Calcium Regulation of Gelsolin and Adseverin: A Natural Test of the Helix Latch Hypothesis†

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ABSTRACT: The gelsolin family of actin filament binding proteins have highly homologous structures. Gelsolin and adseverin, also known as scinderin, are the most similar members of this family, with adseverin lacking a C-terminal helix found in gelsolin. This helix has been postulated to serve as a calcium-sensitive latch, keeping gelsolin inactive. To test this hypothesis, we have analyzed the kinetics of severing by gelsolin, adseverin, and a gelsolin truncate which lacks the C-terminal latch. We find that the relationship between severing rate and calcium ion concentration differs between gelsolin and adseverin, and suggest that calcium controls one rate-limiting step in the activation of adseverin and two in the activation of gelsolin. In contrast, both proteins are activated equally by protons, and have identical severing kinetics at pHs below 6.3. The temperature sensitivity of severing by adseverin and gelsolin is remarkably different, with gelsolin increasing its severing rate 8-fold per 10 °C increase in temperature and adseverin increasing its rate only 2-fold per 10 °C increase in temperature. Analysis of the gelsolin construct lacking the C-terminal helix demonstrates that this helix is responsible for the regulatory differences between gelsolin and adseverin. These results support the C-terminal latch hypothesis for the calcium ion activation of gelsolin.

The actin cytoskeleton is involved in processes as diverse as cell movement and cell shape (*1*−*3*), regulation of ion channels (*4*), exo- and endocytosis (*5*, *6*), and intracellular sorting of RNA (*7*−*9*). Not surprisingly, a growing number of proteins that modulate actin filament dynamics have been identified (*10*). One class of proteins postulated to be critical to the rapid remodeling of actin filaments in the cell are those that sever actin filaments (*11*, *12*). To date, all proteins that sever actin filaments efficiently are homologues of gelsolin, the protein first identified to have actin filament severing activity (*13*).

In humans, family members include gelsolin [for review, see (*14*, *15*)], adseverin/scinderin (*16*, *17*), villin (*18*), supervillin (*19*), flightless (*20*), advillin/DOC6 (*21*, *22*), and cap-G (*23*, *24*). Of these, gelsolin (*25*), villin (*26*), and adseverin (*16*, *27*) have been documented to sever actin filaments and cap their high-affinity end in the presence of calcium ions. Cap-G, in contrast, has only actin filament capping activity (*23*). The actin binding activities of the other mammalian family members remain to be characterized in detail.

Recent analysis of adseverin and gelsolin expression in mice demonstrates that they have both unique and overlapping expression patterns within organs such as the kidney and intestine (*28*). These proteins are well-known to be activated by calcium to sever actin filaments and inhibited by phosphoinositide lipids, in vitro (*25*, *27*, *29*). This raises the question of why particular cells would express, one, the other, or both of these structurally similar actin severing proteins (*30*−*32*). These proteins are 59% identical at the amino acid level (mouse gelsolin compared with mouse adseverin), with a greater degree of homology allowing conservative substitutions. One significant difference between these two actin severing proteins is the absence of a helix at the C-termini of adseverin found in gelsolin. In the crystal structure of inactive gelsolin, this helix reaches over and contacts the actin binding helix of domain 2. This interaction led to the hypothesis that this helix could function as a latch, keeping gelsolin inactive in the absence of calcium (*33*).

In this work we demonstrate that the C-terminal helix of gelsolin acts as a latch, as proposed by Burtnick and colleagues (*33*). The presence of the C-terminal latch helix slows the kinetics of gelsolin activation by calcium ions, and renders the activation process remarkably temperature sensitive. Adseverin acts similarly to latch deficient gelsolin. The latch helix is not the sole structural regulator of gelsolin activation, as its absence has no effect on the kinetics of severing induced by low pH.

EXPERIMENTAL PROCEDURES

Materials. All buffers, salts, and nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO). Rhodamine

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phalloidin was purchased either from Sigma or from Molecular Probes (Eugene, OR).

Actin. Monomeric (G)[1] actin was purified from an acetone powder of rabbit skeletal muscle as previously described (*34*, *35*). Actin concentrations were determined from the absorbance at 290 nm using an extinction coefficient of 0.62 mL mg^{-1} cm^{-1}. Actin was polymerized at concentrations of 15−30 μM in solutions containing 150 mM KCl, 20 mM HEPES, pH 7.4, 0.5 mM ATP, 0.2 mM DTT, 2 mM $MgCl_2$, and 0.2 mM $CaCl_2$ (F-buffer) 1 h before use.

