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Ca^{2+} and Calmodulin Regulate the Binding of Filamin A to Actin Filaments*

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Fumihiko Nakamura[1], **John H. Hartwig, Thomas P. Stossel, and Pawel T. Szymanski**

From the Hematology Division, Brigham and Women's Hospital, Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115

Filamin A (FLNa) cross-links actin filaments (F-actin) into three-dimensional gels in cells, attaches F-actin to membrane proteins, and is a scaffold that collects numerous and diverse proteins. We report that Ca^{2+}-calmodulin binds the actin-binding domain (ABD) of FLNa and dissociates FLNa from F-actin, thereby dissolving FLNa·F-actin gels. The FLNa ABD has two calponin homology domains (CH1 and CH2) separated by a linker. Recombinant CH1 but neither FLNa nor its ABD binds Ca^{2+}-calmodulin in the absence of F-actin. Extending recombinant CH1 to include the negatively charged region linker domain makes it, like full-length FLNa, unable to bind Ca^{2+}-calmodulin. Ca^{2+}-calmodulin does, however, dissociate the FLNa ABD from F-actin provided that the CH2 domain is present. These findings identify the first evidence for direct regulation of FLNa, implicating a mechanism whereby Ca^{2+}-calmodulin selectively targets the FLNa·F-actin complex.

Filamin A (FLNa)[2] is the dominant mammalian nonmuscle isoform of a widely distributed family of actin-binding proteins. FLNa, originally named "actin-binding protein" or ABP-280, is the most potent actin filament (F-actin) cross-linking protein known, and its power resides in its ability to promote perpendicular branching of actin filaments into three-dimensional gelled networks. The physiological relevance of this actin gelation property of FLNa is manifest by the surface instability and the lack of translational locomotion capacity of cells lacking the protein. FLNa is also a scaffold for numerous binding partners that include transmembrane proteins, components of intracellular signal transduction pathways, and other cellular constituents (1, 2).

The structural requirements for actin cross-linking by FLNa include dimerization at the C terminus of its subunits and a stretch of 278 amino acids designated the actin-binding domain (ABD) at the N-terminal of each dimeric subunit. The potency of the FLNa F-actin cross-linking activity resides in the large size (280 kDa) of the subunits composed of a string of 24 β-pleated sheet repeats, because much higher concentrations of other dimeric F-actin cross-linking proteins with nearly identical ABDs and binding affinities for F-actin are necessary to create gels of actin filaments of identical length.

Although regulation of FLNa dimerization, the ABD, or the structure of the intervening β-sheet repeats could affect cross-linking of the F-actin of FLNa, purified FLNa constitutively cross-links actin filaments *in vitro*, and no direct regulation of this activity has emerged in 30 years of research on this protein. Therefore, the working hypothesis for cellular control of actin cross-linking by FLNa has been that FLNa binding partners such as Rac, Rho, Cdc42, and other signaling components produce linear actin assembly on the FLNa template that orients the filaments formed into a three-dimensional cross-linked gel. Actin filament-severing proteins such as members of the gelsolin and actin-depolymerizing factor/cofilin family could dismantle such actin networks (3, 4). However, although plausible, this mechanism does not explain why a large fraction of cellular FLNa is free of F-actin *in vivo*. In this paper we present the first evidence for a direct regulation of the FLNa·F-actin interaction by the ubiquitous abundant calcium-binding protein, calmodulin (CaM).

MATERIALS AND METHODS

Proteins—Rabbit skeletal actin and human recombinant FLNa were prepared as described previously (5). HA-CaM was expressed and purified as described (6). Recombinant CaM was cloned from an IMAGE human cDNA clone (source identifier 4801400) into pET-23a(+) (EMB Biosciences, Inc.), expressed in *Escherichia coli* BL21(DE3), and purified as described (7). Bovine CaM was purchased from Sigma (catalog number P1431). Fragments of human FLNa were cloned by PCR and expressed as GST·His_6 or maltose-binding protein·His_6-tagged fusion proteins in *E. coli* using a modified pGEX-4T1-HT(a, b, or c) or pMALc-HT(a, b, or c) plasmid, respectively. The pGEX-4T1-HT(a, b, or c) were made by inserting the BglII/NotI fragment of a PCR product amplified from pFASTBAC-HT(a, b, or c) (Invitrogen) with two primers, 5′-GAAGATCTATGTCGTACTACCATCAC-3′ and 5′-CTAG-TACTTCTCGACAAGCTTGG-3′, into BamHI/NotI sites of pGEX-4T1 (Amersham Biosciences). The pMALc-HT(a, b, or c) were made using similar procedures but inserted into BamHI/XbaI sites of pMALc (New England BioLabs). An acidic sequence of FLNa, ^{156}DEEEDEE^{162}, was deleted using the QuikChange site-directed mutagenesis kit (Stratagene). The fusion proteins were affinity purified using glutathione-Sepharose 4 Fast Flow, Amylose resin or nickel-nitrilotriacetic acid agarose. For some experiments, the tags were cleaved with His_6·tobacco etch virus (TEV) protease at room temperature for 1 h. GST·His_6 tag, maltose-binding protein·His_6 tag, and His_6·TEV were removed by passing the solution supplemented with 3 mM $MgCl_2$ and 5 mM imidazole through a nickel-nitrilotriacetic acid agarose column. The flow-through fractions were concentrated using an Amicon Ultra-15 (Millipore) and applied onto a Superdex 75 HR 10/30 (Amersham Biosciences) equilibrated with 10 mM Tris-HCl, 100 mM NaCl, and 1 mM dithiothreitol, pH 7.4. The highly purified proteins were stored at 4 °C.

Antibodies—Anti-FLNa monoclonal antibodies were prepared by hybridoma techniques. Approximately 15–20 μg of purified recombi-

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[1] To whom correspondence should be addressed: Hematology Division, Brigham and Women's Hospital, 6F Karp Family Research Laboratories, 1 Blackfan Circle, Boston, MA 02115. Tel.: 617-355-9000; Fax: 617-355-9016; E-mail: fnakamura@rics.bwh.harvard.edu.

[2] The abbreviations used are: FLNa, filamin A; ABD, actin-binding domain; ABS, actin-binding site; CaM, calmodulin; CBB, Coomassie Brilliant Blue; CH, calponin homology; GST, glutathione *S*-transferase; HA, hemagglutinin; mAb, monoclonal antibody; N-ABD, N-terminal actin-binding domain; C-ABD, C-terminal actin-binding domain.

nant *Homo sapiens* FLNa proteins, dissolved in phosphate-buffered saline, were mixed with complete Freund's adjuvant and injected intraperitoneally to BALB/c mice. The spleen of the immunized mouse was macerated, and the spleen cells were fused with mouse myeloma Sp20 cells in the presence of a Hybrimax polyethylene glycol solution (Sigma-Aldrich). The fused cells were plated onto 96-well plates and cultured in Optimum medium. The standard hypoxanthine-aminopterin-thymidine selection procedure was applied to select the hybridoma clones. After 10 days, hybridoma supernatants were tested for reactivity with recombinant *H. sapiens* FLNa by an enzyme-linked immunosorbent assay. To further select antibodies, the media were also tested by immunoblotting and immunostaining. Cells from the positive wells were cloned at least three times by limiting dilution and adapted to the serum-free medium Hybridoma-SFM (Invitrogen). The antibody subtypes were determined using a kit (Roche Applied Science). Monoclonal IgG or IgM antibodies were purified from serum-free hybridoma supernatant by GammaBind G-Sepharose (Amersham Biosciences) or KAPTIV-M (Genomics One International, Buffalo, NY) affinity chromatography. Anti-HA mouse mAb (clone 12CA), anti-GST rabbit polyclonal antibody (Sigma), goat horseradish peroxidase-conjugated anti-mouse, or anti-rabbit IgG (Bio-Rad) were used for immunoblotting.

