Visual Insight into How Low pH Alone Can Induce Actin-severing Ability in Gelsolin under Calcium-free Conditions

Gelsolin is a key actin cytoskeleton-modulating protein primarily regulated by calcium and phosphoinositides. In addition, low pH has also been suggested to activate gelsolin in the absence of Ca$^{2+}$ ions, although no structural insight on this pathway is available except for a reported decrement in its diffusion coefficient at low pH. We also observed a 1.6-fold decrease in the molecular mobility of recombinant gelsolin when buffer pH was lowered from 9 to 5. Analysis of the small angle x-ray scattering data collected over the same pH range indicated that the radius of gyration and maximum linear dimension of gelsolin molecules increased from 30.3 to 34.1 Å and from 100 to 125 Å, respectively. Models generated for each dataset indicated that similar to the Ca$^{2+}$-induced process, low pH also promotes unwinding of this six-domain protein but only partially. It appeared that pH is able to induce extension of the G1 domain from the rest of the five domains, whereas the Ca$^{2+}$-sensitive latch between G2 and G6 domains remains closed. Interestingly, increasing the free Ca$^{2+}$ level to merely 40 nM, the partially open pH 5 shape “sprung open” to a shape seen earlier for this protein at pH 8 and 1 mM free Ca$^{2+}$. Also, pH alone could induce a shape where the g3-g4 linker of gelsolin was open when we truncated the C-tail latch from this protein. Our results provide insight into how under physiological conditions, a drop in pH can fully activate the F-actin-severing shape of gelsolin with micromolar levels of Ca$^{2+}$ available.

Gelsolin, a multifunctional protein, is involved mainly in the reorganization of the actin cytoskeleton to control intracellular movements essential for cell growth, proliferation, and differentiation. It is expressed both as an intracellular (80.5 kDa) as well as a secreted extracellular isoform (83 kDa), each composition of six domains (G1 through G6) (1). Each molecule of gelsolin can bind six Ca$^{2+}$ ions, which induce large scale structural changes in the protein allowing three of the six domains to bind actin, monomeric globular actin (G-actin) as well as filamentous actin (F-actin) (2). Playing seemingly contradictory roles, each gelsolin molecule can bind two G-actin molecules to nucleate actin polymerization, or else it can bind to F-actin and sever the filament by weakening non-covalent bonds between the actin subunits (3). Another important role played by gelsolin during severing is remaining attached to the barbed/rapidly polymerizing end of filamentous actin as a cap to prevent its subsequent elongation (3, 4). Although direct binding with Ca$^{2+}$ activates gelsolin, phosphoinositides, particularly polyphosphoinositide 4,5-bisphosphate (PIP2), cause “deactivation” of gelsolin (5). The uncapping of gelsolin from these filaments leaves polymerization-competent ends free to rebuild the cytoskeleton, exhibiting an alternative way of promoting actin polymerization by gelsolin. The extracellular gelsolin, the plasma form, on the other hand, is an important component of the actin-scavenging system primarily involved in the rapid severing and removal of actin filaments released from dead cells into the bloodstream (6). That way, it protects essential organs from otherwise toxic F-actin, whose release goes up substantially during injury (6). Importantly, depleted levels of plasma gelsolin were found in many intensive care patients (7), and administration of exogenous gelsolin in mouse models of burn and sepsis showed a remarkable rate of recovery relative to placebo, promoting the concept of “gelsolin replacement therapy” (8). The existing understanding is that to stimulate its actin modulation functions, gelsolin primarily binds to Ca$^{2+}$ ions and gets activated, or in other words, undergoes shape changes that make it actin binding-competent (2, 4).

Visual insight into the Ca$^{2+}$ activation process could be obtained from synchrotron footprinting (9) and small angle x-ray scattering (SAXS) data analyses (2). Ab initio modeling of the global structure of gelsolin in buffers varying in levels of free Ca$^{2+}$ revealed the critical role played by flexible linkers between homologous domains in the opening of the molecule (2). Placement of the crystal structures of full-length gelsolin resolved under Ca$^{2+}$-free conditions and three domain N- and C-terminal halves of gelsolin resolved under the Ca$^{2+}$-activated actin-bound form, inside the volumes of the SAXS data-based models, showed that the G1 domain orients itself differentially in the Ca$^{2+}$-activated actin versus non-actin-bound state (2).
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Additionally, overlaying the SAXS-based model of the fully Ca$^{2+}$-activated gelsolin across the cross-section of the Holmes model of F-actin led to a proposition that although G2-G6 domains latch on to the F-actin, the g1-g2 linker "brings in" the distally positioned G1 domain into the actin-actin contact points, which leads to disruption of the filament. Supporting this notion, the functional role of residues composing the g1-g2 linker was confirmed by grafting "gain of function" in the non-severing gelsolin family protein, CapG (10). Interestingly, PIP2 has been shown to regulate Ca$^{2+}$-mediated gelsolin activity by inducing a conformational change, which disrupts the local rearrangements required to permit G1 and G2 to bind the actin molecule to cap the barbed end of F-actin, thus causing the uncapping of the filaments and favoring actin polymerization (11).

In addition to the above mentioned regulation of gelsolin activity by Ca$^{2+}$ ions and PIP2, acidic pH (~6) has also been shown to enable actin assembly/disassembly function of gelsolin (12–14). It was shown that even when intracellular Ca$^{2+}$ concentrations were about subnanomolar range, pH value close to 6 allowed gelsolin/G-actin binding as estimated by an increase in fluorescence intensity of labeled actin, with the rate constant of 15. Other reports also showed that the lowering of pH (~6) in the absence of calcium caused activation of gelsolin because with decrement in buffer pH, both filament severing as well as nucleating activities of gelsolin were substantially potentiated (13, 15). At a pH <6.0, the gelsolin activation has been found to require either no Ca$^{2+}$ or substantially reduced levels of Ca$^{2+}$ to exhibit weak/moderate activity similar to that observed at Ca$^{2+}$ concentration of 200 μM at pH 7 (13). Decrement in diffusion coefficient values measured by dynamic light scattering (DLS) supported that lower pH induces opening of the solution structure of gelsolin (13), similar to that seen as a function of increase in Ca$^{2+}$ ions without changing buffer pH (16). Additionally, biochemical studies supported that low pH leads to a cascade of ion-pair exchanges in gelsolin, which causes disruption of interdomain bonds, but these events were concluded to be distinct from those induced by Ca$^{2+}$ binding (15). Importantly, one of the first events of Ca$^{2+}$-dependent activation, the opening of the C-tail linker (interaction between the C-tail of the G6 and G2 domain), was suggested to be absent in the pH-mediated activation process. Overall, the literature suggests that although lower pH can override the need of Ca$^{2+}$ ions by gelsolin to adopt an open active conformation, the conformational changes that enable the activation by lower pH are possibly different from those followed during Ca$^{2+}$ activation. Because extension of the g1-g2 linker is an essential prerequisite for the actin binding/severing function of gelsolin, we acquired and analyzed SAXS data from samples of this protein under varying pH conditions to affirm that lower pH can actually induce an alteration of global structure leading to a shape where the G1 domain extends out into space enabled by unfolding of the g1-g2 linker. Apart from this similarity, lack of an extended g3-g4 linker was observed in the pH-induced gelsolin model as opposed to that seen in the models of Ca$^{2+}$-activated gelsolin (2), suggesting that pH activation may not fully open the protein, possibly due to retention of native-like G2-G6 interactions.