Recombinant Gelsolins. Gelsolin exists both in cytoplasmic and in extracellular forms, generated by differential splicing of a single gene. Native plasma gelsolin contains, and cytoplasmic gelsolin lacks, a disulfide bond between cysteines 188 and 201 (*36*). The oxidized recombinant plasma gelsolin severing rate at various free calcium concentrations is indistingquishable from plasma gelsolin purified from human blood (*37*). Oxidized recombinant human plasma gelsolin was expressed in *E. coli* and purified by chromatography on Q-Sepharose, with further purification on SP-Sepharose and the disulfide bond in domain 2 oxidized as described (*36*). After purification, gelsolin was extensively dialyzed in 75 mM KCl, 10 mM HEPES, pH 7.4, and 0.2 mM EGTA before use.

The cDNA for mouse cytoplasmic gelsolin was obtained from C. Dieffenbach and colleagues (*38*) and subcloned into the *Eco*RI site of pMW172. This generated a protein whose N-terminal methionine was followed by residue 6 of mouse cytoplasmic gelsolin with the sequence VVEHP deleted from the N-terminus. Mouse cytoplasmic gelsolin was expressed and purified as described above for human plasma gelsolin (*36*) without oxidization. The absence of a disulfide bond in recombinant mouse cytoplasmic gelsolin was determined as previously described (*37*).

Recombinant Gelsolin Truncate 744. The gelsolin truncate lacking the C-terminal helix was prepared essentially as described (*39*). This truncate terminates at the start of the final helix of native gelsolin, which is highlighted in Figure 4A. Briefly, PCR was used to generate a novel restriction site at the appropriate position, and cloned into pGEMT-easy vector (Promega). Recombinant protein was expressed in bacteria, purified by anion and cation exchange chromatography, and dialyzed against 10 mM HEPES, pH 7.4, 75 mM KCl, 0.2 mM EGTA, and 1 mM 2-mercaptoethanol before use. Protein concentration was determined using the BCA method (Pierce, Rockford, IL).

Recombinant Murine Adseverin. Recombinant murine adseverin was expressed in bacteria and purified essentially as described (*28*). Purified adseverin was then dialyzed against a buffer containing 75 mM KCl, 10 mM HEPES, pH 7.4, 0.2 mM EGTA, and 1 mM 2-mercaptoethanol.

Functional Assays. The gelsolin family of actin severing proteins displace rhodamine phalloidin from actin filaments during severing (*40*). We measured the kinetics of severing under nonequilibrium conditions where calcium binding and subsequent protein conformational change are the rate-limiting steps in the reaction (*40*−*43*).

The specific severing activity of gelsolin and adseverin preparations was measured as described previously (*40*). 100% activity is equal to 2 mol of phalloidin being displaced by 1 mol of severing protein. For the experiments described, only protein preparations which displaced more than 1.5 mol of phalloidin per mole of severing protein (a specific activity of 75% or greater) were used.

The severing rates of actin filaments by gelsolin or adseverin were calculated under conditions differing in free calcium concentration, pH, or temperature. In our previous work, the loss in phalloidin fluorescence over time was modeled as a simple second-order process carried out under psuedo-first-order conditions (excess actin to gelsolin) (*40*). As discussed in our previous work, the reaction mechanism is likely to be much more complicated (*40*), which has been borne out by more recent studies (*41*−*43*). Since we are measuring the combined reactions of gelsolin or adseverin binding calcium, binding to the filament, and severing, we have presented our data in the form of the rate of fluorescence loss per second at equal concentrations of severing proteins. Under our experimental conditions, calcium binding and activation of the severing protein is the rate-limiting step of these reactions (*40*, *43*)

