Identification of a Functional Switch for Actin Severing by Cytoskeletal Proteins

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Actin severing is vital for the organization of the actin cytoskeleton during cell motility. Severing of F-actin by the homologous proteins villin and gelsolin requires unphysiologically high calcium concentrations (20–200 μM). Here we demonstrate that high calcium releases an autoinhibited conformation in villin that is maintained by two low affinity calcium binding sites (aspartic acids 467 and 715) that interact with a cluster of basic residues in the S2 domain of villin. Mutation of either of these sites as well as tyrosine phosphorylation alters the conformation of villin resulting in a protein that can sever actin in nanomolar calcium. These results suggest that tyrosine phosphorylation rather than high calcium may be the mechanism by which villin and other related proteins sever actin in vivo.

EXPERIMENTAL PROCEDURES

Materials

Escherichia coli competent cells BL21 and TKX1 and QuikChange site-directed mutagenesis kit were from Stratagene; prokaryotic expression vector pGEX-2T was from Amersham Biosciences; glutathione-Sepharose 4B Fastflow was from Amersham Biosciences; GelCode Blue was from Pierce; Staphylococcus aureus V8 protease was obtained from Sigma; monoclonal antibodies to villin, and phosphotyrosine (clone PY-20), were from Transduction Laboratories; muscle actin polymerization kit and the actin binding kit were from Cytoskeleton; all other chemicals were from Sigma or Invitrogen.

Methods

Calcium Binding Site Mutants—Substitution mutants of villin were generated by designing complimentary primers to introduce a leucine for aspartic acid at Asp(467) and Asp(715) in full-length human villin cDNA using QuikChange site-directed mutagenesis kit as recommended by the manufacturer. The introduction of the desired codons was verified by sequencing.

Expression and Purification of Recombinant Villin Proteins—Full-length or mutant villin cDNA (human) cloned in pGEX-2T was expressed in the E. coli BL21 cells as described earlier (1). Glutathione S-transferase-tagged recombinant proteins were obtained by induction of the villin gene using isopropyl-β-D-thiogalactopyranoside. Tyrosine-phosphorylated villin protein was obtained as described before.

Circular Dichroism (CD) Measurements—CD spectra were collected from 191 to 260 nm with data collection for 5-s averaging in 1-nm increments with three repeats at 25 °C essentially as described before (2). An AVIV model 62S spectropolarimeter equipped with electronic temperature control and a 1-cm path length quartz cuvette was used for CD studies. Mean molar ellipticity per residue was calculated by (θ/10)/[(c×n×l)/1000], units are degrees cm²/demolc. θ is ellipticity in millidegrees; c is concentration in moles/liters; n is number of amino acid residues per protein; and l is path length in centimeters. The K2d program was used for estimation of secondary structure from CD data. K2d provides estimated structure and the error estimate (kaleal.ugr.es/k2d.htm).

Quenching of Intrinsic Tryptophan Fluorescence—Tryptophan fluorescence spectra of wild-type, mutant, and tyrosine-phosphorylated villin proteins were recorded using a FluoroMax3 spectrophotometer as described before (2). Ca2⁺ (final concentration between 0 and 1.0 mM) was added in 0.5-μl increments, and the fluorescence spectra were recorded 5 min after each addition. The emission scan was recorded with an excitation at 290 nm.

Proteolysis of VIL/WT, VIL/D467L, and VIL/D715L—Villin proteins were digested for 90 min with S. aureus V8 protease (1:100, w/w) as described before (3). The proteolytic fragments were separated by 12% SDS-PAGE and stained with GelCode Blue.

Measurement of Actin Depolymerization Kinetics—The ability of villin to sever actin filaments was determined by its effect on the rate and extent of decrease in fluorescence of pyrene-labeled actin as described before (4).