Actin Filament Sedimentation Assay—Purified FLNa or FLNa fragments were mixed with or without CaM and G-actin (5 or 10 μM) in solution F (20 mM Tris-HCl, pH 7.4, 0.5 mM Na_2ATP, 5 mM $MgCl_2$, 120 mM NaCl, and 0.2 mM dithiothreitol) with 2 mM $CaCl_2$ or 5 mM EGTA for 1 h at 25 °C. The actin filaments were then sedimented by centrifugation at 150,000 × *g* for 20 min at 25 °C. Proteins in the supernatants and pellets were then solubilized in SDS gel sample buffer and subjected to SDS-PAGE. Polypeptides in the gel were visualized by Coomassie Brilliant Blue (CBB) staining or immunoblotting.

Measurements of Apparent Viscosity and Gelation—Apparent viscosity and gelation were determined using a miniature falling ball viscometer as described previously (5).

HA-Calmodulin Blot Overlay Assay—A CaM overlay assay was performed in the presence of either 2 mM $CaCl_2$ or 5 mM EGTA as described by Szymanska *et al.* with slight modifications (6). Purified FLNa or FLNa constructs (2 pmol) were spotted onto polyvinylidene difluoride membranes and blocked for 1 h at room temperature in 3% bovine serum albumin in TTBS (20 mM Tris-HCl, pH 7.4, 110 mM NaCl, 5 mM $MgCl_2$, and 0.1% Tween 20). In some experiments, the blots were soaked in methanol/acetic acid/water (50:10:40) (v/v) for 10 s followed by methanol for 10 s to denature proteins. The membranes were then rinsed with water before blocking. The blots were probed with 5 μg/ml HA-CaM in TTBS for 1 h. The blot was washed three times (10 min/wash) with TBST, and bound HA-CaM was detected with an anti-HA mAb and horseradish peroxidase-conjugated anti-mouse IgG.

HA-Calmodulin Pull-down Assay—FLNa or GST·FLNa fragments (0.1 μM) were incubated for 1 h at room temperature with 20 μl (50% slurry) of CaM-agarose (Sigma) or 0.2 μM HA-CaM in 200 μl of TTBS in the presence of either 2 mM $CaCl_2$ or 5 mM EGTA, and then 20 μl (50% slurry) of anti-HA mAb-agarose beads (Santa Cruz Biotechnology Inc.) was added. After 1 h, the slurry was centrifuged and washed four times with TTBS containing either $CaCl_2$ or EGTA. The polypeptides bound to the beads were solubilized in SDS sample buffer and analyzed by SDS-PAGE and immunoblot.

Measurements of Affinity to Actin Filaments—Affinity to actin filaments was measured by a sedimentation assay. Five or 10 μM G-actin was incubated with increasing concentrations of FLNa fragments in a solution containing 20 mM Tris-HCl, pH 7.4, 0.5 mM Na_2ATP, 2 mM $MgCl_2$, 120 mM NaCl, 0.2 mM dithiothreitol, and 0.5 mM EGTA for 2 h at 25 °C. The actin filaments were then sedimented by centrifugation at 150,000 × *g* for 20 min at 25 °C. Proteins in the supernatants and pellets were then solubilized in SDS gel sample buffer and subjected to SDS-PAGE. Polypeptides in the gel were visualized by CBB staining, and the band intensities were quantified by densitometry.

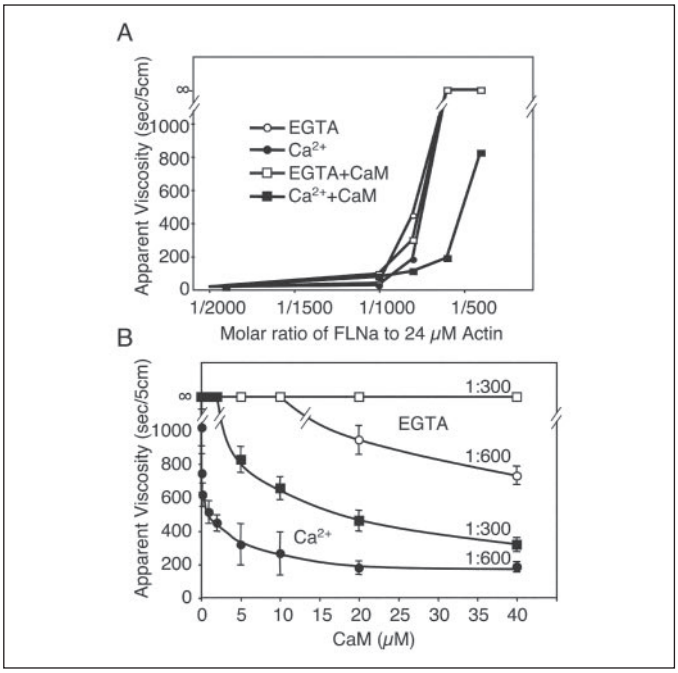

FIGURE 1. **Effect of CaM on the gelation of F-actin by FLNa.** *A*, gel points of 24 μM actin polymerized with various concentrations of FLNa in the presence or absence of holocalmodulin or apocalmodulin. *B*, apparent viscosity of 24 μM actin polymerized with FLNa (*circles*, 0.04 μM; *squares*, 0.08 μM) and increasing concentrations of CaM in the presence of 5 mM EGTA (*open symbols*) or 2 mM $CaCl_2$ (*closed symbols*).

Titration of Free Calcium Ion—Ca^{2+}-EGTA solutions were prepared as described previously (8). The concentration of free calcium was determined using Fura-2 and calcium calibration buffer kit in accordance with the manufacturer's instructions (Molecular Probes).

Data Base Analysis—Secondary or tertiary structures of FLNa were predicted by data base analysis available on web sites. For homology searches, protein-protein BLAST data base was used. For secondary structure prediction, the sites used were those of the Pôle BioInformatique Lyonnais network protein sequence analysis server (npsa-pbil.ibcp.fr) and the PredictProtein server of the Columbia University Bioinformatics Center (cubic.bioc.columbia.edu/predictprotein/). For three-dimensional structure prediction, the SWISS-MODEL automated comparative protein modelling server (swissmodel.expasy.org//SWISS-MODEL) and the 3D-Jigsaw comparative modelling server (www.bmm.icnet.uk/servers/3djigsaw/) were used. To predict the CaM-binding site, the Ontario Cancer Institute's calmodulin target data base (calcium.uhnres.utoronto.ca/ctdb/flash.htm) was examined. The protein charge plot was carried out by the EMBOSS charge program (emboss.sourceforge.net/). To access these sites, include http:// at the beginning of each address.