For each measurement, F-actin was diluted to 800 nM in buffers containing 1 μM TRITC-phalloidin and the fluorescence increase upon phalloidin binding measured in a Perkin-Elmer (Newton, MA) LS-50b fluorescence spectrophotometer. Addition of gelsolin or adseverin at 200 nM to the phalloidin-saturated filaments reduced the enhanced emission of the TRITC-phalloidin bound to actin filaments. Both the extent of fluorescence loss as well as the initial rate of fluorescence loss were linearly dependent on the concentration of severing protein added [(*40*) and data not shown]. To ensure rapid mixing, reactions were continuously stirred using the stirrer of the LS-50b. Buffers differing in free Ca^{2+} were generated by addition of various concentrations of EGTA, pH 7.4, to F-buffer that contained 1 mM instead of 0.2 mM $CaCl_2$. Ca^{2+} concentrations were measured as previously described (*40*) using Mag Fura 5, or Fura-2 (Molecular Probes, Eugene, OR). In experiments analyzing the effect of pH on the activation of severing activity, the buffer utilized was 150 mM KCl, 2 mM EGTA, 20 mM MES at pH values ranging from 5.5 to 6.5.

RESULTS

To compare the functional properties of adseverin and cytoplasmic and plasma gelsolins, these proteins were cloned and expressed in bacteria. Expression and purification of each protein yielded single bands on SDS−PAGE of the correct molecular mass. The recombinant severing proteins described in this work displaced 2 mol of phalloidin from actin filaments per mole of severing protein (1.9 ± 0.3 phalloidins lost per adseverin, $N = 6$; 2.1 ± 0.4 phalloidins lost per mouse cytoplasmic gelsolin), consistent with previous observations. Recombinant adseverin and cytoplasmic gelsolin had no detectable severing activity in 2 mM EGTA at pH 7.5, consistent with previous reports; however, the gelsolin 744 protein had minor severing activity under these conditions. This activity was only 20% of the total activity in calcium and was very slow, with half-times on the order of 20 min.

Kinetics of Severing and Calcium. Gelsolin and adseverin are both calcium-regulated actin severing proteins, yet

[1] Abbreviations: G-actin, monomeric actin; F-actin, filamentous actin.

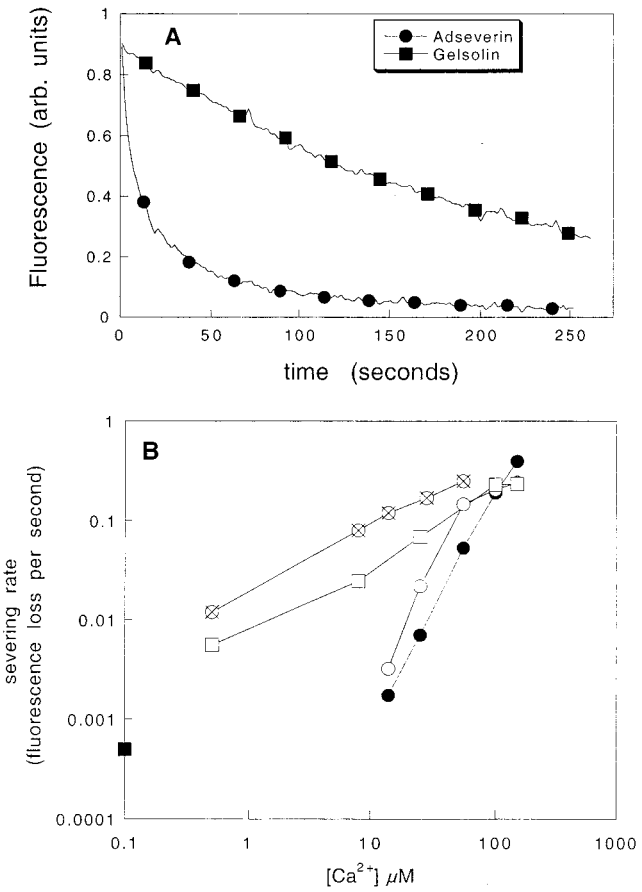

FIGURE 1: The C-terminal helix of gelsolin is responsible for the differences in calcium-dependent severing rates of gelsolin and adseverin. In panel A, the severing of F-actin, measured from the loss of TRITC-phalloidin fluorescence emission as described under Experimental Procedures, by adseverin (closed circles) or recombinant human plasma gelsolin (closed squares) in buffer containing 26 μM free calcium is illustrated. The actin monomer concentration in filaments was 1 μM, and the gelsolin and adseverin concentrations were each 450 nM. In panel B, the apparent rate for the severing of actin filaments by adseverin (open squares), mouse cytoplasmic (closed circles), or human plasma gelsolin (open circles) or by the helix-deficient gelsolin 744 truncate (crossed circles) over a range of free calcium concentrations is illustrated. The closed square indicates the minimal rate of phalloidin loss we attribute to severing activity, defined as a severing rate twice as fast as the intrinsic off-rate of phalloidin. All reactions were carried out at 25 °C. Each point is the average of three or more measurements, and the standard errors of the mean were smaller than the data marker and therefore not plotted.