RESULTS AND DISCUSSION

Villin, gelsolin, fragmin, and adseverin are homologous, calcium (Ca2⁺)-regulated, actin-severing proteins that are involved in actin remodeling and regulation of cell motility. Gene knock-out experiments have implicated the actin severing properties of villin and gelsolin in their motogenic effect (5, 6). However, all these proteins require very high calcium concentrations (7–13). Activation of gelsolin by Ca2⁺ may be modulated by other factors as well (7, 9, 14). Low pH has been reported to override the Ca2⁺ requirements of gelsolin (7); however, the concentration of H⁺ (pH < 6.0) may also never be reached inside the cell. Thus understanding the regulation of actin severing by villin and its related proteins is important. Villin and gelsolin contain segments that display internal homology with each other. In villin, gelsolin and adseverin/scrin there are six such segmental repeats (S1–S6) with S1 most closely related to S4, S2–S5, and S3–S6. The two halves of the protein, the amino-terminal (S1–S3) and carboxyl-terminal (S4–S6) are joined by a long linker region. Interaction of actin with villin is strongly Ca2⁺-dependent (15). Changes in the conformation of villin protein following its association with calcium have led to a hypothetical mechanism referred to as the “hinge mechanism” (16). According to this model, villin bound to Ca2⁺ adopts a local secondary structure that allows another region of the villin protein to rotate out such that it is now exposed to the solution interface (16). In gelsolin, the high calcium concentrations requirements have been attributed to an analogous model referred to as the “tail helix unlaying” that is required to expose the actin side binding and severing domain (S1–S2 and S4) in this protein (10). However, no biochemical or functional studies are available identifying these latch/ hinge or low affinity Ca2⁺ binding sites in either of these proteins.
The Ca\(^{2+}\) concentrations required for actin severing by villin are in the micromolar range, which suggests that functional regulation of villin cannot be modulated by calcium, since intracellular Ca\(^{2+}\) levels may never reach 200 \(\mu\)M (17). We have suggested previously that tyrosine phosphorylation of villin could modulate its ability to regulate the cortical cytoskeleton in submicromolar concentrations of Ca\(^{2+}\) (1). To resolve this paradox, we sought first to identify low affinity Ca\(^{2+}\) binding residues that could allow villin to adopt a head to tail latched/hinge mechanism and secondly to determine whether such a mechanism could be released by tyrosine phosphorylation as opposed to high Ca\(^{2+}\).

Sequence alignment of human villin and gelsolin as well as the crystal structure of gelsolin (18, 19) allowed us to identify three well conserved aspartic acid (Asp) residues (Asp\(^{86}\), Asp\(^{467}\), and Asp\(^{715}\)) that could constitute the low affinity Ca\(^{2+}\) binding sites in villin and function as latch residues. Based on the crystal structure of gelsolin, these three residues could form salt bridges with arginine and lysine residues in the S1/S2 domain of villin (as predicted for gelsolin) thus forming a latch between the S1–S3 or S2–S6 or the linker region between S3–S4 and S2 domain of villin, thus inhibiting access to the actin binding sites in the villin core. Binding of Ca\(^{2+}\) to these low affinity binding sites could release the autoinhibited conformation thus regulating actin severing. On the basis of this, mutants of villin lacking these putative latch residues were first assayed for their ability to bind Ca\(^{2+}\) (17). Using site-directed mutagenesis, each of these aspartic acid residues was mutated to a leucine and expressed as glutathione S-transferase fusion protein (20). Ca\(^{2+}\) binding was determined by recording quenching of the intrinsic tryptophan fluorescence of wild-type and mutant villin proteins. Villin has an emission maximum of 337 nm, and Ca\(^{2+}\) induces a dose-dependent (0 to 1.0 mM) and saturable quenching of the intrinsic tryptophan fluorescence of villin without shifting its emission maxima (Fig. 1A). In contrast, the villin latch mutants VIL/D467L and VIL/D715L show no Ca\(^{2+}\)-dependent quenching of tryptophan fluorescence even at the highest concentration of Ca\(^{2+}\) used (Fig. 1, A and C). VIL/D86L behaved like VIL/WT (supplemental Fig. S1). These data suggest that Asp\(^{467}\) and Asp\(^{715}\) are low affinity Ca\(^{2+}\) binding residues that could function as latch residues. To determine the effect of tyrosine phosphorylation on Ca\(^{2+}\) sensitivity of villin, Ca\(^{2+}\)-dependent change in tryptophan fluorescence was monitored for full-length tyrosine-phosphorylated human villin expressed as a glutathione S-transferase fusion protein (VILT/WT) (1). Tyrosine-phosphorylated villin does not show a Ca\(^{2+}\)-dependent change in tryptophan fluorescence (Fig. 1D). Thus tyrosine phosphorylation of villin may result in a change in the conformation of villin protein such that it is insensitive to Ca\(^{2+}\)-induced changes in its structure.