RESULTS

Ca^{2+}-CaM Inhibits the Gelation of Actin by FLNa—Fig. 1*A* shows the apparent viscosity of 24 μM actin polymerized with various concentrations of FLNa in the presence either EGTA or Ca^{2+} with or without 10 μM CaM. In EGTA the gel point, an abrupt increase in the viscosity of actin, occurred at a FLNa concentration of 0.03 μM, a ratio of ~1 FLNa to 800 actin subunits as described previously (5), and the gel point was unaffected by Ca^{2+}-free CaM. The amount of FLNa required to gel actin in the presence of 2 mM calcium was slightly higher, presumably

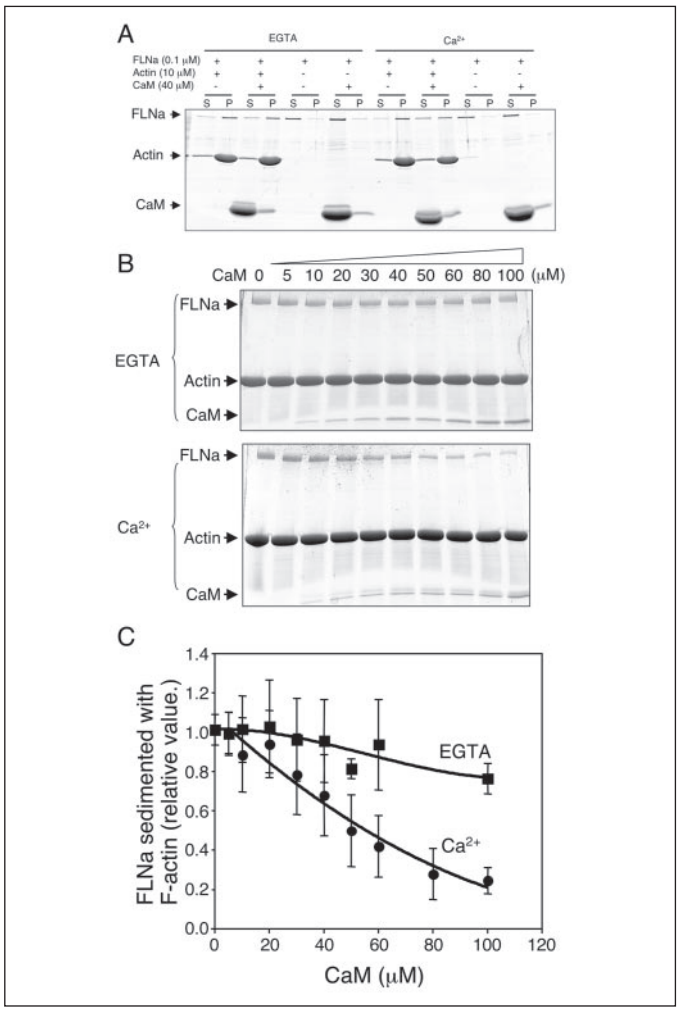

FIGURE 2. **Effect of CaM on the co-sedimentation of FLNa with F-actin.** *A*, recombinant human FLNa (0.1 μM) was incubated for 1 h with 10 μM G-actin and/or 40 μM CaM in the presence of 5 mM EGTA (*left*) or 2 mM $CaCl_2$ (*right*). The protein mixtures were centrifuged at 150,000 $\times$ *g* for 20 min at 25 °C, and the supernatant (*S*) and pellet (*P*) were displayed by SDS-PAGE and stained with CBB. *B*, FLNa (0.32 μM) was mixed with 10 μM G-actin and polymerized for 1 h with the indicated amounts of CaM in the presence of 5 mM EGTA or 2 mM $CaCl_2$. The mixtures were centrifuged, and the pellets were displayed by SDS-PAGE and stained with CBB. *C*, the intensities of CBB-stained FLNa bands in *panel B* were determined by densitometry. The relative intensity of each band compared to that with no CaM was plotted as function of the CaM concentration.

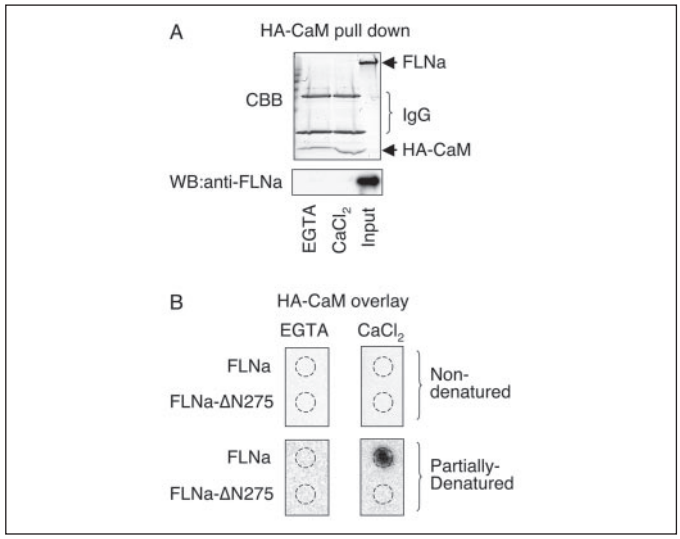

FIGURE 3. **FLNa does not bind CaM.** *A*, recombinant human FLNa (20 pmol) was incubated for 1 h at room temperature with 40 pmol of HA-CaM in 200 μl of TTBS in the presence of 2 mM $CaCl_2$ or 5 mM EGTA. HA-CaM was collected with anti-HA mAb-agarose beads. After washing the beads, bound proteins were resolved by SDS-PAGE and stained with CBB or immunoblotted with an anti-FLNa antibody. *WB*, Western blot. *B*, purified FLNa or a FLNa truncate lacking the 275 N-terminal amino acids comprising the ABD (*FLNa-ΔN275*) (2 pmol) were spotted onto a polyvinylidene difluoride membrane and dried and were or were not denatured with methanol/acetic acid/water (50:10:40) (v/v) for 10 s. The membrane was blocked for 1 h at room temperature in 3% bovine serum albumin in TTBS and reacted with HA-CaM. Bound HA-CaM was detected with an anti-HA mAb and horseradish peroxidase-conjugated anti-mouse IgG.

because actin filaments polymerized in calcium are shorter than the filaments containing Mg^{2+} (9). However, CaM nearly doubled the FLNa concentration required for incipient gelation in the presence of Ca^{2+}. Fig. 1*B* shows the apparent viscosity of 24 μM actin polymerized with 0.04 (1:600) and 0.08 μM (1:300) FLNa in the presence of either EGTA or Ca^{2+} with various concentrations of CaM. Low concentrations of CaM ($\geq$ 0.1 μM) dissolved the F-actin-FLNa gel in the presence of Ca^{2+}.

Ca^{2+}-CaM Inhibits the Binding of FLNa to F-actin—The simplest explanation for inhibition of FLNa-mediated actin gelation by Ca^{2+}-CaM could be interference with the binding of FLNa to actin filaments, and Fig. 2 shows the evidence for this mechanism. Approximately 80% of added FLNa co-sedimented with actin filaments in the presence or absence of free Ca^{2+} (Fig. 2*A*). CaM reduced the binding of FLNa to actin filaments in the presence of Ca^{2+}. CaM did not affect the solubility of FLNa in the absence of F-actin. Increasing concentrations of CaM inhibited the co-sedimentation of FLNa with actin filaments, but only in the presence of Ca^{2+} (Fig. 2*B*). Fig. 2*C* shows that the half-maximal inhibition of the binding of FLNa to actin filaments occurred at 40 μM CaM in the presence of Ca^{2+}. More than 76% of the added FLNa bound to actin filaments in the presence of the same concentration of CaM in the absence of Ca^{2+}.