adseverin lacks the C-terminal helix proposed to act as a calcium-sensitive latch in gelsolin. If this helix functions as proposed, we would hypothesize that adseverin, which lacks this helix, should be more active at equivalent calcium concentrations. We compared the rate of severing by gelsolin and adseverin at a number of free calcium concentrations and calculated an apparent severing rate from the loss of phalloidin fluorescence as described under Experimental Procedures. We find that the kinetics of activation and severing differ dramatically between these two molecules.

At a calcium concentration of 25 μM, the apparent severing rates of gelsolin and adseverin differ by 8-fold (Figure 1A). Adseverin severs rapidly at much lower free calcium concentrations than gelsolin (Figure 1B), with detectable severing activity at calcium concentrations as low as 0.5 μM, while severing by gelsolin requires more than 6 μM calcium. We can detect a small, gelsolin-dependent loss of the enhanced phalloidin fluorescence at concentrations below 6 μM Ca^{2+} (data not shown). However, both the extent of fluorescence loss and the rate of fluorescence loss at these free calcium concentrations are significantly less than at higher calcium concentrations. As these rates approach the intrinsic off-rate of phalloidin, indicated in Figure 1B by the closed square, we are hesitant to conclude that the displacement is due to severing and not some more complicated process such as prevention of annealing due to capping of spontaneously fragmented filaments.

Gelsolin and adseverin also differ in the rate of change of severing kinetics with changing calcium concentration, an indicator of the number of kinetic steps requiring calcium. Adseverin's severing rate increases logarithmically with increasing calcium concentration while the log of gelsolin's severing rate increases with approximately the second power of the change in calcium concentration. This suggests that under our experimental conditions, adseverin activation relies on calcium at a single rate-limiting step. Gelsolin activation, in contrast, relies on calcium binding at two or more steps.

Gelsolin and adseverin differ significantly in the absence of the terminal helix; however, the overall amino acid identity between the two proteins is only 59%. To confirm that the kinetic differences we observe are due to the difference in C-terminal structures, we prepared and analyzed a gelsolin truncate lacking only the C-terminal helix. We find that the calcium sensitivity of severing by the helix-deficient gelsolin construct is very similar to adseverin (Figure 1B). This construct severs rapidly at low calcium concentrations, and the relationship between the severing rate and calcium ion concentration changes logarithmically. The differences we observe are not due to species differences of the proteins, as mouse cytoplasmic gelsolin is much more similar to human plasma gelsolin than to mouse adseverin (Figure 1B).

Differential Temperature Sensitivity of Adseverin and Gelsolin. Normal mammalian body temperature is 37 °C, while a majority of assays are carried out at room temperature for convenience. We measured the severing rate of adseverin and gelsolin at a variety of temperatures, and find that the kinetics of severing by these molecules differ with temperature. As illustrated in Figure 2, the rate of severing by adseverin changes linearly with temperature over the range of 10−50 °C, with an approximately 2-fold increase in reaction rate per 10 °C increase in temperature, much like many other biochemical reactions. Gelsolin's activity is much more sensitive to temperature than adseverin, with the severing rate changing approximately 8-fold per 10 °C increase in temperature. Above 55 °C, both gelsolin isoforms and adseverin aggregate irreversibly [data not shown and (*44*)]. The activation energy of gelsolin activation and severing, solved from plotting these data in the form of an Arrhenius plot (plot not shown), is 21 kcal/mol, while those of adseverin or the helix-deficient gelsolin construct are 6 and 6.8 kcal/mol, respectively.

Similar experiments in buffers containing 16 μM calcium indicate that the rate of severing by gelsolin retains the same 8-fold increase in rate per 10 °C increase in temperature between 25 and 50 °C, but with faster overall kinetics consistent with the higher calcium concentration.

