Modulation of Gelsolin Function

ACTIVATION AT LOW pH OVERRIDES Ca2+ REQUIREMENT*

(Received for publication, October 21, 1992)

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The activation of gelsolin by calcium has been postulated to be involved in the receptor-mediated reorganization of the actin cytoskeleton, but cytoskeletal reorganization can also occur in cells with intracellular Ca2+ and actin filament levels. Fluorescence measurements using Fura-2 show that at pH 7.4, the Ca2+ requirement for gelsolin activation in vitro is higher than previously reported, with half-maximal activation of severing and nucleation occurring at 10 μM Ca2+. The Ca2+ requirement for gelsolin activity decreases at more acid pH and is approximately 3 μM at pH 6.5. At pH below 6.0, gelsolin no longer requires Ca2+ for activity and sever actin filaments, binds two actin monomers, and nucleates filament formation in EGTA-containing solutions. The pH-activated severing activity is inhibited by mixed lipid vesicles containing phosphatidylinositol 4,5-bisphosphate. A Ca2+-sensitive fragment consisting of the first 135 amino acids of human cytoplasmic gelsolin also demonstrates severing activity at pH ≤6.0 in the absence of Ca2+. In contrast, the gelsolin homologs severin and villin maintain Ca2+ regulation of severing activity at low pH. These differences suggest that activation of gelsolin at low pH cannot be explained merely by destabilization of F-actin. The difference in diffusion constants of gelsolin measured at pH 5.5 and 6.5, as determined by dynamic light scattering, suggests that the molecule undergoes a shape change similar to that reported upon binding Ca2+ at neutral pH. These results suggest a mechanism by which gelsolin may be activated in vivo under conditions where Ca2+ transients do not occur.

Maintenance of cell shape and regulation of motility are associated with the formation and rearrangement of cytoskeletal networks (1). Transitions between gel and sol phases in the peripheral cytoplasm, which occur during a variety of morphological changes, can be related to specific changes in the structure of the actin cytoskeleton. Actin filaments are the most abundant and often the exclusive filamentous component of the cortical network of motile cells; therefore, remodeling of this network is likely to be necessary for cell motility. Since the mechanical properties of biopolymer networks are strongly influenced by parameters such as filament length, flexibility, concentration, and the presence and geometry of interfilament cross-links (2), proteins that alter these properties (3) are potentially important for regulating cellular morphology and function.

Gelsolin is an actin-binding protein that is poised to direct transformations of the actin filament system. In vitro, gelsolin regulates the length of filaments by severing the noncovalent bonds between actin subunits within the filament and subsequently capping the severed ends. Gelsolin also promotes nucleation of actin polymerization by forming complexes with actin monomers (4, 5). Both functions are modulated by intracellular messengers. Ca2+ activates gelsolin, and the polyphosphoinositides phosphatidylinositol 4-monophosphate and PIP2 inhibit it (5). The concerted changes in these messengers during many types of cell activation support the idea that differential regulation of gelsolin function by receptor-mediated signaling can lead to cytoskeletal reorganization.

The severing function of gelsolin could lead to solation of actin networks and fluidization of the cell cortex. The nuclearization of actin oligomers by gelsolin and removal of the gelsolin cap by membrane phosphoinositides could promote growth of actin filaments at the leading edge of the cell, where actin polymerization occurs (6). The observation of a direct relation between the rate of motility and the level of gelsolin expression in stably transfected fibroblast cell lines further strengthens the link between gelsolin function and cell motility in vivo (7).

Studies of intact cells suggest that the actin cytoskeleton can be remodeled under conditions where gelsolin activity, as predicted solely by its reported in vitro sensitivity to Ca2+ and polyphosphoinositide concentrations, does not correlate with the observed changes in actin filament organization. Microinjection of gelsolin and micromolar Ca2+ into fibroblasts failed to disrupt actin filaments in stress fibers or to immobilize gelsolin on the actin network, as might have been expected if gelsolin severed and bound the ends of actin filament fragments (8, 9), and a variable effect was noted, depending on the source of the gelsolin (10). Furthermore, although preventing a rise in cytosolic calcium in human platelets inhibits actin remodeling effects ascribable to gelsolin’s severing function (11), some other cellular actin reorganizations in which gelsolin would be predicted to be involved are observed under conditions at which bulk intracellular Ca2+ concentrations are maintained below micromolar levels (12-14). Moreover, recent studies of Ca2+ binding by gelsolin suggest that the affinity of gelsolin for Ca2+ in physiologic conditions may be boosted by micromolar BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; DTT, dithiothreitol; Pipes, 1,4-piperazinediethanesulfonic acid.