MATERIALS AND METHODS

Cloning and Purification of Recombinant Gelsolin—Recombinant gelsolin was expressed in Escherichia coli and purified using anion exchange chromatography followed by gel filtration. The plasmid harboring the gene for recombinant protein was created by PCR amplification of gelsolin cDNA with the primers GLF1 (5’-CCTCTAGATGGCCACTGCGTCGCGGGGGC-3’) and GLR1 (5’-AACCTGAGTCGGCAAGGCAAGCTCGAGGATC-3’) and PfuUltra high fidelity DNA polymerase (Fermentas, India) using clone sequence BC026033.1 provided in vector pOTB7 obtained from ATCC as template. The amplified PCR product was digested with XbaI and XhoI and subcloned into the vector backbone of pET303/CT-His (Invitrogen). In the reverse primer, a stop codon was included to eliminate the His tag at the C terminus. The plasmid pET303/Gelsolin was used to transform E. coli (BL21 (DE3)) for the heterologous protein expression. The sequence of subcloned gelsolin DNA was verified by automated DNA sequencing (Applied Biosystems).

Transformed bacteria carrying pET303/Gelsolin were grown in LB broth (Merck) to a density of A$_{600}$ = 0.5 followed by induction of recombinant protein expression with 1 mM isopropyl-β-D-thiogalactoside for 4–5 additional hours. Bacterial cells were harvested (~3 g/liter) and resuspended in lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, with 1X protease inhibitor mixture (Calbiochem). The cleared lysate was subjected to ammonium sulfate precipitation (35 and 50% saturation) as described previously with slight modifications (17). The appropriate amount of ammonium sulfate was added to get 35% saturation with continuous stirring at 4 °C until fully dissolved, and the sample was incubated for 30 min at 4 °C and centrifuged at 13,000 × g for 15 min. Additional ammonium sulfate was added to the supernatant to get 50% saturation, and the supernatant was kept for 1 h at 4 °C and then centrifuged at 13,000 × g for 15 min. After ammonium sulfate precipitation, the pellet was dissolved in equilibration buffer (buffer A; 45 mM NaCl, 25 mM Tris-HCl, pH 8, and 1 mM EGTA) and dialyzed extensively (at least six changes) against buffer A to remove the excess salt. The dialyzed sample was purified by the weak anion exchange chromatography (DE52, Whatman) as follows. Sample was applied onto a column equilibrated with buffer A followed by washing with two column volumes of the buffer A and one column volume of buffer B (containing 30 mM NaCl, 25 mM Tris-HCl, pH 8, and 0.1 mM EGTA). Finally, the protein was eluted using 2 mM Ca$^{2+}$ in the elution buffer (buffer C: 30 mM NaCl, 25 mM Tris-HCl, pH 8, and 2 mM CaCl$_2$). The eluted protein from DE52 column was concentrated using membrane concentrators (30-kDa cut-off; Millipore). The concentrated sample was further subjected to an S200 gel filtration step using an FPLC system (AKTA Prime, GE Healthcare). Based on the retention time of known proteins, the peak corresponding to molecular mass ~80 kDa was collected from FPLC. Its identity was confirmed by the predominant peak corresponding to ~82.9 kDa in the MALDI-TOF experiment (Voyager, Applied Biosystems). The purity was ascertained by 10% SDS-PAGE.
containing molecular mass markers (Fermentas). Concentration of the purified protein was measured using UV absorption at $A_{280} \sim 1.4$ corresponding to 1 mg/ml (U-2900 spectrophotometer, Hitachi). When required, the FPLC purified protein was again concentrated using membrane filters.

Enterokinase digestion of purified gelsolin was carried out to cleave the C-terminal tail region of the protein as follows. The protein (1.2 mg) was dialyzed against 25 mM phosphate buffer, pH 7, containing 45 mM NaCl and 1 mM CaCl$_2$ prior to digestion using 20 ng of enterokinase (New England Biolabs) for 16 h at 20 °C. Lysozyme (10 mg/ml) was added to the reaction mixture as bait for nonspecific digestion. The digestion was inhibited by adding imidazole to a final concentration of 150 mM, and the protein was quickly FPLC-purified using buffer A to remove the enterokinase. The purified protein was concentrated using membrane filters for SAXS analysis. Monodispersity of the FPLC-purified truncated gelsolin used for SAXS experiments can be seen from the SDS-PAGE and MALDI-TOF results shown in supplemental Fig. S1.

**DLS**—Prior to DLS experiments, FPLC-purified gelsolin was centrifuged at 60,000 × g (using Beckman TLA 120.2) for 1 h to remove any soluble aggregates. The protein solution with final concentration of ~6 mg/ml in various buffers was used to find the hydrodynamic diameters at various pH values (25 mM Tris-HCl, pH 9, and 8, 25 mM phosphate buffer, pH 7, 6.5, and 6, 25 mM NaOAc buffer, pH 5.5 and 5; all buffers also contained 40 mM NaCl and 1 mM EGTA). A probe-based pH meter capable of correcting for the temperature effects from EUTECH Instruments was used to measure the pH of the buffers. Similarly, a gelsolin sample with final concentration close to 6 mg/ml was used to measure DLS profiles of gelsolin molecule as a function of increasing Ca$^{2+}$ concentration in buffer. The intensity autocorrelation functions were measured using a microcell (volumes ~60 μl) at an angle of 165° in DelsaNanoC equipped with a Peltier-based thermostat (Beckman Coulter). 200 data points were collected for each sample for analysis of the measured autocorrelation functions using the Delsa Nano software, version 2.21. The diffusion coefficients of the scattering particles were calculated from the slope of a plot of the average decay constant versus the inverse scattering vector. Both titrations were repeated three times to compute errors.

