Calcium-sensitive Activity and Conformation of Caenorhabditis elegans Gelsolin-like Protein 1 Are Altered by Mutations in the First Gelsolin-like Domain

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The gelsolin family of actin regulatory proteins is calcium-regulated. The gelsolin family of actin-severing/capping proteins is calcium-regulated. Results: Two acidic residues of Caenorhabditis elegans gelsolin-like protein 1 (GSNL-1) were important for calcium regulation. Mutation at these residues sensitized GSNL-1 for calcium. Conclusion: The two acidic residues are important to maintain normal calcium sensitivity of GSNL-1. Significance: These residues are conserved, suggesting their importance in calcium regulation of the gelsolin family.

The gelsolin family of actin regulatory proteins is a major class of Ca2+-dependent actin filament-severing and -capping proteins that regulate dynamic reorganization of the actin cytoskeleton (1–4). Gelsolin is inactive in the absence of Ca2+, whereas Ca2+ exposes its actin-binding sites and activates its actin filament-severing and barbed end-capping functions. Gelsolin has six repeats of homologous domains of 100–120 amino acids, which are denoted as gelsolin-like domains (G domains) or segments (G1–G6). A Ca2+-free inactive form of gelsolin has a compact globular fold with several interdomain connections, such that actin-binding sites are masked (5). Upon Ca2+ activation, large structural changes are induced (6), and actin-binding sites are exposed (7–9). The C-terminal helical tail binds to the helix in G2, thereby acting as a latch that holds a closed conformation (5). Ca2+-dependent release of the C-terminal latch is one of the mechanisms to open up gelsolin (10–12). However, release of the C-terminal latch is not sufficient for full activation of gelsolin, and the mechanism of Ca2+ activation of the gelsolin family is not completely understood.

The presence of another latch mechanism between G1 and G3 has been suggested, but it has not been extensively characterized. In an inactive form of gelsolin, G1 and G3 make a close contact by forming a continuous β-sheet and masking actin-binding residues in the long helix of G1 (5). However, the structure of an actin-bound form of G12G3 indicates that G1 is moved away from G3 to bind to actin and that G2 and G3 make a close association (9). Ca2+ binding to G2 is implicated in stabilizing the G2-G3 association (13). However, the precise nature of the G1-G3 latch is not clearly understood.

We have previously reported that Caenorhabditis elegans gelsolin-like protein 1 (GSNL-1), which has only four G domains (G1–G4), severs actin filaments and caps barbed ends in a similar manner to gelsolin (14). However, unlike gelsolin, GSNL-1 remains bound to the side of actin filaments and does not nucleate actin polymerization (14). Analysis of the domain-function relationship of GSNL-1 shows that G1 and the linker between G1 and G2 are sufficient for actin filament severing in a similar manner to the equivalent part of vertebrate gelsolin (15). However, an F-actin-binding site of GSNL-1 is present in G3-G4 (15), whereas G2 of vertebrate gelsolin binds to F-actin (16). Moreover, the G12G3 fragment of GSNL-1 severs...
Calcium Regulation of GSNL-1

actin filaments in a Ca\(^{2+}\)-dependent manner (15), whereas the equivalent fragments of vertebrate gelsolin exhibit Ca\(^{2+}\)-independent actin-severing activity (17, 18). Because G4 of GSNL-1 is much shorter than G4–G6 of gelsolin, the C terminus of GSNL-1 may not extend to G2 to function as a latch. This may be the reason why a Ca\(^{2+}\) regulation system is contained within G1/G2/G3 of GSNL-1.

In this study, we originally attempted to identify sequences that are critical for actin filament-severing activity of GSNL-1. Previously, Southwick (19) reported that human CapG, a member of the gelsolin family lacking actin-severing activity, can be converted to an actin filament-severing protein by introducing short gelsolin sequences in G1 and the G1–G2 linker. The critical sequence in G1 is “LDDY” at the C-terminal end of the long helix, whereas the two acidic residues are not conserved in CapG (20). GSNL-1 has “IDDS” at the equivalent position (14). Therefore, we reasoned that the two aspartic acid residues are important for actin-severing activity of GSNL-1. Unexpectedly, we found that these acidic residues are dispensable for actin-severing activity of GSNL-1 but rather important for Ca\(^{2+}\)-dependent actin-severing activity (17, 18). Because G4 of GSNL-1 may not extend to G2 to function as a latch, this sequence is an important determinant for maintaining active and inactive conformations of the gelsolin family.

EXPERIMENTAL PROCEDURES

**Proteins and Materials**—Actin was purified from rabbit skeletal muscle acetone powder (Pel-Freeze Biologicals) as described (21). Pyrene-labeled actin was prepared as described (22). Bacterially expressed full-length GSNL-1 was purified as described previously (14). Phosphatidylinositol 4,5-bisphosphate (PIP2) (P-4516, Echelon Biosciences) was suspended in water at 1 mg/ml with brief sonication and stored at −20 °C. Bovine pancreas chymotrypsin, which had been treated with 1-chloro-3-tosylamido-7-amino-2-heptanone, was purchased from Worthington Biochemical Corp., suspended in 1 mM HCl at 1 mg/ml, and stored at −80 °C.