Analysis of severing by the gelsolin 744 truncate shows a much reduced temperature sensitivity compared with full-

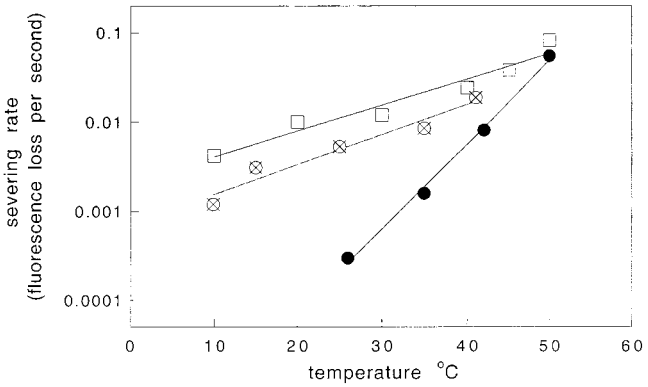

FIGURE 2: Gelsolin's and adseverin's severing rates differ markedly with temperature. The severing rate of recombinant human plasma gelsolin (closed circles) and adseverin (closed squares) or the helix-deficient gelsolin construct (crossed circles) was measured at different temperatures and the severing rate calculated as described under Experimental Procedures. The free calcium concentration was 8 μM for all measurements. The exact temperature of the reaction vessel was measured with a thermocouple probe after the reaction was complete. Each point is the mean of at least three measurements. The standard errors of the mean were smaller than the data marker.

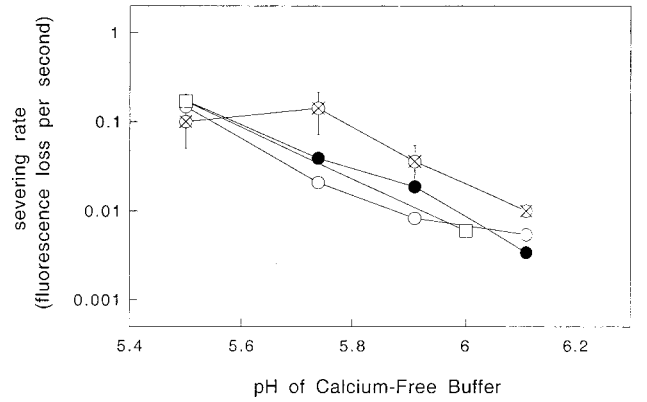

FIGURE 3: Adseverin and gelsolin both sever in the absence of calcium at pH <6.5. The severing rate of adseverin (open squares), mouse cytoplasmic (closed circles), or human plasma gelsolin (open circles) or by the helix-deficient gelsolin 744 truncate (crossed circles) was measured at different pHs in the presence of the calcium chelator EGTA as described under Experimental Procedures. All experiments were carried out at 25 °C. Each point is the average of three measurements. Standard errors of the mean are indicated by the error bars.

length gelsolin (Figure 2). The change in rate with changing temperature is similar to that of adseverin, with approximately 2-fold increase per 10 °C change. This indicates that the C-terminal helix is also responsible for the unique temperature sensitivity of gelsolin.

Activation of Adseverin and Gelsolin by Protons. Previous work demonstrated that gelsolin could be activated in the absence of calcium by elevation of proton concentrations to pH 6 or below (*35*). Villin and severin, two other members of the gelsolin superfamily, did not show this activation. The report that pH and calcium modulated adseverin's interaction with lipids (*45*) inspired us to determine whether protons could activate adseverin's severing activity in the absence of calcium. As illustrated in Figure 3, both gelsolin and adseverin sever filaments when the pH is dropped to 6.1 or below in the absence of significant free calcium concentrations (less than 100 nM). The kinetics of severing are equal for the two different proteins, in contrast to their behavior at equivalent levels of calcium. The gelsolin C-terminal helix truncate showed very similar kinetics with pH activation, and the relationship between pH and severing rate changed similarly. There was no alteration in phalloidin association and dissociation rates in the absence of gelsolin or adseverin over this pH range [(*46*) and data not shown].

DISCUSSION

Actin filament severing and capping proteins of the gelsolin superfamily can strongly alter the organization and dynamics of actin in vitro and in vivo (*47*, *48*). The regulation of these molecules is tightly coupled to cellular second messengers such as calcium (*13*) and phosphoinositide lipids (*29*). Understanding the structural basis for the regulation of these molecules is critical to understanding both their mechanisms of action and their cellular function.

