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Molecular Basis of Filamin A-FilGAP Interaction and Its Impairment in Congenital Disorders Associated with Filamin A Mutations

Fumihiko Nakamura1*, Outi Heikkinen2, Olli T. Pentikäinen3, Teresia M. Osborn1, Karen E. Kasza4, David A. Weitz4, Olga Kupiainen2, Perttu Permi5, Ilkka Kilpeläinen2, Jari Ylänne3, John H. Hartwig1, Thomas P. Stossel1

1 Translational Medicine Division, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, United States of America, 2 Laboratory of Organic Chemistry, Department of Chemistry, University of Helsinki, Helsinki, Finland, 3 Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland, 4 Department of Physics & SEAS, Harvard University, Cambridge, Massachusetts, United States of America, 5 Program in Structural Biology and Biophysics, Institute of Biotechnology, University of Helsinki, Helsinki, Finland

Abstract

Background: Mutations in filamin A (FLNa), an essential cytoskeletal protein with multiple binding partners, cause developmental anomalies in humans.

Methodology/Principal Findings: We determined the structure of the 23rd Ig repeat of FLNa (IgFLNa23) that interacts with FilGAP, a Rac-specific GTPase-activating protein and regulator of cell polarity and movement, and the effect of the three disease-related mutations on this interaction. A combination of NMR structural analysis and in silico modeling revealed the structural interface details between the C and D β-strands of the IgFLNa23 and the C-terminal 32 residues of FilGAP. Mutagenesis of the predicted key interface residues confirmed the binding constraints between the two proteins. Specific loss-of-function FLNa constructs were generated and used to analyze the importance of the FLNa-FilGAP interface perturbs cell spreading. FilGAP does not bind FLNa homologs FLNb or FLNc establishing the importance of this interaction to the human FLNa mutations. Tight complex formation requires dimerization of both partners and the correct alignment of the binding surfaces, which is promoted by a flexible hinge domain between repeats 23 and 24 of FLNa. FLNa mutations associated with human developmental anomalies disrupt the binding interaction and weaken the elasticity of FLNa/F-actin network under high mechanical stress.

Conclusions/Significance: Mutational analysis informed by structure can generate reagents for probing specific cellular interactions of FLNa. Disease-related FLNa mutations have demonstrable effects on FLNa function.

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* E-mail: fnakamura@rics.bwh.harvard.edu

Introduction

Filamin A (FLNa), encoded in humans and mice by a gene on the X chromosome, is an abundant and ubiquitously expressed non-muscle isoform of a family of actin-cross-linking proteins [1]. Human melanoma cells lacking FLNa protein have unstable plasma membranes, do not polarize or undergo locomotion, and lack functional readouts for many of the identified FLNa-binding partners, but restoring normal levels of FLNa in these deficient cells rescues these functions [2–4]. Mutations of the FLNa gene were first identified in human periventricular nodular heterotopia (PVNH), an X-linked neuronal migration disorder that predominantly affects females and results in embryonic lethality in hemizygous males [5]. FLNa mutations are also associated with a group of X-linked skeletal anomalies including frontometaphyseal dysplasia (FMD) and cardiovascular defects such as familial cardiac valvular dystrophy, the most common indication for valvular surgery [6–8]. Complete loss of Flna in mice results in embryonic lethality with bleeding and cardiovascular malformations [9,10]. This wide range of phenotypes is presumably attributed to alterations of FLNa association with F-actin and its binding partners, obstructing analysis of mechanisms underlying FLNa pathogenesis.

FLNa is a dominant isoform of FLN family proteins (a, b and c) and all isoforms are dimers of 270–280 kDa subunits that have N-terminal spectrin-related actin-binding domains (srABD) separated from C-terminal dimerization domain by 23 Ig repeats organized as linear rod-like strands. Two flexible hinges separate Ig repeats 15 and 16 and 23 and 24 [1,11]. FLNa cross-links F-actin to form orthogonal networks that are responsible for cellular integrity and...
mechanics and attaches to membrane receptors including adhesion molecules and ion channels. FLNa is also a scaffold for numerous intracellular signaling intermediates. One of these, FilGAP, has a pleckstrin homology domain for membrane lipid binding, a GTPase-activating protein (GAP) domain, and a coiled-coil domain responsible for FLNa binding [12]. FilGAP specifically inactivates Rac in vitro, and this activity requires its FLNa association. FilGAP controls cell polarity and movement downstream of ROCK (Rho-kinase) [12].