To determine whether the latch mutants are functional and to determine the Ca\(^{2+}\) requirements of these mutants as well as of tyrosine-phosphorylated villin to sever actin, we assessed their ability to sever actin in vitro by pyrene-actin depolymerization assays (4). VIL/WT severs actin efficiently at 200 \(\mu\)M (Fig. 2A) consistent with previous reports (11). Interestingly, tyrosine phosphorylation of villin allows it to sever actin very effectively at nanomolar Ca\(^{2+}\) levels (Fig. 2A). Tyrosine phosphorylation abolishes the high Ca\(^{2+}\) requirements for actin severing by villin and reduces the Ca\(^{2+}\) dependence by more than 4000-fold. In contrast, VIL/D467L and VIL/D715L mutants are constitutively active at physiological Ca\(^{2+}\), concentrations and can sever actin just as effectively at much lower Ca\(^{2+}\) concentrations of 50 \(\mu\)M (Fig. 2B). None of the villin proteins,

**FIG. 1.** Calcium binding by wild-type, mutant, and tyrosine-phosphorylated villin proteins. A, tryptophan fluorescence emission spectra of villin. 0.3 \(\mu\)M of CaCl\(_2\) (0–1 mM) solution was added sequentially to full-length human villin (VIL/WT, 0.5 \(\mu\)M) and the fluorescence recorded using a FluoroMax3 spectrofluorometer. Villin has an emission maximum at 337 nm, and calcium induces a dose-dependent quenching of the intrinsic tryptophan fluorescence of villin. B, calcium binding to the villin mutant VIL/D467L (B), VIL/D715L (C), and full-length tyrosine-phosphorylated villin (VILT/WT, D).

**FIG. 2.** VIL/D467L, VIL/D715L, and VILT/WT sever actin in nanomolar calcium. A, actin severing by full-length tyrosine-phosphorylated and non-phosphorylated villin at different calcium concentrations (0–200 \(\mu\)M). B, actin severing by wild-type villin (VIL/WT) was compared with the villin mutant proteins VIL/D467L and VIL/D715L at 50 \(\mu\)M Ca\(^{2+}\). Pyrene-\(\alpha\)-actin (1 \(\mu\)M) in the presence of wild-type or mutant villin proteins (60 \(\mu\)M) was diluted to 0.1 \(\mu\)M in actin-polymerizing buffer, and the decrease in fluorescence intensity was followed over time. Control represents the depolymerization of actin in the absence of villin. Fluorescence was recorded every 15 s. Values represent the mean of three independent experiments.
Identification of a Functional Switch for Actin Severing

wild-type or mutant, sever actin in the absence of Ca$^{2+}$, consistent with previous observations that villin requires calcium for its association with actin (21). Therefore, actin severing remains a Ca$^{2+}$-dependent process, suggesting the presence of additional high affinity Ca$^{2+}$ binding sites in villin. The latch mutants and tyrosine phosphorylation had no effect on the actin capping activity of villin (supplemental Fig. S2). Together these data suggest that binding of Ca$^{2+}$ to low affinity Ca$^{2+}$ binding sites, namely Asp$^{467}$ and Asp$^{715}$ as well as tyrosine phosphorylation of villin regulate the Ca$^{2+}$-dependent actin severing activity of villin.

