding to gelsolin induces conformational changes determined by tryptophan fluorescence intensity

Gelsolin was titrated with $CaCl_2$ in the presence of EGTA to various free Ca^{2+} concentrations. Fig. 1 shows the tryptophan fluorescence intensity of gelsolin as a function of free Ca^{2+} concentration. The symbols represent individual titrations, which were done in the presence (*circles*) or absence (*squares*) of 2 mM $MgCl_2$. The lines are fits to the data, using two hyperbolic terms yielding K_d values of 0.05 (± 0.01) μM and 6.0 (± 3.6) μM in the presence of 2 mM $MgCl_2$ (*circles, solid line*) or K_d values of 0.03 (± 0.01) μM and 8.3 (± 1.7) μM in the absence of $MgCl_2$ (*squares, dashed line*). The presence of 2 mM $MgCl_2$ did not significantly affect the apparent equilibrium dissociation constants for Ca^{2+}. The data indicate that gelsolin undergoes conformational changes induced by Ca^{2+} binding at two separate sites, although the stoichiometry of Ca^{2+} binding at each site could not be determined.

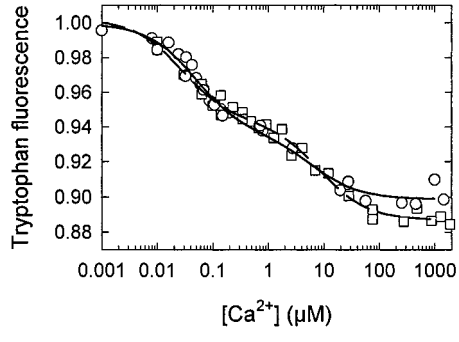

FIGURE 1 Titration of gelsolin tryptophan fluorescence with Ca^{2+}. The symbols represent data for titrations of 0.75 μM gelsolin, in 10 mM HEPES, pH 7.0, 100 mM KCl, 0.1 mM EGTA. The lines are fits to the data, using two hyperbolic terms with apparent K_d values of 0.05 (±0.01) μM and 6.0 (±3.6) μM in the presence of 2 mM $MgCl_2$ (—○—) or K_d values of 0.03 (± 0.01) μM and 8.3 (±1.7) μM in the absence of $MgCl_2$ (– – □ – –).

Cosedimentation of gelsolin with F-actin

To investigate the binding of gelsolin at approximately physiologic protein concentrations, we incubated 0.2 μM gelsolin with 80–100 μM F-actin at various free Ca^{2+} concentrations. The samples were centrifuged and the supernatant contents analyzed by densitometric scanning of Coomassie blue-stained SDS-PAGE gels. The results in Fig. 2 show the fraction of gelsolin in the supernatants as a function of free Ca^{2+} concentration; the line is a fit to the data with an apparent K_d of 0.14 ($\pm$ 0.04) μM. These results suggest that significant binding of gelsolin to F-actin may occur in vivo, where the resting intracellular free Ca^{2+} concentration is in the range of 0.1–1 μM.

Gelsolin-induced depolymerization of F-actin

Fig. 3 shows the results of gelsolin-induced actin depolymerization experiments. Fig. 3 *A* shows typical time courses for actin depolymerization induced by severing and barbed-end capping of F-actin by gelsolin at the indicated free Ca^{2+} concentrations. The inset shows the first derivatives of the depolymerization time courses; the increasingly negative derivative for the 5 and 10 μM Ca^{2+} samples indicates that the depolymerization accelerates during the first few minutes. We interpret this as due to the slow severing of actin filaments during the depolymerization time course, with formation of more pointed actin filament ends and, thus, an increasing depolymerization rate. Fig. 3 *B* shows the values determined for the initial depolymerization rates as a function of free Ca^{2+} concentration; the line is a binding function fit to the data with half-saturation at 37 μM Ca^{2+}. Fig. 3 *C* shows the steady-state fluorescence values after 18 h of incubation; a fit to the data with the Hill equation yields K_d = 0.42 ($\pm$ 0.02) μM and n = 2.5 ($\pm$ 0.2). Thus, even though the actin depolymerization rates suggest that half-maximum gelsolin activity requires ~40 μM Ca^{2+}, given enough time, gelsolin activity is evident even at free Ca^{2+} concentration below 1 μM.

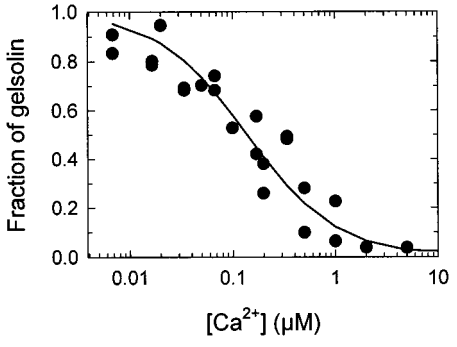

FIGURE 2 Cosedimentation of gelsolin with F-actin. Gelsolin (0.2–0.3 μM) and F-actin (80–100 μM) were mixed in the presence of various free Ca^{2+} concentrations and centrifuged. The gelsolin in the supernatant was assayed by scanning densitometry of Coomassie blue-stained SDS-PAGE gels. The line is a binding curve fit to the data with K_d = 0.14 ($\pm$ 0.04) μM.