**Site-directed Mutagenesis and Production of GSNL-1 Variants**—Site-directed mutagenesis to convert Asp-83 and Asp-84 of GSNL-1 to alanines was performed on expression vectors for full-length GSNL-1 (pGEX-GSNL-1) (14) and the G1b fragment (pGEX-G1b) (15) by a QuikChange mutagenesis kit (Stratagene) using a forward primer 5’-AATCGGCAATCTCTACAGTCTT-3’ and a reverse primer 5’-GTTGGAATTCAGAGAAGCGTCAATCTCTACAGTCTT-3’. D83A and D84A single mutations were introduced into full-length GSNL-1 (pGEX-GSNL-1) using the following primers: D83A-forward 5’-AAGACTGTGAGATTGCCGATTCTCTCGGTGGAATTCCA-3’, D83A-reverse 5’-AATCGGCAATCTCTACAGTCTT-3’, D84A-forward 5’-AAGACTGTGAGATTGCCGATTCTCTCGGTGGAATTCCA-3’, and D84A-reverse 5’-GTTGGAATTCAGAGAAGCGTCAATCTCTACAGTCTT-3’. The sequences of protein coding regions were verified by DNA sequencing. Both wild-type and mutant full-length GSNL-1 and the G1b fragment were expressed as fusion proteins with glutathione S-transferase (GST) in *Escherichia coli* strain BL21(DE3) and purified as described previously for wild-type proteins (14, 15). Briefly, *E. coli* lysates were applied to a glutathione Uniflow (Clontech) column to adsorb the GST fusion proteins, and then the GSNL-1 variants were cleaved by thrombin (Roche Applied Science) on beads and eluted from the column. They were dialyzed against F-buffer (0.1 mM KCl, 2 mM MgCl\(_2\), and 20 mM HEPES-NaOH (pH 7.5)) containing 50% glycerol and stored at −20 °C. Protein concentrations were determined by densitometry of Coomassie Blue-stained gels after SDS-PAGE using actin as a standard.

**Direct Observation of Actin Filaments by Fluorescence Microscopy**—Microscopic observation of actin filament severing was performed essentially as described previously (15). Unlabeled G-actin was co-polymerized with DyLight 549-labeled G-actin at 2 μM total actin (20% labeled) for 2 h in F-buffer. Labeled actin was diluted to 0.4 μM in F-buffer with or without GSNL-1 variants in the presence of various concentrations of CaCl\(_2\) and EGTA and incubated for 5 min at room temperature. Free Ca\(^{2+}\) concentrations were calculated by CHELATER (23). Then, the reactions were put on a nitrocellulose-coated coverslip, and immobilized actin filaments on the coverslip were observed by epifluorescence using a Nikon TE2000 microscope with a 60× Plan Apo objective (oil, numerical aperture of 1.4). Images were captured by a SPOT RT monochrome CCD camera (Diagnostic Instruments) and processed by IPLab (BD Biosciences) and Adobe Photoshop CS2. Measurements of filament length were performed with IPLab.

**Determination of Critical Concentration of Actin**—Varying concentrations of pyrene-labeled G-actin (20% labeled) were polymerized overnight at room temperature in the presence of constant concentrations (50 nM) of GSNL-1 variants in F-buffer containing various concentrations of free Ca\(^{2+}\) as calculated by CHELATER (23). Fluorescence intensity of pyrene (excitation at 366 nm and emission at 384 nm) at the steady state was measured with a PerkinElmer Life Sciences LS50B fluorescence spectrophotometer.

**F-actin Sedimentation Assays**—F-actin sedimentation assays were performed as described (24) with some modifications. Varying concentrations of GSNL-1 variants were added to 5 μM F-actin in F-buffer plus 0.2 mM dithiothreitol. After incubation for 1 h at room temperature, the mixtures were ultracentrifuged by a Beckman TLA100 rotor at 436,000 × g for 15 min at 4 °C. Supernatant and pellet fractions were adjusted to the same volumes and subjected to SDS-PAGE and staining with Coomassie Brilliant Blue R-250 (National Diagnostics). Gels were scanned by an Epson Perfection V700 photo scanner at 300 dpi, and band intensity was quantified using ImageJ.