*This work was supported by United States Public Health Service Grants AR38910 and HL07680 and by the Whitaker Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§The abbreviations used are: PIP2, phosphatidylinositol 4,5-bisphosphate; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; DTT, dithiothreitol; Pipes, 1,4-piperazinediethanesulfonic acid.
weaker than originally estimated (15). These results suggest that the activation of gelsolin by Ca$^{2+}$ (4, 16, 17) may be modulated by other factors in vivo, allowing both activation at low Ca$^{2+}$ and inactivation at high Ca$^{2+}$ levels.

This report describes data showing that the micromolar bulk concentrations of free calcium reported to occur during some kinds of cell stimulation are insufficient by themselves to activate gelsolin and that changes in pH may participate in gelsolin activation.

**MATERIALS AND METHODS**

**Proteins**—Rabbit skeletal muscle actin (18) and human plasma gelsolin (19) were purified by published methods, and actin was labeled with pyrene iodocetamide as previously described (20). Macrophage gelsolin was the generous gift of C. Chaponnier. Villin and villin fragments were prepared by the methods of deArruda et al. (21) and generously provided by Tomoko Nakayama, Whitehead Institute, Massachusetts Institute of Technology. Severin was purified by the method of Eichinger (22) from Dictyostelium discoideum strain AX2 kindly provided by Dres. Angela Nogel and Michael Schleicher, Max-Planck-Institute, Martinsried. G-actin was prepared by dialysis in solutions containing 2 mM Tris, pH 8.0, 0.2 mM CaCl$_2$, 0.2 mM diithiothreitol (DTT), 0.5 mM ATP (buffer A) and polymerized by the addition of 2 mM MgCl$_2$ and 150 mM KCl (to form buffer B). Gelsolin was stored frozen in 10 mM Tris, 150 mM NaCl, 1 mM EGTA, pH 7.4 (TBS-EGTA). Fura-2 was obtained from Molecular Probes (Eugene, OR). Monoclonal anti-gelsolin antibody was a gift from C. Chaponnier and was immobilized on CNBr-activated Sepharose as described (23).

**Synthesis and Purification of N-terminal Ca$^{2+}$-sensitive Gelsolin Fragment 26-160**—Polymerase chain reaction mutagenesis of the plasmid encoding PG160, which contains residues 1–160 of plasma and pH 6.50 were calculated from the above relation by determining Fragment 26–160“Polymerase chain reaction mutagenesis of the addition of Met-Gly-Ser at its N terminus.

Fluorescence Assays of Actin Filament Nucleation and Severing—The ability of gelsolin to nucleate actin assembly or to sever actin filaments was determined by its effect on the rate and extent of increase or decrease, respectively, of fluorescence of pyrene-labeled actin. The rate of fluorescence increase or decrease, proportional to the depolymerization rate, depends on the number of ends and, therefore, on the number of cuts introduced by gelsolin.

Dynamic Light Scattering—The hydrodynamic diameters of gelsolin at various pH values were calculated from the intensity autocorrelation functions measured by dynamic light scattering using a Brookhaven Instruments BI30AT apparatus. Gelsolin was centrifuged at 125,000 × g for 90 min to remove aggregated protein and diluted to a concentration of 0.65 mg/ml in 150 mM NaCl, 10 mM Tris-HCl, 1 mM EGTA, pH 7.4. Subsequent measurements at pH 5.5 were done by mixing 1 ml of 0.65 mg/ml gelsolin in TBS-EGTA with 200 μl of filtered 0.3 M Pipes, 0.1 M Hepes, 10 mM EGTA, pH 5.5. Dynamic light scattering measurements were made on a 1-ml sample in cylindrical scattering tubes at angles from 60 to 120 degrees. The diffusion constant $D$ was calculated from the slope of a plot of the average decay constant, determined from the autocorrelation function by a single exponential fit versus the inverse scattering vector, and the diameter $d$ was calculated from the expression, $d = \frac{kT}{6\pi\eta D}$, where $k$ is Boltzmann’s constant, $T$ is the absolute temperature (298 K), and $\eta$ is the solvent viscosity (0.89 centipoise) (31).