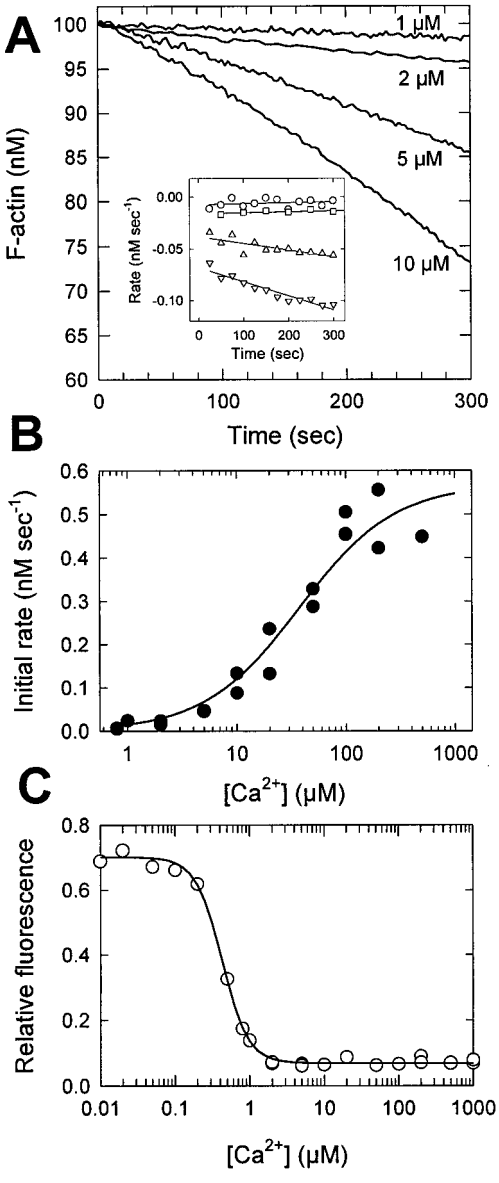

FIGURE 3 Depolymerization of actin induced by gelsolin. (*A*) Time courses for depolymerization of pyrene-F-actin after the addition of 10 nM gelsolin at various free Ca^{2+} concentrations. The inset shows the first derivative of the depolymerization time courses for the 1, 2, 5, and 10 μM Ca^{2+} samples (*from top to bottom, respectively*). The initial rates of depolymerization are plotted in *B* as a function of the free Ca^{2+} concentration. The line is a fit to a binding equation with a half-maximum initial rate at 37 ($\pm$ 8) μM free Ca^{2+} concentration. (*C*) Values for the pyrene-actin fluorescence after 18 h of incubation, and the line is a fit by the Hill equation with half-maximum actin depolymerization at 0.42 ($\pm$ 0.02) μM, and n = 2.5 ($\pm$ 0.2).

Measurements of F-actin severing by gelsolin

Because gelsolin-induced actin depolymerization assays indicate that the time courses for severing and depolymerization overlap, determining the amount and rate of severing by gelsolin by such assays is somewhat ambiguous. We have therefore employed alternative techniques to measure severing. Dynamic light scattering (DLS) measurements do not

perturb the sample and provide an estimate of polymer size. We have also used the rate of nucleated actin polymerization as an assay for the number of polymers in solution. Results obtained using these two independent methods correlate well with each other, as described below.

Dynamic light scattering measurements of F-actin severing by gelsolin

The time course of F-actin severing by gelsolin was followed using DLS to determine the translational diffusion coefficient, *D*, which is inversely proportional to the length of the polymers. The time course of *D* can be followed only for relatively low Ca^{2+} concentrations and slow severing rates because each measurement of *D* requires ~20 s. Fig. 4 *A* shows representative data obtained at several free Ca^{2+} concentrations with 5 μM F-actin and 0.25 μM gelsolin. The observed severing rate constants decrease markedly with decreasing free Ca^{2+} concentration, but the final values obtained for *D* indicate that the extent of the severing reaction did not vary with free Ca^{2+} concentration. The presence of phalloidin had little effect on the rate constant for severing (data not shown).

Monomeric actin add-on assay of F-actin severing by gelsolin

To assay for severing on a faster time scale than could be achieved with DLS measurements, the observed rate constant for polymerization of pyrene-actin onto the pointed ends of actin filaments was used to assay for the number concentration of actin polymers. Fig. 4 *B* shows the time course for severing of 1 μM F-actin by 0.05 μM gelsolin at the indicated free Ca^{2+} concentrations. The lines are fits to the data by a single-exponential function.