**Synchrotron SAXS Experiments**—Gelsolin (~6 mg/ml) was stored in EGTA-containing Tris buffer at pH 8 during travel to synchrotron and was dialyzed against buffers of different pH for ~45 min (three changes; 10-, 15-, and 20-min interval) before loading them in capillary for scattering experiments. A probe-based pH meter was again used to confirm that target pH was achieved. Similarly, enterokinase-digested gelsolin (0.5 mg/ml) was also dialyzed against buffers of different pH for scattering experiments. To study the effect of free Ca$^{2+}$ on the SAXS profiles of pH-activated gelsolin molecules, increasing amounts of CaCl$_2$ were added to gelsolin samples dialyzed against the buffers of pH 9 – 5. X-ray scattering data were collected at beam line X9 at the National Synchrotron Light Source (Brookhaven National Laboratory). Low angle scattering data were collected on a charge-coupled detector. The wavelength of the beam was 0.873 Å, and the ratio of the sample-to-detector distance to the diameter of the charge-coupled detector was 20.8. Samples (50 μl) and their matched buffers were exposed for 30 s in a quartz flow cell at 15 °C with a flow rate of ~30 μl/min. All the SAXS experiments were carried out in duplicate. Alongside, SAXS on a hen egg white lysozyme concentration series was also collected under identical conditions to estimate beam intensity at zero angles and thus estimate actual concentration of gelsolin in samples. For each sample, the contribution from buffer was subtracted to obtain the scattering intensity (I) from the protein sample as a function of momentum transfer vector, Q ($Q = (4\pi\sin(\theta))/\lambda$), where λ is the beam wavelength and θ is the scattering angle. Using SDS-PAGE analysis, lack of bands in the lower mass portion and similarity in the migration pattern of the sample of protein collected after exposure of X-rays to the sample, which never traveled out of our laboratory, confirmed that no sample suffered radiation-induced damage during data collection.

SAXS Data Analysis—Guinier approximation was employed to estimate the $R_g$ of the scattering particle. As per this approximation, for a monodisperse sample of globular protein, a plot of ln(I(Q)) versus $Q^2$, where $Q \times R_g \lesssim 1.3$, should be linear and fit into the following Equation 1.

$$\ln[I(Q)] = \ln[I_0] - (R_g^2/3) \times Q^2$$  

(Eq. 1)

$I_0$, defined as the intensity of scattering at zero angles, is directly proportional to the product of molar concentration and molecular mass of the scattering sample and can be approximated by extrapolating SAXS data to Q = 0. $R_g$ is defined as the root mean square of all elemental volumes from the center of mass of the particle to its edge, weighted by their scattering densities, and is characteristic of the overall shape of the molecule.

Similarly, Equation 2 represents the Guinier analysis for rod-like particles, which provides the mean cross-sectional radius of the molecule, $R_C$, and the mean cross-sectional intensity at zero angle, $I(0)$. $Q_0$.

$$\ln[I(Q)] = \ln[I_0] - (R_C^2/2)Q^2$$  

(Eq. 2)

Importantly, using $R_g$ and $R_C$ values, the length of an ellipsoidal structure, L, can be estimated using following relationship.

$$L = [12(R_g^2 - R_C^2)]^{1/2}$$  

(Eq. 3)

For this study, both Guinier approximations were performed using the primus software package (18). Using GNOM45 software (19), indirect Fourier transformation of the scattering data over the measured Q range computed a pairwise distribution function of interatomic vectors, $P(r)$ (Equation 2).

$$P(r) = (1/2\pi) \int I(Q)Q \times r \sin(Q \times r) dQ$$  

(Eq. 4)

$P(r)$ is a histogram of the frequency of vector lengths connecting small volume elements within the entire volume of the scattering particle. The analysis also provided $R_g$ and $I_0$ from the second moment and the start of $P(r)$, respectively. Kratky plots ($I(Q) \times Q^2$ versus Q) of each dataset were prepared to assess the globular or Gaussian chain-like nature of the protein in solution. The CRYSO126 program was used to compare our experimental SAXS profiles with the theoretical scattering profiles computed using structures resolved by x-ray diffraction (20).
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**Structure Reconstruction within SAXS Profiles**—To visualize the pH-induced changes in the global structure of the gelsolin, 10 low resolution models of protein were reconstructed within the shape constraints computed during $P(r)$ analysis using DAMMIN23IQ and averaged using the DAMAVER suite of programs (21). Alternatively, using the chain-ensemble protocol (GASBOR22IQ software (22)), structures were generated for each dataset considering no shape and symmetry bias using 800 and 700 dummy residues for full-length gelsolin and truncated gelsolin, respectively. A Fibonacci grid order of 15 (988 water molecules) was used to model the hydration layer around the chain ensemble. Each calculation was repeated 10 times. Of the 10 models constructed, the structure with the $x^2$ close to 1 between computed $I(Q)$ for the modeled structure versus the experimental data was used for structure interpretation. Using SUPCOMB20, the inertial axes of the resultant low resolution shapes for gelsolin (pH 9, 8, and 7) and known structures from x-ray diffraction were superimposed (23).

**Graphical Analysis and Representations**—The programs RASWIN 2.6UCB, SPDB viewer, and WebLab viewer were used for graphical analysis, manual alignment of models, and figure generation. Data plotting and curve-fitting was done using the OriginLab software.