One possibility is that villin adopts an autoinhibited conformation that both high calcium or tyrosine phosphorylation could release allowing villin to sever at physiological Ca$^{2+}$ levels. The very high Ca$^{2+}$ levels required to sever actin may in fact function to maintain the villin protein in an autoinhibited conformation, and tyrosine phosphorylation may be the physiologically relevant agonist for the activation of the severing

![Diagram of villin structure and function](http://www.jbc.org/)

**Fig. 3.** Changes in the conformation of VIL/WT, VILT/WT, VIL/D467L, and VIL/D715L in response to calcium binding. A, the CD spectra of full-length villin protein in the presence of different concentrations of Ca$^{2+}$ (0–1 mM). The inset shows maximum change in conformation of VIL/WT in the presence of 200 μM free Ca$^{2+}$. B, CD spectra of VIL/D467L compared with VIL/WT. The inset shows no change in conformation of VIL/D467L at 1 mM free Ca$^{2+}$. C, CD spectra of VIL/D715L compared with VIL/WT. The inset shows no change in conformation of VIL/D715L in the presence of 1 mM Ca$^{2+}$. D, CD spectra of VIL/T/WT compared with VIL/WT. The inset shows no change in conformation of VIL/T/WT in the presence of 1 mM Ca$^{2+}$. E, model for high calcium and tyrosine phosphorylation mediated changes in the villin structure and function, as reported earlier (diagram 1) at Ca$^{2+}$ concentrations <100 nM villin nucleates actin filaments via the actin monomer binding sites in domain S1 and S4; at Ca$^{2+}$ concentrations <10 nM villin bundles actin filaments (diagram 2); at Ca$^{2+}$ concentrations <5 μM villin caps actin filaments (diagram 3); at Ca$^{2+}$ concentrations >100 μM or in the presence of tyrosine-phosphorylated villin, the protein adopts an open conformation where the S1 and S2 actin binding sites are accessible allowing villin to sever actin (diagram 4). F, sequence alignment of villin and related proteins of the villin superfamily. The top panel shows alignments of villin sequences from human, mouse, and chicken. The bottom panel shows alignment of homologous sequences in related actin severing proteins.
activity of villin. To determine whether high Ca²⁺ or tyrosine phosphorylation could change the conformation of the villin protein, we measured changes in the secondary structure of the villin protein using circular dichroism spectroscopy in the far-ultraviolet region (2). The CD measurements were made with samples containing VIL/WT, VIL/T7 WT, as well as the latch mutants in the absence or presence of different concentrations of calcium (0–1.0 mM). The CD spectra were fitted to the K2d program to quantify the structural changes in the villin protein as described before (2). The circular dichroism spectrum of human villin shows significant dose-dependent changes in the presence of Ca²⁺ (Fig. 3A). Binding with Ca²⁺ results in a significant increase in the α-helical and a decrease in the β-sheet content of the protein (supplemental Table S1). These data agree with a previous observation made by Hesterberg and Weber (16), where the α-helical content of villin protein was increased at 50 μM Ca²⁺. A comparison of VIL/WT structure with the structure of the latch mutants VIL/D467L and VIL/D715L shows an increase in the α-helical and decrease in the β-sheet content of the villin protein, consistent with a change in the secondary structure of the protein (Fig. 3, B and C). Mutation of the latch residues also makes these proteins insensitive to Ca²⁺-induced structural changes (inserts in Fig. 3, B and C). Tyrosine-phosphorylated villin likewise shows an increase in the α-helical content and a decrease in the β-sheet structure (Fig. 3D and supplemental Table S1). Unlike VIL/WT, tyrosine-phosphorylated villin shows no Ca²⁺-dependent change in the secondary structure (Fig. 3D and supplemental Table S1). Thus tyrosine phosphorylation also makes villin insensitive to Ca²⁺-induced structural changes. These data show that both high Ca²⁺ as well as tyrosine phosphorylation induce global changes in the secondary structure of the villin, which regulate the ligand binding properties of villin (Ca²⁺) as well as the actin-modifying functions of villin (actin severing).