Summary of severing rate constant measurements

Fig. 4 *C* shows the observed severing rate constants, k_{sev}, determined from DLS experiments (*circles*) and monomeric actin add-on experiments (*triangles*) at various free Ca^{2+} concentrations. All experiments used a gelsolin:actin molar ratio of 1:20. The results for the two independent methods show good correlation with one another; the log-log slope for the add-on data alone is 1.91 ($\pm$ 0.23) and for DLS data alone is 1.62 ($\pm$ 0.25). The solid line is a linear fit to the log-log plot of all of the data with a slope of 1.85 ($\pm$ 0.18). These results indicate that gelsolin can sever F-actin at micromolar free Ca^{2+} concentrations, although at very slow rates, and the Hill coefficient of 1.85 suggests that more than one Ca^{2+} binding site affects the severing rate.

Fluorescence assays for the binding of gelsolin to phalloidin-stabilized F-actin

The association of gelsolin with F-actin in the presence of phalloidin was detected by monitoring the quenching of pyrene-F-actin fluorescence intensity or the quenching of TRITC-phalloidin fluorescence intensity (Allen and Janmey, 1994; Kinosian et al., 1996). The presence of phalloidin or TRITC-phalloidin prevents depolymerization of F-actin, which would otherwise result from capping of actin filament barbed ends by gelsolin (Kinosian et al., 1996). At micromolar Ca^{2+} concentrations, the association of gelsolin with phalloidin-stabilized F-actin is biphasic. Fig. 5 *A* shows representative time courses for pyrene-F-actin binding by gelsolin in the presence of various free Ca^{2+} concentrations as indicated. The lines are fits to the data using

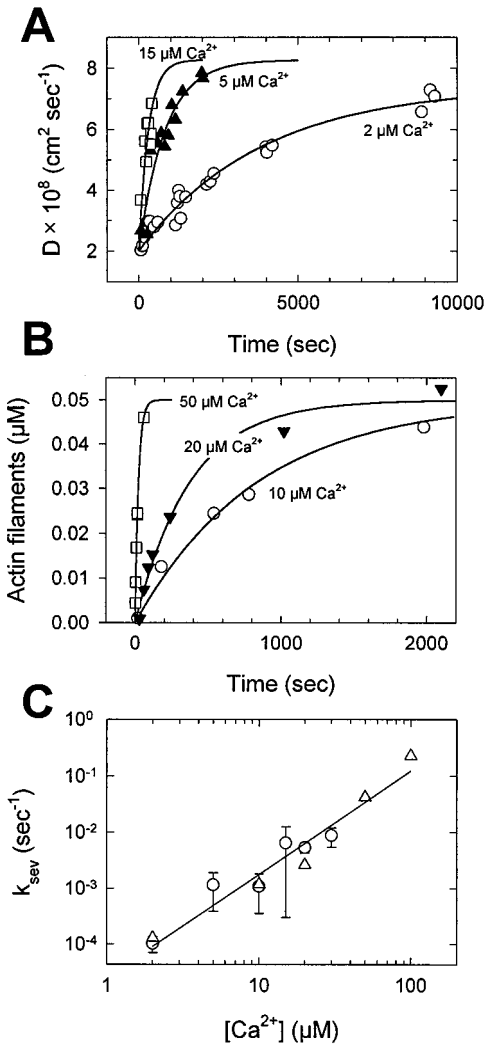

FIGURE 4 Severing of F-actin by gelsolin. (*A*) Representative time courses of the translational diffusion coefficients for actin polymers measured by dynamic light scattering. Gelsolin at a molar ratio of 1:20 was added to solutions of 5–10 μM F-actin in the presence of various concentrations of free Ca^{2+}, as indicated. Lines are exponential fits to the data. (*B*) Time course data for actin monomer add-on assays (as described in Materials and Methods) in the presence of various free Ca^{2+} concentrations, as indicated. Lines are exponential fits to the data. (*C*) The values for the observed severing rate constant, k_{sev}, as determined from DLS (○; error bars represent the standard deviation for two to six determinations) and by monomer add-on experiments (△). The line is a linear regression to the log-log plot with a slope of 1.85 ($\pm$ 0.18).