**Chymotryptic Digestion**—Wild-type or mutant GSNL-1 (0.1 mg/ml) was incubated with 0.002 mg/ml chymotrypsin in F-buffer in the presence of 0.1 mM CaCl\(_2\) or 0.2 mM EGTA at room temperature. Reactions were stopped by mixing with equal volumes of SDS-sample buffer (2% SDS, 80 mM Tris-HCl, 5% β-mercaptoethanol, 15% glycerol, 0.05% bromphenol blue, pH 6.8) and heating at 98 °C for 3 min. Digestion patterns were analyzed by SDS-PAGE. Amounts of intact proteins at time 0 was set as 100%, and remaining intact proteins at each time point were quantified by densitometry. To determine the N-terminal sequence of a 30-kDa chymotryptic fragment, GSNL-1(D83A/D84A) was digested with chymotrypsin in the...
absence of CaCl₂ for 20 min, applied to SDS-PAGE, and blotted onto a polyvinylidene difluoride membrane (Millipore). The polypeptide bands were visualized by staining with Coomassie Brilliant Blue R-250. The 30-kDa band was excised and subjected to Edman degradation-based N-terminal peptide sequencing using an automated protein sequencer (Procise 491-HT, Applied Biosystems, Foster City, CA).

Non-denaturing Polyacrylamide Gel Electrophoresis—Non-denaturing polyacrylamide gel electrophoresis to examine PIP2 binding was performed as described (25, 26). GSNL-1 variants were incubated with PIP2 in G-buffer (0.2 mM CaCl₂, 0.2 mM ATP, 0.2 mM DTT, 2 mM Tris-HCl (pH 8.0)) for 30 min at room temperature. The samples were supplemented with 0.25 volume of a loading buffer (50% glycerol, 0.05% bromphenol blue) and electrophoresed using a Bicine/triethanolamine buffer system at 4 °C. The proteins were visualized by staining with Coo massie Brilliant Blue R-250 (National Diagnostics).

RESULTS

Site-directed Mutagenesis of GSNL-1 at Asp-83 and Asp-84—Comparison of sequences of the actin-binding helix in the first G-domains (G1s) of human CapG, human gelsolin, and C. elegans GSNL-1 indicated that C. elegans GSNL-1 has a very similar sequence to gelsolin (Fig. 1A). In particular, two aspartic acids at positions 85 and 86 of human gelsolin are conserved in GSNL-1 at positions 83 and 84 but not in CapG (Fig. 1A). Indeed, this region is one of the elements in the gelsolin sequence to confer actin-severing activity to CapG in sequence-swapping experiments and is designated as a “severing loop” (19, 20). This comparison suggests that these acidic residues play an important role in the actin filament-severing activity of gelsolin and GSNL-1. To investigate the roles of these residues, we generated mutant forms of GSNL-1 by converting both Asp-83 and Asp-84 into alanines (GSNL-1(D83A/D84A)) and converting each aspartic acid residue into alanine (GSNL-1(D83A) and GSNL-1(D84A)) (Fig. 1, A and B). We also introduced the D83A/D84A mutation into the G1b (residues 1–131 of GSNL-1 containing G1 and the G1-G2 linker) fragment, which is the smallest fragment that exhibits strong calcium-dependent actin filament-severing activity (15). These GSNL-1 variants were bacterially expressed and purified as described under “Experimental Procedures,” and their activities were analyzed in vitro.

Calcium-sensitive Actin Filament-severing Activity of GSNL-1 Is Enhanced by Mutations at Asp-83 and Asp-84—We first tested the calcium-dependent actin filament-severing activity of GSNL-1 variants by direct observation of fluorescently labeled actin filaments (Fig. 2) and quantitative measurements of filament length (Fig. 2, H–K). Unexpectedly, GSNL-1(D83A/D84A) exhibited stronger Ca²⁺-dependent actin-severing activity than wild-type GSNL-1 (Fig. 2). DyLight 549-labeled actin filaments were incubated with 20 nM wild-type GSNL-1 or GSNL-1(D83A/D84A) for 5 min, and the actin filaments were observed by fluorescence microscopy. Actin alone (control) did not show a significant difference in the absence or presence of Ca²⁺ (Fig. 2A). In the presence of 5 mM EGTA or at pCa 6, wild-type GSNL-1 did not sever filaments (Fig. 2, B, C, H, and I), whereas GSNL-1(D83A/D84A), GSNL-1(D83A), and GSNL-1(D84A) slightly shortened filaments (see Fig. 2, H and I, for quantitative results), suggesting that these mutants had weak Ca²⁺-independent actin-severing activity. At pCa 5 and pCa 4, wild-type GSNL-1 severed filaments (Fig. 2, B, I, and K), and GSNL-1(D83A/D84A) showed stronger severing activity (Fig. 2, C, J, and K). However, single mutants, GSNL-1(D83A) and GSNL-1(D84A), showed slightly weaker severing activity at pCa 5 than wild type (Fig. 2J) and similar severing activity to wild-type GSNL-1 at pCa 4 (Fig. 2K). These results indicate that Asp-83 and Asp-84 are not required for actin filament-severing activity of GSNL-1. Rather, the D83A/D84A mutation enhanced Ca²⁺ sensitivity for actin filament severing. Single mutations, D83A and D84A, had minor effects on Ca²⁺ sensitivity, suggesting that Asp-83 and Asp-84 cooperatively regulate Ca²⁺ sensitivity of GSNL-1.