**RESULTS AND DISCUSSION**

**Decrease in Diffusion Coefficient at Low pH**—Although it has been reported earlier that diffusion coefficient of gelsolin is substantially reduced when compared in buffers containing pH 7.4 and 5.5 (13), to examine the extent and profile of the pH-induced change, we carried out DLS experiments in buffers varying in pH from 9 to 5 but containing the same ionic strength. Analysis of the back-scattering data suggested that the diffusion coefficient of the protein molecules decreased from $\sim 4.7 \times 10^{-7}$ to $\sim 2.8 \times 10^{-7}$ cm$^2$/s when subjected to buffer conditions varying in pH sequentially from 9 to 5. Fig. 1 (left) summarizes how lowering of pH from 9 to 7 caused no detectable change in the diffusion coefficient, but at pH values below 7, the measured diffusion coefficient values started displaying a significant decrement relative to their values at pH 8/9. Earlier, Lamb et al. (13) reported that upon lowering buffer pH from 7.4 to 5.5, the diffusion coefficient of the gelsolin decreased from $3.2 \times 10^{-7}$ to $2.15 \times 10^{-7}$ cm$^2$/s, concluding a restructuring of this protein to a more elongated shape at lower pH. It is important to highlight here that although earlier authors reported (13) a relative decrement in diffusion coefficient by a factor of $\sim 1.5$ over the pH range of 7.4 to 5.5, the relative decrement observed in our study amounted to a factor of $\sim 1.6$ over the pH range of 9–5. Slightly higher values observed by us could partly be due to the wider range of pH studied here. A sigmoidal growth pattern best-fitted to the relative decrease in diffusion coefficient as a function of pH implied that 50% change occurs when pH nears 6.4 $\pm$ 0.1. Moreover, the pH-dependent opening in gelsolin was found to be reversible in our DLS studies when the pH was again raised from 5 to 9 with half-change close to pH 6 (data not shown). Overall, we found that lowering of pH clearly induced a progressive decrement in the molecular mobility, which in the absence of any pH-dependent self-association is a strong indicator of increase in the hydrodynamic size of the molecule.

Interestingly, when compared with the pH-dependent experiment, the diffusion coefficient for gelsolin decreased more significantly, from $\sim 4.5 \times 10^{-7}$ to $\sim 2.5 \times 10^{-7}$ cm$^2$/s, as a function of increasing Ca$^{2+}$ ions in buffer whose pH was kept constant at 8 (Fig. 1, right). Keeping the final gelsolin concentration close to 6 mg/ml, the [Ca$^{2+}$]/[gelsolin] ratio was increased from 0 (EGTA) to $\sim 80$. Similar to earlier published DLS (16) and SAXS (2) studies, mobility of gelsolin in our experiments was affected by very low amounts of Ca$^{2+}$ ions in buffer. Even [Ca$^{2+}$]/[gelsolin] values close to 0.1 could bring detectable slow down in the particle velocities. Upon increasing the [Ca$^{2+}$]/[gelsolin] ratios, the decrement in diffusion coefficient values was around 1.8 times relative to the value under EGTA conditions, a value that is about 12% more than the pH-induced effect. It is pertinent to discuss here that the Ca$^{2+}$-induced shape changes in the gelsolin molecule, as tracked by synchrotron footprinting (9) and SAXS data analyses (2), upheld a three-state process of activation of this protein. Knowing this fact, we tried to fit both the two-state as well as the three-state model for the changes seen in the DLS experiments upon increasing Ca$^{2+}$ concentration (Fig. 1, right). As per our three-state approximation, the second state is achieved by [Ca$^{2+}$]/[gelsolin] ratio of $\sim 0.04$ and remains stable until the Ca$^{2+}$-protein ratio exceeds 2. Earlier, SAXS data-based analysis indicated that the second state is reached by [Ca$^{2+}$]/[gelsolin]...
ratio of ~0.004 and is stable until the ratio is about 0.4, with median value at 0.04 (2). On the other hand, the sigmoidal fit to a two-state model of Ca$^{2+}$ activation suggested that 50% activation occurred when [Ca$^{2+}$]/[gelsolin] ratio is ~2 or at a Ca$^{2+}$ concentration of ~5 μM, a value comparable with an earlier publication, which reported that half-change occurs at ~3 μM (16). Considering limitations of the laser-based light scattering technique versus SAXS, we feel that the two-state model is probably adequate to interpret DLS data.

As seen earlier from SAXS data analysis and modeling, although one molecule of gelsolin can at most bind six Ca$^{2+}$ ions, the complete conformational shift in the population toward a shape that closely resembled that seen from crystal structures occurred well past [Ca$^{2+}$]/[gelsolin] ratio of ~6 (2). We also observed that the mobility of protein kept decreasing on increasing Ca$^{2+}$ levels in buffer, eventually saturating at [Ca$^{2+}$]/[gelsolin] ratio ~70 (Fig. 1, right). Importantly, for the sample having [Ca$^{2+}$]/[gelsolin] ratio ~6, the relative decrement was about 1.6, which was actually very close to the decrement measured for the molecules at pH 5 under Ca$^{2+}$-free conditions. Overall, our DLS experiments concluded that low pH can induce an opening in the preferred solution conformation of gelsolin, by a factor of ~1.6 times, which is comparable with the shape adopted by partially Ca$^{2+}$-activated protein because excess Ca$^{2+}$ ions could open the molecular dimensions further up to a factor of 1.8.

SAXS Data Analysis—Measured SAXS $I(Q)$ data as a function of $Q$ collected from samples of gelsolin are presented in Fig. 2A. Lack of “upturned” intensity profile in the low $q$ region confirmed complete lack of aggregation in the samples during data collection (24). To probe the inherent flexibility in the protein molecules in solution and understand globular versus Gaussian chain-like nature of the scattering species, we analyzed the Kratky plots of the acquired SAXS intensity data (25). The peak profile of the Kratky plots supported that a globular nature is preferred by the gelsolin molecules under all pH conditions studied here (Fig. 2B). Alternatively, a hyperbolic Kratky plot would have concluded a Gaussian chain-like behavior resulting from the molecules adopting solution structures comparable at larger dimensions but differing significantly in smaller features or high conformational polydispersity in the molecules. Although the area under the peak(s) with maxima at

![SAXS data analysis](image-url)
was similar to those observed earlier for gelsolin samples at pH 8 under EGTA conditions, the values for the sample at pH 5 were similar to the gelsolin molecules at pH 8 supplemented with free $pCa$ level of 7.4 ($\sim 40$ nM) (2). These results, along with the observations from DLS data, suggested a partial opening of the gelsolin molecules at pH 5 as the molecular dimensions were found to be intermediary of the dimensions of fully opened gelsolin molecules at free $pCa$ level of 3 ($\sim 1$ mM) (2).