Limited proteolysis was also used to compare the conformational changes induced by Ca²⁺ during unbinding of the hinge mechanism. S. aureus V8 cleaves villin to generate large amino-terminal fragments, the villin core (87 kDa) and the villin headpiece of 8.5 kDa. Further proteolysis produces fragments of 51, 44, and 30 kDa (supplemental Fig. S4). V8 maximally cleaves VIL/WT in the presence of calcium but not in the presence of EGTA. In contrast, VIL/W7/WT, VIL/D467L, and VIL/D715L are cleaved at these sites even in the presence of EGTA. These data then support the idea that V8 digests villin in a Ca²⁺-dependent manner, and tyrosine phosphorylation or latch site mutations in villin generate conformational changes that are comparable with those generated by Ca²⁺ in full-length villin.

Villin contains a cluster of arginine and lysine residues in S2 that have been identified as the F-actin side binding and actin severing domain in villin (22). Since we speculate that if Asp⁶⁶⁷ and Asp⁷¹⁵ can form a latch with this domain in villin, then mutation in the S2 domain should similarly release the inhibitory effect of the latch. As shown in supplemental Table S1, deletion of all the positive residues in this domain (amino acids 138–146) results in a villin mutant protein (ΔPβ2) that adopts a different conformation compared with VIL/WT. ΔPβ2 has a higher α-helical content, lower β-sheet structure, and an increase in the random coil content of the protein, consistent with significant structural changes in the villin mutant protein (supplemental Table S1). These data are in agreement with the idea that Asp⁶⁶⁷ and Asp⁷¹⁵ form a latch with the cluster of basic residues in S2 domain of human villin. We have demonstrated previously that mutation of R138A, R145A, and R146A in villin does not alter the conformation of the protein (2). This suggests that perhaps no single residue in this domain but rather the cluster of positive charge in PB2 contributes to the formation of the latch. These data show that both tyrosine phosphorylation as well as mutation of latch residues result in a rearrangement of the villin conformation that no longer requires high Ca²⁺ levels for its activity. We speculate that this new conformation of villin is a more open structure where the villin F-actin side binding site is exposed allowing the villin protein to bind and sever F-actin in physiologically relevant Ca²⁺ concentrations (Fig. 3 E). Such a model would agree with the reported Ca²⁺-induced increase in the Stoike volume of the villin protein (16).

Regulation of actin severing by tyrosine phosphorylation is likely to extend to other proteins of the villin family including gelsolin (Fig. 3F), which have been shown to be tyrosine-phosphorylated in vitro (23, 24) and in vivo (23, 25). Since both villin and gelsolin are tyrosine-phosphorylated we hypothesize that tyrosine phosphorylation of this family of proteins may abrogate the high Ca²⁺ requirements for actin severing and may in fact be a molecular mechanism shared by this family of proteins. The reduced calcium affinity of villin may possibly be physiologically relevant, since the intestine is the primary site of calcium absorption, allowing villin to maintain actin bundles not only under physiological calcium concentrations but also at elevated calcium concentrations (less than 100 μM).