By contrast, in the G1b fragment containing only G1 and the G1-G2 linker, both the wild type and the D83A/D84A mutant were weakly activated at pCa 6 and pCa 5 (Fig. 2, F, G, I, and J) and more strongly at pCa 4 (Fig. 2, F, G, and K). The activities were indistinguishable between wild-type G1b and G1b(D83A/
Calcium Regulation of GSNL-1

A-G: Images showing the effect of EGTA, pCa6, pCa5, and pCa4 on the control and GSNL-1 (DD/AA), GSNL-1 (D83A), GSNL-1 (D84A), G1b, and G1b (DD/AA) treatments.

H-K: Bar graphs illustrating the length (μm) of the control, GSNL-1, GSNL-1 (DD/AA), GSNL-1 (D83A), GSNL-1 (D84A), G1b, and G1b (DD/AA) treatments under the influence of EGTA, pCa6, pCa5, and pCa4, respectively. Statistical significance is indicated by asterisks.
D84A) at pCa 6 and pCa 5 (Fig. 2, F, G, I, and J), whereas G1b(D83A/D84A) showed slightly weaker activity than wild-type G1b at pCa 4 (Fig. 2K). These results suggest that Asp-83 and Asp-84 are not involved in the activation process of the G1b fragment, suggesting that other parts of the molecule are involved in the Asp-83/Asp-84-dependent activation of GSNL-1.

**Mutations in Asp-83 and Asp-84 Alter Susceptibility of GSNL-1 to Chymotryptic Digestion**—To determine whether a conformational change of GSNL-1 is associated with the mutations, we determined susceptibility of wild-type and mutant GSNL-1 to proteolysis by chymotrypsin (Fig. 3 and supplemental Fig. 1). Wild-type GSNL-1 was digested by chymotrypsin more rapidly in the presence of Ca$^{2+}$ (Fig. 3, black circles) than in the absence of Ca$^{2+}$ (Fig. 3, white circles), suggesting that Ca$^{2+}$ induced a conformational change of GSNL-1 to a loose or open conformation. GSNL-1(D83A/D84A) was much more susceptible to chymotrypsin regardless of the presence of Ca$^{2+}$ (Fig. 3, black squares) or absence of Ca$^{2+}$ (Fig. 3, white squares). Because GSNL-1(D83A/D84A) is inactive in actin severing in the absence of Ca$^{2+}$ (Fig. 2H), these results indicate that the D83A/D84A mutation induces a Ca$^{2+}$-independent open conformation of GSNL-1 while maintaining Ca$^{2+}$-dependent actin-binding sites. These results are consistent with the studies on gelsolin in which nanomolar concentrations of Ca$^{2+}$ initiate unlatching of gelsolin while keeping its actin-binding sites inaccessible (10). It should also be noted that GSNL-1(D83A/D84A) was digested much more rapidly than Ca$^{2+}$-bound wild-type GSNL-1. This suggests that Ca$^{2+}$ stabilizes an open conformation of GSNL-1, whereas the D83A/D84A mutation disrupts this mechanism.

Specific roles of Asp-83 and Asp-84 in conformational stability of GSNL-1 were examined in single mutants. Interestingly, GSNL-1(D83A) was more susceptible to chymotrypsin in the absence of Ca$^{2+}$ (Fig. 3, white triangles) than in the presence of Ca$^{2+}$ (Fig. 3, black triangles). In the absence of Ca$^{2+}$, GSNL-1(D83A) was digested rapidly (Fig. 3, white triangles) in a similar manner to GSNL-1(D83A/D84A), whereas in the presence of Ca$^{2+}$, GSNL-1(D83A) was as stable as wild-type GSNL-1 (Fig. 3, black triangles). Therefore, the Asp-83 mutant could not maintain a Ca$^{2+}$-free closed conformation but was able to maintain a Ca$^{2+}$-stabilized conformation. By contrast, Ca$^{2+}$-free GSNL-1(D84A) had similar stability to Ca$^{2+}$-free wild-type GSNL-1 (Fig. 3, white diamonds), whereas Ca$^{2+}$-bound GSNL-1(D84A) was as unstable as GSNL-1(D83A/D84A) (Fig. 3, black diamonds). Therefore, the Asp-84 mutant had a Ca$^{2+}$-free closed conformation but could not retain a Ca$^{2+}$-stabilized conformation. These results indicate that both Asp-83 and Asp-84 are required to regulate Ca$^{2+}$-dependent conformational changes of GSNL-1 and that Asp-83 and Asp-84 have specific roles in maintaining Ca$^{2+}$-free and Ca$^{2+}$-bound conformations, respectively.