**Open Structure of Gelsolin at Low pH**—Both uniform density and chain-ensemble modeling approaches were used to model the solution structure of gelsolin under different pH. Initially, to understand the global structural features, 10 different solutions modeled using the DAMMIN program for each dataset were averaged using the DAMAVER suite of programs (Fig. 3). Alongside uniform density models, chain-ensemble models were also generated using 800 dummy residues to gain insight on domain shapes interconnected by linkers. As expected from DLS and SAXS data analysis, the global structure of gelsolin increased in the molecular dimensions upon lowering pH below 7. Comparison of the theoretical SAXS profile calculated using the crystal structure of gelsolin under $Ca^{2+}$-free conditions (PDB ID: 1D0N chain A) with the experimentally measured SAXS profiles (over a Q range 0.005–0.22 Å$^{-1}$) at pH 9, 8, and 7 yielded $\chi$ values of 1.5, 1.3, and 1.6, respectively, indicating a high similarity between the crystal structure resolved for gelsolin under EGTA conditions and the structure preferred by gelsolin at these pH values. This observation was visually confirmed upon analyzing the automated superimposition of a monomer from the crystal structure of $Ca^{2+}$-free gelsolin with the models generated for the protein at pH 9, 8, and 7, implying that gelsolin maintains a tight compact globular structure in this pH range similar to its structure seen in diffractable form (PDB ID: 1D0N) (Fig. 3). In contrast, the models solved within the shape constraints present in the SAXS profiles of the samples at pH 6 and 5 showed that the globular compact gelsolin had started to open up from both ends as a result of lower buffer pH, and the opening remained progressive from pH 6 to 5. In corroborating with molecular dimensions, the shape profile of the gelsolin molecules at pH 5 was found to be matching best with that of previously reported gelsolin molecules at pH 8 supplemented with $\sim 40$ nM of free $Ca^{2+}$ ions, again supporting the notion that the low pH might lead to a partial opening of the gelsolin molecule when compared with the $Ca^{2+}$-activated gelsolin molecules.

In the absence of any crystal structure of pH-activated gelsolin, we placed crystal structures of gelsolin under EGTA conditions and its $Ca^{2+}$-activated halves (minus the bound actin) to interpret the pH-induced domain opening in our low resolution models (Fig. 4). Automated superimposition of the crystal structure of full-length gelsolin solved under EGTA conditions (PDB ID: 1D0N chain A) over the model of gelsolin at pH 8 helped us in understanding the possible location of the G1-G3 (red) and G4-G6 (blue) halves within the volume of gelsolin at pH 8. To infer similar information about the pH 5-induced open structure of the gelsolin, we manually placed open domain structures of the N- and C-terminal halves of gelsolin resolved as $Ca^{2+}$-activated forms bound to actin. While aligning crystal structures inside the volume of SAXS data-based model, a

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### TABLE 1

Summary of the structural parameters deduced for the global structure of recombinant gelsolin in buffers containing varying pH

| pH | Guinier Analysis | Indirect Fourier Transformation |
|----|-----------------|--------------------------------|
|    | $R_G$ | $R_C$ | $L$ | $D_{\text{max}}$ | $R_G$ | $I_0$ |
| 9  | 30.0 ± 0.04 | 17.1 ± 0.01 | 86 | 105 | 30.3 ± 0.02 | 94.5 |
| 8  | 30.4 ± 0.03 | 17.4 ± 0.02 | 86 | 100 | 30.1 ± 0.02 | 94.4 |
| 7  | 30.0 ± 0.04 | 17.1 ± 0.04 | 85 | 100 | 30.4 ± 0.02 | 100.6 |
| 6  | 31.1 ± 0.04 | 16.4 ± 0.01 | 92 | 111 | 31.5 ± 0.03 | 97.3 |
| 5  | 33.7 ± 0.03 | 15.4 ± 0.01 | 105 | 125 | 34.5 ± 0.05 | 97.9 |

$\sim Q = 0.061–0.059$ Å$^{-1}$ (from pH 9 to 5) remained almost constant, the peak width at half-height increased about 1.5 times for the scattering species as a function of lowering pH from 8 to 5, again supporting an increment in the molecular dimensions. Guinier approximation for globular particles showed a systematic increment in the $R_G$ values of the predominant solution structure of the protein as a function of lowering of the buffer pH (Fig. 2, inset, and Table 1). The calculated $R_G$ remained more or less close to 30 Å for the protein at pH 9–7, and the value increased to 30.7 and 33.7 Å upon further lowering the buffer pH to 6 and 5, respectively. Presuming a rod-shape for the global structure of the protein, Guinier approximation for rod-like particles suggested that the cross-sectional radius of gyration ($R_G$) of the molecules decreased from $\sim 17$ to 15 Å with lowering of buffer pH (Table 1). Employing the relationship between $R_G$ and $R_C$ (Equation 3), analysis of only the low angle scattering data suggested that the maximum dimension of the gelsolin molecule increases by about 22% upon lowering its buffer pH from 9 to 5.

A similar trend was observed upon deducing the structural parameters of the global structure of the protein molecules by carrying out indirect Fourier transformation of much wider data ($Q$ used from 0.005 to 1 Å$^{-1}$) (Table 1). Assuming zero probability of finding an interatomic vector of length 0 Å and longer than $D_{\text{max}}$, good solutions were obtained for the protein molecules at $D_{\text{max}}$ close to 100 Å for samples at pH 9, 8, and 7 (Fig. 2C). On the other hand, same analysis of the $l(Q)$ data collected from samples at pH 6 and 5 indicated a clear increment in the $D_{\text{max}}$ to 111 and 125 Å, respectively. Along with the increase in $D_{\text{max}}$, the $R_G$ of the molecules increased modestly from 30 to 34.1 Å. The estimated $I_0$ values of $\sim 97 \pm 3$ units suggested that the concentration of the scattering species was similar in all five samples, and during $P(r)$ analyses, we did not grossly under- or overestimate the molecular dimensions (Table 1). In comparison with the $I_0$ value extrapolated for 1 mg/ml of lysozyme sample collected under identical conditions, exposure times, and scaled transmission counts, the final concentration of gelsolin during our SAXS experiments was about 5.7 mg/ml.