In the crystal structure of calcium-free gelsolin, the C-terminal helix was observed to contact the putative actin filament binding helix (*33*). This observation led to the hypothesis that that the C-terminal helix might function as a latch, opening in the presence of calcium to allow function. We have tested this hypothesis by analyzing the calcium dependence of the severing rate of gelsolin and two gelsolin like molecules that lack the C-terminal helix: adseverin/scinderin and an artificial gelsolin which terminates just before the latch helix. We find that the calcium sensitivity of the severing rate and the number of calcium-dependent kinetic processes vary between these molecules in a manner consistent with the latch hypothesis. We also find that the latch is responsible for the unusual temperature sensitivity of gelsolin.

Calcium Regulation of Structure and Function. The crystal structure of gelsolin (*33*) confirmed predictions, based on sequence analysis and proteolytic digestion, that gelsolin and adseverin consists of six homologous domains (*30*−*32*, *49*). Each domain folds as a separate structural element, and different functions have been ascribed to each domain. Domains 1 and 4 bind actin monomers (*50*−*52*), and domain 2 contains the actin filament binding domain (*53*). Actin filament severing requires domains 1 and 2, while nucleation of pointed end polymerization requires domains 2 through 6 (*54*, *55*). The domains necessary for capping, preventing the addition of actin monomers to the high-affinity or barbed end of the actin filament, are less clear. Several isolated domains have capping activity though at lower affinities than that seen in the parent protein [for review, see (*15*)].

Analysis of the regulation of proteins of the gelsolin superfamily has not progressed as far as has the functional analysis. Calcium regulation requires the C-terminal half of the molecule (*49*), and domains 1−3 of gelsolin sever rapidly at extremely low free calcium ion concentrations (*42*, *54*). The idea that calcium regulation resides in the C-terminal half of these molecules is further supported by the observation that there are significant calcium-induced changes in protein shape in the C-terminal half, but not in the N-terminal half, of gelsolin (*56*). Monomer binding by domain 4 of gelsolin is calcium-sensitive (*52*). To complicate matters however, the minimal piece of gelsolin which retains severing activity, domains 1 and a small piece of domain 2 (amino acids 1−160), requires calcium for activity (*54*).

Sequence analysis as well as the crystal structure demonstrates that gelsolin has no classic calcium binding sites

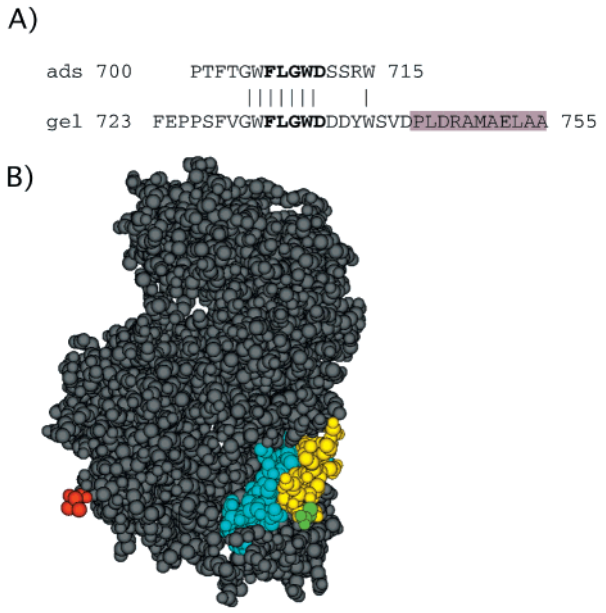

FIGURE 4: Homology of adseverin and gelsolin in a region necessary for calcium regulation. The sequences of the C-termini of gelsolin and adseverin are listed above in panel A. The C-terminal latch helix in gelsolin is shaded in gray, and the residues identified as being necessary to maintain calcium regulation are identified in boldface type. In the space-filling diagram (B), the C-terminal helix is identified in yellow, and the helix in domain 2 is identified in blue. The N-terminal and C-terminal amino acids are red and green, respectively.

such as EF hands. In the intact molecule, multiple calcium binding sites differing in affinity have been identified. Depending on the study, two classes of sites exist with multiple calcium binding sites with affinities on the order of 100 nM and a second class of binding sites for calcium with a measured affinity of 3−10 μM [for review, see (*15*)]. Structural analysis of protein shape and organization also provides evidence for calcium-induced conformational changes which occur at hundreds of micromolar free calcium (*57*, *58*). The relevance of these binding sites to function remains unclear.