Chymotryptic digestion of both wild-type and mutant GSNL-1 yielded a relatively stable 30-kDa fragment (supplemental Fig. 1, arrowheads). The N-terminal sequence of this fragment was determined as GSGMTSDL, which corresponded to residues 1–7 (MGMTSDL) of GSNL-1 plus G5 at the N terminus as a remnant after cleavage of GST by thrombin. Based on the size of this fragment, the C-terminal end is likely to be Trp-244 (yielding a 28-kDa fragment) or Trp-254 (yielding a 29-kDa fragment), which are located in the G2-G3 linker. This result suggests that the G1-G2 portion of GSNL-1 is relatively stable, and a conformational change occurs at or near the G2-G3 linker.

**Mutations in Asp-83 and Asp-84 Alter Calcium Sensitivity of GSNL-1 for Barbed End Capping**—Next, we tested the effect of the mutations on the Ca$^{2+}$-dependent barbed end-capping activity of GSNL-1. We determined the effects of wild-type or mutant GSNL-1 on the critical concentration ($C_c$) of actin at various free Ca$^{2+}$ concentrations (Fig. 4). The $C_c$ of uncapped actin is 0.1–0.2 μM, and the amounts of polymerized actin will be linearly correlated with total actin concentrations above the $C_c$. However, because the $C_c$ at the barbed end (0.1 μM) is much lower than that at the pointed end (0.5 μM), the $C_c$ value of barbed end-capped actin will be near the $C_c$ value at the pointed end (0.5 μM), and polymerized actin will be increased above this higher $C_c$. In the absence of free Ca$^{2+}$ (0.2 mM EGTA, Fig. 4A), wild-type and mutant GSNL-1 did not alter the $C_c$. GSNL-1(D83A/D84A) at pCa 6 and pCa 5 (Fig. 2, F, G, I, and J), whereas G1b(D83A/D84A) showed slightly weaker activity than wild-type G1b at pCa 4 (Fig. 2K). These results suggest that Asp-83 and Asp-84 are not involved in the activation process of the G1b fragment, suggesting that other parts of the molecule are involved in the Asp-83/Asp-84-dependent activation of GSNL-1.
Calcium Regulation of GSNL-1

FIGURE 4. Effects of GSNL-1 variants on the critical concentration of actin at various free Ca\(^{2+}\) concentrations. Varying concentrations (0.1–1.0 \(\mu\)M) of actin (20% pyrene-labeled) were polymerized without any additional protein (black squares) or with GSNL-1 (black circles), GSNL-1(D83A/D84A) (GSNL-1(DD/AA)) (white circles), GSNL-1(D83A) (black diamonds), or GSNL-1(D84A) (white diamonds) in the presence of 0.2 mM EGTA (A) or various concentrations of free Ca\(^{2+}\) (B–G). After overnight incubation, the pyrene fluorescence (arbitrary units (AU)) was measured and plotted as a function of the total actin concentrations.

1(D83A/D84A) and GSNL-1(D84A) slightly lowered the pyrene fluorescence (Fig. 4A, white circles and white diamonds). This may be due to weak interaction between GSNL-1(D83A/D84A) or GSNL-1 (D84A) with actin in the absence of free Ca\(^{2+}\), but the mechanism of this effect is currently unclear. By testing various free Ca\(^{2+}\) concentrations, GSNL-1(D83A/D84A) was capable of shifting the \(C_a\) at pCa < 6 (Fig. 4, D–G, white circles), whereas the capping activity of wild-type GSNL-1 was partially activated at pCa 5 (Fig. 4E, black circles) and fully activated at pCa < 4 (Fig. 4, F and G, black circles). However, single mutations, D83A and D84A, caused different effects on Ca\(^{2+}\) sensitivity. GSNL-1(D83A) was partially activated at pCa 5 (Fig. 4E, black diamonds) and fully activated at pCa 4 (Fig. 4F, black diamonds) in a similar manner to wild type. By contrast, GSNL-1(D84A) required pCa 4 for partial activation (Fig. 4F, white diamonds) and pCa 3 for full activation (Fig. 4G, white diamonds). Thus, although the D83A/D84A double mutation enhances Ca\(^{2+}\) sensitivity of GSNL-1, single mutations had no or negative effects on Ca\(^{2+}\) sensitivity. These results suggest that Asp-83 and Asp-84 play distinct roles in regulating Ca\(^{2+}\) sensitivity of GSNL-1 for its barbed end-capping activity.