Sorting out the contributions of these numerous interactions to in vivo function requires structural information to enable use of point mutant FLNa or partners lacking specific activities that are otherwise fully functional. Here we describe the structure of the FLNa/FilGAP complex and use the information to engineer mutant protein incapable of expressing FilGAP function in vivo. We also show that FilGAP interacts exclusively with FLNa among its homologs and specific disruption of the FLNa-FilGAP interaction perturbs cell spreading. Furthermore, we show that two FLNa mutations found in PVNH and FMD patients disrupt the folding of FLNa Ig repeat (IgFLNa) 23 and abolish FilGAP binding, establishing a link between downstream signaling of Rac and the patients’ pathogenesis.

Results

Identification of the FLNa-binding interface with FilGAP

Most of the protein constructs used for in vitro binding assay were obtained in good yield and purity (Figure S1A). Figure 1A shows a schematic diagram of FilGAP structure and demonstrates that the C-terminal 100 residues (649–748 amino acid, aa) of FilGAP tagged to a glutathione-S-transferase-hexahistidine (GST-His) interact with purified full-length FLNa in vitro, consistent with previous results [12]. Further deletion constructs identified that the last 32 residues (717–748aa, FilGAPC32), but not residues 649–729 of the predicted coiled-coil domain by EMBOSS COILS, are the FLNa-binding site (Figure 1A). FilGAPC32 tagged to a masking binding protein followed by hexahistidine (MBP-His), however, did not pull down with FLAG-FLNa (Figure 1B), and a synthetic peptide of FilGAPC32 immobilized on Sepharose also did not precipitate FLNa (data not shown). Analytical gel filtration demonstrated that full-length FilGAP is a dimer and FilGAP lacking residues 649–725, FLNa association. FilGAP controls cell polarity and movement downstream of ROCK (Rho-kinase) [12].

In silico model of the IgFLNa23-FilGAP complex

The CD faces of FLNa domains are common binding sites in other known filamin interactions, including platelet glycoprotein (GP) Ibα binding to IgFLNa17 [14] and integrin β subunit cytoplasmic tail binding to IgFLNa21 [15, 16]. As FilGAP also interacted with the CD face of IgFLNa23, and the amino acid sequence of FilGAPC32 could be aligned to the β-strand forming filamin-interacting peptides of GPIbα and integrins (Figure 3D), we modeled the FilGAPC32-IgFLNa23 interaction based on the complex between IgFLNa17 and a GPIbα peptide (Figures 3C and S4). To verify that the interaction site with FilGAP is on the CD face of IgFLNa23 we next mutated the hydrophobic M2474 to negatively charged glutamate. Indeed, the point mutation M2474E in IgFLNa23 abolished the interaction of full length FLNa with recombinant FilGAP C-terminal fragment and with full length FilGAP in vitro as predicted (Figure 4A). However, the mutant filamin fully retained F-actin gelation activity, and its morphology is indistinguishable from wild-type FLNa in electron micrographs (Figure S2). NMR spectra also showed that M2474E in IgFLNa23 was fully folded (Figure S3). Thus, this mutation further confirms that also in the context of full length FilGAP and FLNa, the major FilGAP binding site is at the CD face of IgFLNa23.

To confirm the position of the β-strand forming segment of FilGAP we mutated G730 and V734 to bulky aromatic residues. G730W and V734Y substitutions perturbed the interaction of FilGAP C-terminal fragments with the full-length FLAG-tagged FLNa, confirming that these residues are indeed oriented towards the interface (Figure 4B). The effect of V734Y substitution also suggests that we modeled the β-strand interface in correct position of FilGAP, since in our model this residue at the end of the β-strand.

The point mutation T728V had no effect in the interaction (Figure 4B).
Effect of FLNa-FilGAP interaction on cell spreading

To study the effects of FLNa and FilGAP mutations in vivo, full length proteins were expressed in filamin-deficient M2 cells. Point mutations of FLNa(M2474E) and FilGAP(V734Y) confirm that these residues are critical for the binding partnering interaction in vivo (Figure 5A). Transfection of either wild-type or V734Y mutant hemaglutin (HA)-FilGAP in FLNa-null M2 cells suppressed spreading on fibronectin-coated coverslips (Figure 5, B and C).
Co-expression of EGFP-FLNa with wild-type FilGAP diminished the effect of FilGAP, but expression of the mutant FilGAP did not have this effect (Figure 5, B and C).