CaM Does Not Bind Native Full-length FLNa but Binds the ABD of Denatured FLNa—The results of both HA-CaM pull-down and HA-CaM overlay assays were inconsistent with a mechanism whereby Ca^{2+}-CaM inhibits F-actin binding by competitive ligation to native FLNa, because the binding of recombinant FLNa to CaM was minimal (Fig. 3, *A* and *B*). FLNa partially denatured with methanol/acetic acid, however, bound CaM in a calcium-dependent fashion. An identically treated FLNa truncate lacking its 275 N-terminal amino acids comprising the ABD, which did not bind F-actin (data not shown), did not bind CaM (Fig. 3*B*), suggesting that binding occurs with first 275 amino acids of FLNa.

Localizing the CaM-binding in the N-terminal Half of the FLNa ABD (N-ABD)—Fig. 4 shows that the a GST·His_6 construct encompassing the N-terminal half of the FLNa ABD, N-ABD, binds to CaM in a Ca^{2+}-dependent manner, whereas a corresponding construct containing the ABD C-terminal half (C-ABD) does not. A GST fusion protein including the entire ABD, like full-length FLNa, did not bind to CaM. The ABD of FLNa contains two calponin homology (CH) domains, CH1 (Ile^{44}–Tyr^{148}) and CH2 (Pro^{168}–Phe^{264}), which each contain four principal α helices connected by loops or shorter helices (10). Based on these predictions and because many known CaM-binding proteins possess a relatively short α helical region, fusion proteins representing smaller fragments of the FLNa ABD illustrated in Fig. 4, *C* and *E*, were generated and tested for their CaM-binding activity in the presence of EGTA or Ca^{2+} (Fig. 4*D*). As shown in Fig. 4*E*, the minimum CaM binding site was narrowed down to a region between Thr^{50} and Arg^{96} within the CH1 domain. Removal of Thr^{50}–Val^{60} from this sequence completely eliminated the CaM-binding activity of this fragment, and a shorter fragment containing Thr^{50}–Val^{60} (Thr^{31}–Ala^{65}) did not bind to CaM either. Removal of a highly positively charged sequence, ^{87}KKMHRKHNQR^{96} from the putative CaM-binding fragment diminished its CaM-binding activity, and smaller fragments containing this region (Ser^{61}–Ser^{118} or

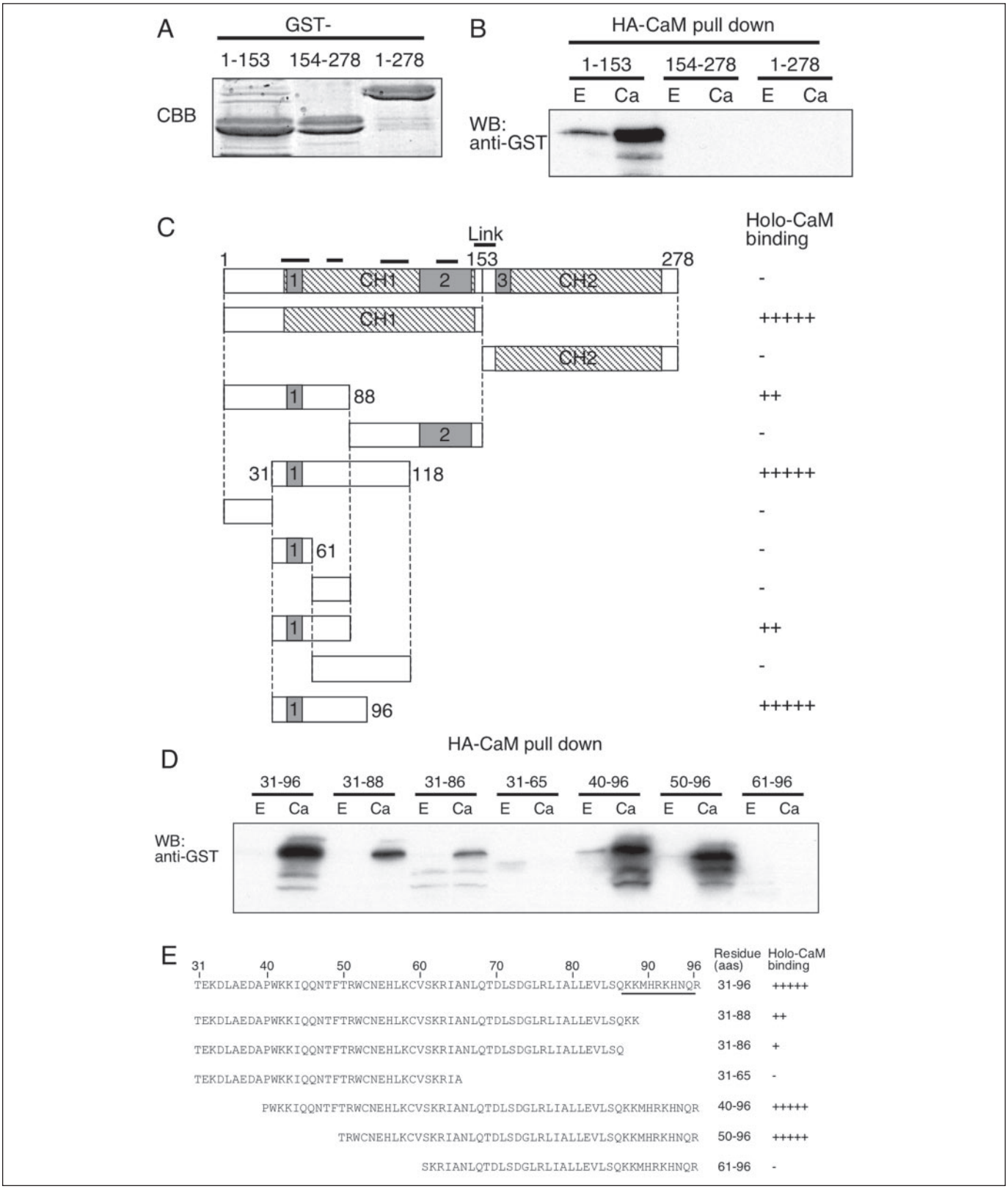

FIGURE 4. **CaM binds to the N-terminal half of FLNa ABD.** *A*, CBB-stained SDS-PAGE gel of purified GST·FLNa constructs used in the experiments. *B*, HA-CaM pull-down assays with the purified GST·FLNa constructs in the presence of 5 mM EGTA (*E*) or 2 mM $CaCl_2$ (*Ca*). HA-CaM (40 pmol/200 μl = 0.2 μM) was mixed with the same input amount of GST·FLNa constructs (20 pmol/200 μl = 0.1 μM), and then HA-CaM was collected with anti-HA mAb-agarose beads. After washing the beads, a bound GST fusion protein was detected by immunoblot with anti-GST polyclonal antibodies. *WB*, Western blot. *C*, dissection of the FLNa CaM-binding domain. Predicted α-helix domains are indicated by the *solid line*. Predicted actin filament-association sites (*1*, *2*, and *3,* in *shaded boxes*), CH1 and CH2 domains, and the link domain are indicated. The minimum CaM-binding domain was determined using HA-CaM pull-down assays. *D*, HA-CaM pull-down assay with GST fusion proteins of FLNa constructs within the sequence from Thr^{31} to Arg^{91}. The assay was performed as for *panel B*. *E*, amino acid sequence of the FLNa constructs tested. The essential CaM-binding domain is *underlined*, and the positively charged region is *double underlined*. The relative intensity of the band in *panel D* is indicated from strong (+++++) to none (−).