D83A/D84A Mutation Alters Phosphoinositide Sensitivity of GSNL-1—We previously demonstrated that actin-severing activity of GSNL-1 can be negatively regulated by PIP2 and that PIP2 binds to G1-G2 (15). We tested whether the D83A/D84A mutation influences PIP2 sensitivity of GSNL-1. In the full-length GSNL-1 protein, GSNL-1(D83A/D84A) was less sensitive to PIP2 than wild-type GSNL-1 (Fig. 5, A and B). Actin filament severing was examined by sedimentation assays using ultracentrifugation. Wild-type GSNL-1 severed actin filaments and decreased pelletable actin in the presence of Ca\(^{2+}\) (Fig. 5A, black circles) but not in the presence of EGTA (Fig. 5A, black triangles). PIP2 (200 \(\mu\)M) nearly completely inhibited this activity (Fig. 5A, white circles). GSNL-1(D83A/D84A) also exhibited similar Ca\(^{2+}\)-dependent actin-severing activity (Fig. 5B, black circles). PIP2 strongly inhibited this activity in the presence of up to 2 \(\mu\)M GSNL-1(D83A/D84A) but only partially inhibited this activity when GSNL-1(D83A/D84A) was present at a high concentration (5 \(\mu\)M) (Fig. 5B, white circles). Interestingly, in the G1b fragment, the D83A/D84A mutation had an opposite effect and enhanced PIP2 sensitivity (Fig. 5, C and D). Wild-type G1b was nearly completely inhibited at low concentrations (up to 2 \(\mu\)M) but only partially inhibited by PIP2 when G1b was present at a high concentration (5 \(\mu\)M) (Fig. 5C, white circles), whereas G1b(D83A/D84A) was nearly completely inhibited by PIP2 (Fig. 5D, white circles).

Examination of direct binding of GSNL-1 variants with PIP2 by non-denaturing gel electrophoresis indicated that the D83A/D84A mutation enhanced its binding in both full-length and G1b forms (Fig. 6). In the presence of increasing concentrations of PIP2, the band of full-length wild-type GSNL-1 was shifted to an upper band in the presence of Ca\(^{2+}\) (Fig. 6A) or a lower band in the presence of EGTA (Fig. 6E), as reported previously (15). Ca\(^{2+}\) slightly enhanced their binding as the amount of unbound GSNL-1 was less in the presence of Ca\(^{2+}\) at 50 \(\mu\)M PIP2 than in the absence of Ca\(^{2+}\). The band of GSNL-1(D83A/D84A) was shifted to an upper band in the presence of Ca\(^{2+}\) when PIP2 was at 10–50 \(\mu\)M (Fig. 6B). However, at 100 \(\mu\)M PIP2, the band was shifted to a lower band (Fig. 6B). In the absence of Ca\(^{2+}\), the band of GSNL-1(D83A/D84A) was shifted to lower bands (Fig. 6F), and the shift occurred at lower PIP2 concentrations than that of wild-type GSNL-1 (compare Fig. 6, A, B, and D). The band of GSNL-1(D84A) was nearly completely inhibited by PIP2 (Fig. 6D, white circles). The data suggest that the D83A/D84A mutation enhances PIP2 sensitivity of GSNL-1, which may contribute to the increased Ca\(^{2+}\) sensitivity of the mutant protein.
E and F), suggesting that GSNL-1(D83A/D84A) binds to PIP2 with a high affinity in the absence of Ca\(^{2+}\). Wild-type G1b did not show a shift in the presence of Ca\(^{2+}\) (Fig. 6C), whereas it was shifted to upper smear bands in the presence of EGTA at 100 \(\mu M\) PIP2 (Fig. 6G). G1b(D83A/D84A) also showed a band shift only in the absence of Ca\(^{2+}\) (Fig. 6, D and H), and the shift of G1b(D83A/D84A) occurred at lower PIP2 concentrations than that of wild-type G1b (compare Fig. 6, G and H). Thus, the D83A/D84A mutation enhanced the PIP2-GSNL-1 binding in both full-length and G1b forms, which indicates that weaker PIP2 sensitivity of full-length GSNL-1(D83A/D84A) is not simply due to an alteration in its affinity with PIP2.

**DISCUSSION**

In this study, we demonstrated that Asp-83 and Asp-84 of *C. elegans* GSNL-1 are dispensable for actin filament-severing activity but important for Ca\(^{2+}\)-dependent activation and conformational changes of GSNL-1. Full-length GSNL-1 with a D83A/D84A mutation was activated at lower Ca\(^{2+}\) concentrations than wild-type GSNL-1, whereas single mutations at Asp-83 and Asp-84 caused minor effects on activity. Mutations at Asp-83 and Asp-84 caused remarkable changes in the conformation as determined by susceptibility to chymotrypsin digestion, suggesting specific roles for Asp-83 and Asp-84 in maintaining Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound conformations, respectively. Because these aspartic acid residues are conserved in vertebrate gelsolin, we expect that they have similar regulatory functions for Ca\(^{2+}\)-dependent properties of vertebrate gelsolin.