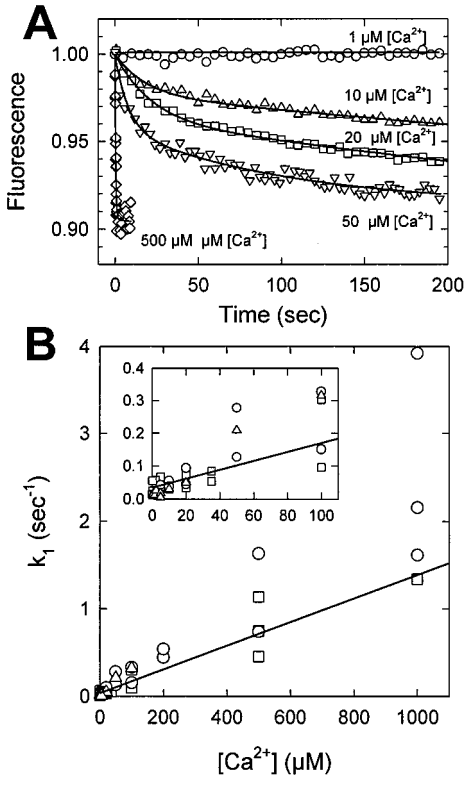

FIGURE 5 Association of gelsolin with phalloidin-stabilized F-actin in the presence of various concentrations of free Ca^{2+}. Samples contain 100 mM KCl, 2 mM $MgCl_2$, 0.2 mM ATP, 10 mM HEPES, pH 7.0, 1 mM EGTA, and various concentrations of $CaCl_2$. (*A*) Representative time course data for 10 nM gelsolin and 100 nM pyrene-F-actin/phalloidin at the indicated free Ca^{2+} concentrations. (*B*) The symbols represent the pseudo-first-order rate constants for the association of gelsolin and F-actin (k_1 from Eq. 3) for 10 nM gelsolin and 100 nM F-actin (○), 100 nM gelsolin and 10 nM pyrene-F-actin (□), and 100 nM gelsolin and 10 nM F-actin/TRITC-phalloidin (△). The line is a linear regression to the data with a slope of $2 \times 10^3\ M^{-1}\ s^{-1}$. The inset shows the same data at the lower range of Ca^{2+} concentrations.

Eq. 3. Fig. 5 *B* shows the observed pseudo-first-order rate constant for association of gelsolin with F-actin (k_1 from Eq. 3), determined for a range of free Ca^{2+} concentrations. A linear regression for the data yields a slope of $2 \times 10^3\ M^{-1}\ s^{-1}$. The data are consistent with either a slow activation of gelsolin by Ca^{2+} binding or a third-order process in which Ca^{2+}, F-actin, and gelsolin form a complex with a rate constant of $2 \times 10^3\ M^{-1}\ s^{-1}/10^{-7}\ M = 2 \times 10^{10}\ M^{-2}\ s^{-1}$.

DISCUSSION

In the present study, we have sought to reconcile conflicting reports on the Ca^{2+} concentration dependence of gelsolin activity and to correlate the functional properties of gelsolin with the recent structural data reported for gelsolin (Burtnick et al., 1997; McGough et al., 1998; Pope et al., 1997). Our data suggest that at physiologic ionic strength, and at Ca^{2+} and protein concentrations approximating those found intracellularly, gelsolin functions to sever and cap actin filaments. Although the rate of gelsolin binding and severing of F-actin at micromolar free Ca^{2+} concentrations appears to be orders of magnitude less than maximum, the very high affinity of gelsolin for F-actin drives the reaction toward completion. Thus the apparent contradictions in the reported Ca^{2+} sensitivity for gelsolin (Allen and Janmey, 1994; Lamb et al., 1993; Yin and Stossel, 1979) can be reconciled when one considers that the rate of severing varies greatly over a wide range of free Ca^{2+} concentration, whereas the extent of the reaction is complete. Moreover, different assays for gelsolin activity can produce different apparent results.

Ca^{2+}-induced conformational changes in gelsolin

A variety of studies have found that gelsolin contains three Ca^{2+}-binding sites: a site located on G5–6 binds with a $K_d = 30$ nM, a site located on G4–5 binds with $K_d = 2\ \mu M$, and a site on G2 that binds with a $K_d = 3\ \mu M$ in the absence of actin, but which becomes "trapped" in the actin-gelsolin complex (Pope et al., 1995; Pope et al., 1997; Weeds et al., 1995). We find the Ca^{2+}-induced tryptophan fluorescence intensity changes in gelsolin are consistent with two Ca^{2+}-binding sites with K_d values of 0.04 μM and 7 μM. The higher affinity site appears to correlate with a reported change in hydrodynamic radius that was half-maximum at 30 nM Ca^{2+} (Pope et al., 1997). This Ca^{2+}-induced conformational change is thought to result from the dissociation of G6 from G2 and an opening up of the gelsolin molecule (Burtnick et al., 1997; Pope et al., 1997). The fluorescence intensity decrease resulting from the binding of Ca^{2+} at the 7-μM site may reflect the unlatching of G1 and G3, resulting in the exposure of the G1 actin-binding site, producing half-maximum G-actin binding at 3 μM Ca^{2+} (Pope et al., 1997). Alternatively, G4–6 has been shown to undergo Ca^{2+}-induced conformational changes (Hellweg et al., 1993; Pope et al., 1997); the tryptophan fluorescence quenching ($K_d = 7\ \mu M\ Ca^{2+}$) may be due in whole or in part to the Ca^{2+}-binding site located on G4–5, which regulates the binding of actin by G4 (Pope et al., 1995).