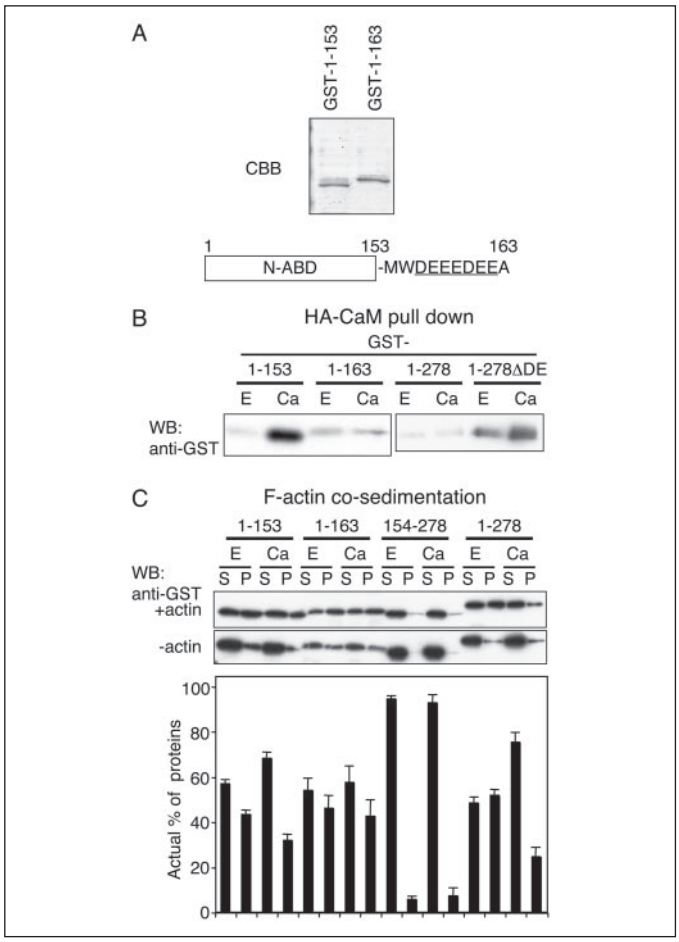

FIGURE 5. **Addition of the acidic region that lies between the two CH domains to N-ABD prevents CaM-binding.** *A*, CBB-stained SDS-PAGE gel of purified GST·1–153 and GST·1–163 fusion proteins. The amino acid sequence between Met[154] and Ala[163] of FLNa added is *underlined*. *B*, HA-CaM pull-down assay of GST·1–153, GST·1–163, GST·1–278, and GST·1–278ΔDE ([156]DEEEDEE[162] deleted). The assay was performed as for Fig. 4*B* (*E*, EGTA; *Ca*, $CaCl_2$). *C*, actin filament co-sedimentation assay of GST·ABD fusion protein constructs in the presence of CaM. Purified GST fusion proteins (0.1 μM) were mixed with (*top gel*) or without (*bottom gel*) 10 μM G-actin and 80 μM CaM for 1 h in the presence of 5 mM EGTA (*E*) or 2 mM $CaCl_2$ (*Ca*). The mixture was centrifuged, and the supernatant (*S*) and pellet (*P*) were displayed by SDS-PAGE. The fusion proteins were detected by immunoblotting with anti-GST polyclonal antibodies. *WB*, Western blot. The graph shows the percent of each protein in the supernatant or pellet. The amount of protein that bound to actin was calculated by determining the intensity of each band and subtracting the amount of it sedimented without actin.

Ser[61]–Arg[96]) did not bind to CaM. These data indicate that the Thr[50]–Val[60] stretch is essential for CaM-binding activity and that the basic region between positions 87 and 96 augments the binding, but also that each segment alone is not sufficient to promote binding. This CaM-binding site of FLNa is apparently unique in that no matches of its sequence to the calmodulin target data base (11) were detectable.

A Negatively Charged Site Linking the CH1 and CH2 Domains of the FLNa ABD Is Sufficient to Suppress the CaM Binding Activity—Our findings suggest that the C-terminal portion of the FLNa ABD is responsible for the inability of the intact FLNa ABD to bind CaM. The connecting segment between CH1 and CH2 ([156]DEEEDEE[162]), which is filamin family-specific, is highly acidic (Fig. 5*A*). This negatively charged sequence could complement the positively charged region (residues 87–96 amino acids) within the CaM-binding site in the N-ABD and might affect CaM-binding activity. Consistent with this hypothesis, the N-ABD extended by 10 C-terminal amino acids, including all of the positively charged residues, is unable to bind CaM, and deletion of the charged residues from the entire FLNa ABD enables it to bind to CaM (Fig. 5*B*).

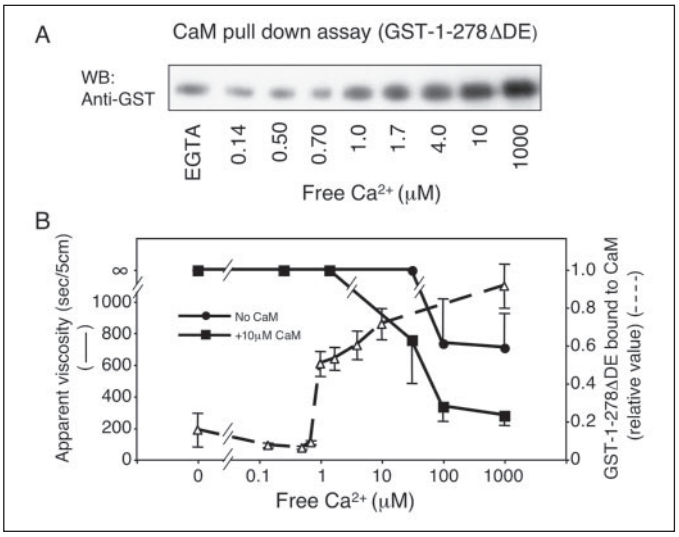

FIGURE 6. **Effect of Ca^{2+} concentration on the binding of CaM to the FLNa ABD and on the gelation of F-actin/FLNa in the presence or absence of CaM.** *A*, CaM pull-down assay of GST·1–278ΔDE ([156]DEEEDEE[162] deleted) at increasing concentrations of free calcium. The assay was performed as for Fig. 4*B*, except that free calcium was increased as indicated. *WB*, Western blot. *B*, apparent viscosity of 24 μM actin polymerized with FLNa (0.04 μM) in the presence (*closed squares*) or absence (*closed circles*) of CaM (10 μM) at increasing concentrations of free calcium. The relative amount of GST·1–278ΔDE that bound to CaM was determined by densitometry (*panel A*) and plotted (*open triangles*).