Although the structure of *C. elegans* GSNL-1 has not been solved, the crystal structure of a Ca\(^{2+}\)-free inactive form of equine gelsolin (PDB accession number 1D0N) indicates that the equivalent aspartic acid residues (Asp-85 and Asp-86) at the C-terminal end of the actin-binding helix of G1 are located near G3 (5) (Fig. 7). In particular, Asp-86 (equivalent to Asp-84 in GSNL-1) forms a salt bridge with Lys-305, which is located at the N-terminal end of a long helix in G3 (Fig. 7), suggesting that they function as a latch between G1 and G3 to stabilize their binding in its inactive conformation. The crystal structure of a G-actin-bound form of the gelsolin G1G2G3 fragment shows that G1 is separated from G3 in its active form (9), suggesting that dissociation of G1 from G3 is an important step during Ca\(^{2+}\) activation of gelsolin. Sequence alignment shows that Lys-309 of *C. elegans* GSNL-1 is equivalent to Lys-305 of vertebrate gelsolin and may bind to Asp-84 (equivalent to Asp-86 of gelsolin). We attempted to test whether an alanine mutation of Lys-309 causes a similar effect on Ca\(^{2+}\) sensitivity of GSNL-1 to...
the D83A/D84A mutation. However, the mutant protein was insoluble when it was expressed in *E. coli*, and we were not able to characterize the effect of this mutation. The D84A single mutation did not disrupt the Ca\(^{2+}\)/H\(_{11001}\)-free inactive conformation (Fig. 3), suggesting that other residues also contribute to the G1-G3 latch. By contrast, Asp-85 (equivalent to Asp-83 in GSNL-1) is not exposed on the surface in the closed conformation (Fig. 7) and may be involved in keeping the internal integrity of the protein. This hypothesis is supported by our data that the D83A single mutation in GSNL-1 specifically disrupted a Ca\(^{2+}\)/H\(_{11001}\)-free conformation. Intriguingly, CapG does not have charged amino acids at equivalent positions in either G1 or G3, suggesting that CapG uses a different mechanism for Ca\(^{2+}\) activation. To uncover the latch mechanism between G1 and G3, we would need an atomic structure of GSNL-1 and/or similar mutagenesis studies on these residues in vertebrate gelsolin. The crystal structure of gelsolin G1-actin complex shows that Asp-85 (reported as Asp-109 in Ref. 27) (equivalent to Gly-83 in GSNL-1) is a part of an intermolecular Ca\(^{2+}\)-binding site between actin and gelsolin G1 (27). Biochemical activities of G1 of GSNL-1 are very similar to those of gelsolin G1 (15, 28). Therefore, our results that D83A/D84A and D83A mutations did not impair actin-severing activity of GSNL-1 suggest that the intermolecular Ca\(^{2+}\) binding is not required for actin filament severing. However, the D83A/D84A mutation in the G1b fragment of GSNL-1 slightly reduced actin-severing activity (Fig. 2). Thus, the intermolecular Ca\(^{2+}\) may stabilize binding of G1b to actin, but this may not be required in the full-length GSNL-1, which has other G domains to augment the severing activity.

The D83A/D84A mutation also affected PIP2 sensitivity of GSNL-1. However, the mutation had a different effect on PIP2 sensitivity and PIP2 binding. Full-length GSNL-1 became less sensitive to PIP2 for inhibition of actin severing by the mutation, whereas G1b became more sensitive by the mutation. In contrast, PIP2 binding was enhanced by the mutation in both full-length and G1b forms. Our interpretation is that the D83A/D84A mutation altered the surface charge and the protein conformation, and these two alterations differently affected PIP2

\[^3\text{Z. Liu and S. Ono, unpublished data.}\]
sensitivity and PIP2 binding. The D83A/D84A mutation removes acidic residues that potentially repel PIP2, which is also acidic. Therefore, such a change in the surface charge of GSNL-1 can enhance binding of PIP2 to GSNL-1. PIP2 inhibition of actin-severing activity of the G1b fragment may be due to a competition between actin and PIP2 for binding to G1b. However, PIP2 inhibition of full-length GSNL-1 may require proper conformation of the protein. PIP2 may promote an inactive conformation of GSNL-1, whereas GSNL-1(D83A/D84A) may be unable to form such a conformation due to lack of stable interaction between G1 and G3. To test this hypothesis, additional analysis of the binding between PIP2 and GSNL-1 and studies on the effects of PIP2 on the structure of GSNL-1 will be needed.