The sigmoidal growth curves best-fitted on the estimated $D_{\text{max}}$ and $R_G$ values suggested that 50% change occurred at pH values close to 5.9 ± 0.4 and 5.6 ± 0.4, respectively (Fig. 2D). The mid-point of change in the values of $D_{\text{max}}$ and $R_G$ coincides with the theoretical pI of gelsolin $\sim 5.7$. More importantly, the pattern of the pH-dependent change in the $D_{\text{max}}$ and $R_G$ values can be confidently fitted to only a two-state model, unlike the three-state model proposed for $Ca^{2+}$ activation of gelsolin (2). Although the $D_{\text{max}}$ and $R_G$ values of gelsolin samples at pH 9–7
knowledge-based approximation was made that the C-terminal G4–G6 half adopts a trilobal triangular shape in the presence of Ca\(^{2+}\) ions, and the shape is independent of whether it is bound to actin or not (26, 27). Similarly, knowing the importance of extension of the g1–g2 linker to achieve the ability of gelsolin to sever F-actin in the presence of Ca\(^{2+}\) ions, we concluded that extended unoccupied volume in our model of gelsolin at low pH conditions in the absence of Ca\(^{2+}\) ions and bound actin must be residing in the G1 domain of gelsolin (Fig. 4). Importantly, a low \(\chi^2\) value of only 1.5 was observed between the experimental and calculated SAXS profile of the open N- and C-terminal halves (as placed within the volume of the SAXS data-based model, Fig. 4). Overall, the molecular shape of the pH-induced model toward the C terminus was much more compact when compared with that of the fully Ca\(^{2+}\)-activated model of gelsolin (2), implying an unopened g3–g4 linker in these molecules. Moreover, the g3–g4 linker being much longer (40–53 amino acids depending on its Ca\(^{2+}\)-bound state (28, 29)) than g4–g5 and g5–g6 linkers, if open, can be seen as an additional volume between N- and C-terminal halves of the protein as observed earlier (2). This additional volume interconnecting the trilobal volume of the N- and C-terminal halves was completely missing in our models solved for pH-activated gelsolin. Thus, despite the similarity of opened g1–g2 linker, an important difference observed between our structural models of pH-activated gelsolin and the previously reported fully Ca\(^{2+}\)-activated model of gelsolin was the unopened g3–g4 linker, which connects the G1–G3 and G4–G6 halves of this protein. The compact packing of the G2–G6 domains in the structure solved for pH 5 could be due to the close C-tail latch, which "locks" G2/G6 domains and is sensitive only to Ca\(^{2+}\) ions. This observation corroborates with an earlier report that the opening of the C-tail latch, which is one of the first events of Ca\(^{2+}\)-dependent gelsolin activation, might be absent in the pH-mediated activation process (15).

**Can Low pH Open up g3-g4 Linker if G2/G6 Interdomain Contacts Are Disrupted?**—To confirm that low pH-induced activation of gelsolin molecules is structurally partial, we investigated whether an additional increment can be induced in the molecular dimensions of gelsolin molecules by adding free Ca\(^{2+}\) to samples pre-equilibrated with lower pH. \(D_{\text{max}}\) values of the protein molecules increased rapidly upon increasing free Ca\(^{2+}\) levels from 0 to 500 nM (Fig. 5A), although most of the changes were achieved by 40 nM for samples at pH 6 and 5. The \(D_{\text{max}}\) values for samples at pH 8 at increasing levels of free Ca\(^{2+}\)
were in good comparison with values published earlier (2). Interestingly, as reported earlier that lowering of pH overrides the need for Ca\(^{2+}\) ions to activate gelsolin (13), we also observed that \(D_{\text{max}}\) values comparable with activation by 1 mM free Ca\(^{2+}\) ions at pH 8 were achieved by only 40 nm of Ca\(^{2+}\) ions in the case of sample pre-equilibrated at pH 5 (2). The average scattering shapes of gelsolin molecules in buffers at pH 7–5 containing 40 nm Ca\(^{2+}\) ions are shown in supplemental Fig. S2.

Interestingly, placement of Ca\(^{2+}\)-activated G1-G3 and G4-G6 halves of gelsolin inside the volume of the SAXS data-based chain-ensemble model of gelsolin at pH 5 and 40 nm Ca\(^{2+}\) highlighted the unoccupied volume separating the two halves of gelsolin (Fig. 5B). Although this placement showed a relatively higher \(\chi^2\) value of 1.9 between the experimental and calculated SAXS profiles, it clearly demonstrated that the g3-g4 linker can also extend with low pH if the C-tail latch can be opened, which in turn requires nanomolar levels of Ca\(^{2+}\) ions to unlock (30).

To further demonstrate that pH alone can lead to opening of both g1-g2 linker as well as g3-g4 linker in the absence of its calcium-sensitive C-tail latch, we generated a tail-less gelsolin by truncating gelsolin at position 693 by digesting gelsolin with enterokinase (the site being intrinsically present in the primary sequence). SAXS data analysis of the truncated gelsolin in buffers with varying pH showed that the shape parameters of this protein increased rapidly with lowering of buffer pH (Fig. 6A). In fact, the structural parameters of this truncated protein at pH 5 were comparable with those derived for the full-length protein at pH 8 and 1 mM free Ca\(^{2+}\) (2). Structure reconstruction of this C-tail lacking protein showed that the compact shape of this protein at pH 8 opens up drastically as buffer pH reaches 5, in the absence of Ca\(^{2+}\) ions (supplemental Fig. S3).

Interestingly, placement of Ca\(^{2+}\) ions to activate gelsolin (13), we also observed that pH 5 –5 as a function of increasing Ca\(^{2+}\) ion concentration in buffer is presented. B, two rotated views of the chain-ensemble model solved for gelsolin molecules at pH 5 supplemented with 40 nm free Ca\(^{2+}\) are shown and compared with crystal structures of Ca\(^{2+}\)-activated gelsolin halves to visualize the domain rearrangements in this protein upon sensing low pH and Ca\(^{2+}\) ions. The C’ traces of the N- and C-terminal halves of the molecule in the crystal structures are shown as red and blue tubes, respectively.

The structure restored for the same protein at pH 5 in Ca\(^{2+}\)-free conditions showed that the two halves of the protein could now extend away from each other, which helped us in identifying unoccupied volume between the space residing G3 and G4 domains (Fig. 6B). In accordance with our previous results with Ca\(^{2+}\) and pH-activated gelsolin, it can be safely concluded that the g3-g4 linker very likely resides in this interconnecting volume. Unclear or diffused volumes of domains in current models in comparison with those seen earlier for high Ca\(^{2+}\)-activated gelsolin (2) may be due to additional conformational organization that this protein might undergo upon binding Ca\(^{2+}\) ions or some kind of chaotropic effect enabled by high Ca\(^{2+}\) ion concentrations, which pH either alone or with low levels of Ca\(^{2+}\) cannot induce. Nevertheless, our scattering data and models clearly support that low pH-induced gelsolin can also induce opening of the g3-g4 linker if the calcium-sensitive G2/G6 interdomain interactions are overcome/removed. Although compacted G2-G6 domains at low pH in the absence of free Ca\(^{2+}\) can be further probed using other complementary techniques such as synchrotron footprinting, NMR, and possibly x-ray diffraction, as of now, our data imply that pH activation of gelsolin may have some overlapping intermediary states with the Ca\(^{2+}\)-activated pathway.