F-actin binding by gelsolin

Binding of G2 between actin subdomains 1 and 3 positions G1 so that it may intercalate between two longitudinally associated actin monomers (McGough et al., 1998). The Ca^{2+} concentration at which F-actin binding is at half-maximum, 0.14 μM, is in the range of the Ca^{2+} concentrations at which the initial gelsolin conformational change is at half-maximum, 0.04 μM. Our data (Figs. 1 and 2) suggest that once the G2-G6 latch is released by Ca^{2+} binding (Burtnick et al., 1997; Pope et al., 1997), the G2 F-actin binding site becomes exposed and is able to bind F-actin. Our observed half-maximum F-actin binding occurs at a Ca^{2+} concentration about threefold greater than the initial high-affinity Ca^{2+} binding, so it appears that the Ca^{2+}-

induced conformational change may not be just a simple switch mechanism that turns on F-actin binding. It is possible that in the "open" conformation of gelsolin (with G2-G6 unlatched), G2 binding of F-actin may be sterically hindered or exist in a conformational equilibrium between an exposed and a blocked G2 site. Thus the binding of G2 to the side of an actin filament may require a further conformational shift in the gelsolin molecule beyond the unlatching of G2 and G6.

F-actin severing by gelsolin

The rates of gelsolin-induced depolymerization of actin (Fig. 3 *A*) were found to be difficult to interpret because there is an acceleration in the depolymerization time courses at micromolar free Ca^{2+} concentrations due to ongoing severing. Analysis of depolymerization rates (Fig. 3 *B*) suggests that severing of actin by gelsolin is at half-maximum at ~40 μM Ca^{2+}; however, steady-state measurements (Fig. 3 *C*) show that gelsolin-induced actin depolymerization is at half-maximum at ~0.4 μM Ca^{2+}. Thus, even though the rate of gelsolin interaction with F-actin is slow at micromolar free Ca^{2+} concentrations, the reaction equilibrium favors severing and capping of F-actin and results in complete depolymerization at 2 μM Ca^{2+}. It should be noted that the gelsolin-induced actin depolymerization is dependent on the shift in critical concentration to that of the pointed end when the barbed ends are blocked. It has been shown that 90% of the barbed ends must be capped to produce a half-maximum critical concentration shift (Selve and Wegner, 1986). At steady state, actin nucleation creates more barbed ends; the rate of nucleation and the rate of capping result in a steady-state number of barbed ends. Thus the observed extent of gelsolin-induced actin depolymerization is not directly proportional to gelsolin severing and capping activity and depends on the gelsolin and actin concentrations. The conclusion that severing with capping, rather than capping alone, is the mechanism for actin depolymerization is supported by DLS data (Fig. 4 *A*), which shows that gelsolin severs F-actin in the presence of 2 μM Ca^{2+}.

The contrasting results between Figs. 3 and 4 help to clarify the reasons for conflicting reports concerning Ca^{2+} regulation of gelsolin activity. Gelsolin-induced depolymerization rate measurements (Fig. 3 *B*) imply that severing is regulated by simple binding of Ca^{2+} ions to an activating site on gelsolin. Figs. 3 *A* and 3 *C*, however, show that depolymerization rate measurements are misleading because severing is not complete at the time of measurement. Measurements of severing in Fig. 4 show that the rate of severing of F-actin by gelsolin is regulated by the Ca^{2+} concentration over two orders of magnitude and is approximately proportional to the second power of the Ca^{2+} concentration.

Ca^{2+} regulation of gelsolin activity

We have previously observed that the severing reaction can be stopped by chelation of Ca^{2+} using EGTA, but the pyrene fluorescence quenching that occurs upon binding of gelsolin to F-actin cannot be reversed (Kinosian et al., 1996). Titrations of pyrene-F-actin indicated that the pyrene fluorescence quench obtained with G1–3 binding is equivalent mole per mole with that obtained with gelsolin (Selden et al., 1998). We conclude that the irreversible step in the severing reaction that occurs concomitantly with the pyrene fluorescence quenching is a conformational change in an F-actin subunit caused by the G1 actin-binding site.