In conclusion, we provide biochemical evidence that conserved aspartic acid residues in G1 of GSNL-1 are critical determinants of Ca$^{2+}$-dependent activity and conformations of GSNL-1. The critical function of G1-G3 of GSNL-1 in actin filament-severing activity is similar to that of G1-G3 of gelsolin. Therefore, we expect that equivalent residues in vertebrate gelsolin play similar roles in Ca$^{2+}$ activation as a putative latch between G1 and G3 and conformational determinants. Our results also suggest that G1-G3 of GSNL-1 forms a compact fold in a similar manner to G1-G3 of gelsolin. Currently, we do not know whether the C terminus of GSNL-1 functions as a latch on G2. G4 of GSNL-1 is most closely related to G6 of gelsolin (14). However, the linker between G3 and G4 is much shorter than the equivalent linker in gelsolin. Therefore, it is not clear whether G4 and the C-terminal tail of GSNL-1 have enough size and flexibility to extend to G2. Further structural and biochemical analysis of GSNL-1 should provide interesting information on unique aspects of GSNL-1 as well as conserved properties of the gelsolin family.

REFERENCES

1. Ono, S. (2007) *Int. Rev. Cytol.* 258, 1–82.
2. Sun, H. Q., Yamamoto, M., Mejillano, M., and Yin, H. L. (1999) *J. Biol. Chem.* 274, 33179–33182.
3. Silacci, P., Mazzolai, L., Gauci, C., Stergiopoulos, N., Yin, H. L., and Hayoz, D. (2004) *Cell. Mol. Life Sci.* 61, 2614–2623.
4. McGough, A. M., Staiger, C. J., Min, J. K., and Simonetti, K. D. (2003) *FEBS Lett.* 552, 75–81.
5. Burtnick, L. D., Koepf, E. K., Grimes, J., Jones, E. Y., Stuart, D. L., McLoughlin, P. J., and Robinson, R. C. (1997) *Cell* 90, 661–670.
6. Ashish, Paine, M. S., Perryman, P. B., Yang, L., Yin, H. L., and Krueger, J. K. (2007) *J. Biol. Chem.* 282, 25884–25892.
7. Choe, H., Burtnick, L. D., Mejillano, M., Yin, H. L., Robinson, R. C., and Choe, S. (2002) *J. Mol. Biol.* 324, 691–702.
8. Robinson, R. C., Mejillano, M., Le, V. P., Burtnick, L. D., Yin, H. L., and Choe, S. (1999) *Science* 286, 1939–1942.
9. Burtnick, L. D., Urosev, D., Irobi, E., Narayan, K., and Robinson, R. C. (2004) *EMBO J.* 23, 2713–2722.
10. Pope, B. I., Gooch, J. T., and Weeds, A. G. (1997) *Biochemistry* 36, 15848–15855.
11. Lueck, A., Yin, H. L., Kwiatkowski, D. J., and Allen, P. G. (2000) *Biochemistry* 39, 5274–5279.
12. Lin, K. M., Mejillano, M., and Yin, H. L. (2000) *J. Biol. Chem.* 275, 27746–27752.
13. Nag, S., Ma, Q., Wang, H., Chummarnsilpa, S., Lee, W. L., Larsson, M., Kankan, B., Hernandez-Valladares, M., Burtnick, L. D., and Robinson, R. C. (2009) *Proc. Natl. Acad. Sci. U.S.A.* 106, 13713–13718.
14. Klaavuniemi, T., Yamashiro, S., and Ono, S. (2008) *J. Biol. Chem.* 283, 26071–26080.
15. Liu, Z., Klaavuniemi, T., and Ono, S. (2010) *Biochemistry* 49, 4349–4360.
16. Sun, H. Q., Wooten, D. C., Janmey, P. A., and Yin, H. L. (1994) *J. Biol. Chem.* 269, 9473–9479.
17. Bryan, J., and Hwo, S. (1986) *J. Cell Biol.* 102, 1439–1446.
18. Chaponnier, C., Janmey, P. A., and Yin, H. L. (1986) *J. Cell Biol.* 103, 1473–1481.
19. Southwick, F. S. (1995) *J. Biol. Chem.* 270, 45–48.
20. 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.
21. Pardee, J. D., and Spudich, J. A. (1982) *Methods Enzymol.* 85, 164–181.
22. Koyama, T., and Mihashi, K. (1981) *J. Cell Biol.* 270, 164–181.
23. Schoenmakers, T. J., Visser, G. J., Flik, G., and Theuvenet, A. P. (1992) *Eur. J. Biochem.* 12, 870–874, 876–879.
24. Mohri, K., Vorobiev, S., Fedorov, A. A., Almo, S. C., and Ono, S. (2004) *J. Biol. Chem.* 279, 31697–31707.
25. Safer, D. (1989) *Anal. Biochem.* 178, 32–37.
26. Ono, S., McGough, A., Pope, B. I., Tolbert, V. T., Bui, A., Pohl, J., Benian, G. M., Gernert, K. M., and Weeds, A. G. (2001) *J. Biol. Chem.* 276, 5992–5998.
27. McLoughlin, P. J., Gooch, J. T., Mannherz, H. G., and Weeds, A. G. (1993) *Nature* 364, 685–692.
28. Kwiatkowski, D. J., Janmey, P. A., and Yin, H. L. (1989) *J. Cell Biol.* 108, 1717–1726.