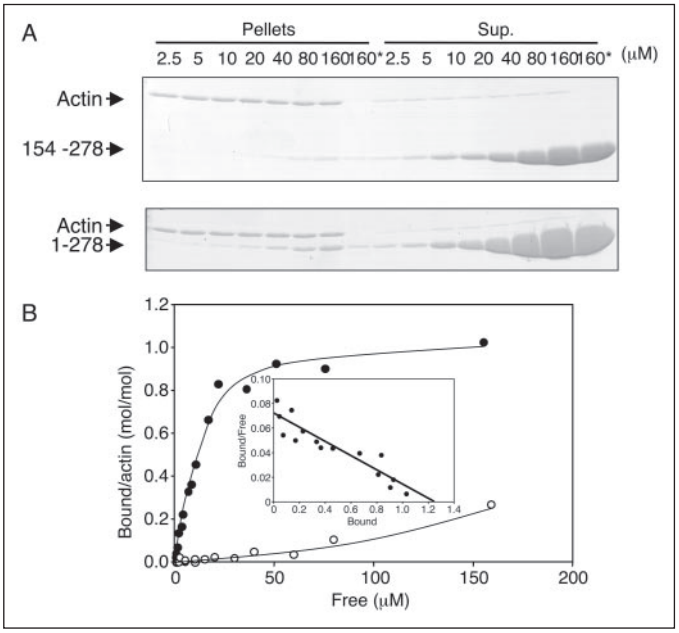

FIGURE 7. **Binding of an intact ABD of FLNa and the subdomain CH2-ABD, a regulatory domain of CaM binding, to actin filaments.** *A*, G-actin (5 μM) (*asterisk*, no actin) was mixed with increasing concentrations of purified proteins and polymerized for 2 h at room temperature. The samples were sedimented by centrifugation. Supernatant (*Sup.*) and pellet fractions were analyzed by 20% SDS-PAGE. The polypeptides were detected by CBB and quantified by densitometry. *B*, the binding of the entire ABD (*closed circles*) to actin filaments was saturated at a molar ratio of 1.2:1 (ABD/actin monomer), whereas that of the C-ABD (*open circles*) did not saturate up to 160 μM. A Scatchard analysis of the binding data (*inset*) reveals that the ABD of FLNa binds actin filaments with a K_d of 17.3 μM ($r = 0.86$).

The CH2 Domain Suppresses the Inhibitory Activity of the Acidic Link between the CH1 and CH2 Domains in the Presence of Actin Filaments—Fig. 5*C* shows that expressed FLNa fragments containing the entire ABD (1–278 amino acids), the N-ABD (1–153 amino acids) and the N-ABD plus the acidic link between CH1 and CH2 (1–163 amino acids) associate with actin filaments, whereas the C-ABD (154–278 amino acids) does not. The results of the binding experiments also demon-

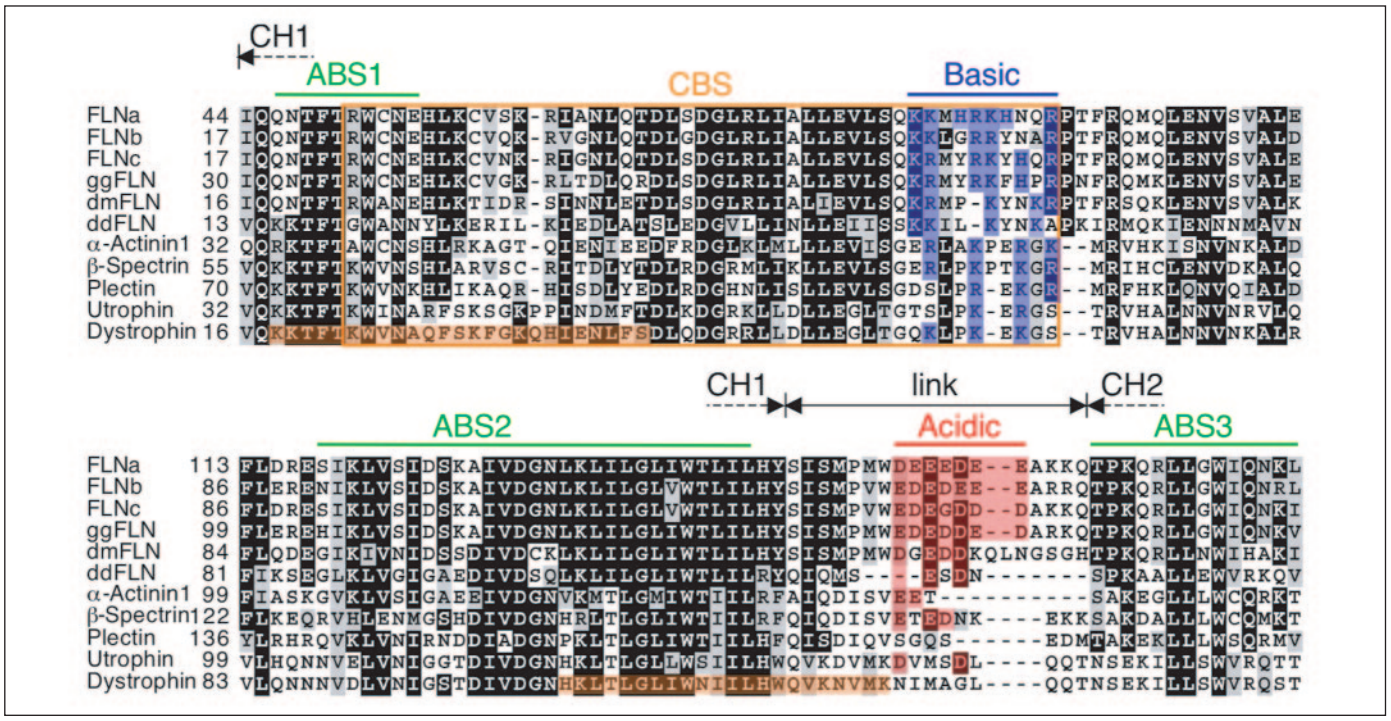

FIGURE 8. **Sequence alignment of a part of ABD of FLNa and that of spectrin superfamily proteins.** The CH1 and the beginning of CH2 are connected with a link (indicated with *arrows*). The CaM-binding site (*CBS*) of FLNa and the corresponding sites of other spectrin superfamily proteins are boxed along with the CaM-binding site of dystrophin marked with an *orange* color (30). Basic amino acids in positively charged site (*blue line*) and acidic amino acids in negatively charged site (*red line*) are marked with *blue* and *red* colors, respectively. ABS1, ABS2, and ABS3 are also delineated (*green line*). The alignment was generated using ClustalW (35). *ddFLN*, *Dictyostelium discoideum* FLN; *dmFLN*, *Drosophila melanogaster* FLN; *ggFLN*, *Gallus gallus* FLN.

strate that the N-ABD is sufficient to regulate actin-binding by CaM. The positively charged CH1-CH2 link desensitizes the N-ABD regulation by CaM; the entire ABD is responsive to CaM, suggesting that the CH2 sequence restores this sensitivity.

CaM Interacts with FLNa at Physiological Ca^{2+} Concentrations—Fig. 6 compares the calcium dose-response for CaM binding to GST·1–278ΔDE (^{156}DEEEDEE^{162} deleted) with that required to solate FLNa·F-actin·CaM networks. GST·1–278ΔDE was used in these experiments because it binds calmodulin in the absence of actin. Binding of CaM to GST·1–278ΔDE required ≥1 μM calcium and was nearly maximal at 10 μM calcium concentrations. The apparent viscosity of F-actin in the presence of FLNa and CaM was significantly decreased in this calcium range. Interpretations of effects at calcium are somewhat complicated because the assembly of actin filaments in >50 μM calcium increases the number of nuclei, leading to shorter filaments (9). The apparent viscosity of FLNa·F-actin, however, was clearly reduced in the presence of CaM.