The "trapped" Ca^{2+} that is resistant to EGTA has been shown to be located at an intramolecular site on G1 in the gelsolin-actin complex (Weeds et al., 1995). This high-affinity Ca^{2+}-binding site exists in the G1-actin complex but is absent in G1 alone (McLaughlin et al., 1993; Weeds et al., 1995); thus the binding of actin by gelsolin creates this high-affinity site. Comparison of the structures of G1-actin and gelsolin suggests that the binding of Ca^{2+} at this G1 site causes a reorientation of the backbone between G1 and G2 as Val^{147} shifts 180° to come into position for coordination of the Ca^{2+} ion (Burtnick et al., 1997; McLaughlin et al., 1993). We postulate that this Ca^{2+}-induced conformational change is the rate-limiting kinetic step for the gelsolin-severing reaction and traps a Ca^{2+} ion in the G1 site. We consider that Ca^{2+} activation of a gelsolin-F-actin complex is not an equilibrium process—a Ca^{2+} ion becomes sterically trapped in the complex—and micromolar Ca^{2+} concentrations affect gelsolin activity. Thus our results for Ca^{2+} regulation of gelsolin binding to F-actin can be explained by a slow rate constant for Ca^{2+} association ($k = 2 \times 10^3\ M^{-1}\ s^{-1}$) or a third-order reaction ($k = 2 \times 10^{10}\ M^{-2}\ s^{-1}$) in which Ca^{2+}, gelsolin, and actin participate as the three reactants. The formation of a gelsolin(G2)-F-actin complex containing a G1-bound Ca^{2+} results in a high-affinity gelsolin(G1, G2, G4)-F-actin complex that is capable of breaking the longitudinal bonds between actin subunits in the filament.

That the Ca^{2+} dependence of the apparent rate of association of gelsolin with F-actin is linear with Ca^{2+} concentration is in agreement with a previous report (Allen and Janmey, 1994), which suggested that the rate-limiting step for gelsolin binding to F-actin was regulated by low-affinity binding of several Ca^{2+} and Mg^{2+} ions to gelsolin. However, their interpretation implies that Ca^{2+} activation of gelsolin severing is in equilibrium, whereas we find that chelation of Ca^{2+} from the solution does not reverse the binding or severing of F-actin by gelsolin. Other recent studies of Ca^{2+}-binding sites on gelsolin with K_d values in the range of 0.03–7 μM (Lin et al., 1997; Pope et al., 1995, 1997; present paper) provide evidence that micromolar Ca^{2+} concentrations bind to gelsolin and affect its conformation and activity. Thus, although we cannot rule out the possible effects of Ca^{2+} ions bound at putative low-affinity sites on gelsolin, they are not needed to explain the data.

We have observed differences in F-actin severing by G1–3 and whole gelsolin that imply that the G4–6 participates in the severing reaction, resulting in a severing efficiency of one severing event per gelsolin molecule compared to one severing event per two G1–3 molecules (Selden et al., 1998). Actin filaments decorated with G2–6 in the presence of Ca^{2+} show kinks that are presumably caused by G2–6 severing of one long-pitch strand of the filament. In contrast, actin filaments decorated with G2–3 do not show such bending or kinking, thus implicating G4–6 in severing (McGough et al., 1998). Paradoxically, at low Ca^{2+} concentrations, F-actin severing by gelsolin is inhibited compared to severing by G1–3. At low Ca^{2+} concentrations, where the Ca^{2+}-binding sites on G1 and G4–5 are not occupied, G4–6 may impede a Ca^{2+}-induced conformational transition thought to reorient G1, G2, and G3 (the release of the G1-G3 latch) (Pope et al., 1997). In the presence of saturating Ca^{2+} concentrations, the Ca^{2+}-regulated actin-binding site on G4 is able to bind an actin subunit in the polymer and enhance severing.

Bound Ca^{2+} apparently affects the interactions between the domains of gelsolin, resulting in regulation of gelsolin activity. Typically, experiments have tested the activity of gelsolin in the presence or absence of Ca^{2+} without testing the effect of low levels of free Ca^{2+}. However, gelsolin in cells most likely contains one bound Ca^{2+} ion and exists in an "open" conformation that functions in a manner different from that of Ca^{2+}-free gelsolin. For example, it has been shown that at pH 7.0, addition of Ca^{2+} to 0.2 μM decreases the K_d for PIP_2 binding to gelsolin by half compared to the K_d in the presence of 1 mM EGTA (Lin et al., 1997). At physiologic protein and Ca^{2+} concentrations (100–500 μM F-actin and 1 μM gelsolin, and 0.2 μM Ca^{2+}), binding of gelsolin to the side of actin filaments could be driven by mass action. These gelsolin-F-actin complexes would then be in place to respond to transient local Ca^{2+} or H^+ concentration increases to rapidly produce severed actin filaments. In stable nonmotile or unactivated cells, gelsolin could slowly sever actin filaments to maintain a low level of filament turnover. In this way, variations in intracellular free Ca^{2+} concentrations in the 10^{-7} to 10^{-6} M range could regulate actin filament lengths and cytoplasmic viscosity.