Immunoprecipitation—Gelsolin and gelsolin-actin complexes were immunoprecipitated by forming 2.5:1 actin:gelsolin mixtures in 60 mM Pipes, 20 mM Hepes, 2 mM MgCl$_2$, 0.5 mM ATP, 0.2 mM DTT, 500 nM CaCl$_2$, pH 7.4. The rate at which actin polymerizes faster than spontaneous, monomeric actin can form, the initial rate of polymerization determined from the rate of fluorescence increase is proportional to the number of pointed end nuclei formed, and, therefore, the relative nucleation activity of gelsolin. For assays of filament severing activity, a sample of F-actin is diluted below its critical monomer concentration into solutions containing gelsolin. Since actin filaments depolymerize only if their ends, the rate of fluorescence decrease, proportional to the depolymerization rate, depends on the number of ends and, therefore, on the number of cuts introduced by gelsolin.

RESULTS

**Ca$^{2+}$ Dependence**—The Ca$^{2+}$ requirements for both severing and nucleating activity are higher than would be expected if the dissociation constant of gelsolin for Ca$^{2+}$ was 1 μM. Fig. 1 shows that half-maximal activation of both severing and nucleating activities at pH 7.4 requires 10 μM free Ca$^{2+}$, and almost no activity is observed at 1 μM Ca$^{2+}$. Similar results were also observed using macrophage gelsolin. These results are in agreement with the most recent measurements of Ca$^{2+}$ binding using either fluorescence polarization (32) or ion exchange chromatography (15) and would predict that at the 500 nM to 1 μM activity of free Ca$^{2+}$ after cell activation in vivo, these functions of gelsolin would be only marginally activated. In contrast, the filament capping activity of gelsolin would be fully active under these Ca$^{2+}$ concentrations (33). At pH 6.75, the Ca$^{2+}$ requirement for severing activity is
Activation of Gelsolin by Calcium and pH

FIG. 1. Calcium dependence of gelsolin severing and nucleating activity. The relative abilities of gelsolin to sever actin filaments (A) (circles, pH 7.4; squares, pH 6.75; triangles, pH 6.5) or to nucleate their assembly from monomers (B) (circles, pH 7.4) as a function of free Ca\(^{2+}\). Methods are described in the text. The vertical error bars reflect the standard deviation of three independent determinations, and the horizontal error bars are an estimate of the accuracy of Ca\(^{2+}\) determination based on the 39% standard deviation of three independent measurements of the affinity of Fura-2 for Ca\(^{2+}\) obtained from the literature as described under "Materials and Methods."

significantly decreased and, at pH 6.5, severing activity is half-maximal at 3 μM but still only marginal at 1 μM.

pH Dependence—The Ca\(^{2+}\) dependence of gelsolin function is not the result of an inherent requirement for Ca\(^{2+}\) at the active site but rather results from a conformational change thought to occur in the C-terminal half of the molecule, which relieves the inhibition of a constitutively active severing site in the N-terminal domain of gelsolin (24, 34–36). Therefore, factors other than Ca\(^{2+}\) that would induce a similar conformational change might also activate the severing function of gelsolin. Fig. 2A shows that lowering the pH activates gelsolin in the absence of Ca\(^{2+}\) ions. In EGTA-containing solutions, where no cutting activity is observed at pH 7, there is an abrupt increase in activity just below pH 6.0 that is maximal at pH 5.7 and equal to the efficiency observed at pH 7 and 200 μM Ca\(^{2+}\). Similarly, actin polymerization is accelerated by gelsolin at low pH in the absence of Ca\(^{2+}\) to levels even greater than those in solutions containing 200 μM Ca\(^{2+}\), pH 7.4 (Fig. 2B). This increase is due mainly to activation of gelsolin, but a slight acceleration of monomer addition to actin filament ends may also occur due to decrease in critical monomer concentration at low pH (37). Activation of severing at low pH is also observed with the smallest N-terminal Ca\(^{2+}\)-sensitive fragment of gelsolin (gelsolin 26-160 (24)) that retains severing activity.

The activation of gelsolin at low pH is a property not shared by the gelsolin homolog, villin which has an even higher requirement of Ca\(^{2+}\) ions at pH 7 (30, 38–40) nor by the D. discoidium protein severin, which is structurally homologous to the N-terminal half of gelsolin and villin (41, 42). Fig. 2A shows that neither villin nor severin expresses severing activity in the absence of Ca\(^{2+}\) between pH 5.4 and 7. The activities of both severin and villin were restored at all pH values upon the addition of 200 μM free Ca\(^{2+}\) (data not shown). These results confirm that the effects of gelsolin on actin polymerization and depolymerization at low pH are due to effects on gelsolin and are not the result of destabilization of the actin filaments. This point is further supported by the finding that both the depolymerization rate of capped filaments and the critical concentration of F-actin in the absence of gelsolin are very similar at pH 5.7 and 7.0. The villin N-terminal half fragment 44T (21) also remained inactive at low pH (data not shown).