REFERENCES

1. Panebra, A., Ma, S. X., Zhai, L. W., Wang, X. T., Rhee, S. G., and Khurana, S. (2001) Am. J. Physiol. 281, C1046–C1058
2. Kumar, N., Zhao, P., Tomar, A., Gales, C. A., and Khurana, S. (2004) J. Biol. Chem. 279, 3096–3110
3. Matsudaïra, P., Jakes, B., and Walker, J. E. (1985) Nature 315, 248–250
4. Zhai, L., Zhao, P., Panebra, A., Guerrero, A. L., and Khurana, S. (2001) J. Biol. Chem. 276, 36163–36167
5. Ferrary, E., Cohen-Tannoudji, M., Pehau-Arnaudet, G., Lapillonne, A., Athman, R., Ruiz, T., Boulouha, I., El Marjou, F., Doye, A., Fontaine, J. J., Anthony, C., Babinet, C., Louvard, D., Jaïser, F., and Robine, S. (1999) J. Cell Biol. 146, 819–830
6. Lu, M., Witte, W., Kwiatkowski, D. J., and Kosik, K. S. (1997) J. Cell Biol. 138, 1279–1297
7. Lam, J. A., Allen, P. G., Tuan, B. Y., and Jamney, P. A. (1993) J. Biol. Chem. 268, 8999–9004
8. Sklyarova, T., De Carle, V., Meerschaert, K., Devriendt, L., Vanboe, B., Bailey, J., Cook, L. H., Gostolas, L., Van Damme, J., Puppe, M., Vandenkerckhove, J., and Gettemans, J. (2002) J. Biol. Chem. 277, 39840–39849
9. Cooper, J. A., Bryan, J., Schwab, B., 3rd, Frieden, C., Loftus, D. J., and Elson, E. (1997) J. Cell Biol. 138, 501–510
10. Lin, K. M., Mejillano, M., and Yin, H. L. (2000) J. Biol. Chem. 275, 27746–27752
11. Northrop, J., Weber, A., Mosecker, M. F., Franzini-Armstrong, C., Bishop, M. F., Dubyk, G. R., Tucker, M., and Walsh, T. P. (1986) J. Biol. Chem. 261, 9274–9281
12. Ditsch, A., and Wegner, A. (1995) Eur. J. Biochem. (Tokyo) 229, 512–516
13. Constantin, B., Meerschaert, K., Vandenkerckhove, J., and Gettemans, J. (1998) J. Cell Sci. 111, 1695–1706
14. Cooper, J. A., Loftus, D. J., Frieden, C., Bryan, J., and Elson, E. L. (1988) J. Cell Biol. 106, 1229–1240
15. Janmey, P. A., and Matsudaïra, P. T. (1988) J. Biol. Chem. 263, 16738–16743
16. Hesterberg, L. K., and Weber, K. (1983) J. Cell Biol. 97, 258–263
17. Villeruel, M. L., and Pulver, H. C. (1989) Annu. Rev. Nutr. 9, 347–376
18. Robinson, R. C., Mejillano, M., Le, V. P., Burtinck, L. D., Yin, H. L., and Choe, S. (1999) Science 286, 1939–1942
19. Choe, H., Burtinck, L. D., Mejillano, M., Yin, H. L., Robinson, R. C., and Choe, S. (2002) J. Mol. Biol. 324, 691–702
20. Zhai, L., Zhao, P., Panebra, A., Zhao, P., Parrill, A. L., and Khurana, S. (2002) Biochemistry 41, 11750–11760
21. Walsh, T. P., Weber, A., Higgins, J., Babinet, C., Louvard, D., Jaisser, F., and Robine, S. (1999) J. Cell Biol. 146, 819–830
22. de Arruda, M. V., Bazari, H., Wallek, M., and Matsudaira, P. (1992) J. Biol. Chem. 267, 36417–36425
23. Q. Wang, X., Du, Q. S., Wu, X. J., Feng, X., Mei, L., McDonald, J. M., and Xiong, W. C. (2003) J. Cell Biol. 160, 565–575
24. De Carle, V., Demol, H. G., Van Damme, J., Gettemans, J., and Vandenkerckhove, J. (1999) Protein Sci. 8, 234–241
25. Yuan, X., and Desiderio, D. M. (2003) J. Proteome Res. 2, 476–487