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REFERENCES

Allen, P. G., and P. A. Janmey. 1994. Gelsolin displaces phalloidin from actin filaments. A new fluorescence method shows that both Ca^{2+} and Mg^{2+} affect the rate at which gelsolin severs F-actin. *J. Biol. Chem.* 269:32916–32923.

Arcaro, A. 1998. The small GTP-binding protein Rac promotes the dissociation of gelsolin from actin filaments in neutrophils. *J. Biol. Chem.* 273:805–813.

Asch, H. L., K. Head, Y. Dong, F. Natoli, J. S. Winston, J. L. Connolly, and B. B. Asch. 1996. Widespread loss of gelsolin in breast cancers of humans, mice, and rats. *Cancer Res.* 56:4841–4845.

Baldassare, J. J., P. A. Henderson, A. Tarver, and G. J. Fisher. 1997. Thrombin activation of human platelets dissociates a complex containing gelsolin and actin from phosphatidylinositide-specific phospholipase Cgamma1. *Biochem. J.* 324:283–287.

Bryan, J., and M. C. Kurth. 1984. Actin-gelsolin interactions. Evidence for two actin-binding sites. *J. Biol. Chem.* 259:7480–7487.

Burtnick, L. D., E. K. Koepf, J. Grimes, E. Y. Jones, D. I. Stuart, P. J. McLaughlin, and R. C. Robinson. 1997. The crystal structure of plasma gelsolin: implications for actin severing, capping and nucleation. *Cell.* 90:661–670.

Davoodian, K., B. W. Ritchings, R. Ramphal, and M. R. Bubb. 1997. Gelsolin activates DNase I in vitro and cystic fibrosis sputum. *Biochemistry.* 36:9637–9641.

Ditsch, A., and A. Wegner. 1995. Two low-affinity Ca^{2+}-binding sites of gelsolin that regulate association with actin. *Eur. J. Biochem.* 229: 512–516.

Estes, J. E., L. A. Selden, and L. C. Gershman. 1981. Mechanism of action of phalloidin on the polymerization of muscle actin. *Biochemistry.* 20:708–712.

Hartwig, J. H. 1992. Mechanisms of actin rearrangements mediating platelet activation. *J. Cell Biol.* 118:1421–1441.

Hartwig, J. H., G. M. Bokoch, C. L. Carpenter, P. A. Janmey, L. A. Taylor, A. Toker, and T. P. Stossel. 1995. Thrombin receptor ligation and activated rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. *Cell.* 82:643–653.

Hellweg, T., H. Hinssen, and W. Eimer. 1993. The Ca^{2+}-induced conformational change of gelsolin is located in the carboxyl-terminal half of the molecule. *Biophys. J.* 65:499–805.

Ismailov, I. I., B. K. Berdiev, V. G. Shlyonsky, C. M. Fuller, A. G. Prat, B. Jovov, H. F. Cantiello, D. A. Ausiello, and D. J. Benos. 1997. Role of actin in regulation of epithelial sodium channels by CFTR. *Am. J. Physiol.* 272:C1077–C1086.

Janmey, P. A., C. Chaponnier, S. E. Lind, K. S. Zaner, T. P. Stossel, and H. L. Yin. 1985. Interactions of gelsolin and gelsolin-actin complexes with actin. Effects of calcium on actin nucleation, filament severing, and end blocking. *Biochemistry.* 24:3714–3723.

Janmey, P. A., K. Iida, H. L. Yin, and T. P. Stossel. 1987. Polyphosphoinositide micelles and polyphosphoinositide-containing vesicles dissociate endogenous gelsolin-actin complexes and promote actin assembly from the fast-growing end of actin filaments blocked by gelsolin. *J. Biol. Chem.* 262:12228–12236.

Kinosian, H. J., L. A. Selden, J. E. Estes, and L. C. Gershman. 1996. Kinetics of gelsolin interaction with phalloidin-stabilized F-actin. Rate constants for binding and severing. *Biochemistry.* 35:16550–16556.

Kothakota, S., T. Azuma, C. Reinhard, A. Klippel, J. Tang, K. Chu, T. J. McGarry, M. W. Kirschner, K. Koths, D. J. Kwiatkowski, and L. T. Williams. 1997. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science.* 278:294–298.

Kouyama, T., and K. Mihashi. 1981. Fluorometry study of *N*-(1-pyrenyl) iodoacetamide-labeled F-actin. Local structural change of actin protomer both on polymerization and on binding of heavy meromyosin. *Eur. J. Biochem.* 114:33–36.

Kurokawa, H., W. Fujii, K. Ohmi, T. Sakurai, and Y. Nonomura. 1990. Simple and rapid purification of brevin. *Biochem. Biophys. Res. Commun.* 168:451–457.