Prior to our earlier report (2) on Ca\(^{2+}\) activation of gelsolin and the current work, Lamb et al. (13) proposed a possible full activation of gelsolin by pH on observing that in the absence of Ca\(^{2+}\) ions, gelsolin at pH 5.7 could sever F-actin as efficiently as gelsolin in a buffer with pH 7.4 and 200 \(\mu\)M Ca\(^{2+}\) ions. In addition, they also reported that starting from monomeric G-actin, actin polymerization was accelerated at low pH (~5.3) in the absence of Ca\(^{2+}\), at levels even higher than those observed at pH 7.4 in the presence of 200 \(\mu\)M Ca\(^{2+}\) (13). In contrast, another group proposed that pH induces partial activation because they observed that at pH 5.7, the affinity of the G4-G6 half toward actin was only 1 \(\mu\)M in comparison with about 30 nm in buffer containing 1 mM Ca\(^{2+}\) and pH 7.4 (15). Bringing clarity to this debate, our present work provides structural insight into how low pH can open up the g1-g2 linker, which is essential for the F-actin-severing function of gelsolin, although mechanically
It still remains to be explored how the partially activated structure might sit across/on F-actin and perform its severing action.

**Physiological Relevance of pH Activation**

Intracellular acidification represents a hallmark of apoptosis in addition to cell shrinkage, membrane blebbing, and DNA fragmentation. A drop in cytosolic pH has been shown from 7.2 ± 0.1 in healthy cells to 6.8 ± 0.1 in pre-apoptotic, caspase-negative cells and 5.7 ± 0.04 in apoptotic, caspase-positive cells (31). During apoptosis, gelsolin undergoes a cleavage by several caspases (32, 33), yielding G1-G3, which severs actin filaments in a manner unregulated by calcium (34). Thus, gelsolin participates in preparing the cell for death by dismantling its actin-based architecture, resulting in morphologic changes and nuclear fragmentation. The lower cytosolic pH in these cells with even nanomolar levels of free Ca²⁺ could provide an alternative way of almost complete gelsolin activation without requiring Ca²⁺ level elevation. Conversely, gelsolin has also been reported to

![Diagram](image)

**FIGURE 6.** A, the increase in $D_{max}$ and $R_g$ values of the gelsolin protein truncated at the 693rd amino acid as a function of low pH alone is plotted here. B, two rotated views of the chain-ensemble models solved for C-terminal truncated gelsolin molecules under pH 8 (top) and 5 (bottom) under EGTA conditions are shown and compared with high resolution structures known from x-ray crystallography to affirm that pH alone can open the protein to its full extent if the G2/G6 interdomain contacts are removed. The Cα traces of the N- and C-terminal halves of the molecule in the crystal structures are shown as red and blue tubes, respectively.
inhibit apoptosis. A proposed mechanism to explain the inhibitory capacity of gelsolin is that gelsolin in complex with phosphoinositides can competitively inhibit caspase-3 (33). The affinity of gelsolin for PIP2 has been shown to be increased by lowering the pH (11), suggesting that the inhibition of apoptosis could be mediated by an increased interaction between full-length gelsolin and PIP2 at lower cytosolic pH as opposed to the pro-apoptotic action of G1-G3. Similar cytosolic acidification has also been documented in other conditions, such as actively growing yeast cells (35), and in chronic alcoholics without carcinoma due to an increased activity of transmembrane Na+/H+ exchange and a more acidic set point (36). It could be that pH-induced gelsolin activation under these conditions results in the necessary actin-cytoskeleton modulation. Because cytosolic acidification has also been suggested to be a part of the trigger for Ca2+ mobilization from intracellular stores in both parietal cells and platelets (37), it might provide an alternative pathway of fine-tuning the gelsolin function by a partial activation of gelsolin prior to its full activation by increase in the intracellular Ca2+ levels. Finally, apart from its actin modulatory functions, gelsolin also exhibits other activities as diverse as working as a crystallin in fish eye (38) and regulating photolytic functions. As of now, it is not clear whether Ca2+ binding and/or lower pH is essential for gelsolin to affect these functions.

In conclusion, even after two decades since the earliest report on pH-mediated regulation of gelsolin activity (12), no visual insight into the pH-induced changes in the structure of the gelsolin has yet been reported other than a possible increase in the molecular dimensions at low pH. This might be due to the immense difficulty posed by researchers in attaining diffraction quality crystals of gelsolin at different pH conditions. Interestingly, the two crystal structures of the Ca2+-activated actin-bound N-terminal G1-G3 half of gelsolin were obtained in buffers containing pH 4.7 (29, 42). The Ca2+ ion at its binding site in the G2 domain is now resolved in the human gelsolin-actin complex (42), but it could not be resolved in the equine gelsolin (29), which led to questions about how the activation/complexation occurred. Now we know that low pH alone can affect shape changes in the N-terminal half of gelsolin that are similar to those induced by Ca2+ ions. It is very likely that pH and Ca2+ ions may work in cooperation as far as the N-terminal half is concerned, which led to stabilization of equine G1-G3-actin complex. On the other hand, the crystal structures of Ca2+-activated G4-G6 halves ± actin were solved at pH 8 and 7.5 (26, 27), but no crystal structure of the G1-G3 half of gelsolin (with or without actin) grown at pH 8 is reported. In the absence of structures from diffraction or from NMR experiments, SAXS data analysis and modeling do offer a way to gain insight into large scale shape changes in the global structure of this protein as a function of pH. Knowing that gelsolin replacement therapy could be a possibility in the future to improve the outcome of critical care patients (43), our work significantly improves the current understanding on how low pH and Ca2+ levels have a synergistic effect in activating the severing ability of this multi-functional therapeutic protein, gelsolin.