The CH2-ABD Has Low Affinity for Actin Filaments but Augments the Binding of the Intact ABD—Qualitative binding studies suggest that both the intact ABD and the CH1-ABD of FLNa bind to actin filaments, whereas the CH2-ABD binds actin weakly. We measured the affinity of the intact ABD and CH2-ABD to actin filaments. These domains were expressed as fusion proteins in *E. coli* and purified, and the GST·His_6 was removed. The ABD and the C-ABD were obtained in high yield and, as monomers, in concentrations up to 300 μM. We could not produce N-ABD protein in good yield because of technical problems that have also been reported with attempts to isolate the corresponding domain of utrophin (12). Fig. 7, *A–C* shows that the ABD of FLNa binds to actin filaments with a 1:1 stoichiometry (mol/mol actin as a monomer) and a K_d of 17.3 μM, whereas the CH2 did not demonstrate saturable binding to actin even at very high concentrations (~160 μM). Although the affinity of N-ABD to actin could not be measured, the GST·His_6·N-ABD sediments with actin filaments, whereas the GST·His_6·C-ABD does not

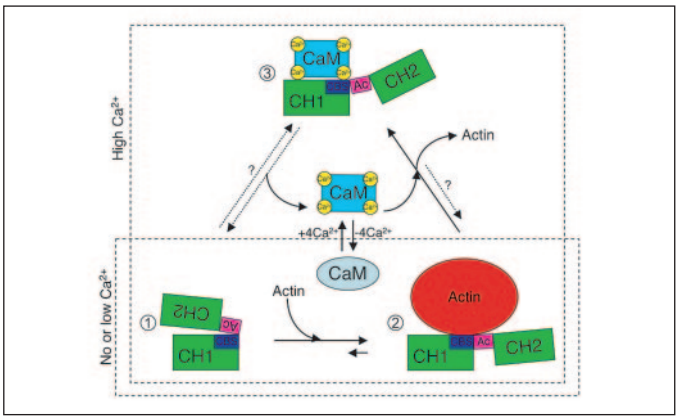

FIGURE 9. **A multistep model for the regulation of the actin filament-binding of FLNa by CaM.** *Step 1*, CaM-binding site (*CBS*) in the CH1 of FLNa is masked by acidic region (*Ac*) within the linker domain in the absence of actin. *Step 2*, the binding of F-actin to FLNa exposes the CaM-binding site. *Step 3*, Ca^{2+} influx activates CaM (holocalmodulin), and holocalmodulin now competes with F-actin for FLNa binding, increasing the gel point of the F-actin solution.

(Fig. 5*C*), implying that the major ABD is located in the N-ABD. Because CaM binds to the N-ABD, the results suggest that Ca^{2+}-CaM directly competes with actin filaments.

DISCUSSION

Phosphorylation of avian gizzard filamin (probably a FLNb homolog, inferring from its DNA sequence) by CaM-kinase II attenuates its actin binding (13). However, such an effect of CaM-kinase II on human FLNa has not been observed.[3] Here, we have obtained the first evidence for a direct regulation of the interaction between FLNa and actin filaments. Calcium-bound calmodulin (holocalmodulin) dissociates F-actin from

[3] F. Nakamura, J. H. Hartwig, T. P. Stossel, and P. T. Szymanski, unpublished observations.

FLNa and inhibits the ability of FLNa to bind and cross-link actin filaments into a three-dimensional gelled network *in vitro*. If this regulation exists within living cells, calcium signaling would have a consistent effect on actin gelation. First, calcium signaling dissolves actin gels by activating actin filament severing and capping proteins of the gelsolin family (3). Second, FLNa mobilized by removal from actin filaments in response to holocalmodulin could initiate a new actin gelation cycle where or when calcium levels fall. Because the binding of FLNa to actin filaments also slows actin depolymerization,[4] calcium-calmodulin would also regulate this aspect of actin dynamics.

Most cells express 1–3 μM FLNa, 50–200 μM F-actin (14), and 30–60 μM CaM (15, 16), although the local concentrations of each at the leading edges of motile cells could be much higher (17). For example, the local concentration of calmodulin around the Ca^{2+} channel has been estimated to be nearly 2.5 mM (18). Bulk measurements of free calcium concentrations in cells have reported increases from 0.1 to ~1 μM following cell activation (19), sufficient to promote the binding of CaM to the FLNa ABD *in vitro*. We determined a half-maximal concentration of ~40 μM CaM to be effective in the presence of 10 μM actin and 0.32 μM FLNa in an actin co-sedimentation assay. This concentration of CaM exists in living cells. Much smaller amounts of CaM were sufficient to induce solation of FLNa·actin gels, most likely because solation can be initiated by dissociation of only one of the two ABDs in a FLNa dimer, whereas inhibition of actin binding requires the inactivation of both ABDs.

Holocalmodulin does not bind FLNa directly. Rather, it seeks out the ABD of FLNa complexed to F-actin, thereby maximizing the specificity of its effect; it removes FLNa from actin filaments but does not prevent FLNa from binding to F-actin. We therefore attempted to achieve some understanding of this complex and interesting mechanism.

The FLNa ABD is representative of many actin filament-binding proteins, including members of the spectrin, α-actinin, dystrophin, plectin, and fimbrin families (20). The conserved ABDs contain two CH subdomains in tandem linked by a short connector sequence (Fig. 8) (21, 22). The first, CH1, has two putative actin-binding sites (ABS1 and ABS2; Fig. 8), and the second, CH2, has one (ABS3). Accumulated evidence points to a hydrophobic stretch in ABS2 essential for actin binding, but the structural requirements for the other two actin binding sites are less clear (23, 24). Measured dissociation constants of isolated spectrin family ABDs for F-actin have been in the micromolar range (as we report in this paper for FLNa) (22, 23, 25). The CH2 subdomain of β-spectrin barely binds to F-actin (22), as we also found for its FLNa counterpart. The affinity of intact FLNa for F-actin (K_d 0.5 μM) (26) is an order of magnitude higher than that of the isolated FLNa ABD. Therefore, although the ABD of FLNa is essential for binding of full-length FLNa to F-actin,[4] other binding sites or the dimeric state of FLNa contribute to the FLNa-F-actin interaction.

Neither intact FLNa nor its ABD bound to holocalmodulin, as determined by sedimentation assays. However, holocalmodulin did bind to partially denatured recombinant FLNa, provided that the protein contained its ABD, suggesting that the FLNa ABD has a cryptic binding site for holocalmodulin. Consistent with this idea, recombinant fragments of the FLNa ABD did bind to holocalmodulin, and from this binding we inferred that residues 50–96 in the CH1 subdomain represent the FLNa binding for calmodulin.