Kwiatkowski, D. J., P. A. Janmey, and H. L. Yin. 1989. Identification of critical functional and regulatory domains in gelsolin. *J. Cell Biol.* 108:1717–1726.

Lamb, J. A., P. G. Allen, B. Y. Tuan, and P. A. Janmey. 1993. Modulation of gelsolin function. Activation at low pH overrides Ca^{2+} requirement. *J. Biol. Chem.* 268:8999–9004.

Lin, K.-M., E. Wenegieme, P.-J. Lu, C.-S. Chen, and H. L. Yin. 1997. Gelsolin binding to phosphatidyl 4,5-bisphosphate is modulated by calcium and pH. *J. Biol. Chem.* 272:20443–20450.

Lind, S. E., P. A. Janmey, C. Chaponnier, T. J. Herbert, and T. P. Stossel. 1987. Reversible binding of actin to gelsolin and profilin in human platelet extracts. *J. Cell Biol.* 105:833–842.

Lu, M., W. Witke, D. J. Kwiatkowski, and K. S. Kosik. 1997. Delayed retraction of filopodia in gelsolin null mice. *J. Cell Biol.* 138: 1279–1287.

Maximov, A. V., E. A. Vedernikova, H. Hinssen, S. Y. Khaitlina, and Y. A. Negulyaev. 1997. Ca-dependent regulation of Na^+-selective channels via actin cytoskeleton modification in leukemia cells. *FEBS Lett.* 412:94–96.

McGough, A., W. Chiu, and M. Way. 1998. Determination of the gelsolin binding site on F-actin: implications for severing and capping. *Biophys. J.* 74:764–772.

McLaughlin, P. J., J. T. Gooch, H. G. Mannherz, and A. G. Weeds. 1993. Structure of gelsolin segment 1-actin complex and the mechanism of filament severing. *Nature.* 364:685–692.

Newman, J., L. A. Day, G. W. Dalack, and D. Eden. 1982. Hydrodynamic determination of molecular weight, dimensions, and structural parameters of Pf3 virus. *Biochemistry.* 21:3352–3358.

Ohtsu, M., N. Sakai, H. Fujita, M. Kashiwagi, S. Gasa, S. Shimizu, Y. Eguchi, Y. Tsujimoto, Y. Sakiyama, K. Kobayashi, and N. Kuzumaki. 1997. Inhibition of apoptosis by the actin-regulatory protein gelsolin. *EMBO J.* 16:4650–4656.

Perrin, D. D., and I. G. Sayce. 1967. Computer calculation of equilibrium concentrations in mixtures of metal ions and complexing species. *Talanta.* 14:833–842.

Pope, B. J., J. T. Gooch, and A. G. Weeds. 1997. Probing the effects of calcium on gelsolin. *Biochemistry.* 36:15848–15855.

Pope, B., S. Maciver, and A. G. Weeds. 1995. Localization of the calcium-sensitive actin monomer binding site in gelsolin to segment 4 and identification of calcium binding sites. *Biochemistry.* 34:1583–1588.

Selden, L. A., H. J. Kinosian, J. Newman, B. Lincoln, C. Hurwitz, L. C. Gershman, and J. E. Estes. 1998. Severing of F-actin by the N-terminal half of gelsolin suggests internal cooperativity in gelsolin. *Biophys. J.* 75: (in press).

Selve, N., and A. Wegner. 1986. Rate constants for capping of the barbed ends of actin filaments by the gelsolin-actin complex. *Eur. J. Biochem.* 155:397–401.

Singh, S. S., A. Chauhan, N. Murakami, and V. P. Chauhan. 1996. Profilin and gelsolin stimulate phosphatidylinositol 3-kinase activity. *Biochemistry.* 35:16544–16549.

Steed, P. M., S. Nagar, and L. P. Wennogle. 1996. Phospholipase D regulation by a physical interaction with the actin-binding protein gelsolin. *Biochemistry.* 35:5229–5237.

Way, M., J. Gooch, B. Pope, and A. G. Weeds. 1989. Expression of human plasma gelsolin in *Escherichia coli* and dissection of actin binding sites by segmental deletion mutagenesis. *J. Cell Biol.* 109:593–605.

Weeds, A. G., J. Gooch, P. McLaughlin, B. Pope, M. Bengtsdotter, and R. Karlsson. 1995. Identification of the trapped calcium in the gelsolin segment 1-actin complex: implications for the role of calcium in the control of gelsolin activity. *FEBS Lett.* 360:227–230.

Wegner, A., K. Aktories, A. Ditsch, I. Just, B. Schoepper, N. Selve, and M. Wille. 1994. Actin-gelsolin interaction. *Adv. Exp. Med. Biol.* 358: 97–104.

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

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