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REFERENCES

1. Kwiatkowski, D. J., Mehrl, R., and Yin, H. L. (1988) J. Cell Biol. 106, 375–384
2. Ashish, Paine, M. S., Perryman, B. P., Yang, L., Yin, H. L., and Krueger, J. K. (2007) J. Biol. Chem. 282, 25884–25892
3. Yin, H. L., and Stossel, T. P. (1979) Nature 281, 583–586
4. Yin, H. L. (1987) Bioessays 7, 176–179
5. Sun, H. Q., Yamamoto, M., Mejillano, M., and Yin, H. L. (1999) J. Biol. Chem. 274, 33179–33182
6. Lee, W. M., and Galbraith, R. M. (1992) N. Engl. J. Med. 326, 1335–1341
7. Wang, H., Cheng, B., Chen, Q., Wu, S., Lv, C., Xie, G., Jin, Y., and Fang, X. (2008) Crit. Care 12, R106
8. Lee, P. S., Waxman, A. B., Cotich, K. L., Chung, S. W., Perrella, M. A., and Stossel, T. P. (2007) Crit. Care Med. 35, 849–855
9. Kissel, J. G., Janney, P. A., Almo, S. C., and Chance, M. R. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 3942–3947
10. Zhang, Y., Vorobiev, S. M., Gibson, B. G., Hao, B., Sidhu, G. S., Mishra, V. S., Yarmola, E. G., Bubb, M. R., Almo, S. C., and Southwick, F. S. (2006) EMBO J. 25, 4458–4467
11. Lin, K. M., Wenegieme, E., Lu, P. J., Chen, C. S., and Yin, H. L. (1997) J. Biol. Chem. 272, 20443–20450
12. Selve, N., and Wegner, A. (1987) Eur. J. Biochem. 168, 111–115
13. Lamb, J. A., Allen, P. G., Tuan, B. Y., and Janmey, P. A. (1993) J. Biol. Chem. 268, 8999–9004
14. Lagarrigue, E., Maciver, S. K., Fantoum, A., Benyamin, Y., and Roustan, C. (2003) Eur. J. Biochem. 270, 2236–2243
15. Lagarrigue, E., Ternent, D., Maciver, S. K., Fantoum, A., Benyamin, Y., and Roustan, C. (2003) Eur. J. Biochem. 270, 4105–4112
16. Pope, B. I., Gooch, J. T., and Weeds, A. G. (1997) Biochemistry 36, 15848–15855
17. Kurokawa, H., Fujii, W., Ohmi, K., Sakurai, T., and Nonomura, Y. (1990) Biochem. Biophys. Res. Commun. 168, 451–457
18. Konarev, P. V., Volkov, V. V., Sokolova, A. V., Koch, M. H. J., and Svergun, D. I. (2003) J. Appl. Crystallogr. 36, 1277–1282
19. Svergun, D. I. (1992) J. Appl. Crystallogr. 25, 495–503
20. Svergun, D., Barberato, C., and Kohl, M. H. (1995) J. Appl. Crystallogr. 28, 768–773
21. Volkov, V. V., and Svergun, D. I. (2003) J. Appl. Crystallogr. 36, 860–864
22. Svergun, D. I., Petoukhov, M. V., and Koch, M. H. (2001) Biophys. J. 80, 2946–2953
23. Koizin, M. B., and Svergun, D. I. (2001) J. Appl. Crystallogr. 34, 33–41
24. Jacques, D. A., and Trendhella, J. (2010) Protein Sci. 19, 642–657
25. Glatter, O., and Kratky, O. (1982) Small Angle X-ray Scattering, Academic Press, New York
26. Choe, S. (2002) J. Mol. Biol. 324, 691–702
27. Narayan, K., Chumamsilpa, S., Choe, H., Irobi, E., Urosev, D., Lindberg, U., Schutt, C. E., Burtnick, L. D., and Robinson, R. C. (2003) FEMS Lett. 552, 82–85
28. Burtnick, L. D., Koenig, E. K., Grimes, J., Jones, E. Y., Stuart, D. I., McLaughlin, P. J., and Robinson, R. C. (1997) Cell 90, 661–670
29. Burtnick, L. D., Urosev, D., Irobi, E., Narayan, K., and Robinson, R. C. (2004) EMBO J. 23, 2713–2722
30. Robinson, R. C., Mejillano, M., Le, V. P., Burtnick, L. D., Yin, H. L., and...
Choe, S. (1999) Science 286, 1939–1942
31. Nilsson, C., Johansson, U., Johansson, A. C., Kågedal, K., and Ollinger, K. (2006) Apoptosis 11, 1149–1159
32. Kothakota, S., Azuma, T., Reinhard, C., Klippel, A., Tang, I., Chu, K., McGarry, T. J., Kirschner, M. W., Koths, K., Kwiatkowski, D. J., and Williams, L. T. (1997) Science 278, 294–298
33. Azuma, T., Koths, K., Flanagan, L., and Kwiatkowski, D. (2000) J. Biol. Chem. 275, 3761–3766
34. Selden, L. A., Kinosian, H. J., Newman, J., Lincoln, B., Hurwitz, C., Gershman, L. C., and Estes, J. E. (1998) Biophys. J. 75, 3092–3100
35. Imai, T., and Ohno, T. (1995) J. Biotechnol. 38, 165–172
36. Spies, C. D., Spies, K. P., Zinke, S., Runkel, N., Berger, G., Marks, C., Helling, K., Blum, S., Müller, C., Rommelspacher, H., and Schaffartzik, W. (1997) Alcohol Clin. Exp. Res. 21, 1653–1660
37. Tsunoda, Y., Matsuno, K., and Tashiro, Y. (1991) Exp. Cell Res. 193, 356–363
38. Xu, Y. S., Kantorow, M., Davis, J., and Piatigorsky, J. (2000) J. Biol. Chem. 275, 24645–24652
39. Steed, P. M., Nagar, S., and Wennogle, L. P. (1996) Biochemistry 35, 5229–5237
40. Ohtsu, M., Sakai, N., Fujita, H., Kashiwagi, M., Gasa, S., Shimizu, S., Eguchi, Y., Tsujimoto, Y., Sakiyama, Y., Kobayashi, K., and Kuzumaki, N. (1997) EMBO J. 16, 4650–4656
41. Hirko, A. C., Meyer, E. M., King, M. A., and Hughes, J. A. (2007) Mol. Ther. 15, 1623–1629
42. Nag, S., Ma, Q., Wang, H., Chumnarnsilpa, S., Lee, W. L., Larsson, M., Kannan, B., Hernandez-Valladares, M., Burtnick, L. D., and Robinson, R. C. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 13713–13718
43. Lee, P. S., Drager, L. R., Stossel, T. P., Moore, F. D., and Rogers, S. O. (2006) Ann. Surg. 243, 399–403