Effect of pH on Gelsolin Conformation—The activation of gelsolin function at low pH coincides with a change in gelsolin structure as detected by its diffusion constant measured by...
FIG. 2. Effect of pH on activation of nucleating and severing activities. A, severing. The severing activity is shown as proportional to the depolymerization rate in arbitrary units observed when 15 μM pyrene-labeled F-actin (pre-capped by a 1:250 or 1:500 molar ratio of gelsolin) is diluted to 200 nM into PHEM buffers at various pH values containing 30 nM gelsolin (circles), 200 nM gelsolin 26-160 (triangles), 160 nM villin (open squares), or 100 nM severin (diamonds). All measurements were made at 23 °C, except for one experiment with gelsolin measured at 37 °C (open circles).

B, nucleation. 6 pM G-actin (33% pyrene-labeled) was polymerized by the addition of PHEM buffers at various pH values in the presence of 44 nM gelsolin, and the maximal polymerization rate proportional to the rate of fluorescence increase is shown relative to the rate in buffer B (200 μM free Ca²⁺).

Dynamic light scattering. The diffusion constant of gelsolin at pH 7.4 is 3.2 × 10⁻⁷ cm²/s, consistent with previous dynamic light scattering measurements (43) and with a hydrodynamic model of gelsolin as a prolate ellipsoid. At pH 5.5, the diffusion constant decreases to 2.15 × 10⁻⁷ cm²/s, indicating an unfolding of the protein to a more elongated shape, which is very similar to the change in diffusion constant observed upon adding micromolar Ca²⁺ ions at pH 7.4.

Formation of Actin-Gelsolin Complexes—The effects of pH on gelsolin function and hydrodynamic shape are consistent with direct measurements of gelsolin/actin binding in the absence of Ca²⁺. Fig. 3 shows that at pH 7.0 in low Ca²⁺, free gelsolin is immunoprecipitated from solutions containing an excess of actin, which is consistent with many previous reports. However, at pH 5.4 and 5.7, gelsolin/actin complexes are precipitated, and the ratio of actin bound to gelsolin is similar to that precipitated at pH 7.0 with 200 μM Ca²⁺. Densitometric scans of the data of Fig. 3 estimate that the relative mass amounts of actin coprecipitating with gelsolin in the absence of Ca²⁺ at pH 5.4, 5.7, and 7.0, respectively, are 96, 71, and 15%. Negligible quantities of actin precipitated with control beads not containing anti-gelsolin antibodies. The complexes formed at low pH in the absence of Ca²⁺ do not dissociate when the pH is raised to 7.4, as judged by either immunoprecipitation experiments or functional assays.

Polyphosphoinositide Regulation at Low pH—Gelsolin that is activated by low pH in the absence of Ca²⁺ remains inhibitable by polyphosphoinositides (Fig. 4), which is consistent
FIG. 4. Inhibition of gelsolin function by PIP$_2$ at high and low pH. Relative severing activity of gelsolin taken as proportional to the depolymerization rate when pyrene-labeled F-actin is diluted to 200 nM in either PHEM buffer, pH 5.6 (open circles) or buffer B, pH 7.6 (closed circles) containing 30 nM gelsolin and various amounts of PIP$_2$.

with previous findings (44, 45) that the gelsolin-polyphosphoinositide interaction is independent of the first ionization state of PIP$_2$ and does not require divalent cations at pH 7.4.

DISCUSSION

The finding that the concentration of Ca$^{2+}$ required to activate gelsolin in vitro is higher than previously reported is consistent with the finding of a lack of effect of microinjected gelsolin under some conditions where intracellular Ca$^{2+}$ concentration would be expected to be near micromolar (8-10, 46). On the other hand, activation of gelsolin at low pH suggests that gelsolin might sever F-actin under conditions where intracellular Ca$^{2+}$ is clamped to near nanomolar levels. Acceleration of actin-gelsolin binding at acid pH was first reported by Selve and Wegner (47) using enhancement of 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl))-actin fluorescence as a probe for binding of actin monomers to gelsolin. The present results are probably due to different structural effects on gelsolin, since no activation of severing activity was detected in the pH range studied by Selve and Wegner (47).