Other actin filament-binding proteins thought to bind and be regulated by holocalmodulin include β spectrin (27), calponin (28), and utrophin (29), although some controversy exists regarding the role of holocalmodulin binding for dystrophin (29, 30) or α-actinin functions (31, 32). Many known calmodulin-binding proteins have basic amphipathic helix structures with positively charged amino acids interspersed among conserved hydrophobic stretches (11, 33). Although such a motif in dystrophin overlaps with the functional calmodulin-binding domain that we identified for FLNa (shown in *red* in Fig. 8), a recombinant polypeptide containing the homologous sequence of FLNa alone bound calmodulin weakly. The addition to this fragment of a basic stretch of amino acids that is unique to the filamin family (shown in *blue* in Fig. 8) resulted in much tighter complexing to holocalmodulin. This cluster of basic amino acids in the FLNa ABD is presumably not the principal site for calmodulin binding, however, because the inferred FLNa calmodulin-binding domain is twice as long as that of most known calmodulin-binding sites, and the basic region augments but is not required for calmodulin binding. Because the FLNa segment Thr^{50}–Val^{60} is critical for calmodulin binding and is homologous to an established calmodulin-binding motif in dystrophin, this region might represent the "authentic" FLNa calmodulin-binding domain, and the basic region may act as a clamp.

The basic subdomain at the C-terminal end of the FLNa calmodulin-binding site also suggests a mechanism as to why holocalmodulin does not bind native intact FLNa or its ABD. Vertebrate filamins have a characteristic acidic region within the linker sequence between their ABD CH domains that could bind to the corresponding basic domain in the CH1. Supporting this hypothesis, this acidic fragment inhibits calmodulin binding to CH1. This inhibition suggests a model for the mechanism by which calmodulin dissociates FLNa from F-actin (Fig. 9). The acidic segment of the FLNa ABD prevents calmodulin binding. However, when bound to F-actin the acidic piece does not block the calmodulin-binding site on FLNa, allowing holocalmodulin to bind the FLNa·F-actin complex and dissociate it by direct competition for the actin-binding site, as has been proposed for dystrophin (34).

Our model suggests that deletions or point mutations of the CaM-binding, basic, or acidic domains could affect FLNa function *in vivo*. However, these mutations might also impair actin binding because the CaM-binding domain of FLNa overlaps the ABD, and mutations could lead to conformational changes of the ABD that would complicate interpretation of any results.

Genetic mutations in FLNa cause a wide range of human diseases, and several point mutations have now been identified in the FLNa ABD (2). None of the mutations, however, are in the calmodulin-binding site identified in this study. Because the regulation of the FLNa-actin interaction requires the participation of other domains in the ABD in addition to the calmodulin-binding site, the point mutations identified in the ABD may cause disease by altering the calmodulin-FLNa interaction.

[4] E. Osborn, J. L. McGrath, F. Nakamura, S. K. Chalos, M. Schleicher Jr., and J. H. Hartwig, submitted for publication.

REFERENCES

1. Stossel, T. P., Condeelis, J., Cooley, L., Hartwig, J. H., Noegel, A., Schleicher, M., and Shapiro, S. S. (2001) *Nat. Rev. Mol. Cell Biol.* **2,** 138–145
2. Feng, Y., and Walsh, C. A. (2004) *Nat. Cell Biol.* **6,** 1034–1038
3. Silacci, P., Mazzolai, L., Gauci, C., Stergiopulos, N., Yin, H. L., and Hayoz, D. (2004) *Cell. Mol. Life. Sci.* **61,** 2614–2623
4. Desmarais, V., Ghosh, M., Eddy, R., and Condeelis, J. (2005) *J. Cell Sci.* **118,** 19–26
5. Nakamura, F., Osborn, E., Janmey, P. A., and Stossel, T. P. (2002) *J. Biol. Chem.* **277,** 9148–9154
6. Szymanska, G., O'Connor, M. B., and O'Connor, C. M. (1997) *Anal. Biochem.* **252,** 96–105
7. Hayashi, N., Matsubara, M., Takasaki, A., Titani, K., and Taniguchi, H. (1998) *Protein Expression Purif.* **12,** 25–28
8. Bers, D. M. (1982) *Am. J. Physiol.* **242,** C404–C408
9. Kinosian, H. J., Selden, L. A., Estes, J. E., and Gershman, L. C. (1991) *Biochim. Biophys. Acta* **1077,** 151–158
10. Stradal, T., Kranewitter, W., Winder, S. J., and Gimona, M. (1998) *FEBS Lett.* **431,** 134–137
11. Yap, K. L., Kim, J., Truong, K., Sherman, M., Yuan, T., and Ikura, M. (2000) *J. Struct.*

Funct. Genomics **1,** 8–14
12. Gimona, M., and Winder, S. J. (1998) *Curr. Biol.* **8,** R674–R675
13. Ohta, Y., and Hartwig, J. H. (1995) *Biochemistry* **34,** 6745–6754
14. Hartwig, J. H., and Shevlin, P. (1986) *J. Cell Biol.* **103,** 1007–1020
15. Szymanski, P. T., Szymanska, G., and Goyal, R. K. (2002) *Am. J. Physiol.* **282,** C94–C104
16. Tansey, M. G., Luby-Phelps, K., Kamm, K. E., and Stull, J. T. (1994) *J. Biol. Chem.* **269,** 9912–9920
17. Radding, W., Williams, J. P., Hardy, R. W., McDonald, J. M., Whitaker, C. H., Turbat-Herrera, E. A., and Blair, H. C. (1994) *J. Cell. Physiol.* **160,** 17–28
18. Mori, M. X., Erickson, M. G., and Yue, D. T. (2004) *Science* **304,** 432–435
19. Berridge, M. J., Lipp, P., and Bootman, M. D. (2000) *Nat. Rev. Mol. Cell Biol.* **1,** 11–21
20. Hartwig, J. H. (1995) *Protein Profile* **2,** 703–800
21. Gimona, M., Djinovic-Carugo, K., Kranewitter, W. J., and Winder, S. J. (2002) *FEBS Lett.* **513,** 98–106
22. Banuelos, S., Saraste, M., and Carugo, K. D. (1998) *Structure* **6,** 1419–1431
23. Winder, S. J., Hemmings, L., Maciver, S. K., Bolton, S. J., Tinsley, J. M., Davies, K. E., Critchley, D. R., and Kendrick-Jones, J. (1995) *J. Cell Sci.* **108**, 63–71
24. Bresnick, A. R., Janmey, P. A., and Condeelis, J. (1991) *J. Biol. Chem.* **266,** 12989–12993
25. Way, M., Pope, B., and Weeds, A. G. (1992) *J. Cell Biol.* **119,** 835–842
26. Hartwig, J. H., and Stossel, T. P. (1981) *J. Mol. Biol.* **145,** 563–581
27. Sobel, J. S., Goldstein, E. G., Venuti, J. M., and Welsh, M. J. (1988) *Dev. Biol.* **126,** 47–56
28. Winder, S. J., Walsh, M. P., Vasulka, C., and Johnson, J. D. (1993) *Biochemistry* **32,** 13327–13333
29. Winder, S. J., and Kendrick-Jones, J. (1995) *FEBS Lett.* **357,** 125–128
30. Jarrett, H. W., and Foster, J. L. (1995) *J. Biol. Chem.* **270,** 5578–5586
31. Tang, J., Taylor, D. W., and Taylor, K. A. (2001) *J. Mol. Biol.* **310,** 845–858
32. Pacaud, M., and Harricane, M. C. (1993) *Biochemistry* **32,** 363–374
33. Rhoads, A. R., and Friedberg, F. (1997) *FASEB J.* **11,** 331–340
34. Levine, B. A., Moir, A. J., Patchell, V. B., and Perry, S. V. (1992) *FEBS Lett.* **298,** 44–48
35. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680