FilGAP specifically interacts with FLNa

Although the overall structure of IgFLNa23 is similar to those of IgFLNb23 and IgFLNc23, immunoprecipitation of full-length FLNb did not pull down full-length FilGAP in vitro (Figure 6A) and the C-terminal of FilGAP interacted only with the C-terminal of FLNa, not FLNb and c (Figure 6B). There are two amino acid changes in the CD strands of FLNa versus FLNb: Ala2461 in FLNa is substituted to Thr in FLNb and Asp2467 is Glu in FLNb. (Figure 6C and D). A mutagenetic analysis demonstrated that the A2461T substitution explains the specificity between FLNa and FLNb (Figure 6E). It is likely that the principal interactions between FLNb and (FLNa:A2461T) and FilGAP are disturbed by the close proximity of two threonine side chains, since threonine is a bulky residue adjacent to the main chain (in Figure 6D wild-type FLNa is shown). In silico modeling suggests that amino acid residues comprising the CD strands of IgFLNc23 do not define the different specificity of FLNa and FLNb. Instead, substitution of Y2483, which is involved in hydrophobic contacts with FilGAP (Leu732 ) and surrounding amino acids in FLNa (Thr2454, Cys2476) to histidine alters the interaction (Figure 6D). It is also possible that Y2483 could donate a hydrogen-bond to the main-chain oxygen of T733 in FilGAP, thus, stabilizing FilGAP binding further. Such an interaction is not possible with a histidine in position 2483.

In vitro pull down experiments confirmed this prediction (Figure 6E).

Effects of disease-related mutations of FLNa on its cross-linking activity and FilGAP binding

A single de novo mutation, 7315C>G of the FLNA gene, which results in PVNH and FMD in females, leads to two transcripts, an L2439M point mutant and a deletion mutant lacking 7 amino acids (Δ7: L2439-G2445) within repeat 23 (Figure 7A) [6,7]. Another de novo mutation in repeat 23 is 7447del9 (or Δ3: Y2483-V2485, Figure 7A) and is associated with FMD in heterozygous females [6]. We tagged these transcripts with EGFP at their N-termini and expressed them in M2 cells with the same transfection
efficiency as wild-type FLNa, but observed no obvious differences in subcellular distribution. The expressed proteins were stable in vivo at least up to 72 h (data not shown). FLAG-tagged-FLNa mutants were expressed in sf-9 cells and purified in good yield (Figure S1A). These mutations do not affect dimerization and mutants were expressed in sf-9 cells and purified in good yield. IgFLNa23 could be obtained due to sample aggregation, suggesting that these deletions disrupt folding of the domain.

Structure of the FLNa-FilGAP complex
Since both the full-length FLNa and FilGAP are prone to aggregate at high concentrations requisite for crystallization (>2 mg/ml, data not shown), electron microscopy was used to study their structure at low concentrations (Figure S8). Purified FilGAP molecules appeared to be flexible dumbbells with two globules (~14.2+/−2.05 nm diameters; n = 50) orienting at various angles. The mixture of FLNa and FilGAP demonstrated that the globules attached to the C-termini of FLNa molecules, consistent with the biochemical findings, whereas FLNaM2474E did not complex with FilGAP. Since gel-filtration revealed that FilGAP molecules are dimers, each globule corresponds to one FilGAP molecule (Figure 8 and Figure S8).

Discussion
We have mapped the binding site between the Rac-specific GTPase-activating protein FilGAP and FLNa. We showed that the residues 723–736 interact with the CD face of IgFLNa23. Biochemical analysis indicated that dimerization of both FLNa and FilGAP and FLNa hinge 2 are required for effective interaction. Furthermore we showed that FilGAP is the first interaction partner that is specific to FLNa, as no binding to FLNb or FLNc was observed. Site-directed mutagenesis revealed that abolishing the FLNa-FilGAP interaction perturbs cell spreading. Disease-causing mutations that disrupt the folding of IgFLNa23 also obliterated FLNa-FilGAP interaction.