The concentrations of Ca$^{2+}$ (10 μM) and H$^+$ (pH <6.0) required to activate gelsolin in purified solutions are higher than have been reported from bulk measurements accompanying most forms of cell activations. One possible way to reconcile these results is to hypothesize the presence of a yet unidentified cofactor that would reduce the Ca$^{2+}$ or the pH requirement analogous to the manner in which phospholipids increase the affinity of the actin-binding protein calpain for Ca$^{2+}$ (48).

An alternative explanation is that although bulk levels of Ca$^{2+}$ and H$^+$ do not reach the levels required to activate gelsolin, local concentrations do. The levels of [Ca$^{2+}$] and pH required to activate gelsolin are approximately 10 times greater than bulk cytoplasmic levels measured using fluorescent dyes but are within the range measured locally by other methods. For example, it has recently been demonstrated that the transfer of un-ionized fatty acids across a phosphatidylcholine bilayer vesicle in a solution maintained at pH 7.4 drops the local pH inside the vesicle by more than 0.3 pH units (49). Such a drop in pH that might occur during rearrangements of membrane lipids resulting from polyphosphoinositide turnover or arachidonate metabolite signaling would lower the Ca$^{2+}$ requirement for gelsolin activation to near the micromolar levels measured in bulk. In addition, there are at least two mechanisms by which locally high Ca$^{2+}$ ion concentrations could occur. First, the opening of Ca$^{2+}$ channels at the plasma membrane or at inositol 1,4,5-trisphosphate-activated intracellular Ca$^{2+}$ storage sites provides access to millimolar pools of free Ca$^{2+}$, and areas near such channels might have higher Ca$^{2+}$ concentrations than the bulk phase. Regions near the plasma membrane of a presynaptic terminal are reported to have Ca$^{2+}$ concentrations between 200 and 300 μM (50). Second, high local concentrations of cations are stabilized by the large negative surface potential of the plasma membrane of most cells. Application of the Gouy-Chapman theory to predict the potential near the bilayer with a 30-nV surface potential in 0.1 M salt suggests that within a few nanometers of the cytoplasmic leaflet of the plasma membrane, the potential is sufficient to increase the local Ca$^{2+}$ concentration 10-fold above its bulk level (51). Therefore, a local Ca$^{2+}$ of 10 μM can be achieved by signals that cause cytosolic Ca$^{2+}$ to rise to 1 μM. Since a significant fraction of gelsolin is localized at the plasma membranes of resting platelets and macrophages (52), such local activation of either the severing or nucleating activity, followed by removal of gelsolin from the newly formed barbed filament ends by membrane polyphosphoinositides represents a potential mechanism to initiate submembrane barbed end actin filament polymerization, which is a common early event in cell activation and locomotion. Acidification of the cytoplasm is often coincident with polymerization of actin in response to cell activation (53-56), but the decreased cytosolic pH is neither necessary (54) nor sufficient (53) to trigger bulk actin assembly. Nevertheless, local changes in pH may be sufficient to alter gelsolin activity, especially near cell membranes, to achieve modulation of cytoskeletal restructuring.

The structural basis for the activation of gelsolin at low pH is not obvious, and there are several apparent possibilities. The finding that the gelsolin 1-135 N-terminal fragment is also activated at low pH implies that the effects of H$^+$ are not due solely to unfolding of the C-terminal half of gelsolin away from its Ca$^{2+}$-insensitive N-terminal half but also may alter directly the N-terminal gelsolin domain necessary for severing activity. The lack of activation at low pH of either severin or the villin N-terminal half suggests that slight differences in primary structure, possibly a histidine, may be responsible for the unique feature of gelsolin. The first domain of gelsolin does contain 1 histidine (His-29 of human plasma gelsolin) but not present in either villin or severin, but whether this residue could participate in regulating gelsolin/actin interactions awaits elucidation of the actin/gelsolin domain-1 complex crystal structure (57). A second possibility that cannot presently be ruled out involves titration of adenine nucleotides. Both ATP and ADP have $K_a$ values between 6.0 and 7.0 (58). The recent reports of ATP binding to gelsolin in the absence of Ca$^{2+}$ (59) suggest that adenine nucleotides bound to either gelsolin or actin may affect how the proteins interact with each other (60).

Acknowledgments—We are grateful to Thomas Stowell and David Kwiatkowski for helpful discussions.

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