The gelsolin helix latch hypothesis suggests that in the absence of bound calcium the C-terminal helix of gelsolin, which in the inactive gelsolin crystal structure associates with the putative actin binding helix of domain 2 (see Figure 4), blocks association with F-actin (*33*). Our results support the basic hypothesis that the C-terminal helix of gelsolin acts to inhibit gelsolin function. Our analysis of severing kinetics at various calcium ion concentrations indicates that unlatching is a significant rate-limiting step in the activation of gelsolin by calcium. The gross alteration in temperature sensitivity of severing in proteins lacking the helix also suggests that the slow step in gelsolin activation is due to dissociation of the latch from its binding partner in domain 2. We suggest that it is the physical dissociation of the two helices which is rate-limiting, though we cannot exclude the possibility that calcium binding to the micromolar binding site regulating the latch is unusually slow.

The latch, however, is only one part of the calcium regulation of gelsolin activation. The absence of a latch greatly alters the calcium sensitivity of gelsolin, but it does not create a calcium-insensitive molecule. The crystal structure of the C-terminal half of gelsolin complexed with actin in the presence of calcium was recently reported (*59*). In this structure, there are substantial rearrangements of the orientation of domains 4−6 compared with their positions in the inactive molecule. These rearrangements of the domains are consistent with the priming component of gelsolin activation, stimulated by binding of calcium to gelsolin's high-affinity binding sites. The C-terminal helix was not resolved in this structure, but the work presented here suggests that it acts as the effector for the micromolar affinity calcium binding site. Recent studies by Lin, Mejillano, and Yin (*39*) come to a similar conclusion using different methods and also demonstrate that helix truncation does not alter the affinity or number of gelsolin's calcium binding sites.

The effector for the remaining 100 nM affinity sites may also reside very near the C-terminus with domain 6. Truncation of four amino acids N-terminal of the helix results in a loss of calcium regulation but not a loss of severing or nucleating activity (*39*, *54*). In the calcium-free gelsolin crystal, these amino acids are in close association with stretches of domains 4 and 6, but are not resolved in the crystal of the C-terminal half of gelsolin complexed with actin (*33*, *59*). These residues are identical in adseverin and gelsolin. The identity in sequence between gelsolin and adseverin in regions necessary for calcium regulation, and the similarity of adseverin and the helix-deficient gelsolin construct suggest that these two proteins undergo similar structural changes upon calcium binding. We speculate that binding of calcium to the 100 nM affinity sites disrupts the interactions holding together the C-terminal domains, and subsequent binding of calcium to the site with micromolar affinity unlatches the domain 6 helix from domain 2.

Activation of Adseverin by pH. In contrast to activation by calcium ions, increasing the concentration of H^+ activates gelsolin, adseverin, and the latch-less truncate of gelsolin to sever with similar kinetics. The rate of severing is very similar, and the relationship between changing the H^+ concentration and the rate of severing is the same. This result suggests that the C-terminal helix is no longer the rate-limiting step in activation under these conditions. Presumably, protonation disrupts the helix interactions between domains 2 and 6, but also alters other, slower processes which become rate-limiting. In our earlier work, we had speculated that histidine 3 (cytoplasmic gelsolin nomenclature) within domain 1 might contribute to the activation by protons (*35*). However, this seems less likely, since our mouse cytoplasmic gelsolin construct lacks this residue yet severs with identical kinetics as full-length human plasma gelsolin containing this residue.

Implications for the Cellular Function of Adseverin and Gelsolin. Due to their high degree of homology, adseverin and gelsolin have been considered as possible functional homologues. While both proteins sever and cap actin filaments in the presence of calcium, the results obtained here suggest that these proteins are likely to function under different cellular conditions. Adseverin is activated by calcium concentrations in the submicromolar range, while gelsolin requires micromolar calcium concentrations. We suggest in cells at 37 °C that adseverin may be activated by rapid calcium transients such as calcium spikes, while gelsolin activation may require sustained elevations of free

calcium to the micromolar range. One balance to the greater severing activity of adseverin at low free calcium ion concentrations may be the greater range of lipids that inhibit its activity. Gelsolin is inhibited specifically by polyphosphoinositide lipids (*29*), while adseverin is also inhibited by phosphatidylinositol and phosphatidylserine (*27, 45*).

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