The FLNa-binding site of FilGAP is followed by the coiled-coil domain
We previously mapped the FLNa-binding domain of FilGAP to its C-terminal 100 residues (649–748aa) which were predicted to form a coiled-coil structure [12]. Although EMNet COILS program predicts three alternative coiled-coil segments (649–725, 648–729, 645–735), the biochemical data suggests that the minimum FLNa-binding site is not a coiled-coil because the last 32 residues (717–748) do not dimerize, and FilGAP lacking residues 648–725 is monomeric. Hence, these data indicate that the FLNa interaction site on FilGAP is outside of the coiled coil region and is available for the FLNa binding.

FilGAP interacts with the CD face of IgFLNa23
NMR titration experiments showed that FilGAP14 peptide (residues 723–736) interact with the CD-face of IgFLNa23. This interaction sequence is similar to the β-strand forming peptides of platelet GPβζ and integrins β2, β3 and β7 that interact with the corresponding faces of IgFLNa17 and IgFLNa21 [14–16]. In the binding interface between FLNa and FilGAP, the amino acids indicated with asterisks in Figure 3D (Phe726, Thr728, Gly730, Leu732, and Val734) face the groove formed between the C and D strands of the IgFLNa repeat, similar to the binding interaction of GPβζ and the β-integrins with FLNa. Further evidence for FilGAP’s use of a similar binding motif arc: 1) Phe726, Leu732, and Val734 in the FilGAP binding site are identical to the corresponding amino acids of GPβζ, which make strong hydrophobic contacts with FLNa [14]; and 2) the amino acid positions corresponding to Thr728 and Gly730 in FilGAP vary between GPβζ and integrin β. Serine occupies this position in GPβζ, and in most of the integrin β chains, and fits within the FilGAP-FLNa interface. The small size of Gly730 would not be expected to disrupt binding.
Based on the NMR analysis and our previous structures of IgFLNa17-GPIbα and IgFLNa21-integrin β7 complexes, we proposed a model for the interaction, and we verified it by introducing point mutations in both repeat 23 of full length FLNa and in FilGAP. The effects of G730W and V734Y mutations on the interaction suggest that these residues point towards the interface, as predicted in our model (Figure 3C). Because the P736 residue is at the end of modeled β strand, alignments where the peptide would be slipped backwards are not possible (Figure 3D). Alternatively, slipping the peptide forward would bring V734 to a position where mutation would not affect the interaction.

Higher order structure of the FLNa-FilGAP complex

Complexing of FilGAP with FLNa is not simply defined by the binding interface, because the interaction is diminished by deletions of 1) dimerization domain IgFLNa24, 2) FLNa hinge 2, and 3) FilGAP coiled-coil domain. The coiled-coil domain of FilGAP is a decamer when fused to an MBP-His tag, presumably because coiled-coil domains aggregate in a variety of conformations [17]. However, full-length FilGAP and FilGAP lacking its coiled-coil domain (residues 649–725) elute as a dimer and a monomer, respectively, on a gel filtration chromatography. Residues 649–729 do not bind FLNa, indicating that the coiled-coil interaction functions only to dimerize FilGAP. These data also suggest that FilGAP domains other than the coiled-coil domain allow dimerization while preventing oligomerization. Although NMR detects interaction of IgFLNa23 with FilGAP peptide, neither monomeric IgFLNa23 nor FilGAP biochemically pulled each other down, indicating that their affinities for each other are very low. Binding strength or avidity of macromolecules is governed by three major factors: the intrinsic affinity of the individual binding interface, the valency of the binding site(s), and geometric arrangement of the interacting components [11,18]. We have previously shown that dimerization of FLNa increases its affinity for F-actin by at least one order of magnitude. Considering that the apparent dissociation constant of FLNa-FilGAP of ~0.2 μM requires (assessed from ref. [11]), FLNa and FilGAP each to be dimers, and IgG antibody molecules bind ~1000 times more strongly than monovalent Fab subfragments to a antigen [18], the dissociation constant of IgFLNa23-FilGAP peptide interaction would be ~200 μM (0.2×~1000), making it difficult to detect in pull down assays. Our data demonstrated that IgFLNa24 and the coiled-coil domain of FilGAP mediate dimerization of FLNa and FilGAP, respectively, and define their geometric arrangements and valencies, thereby
increasing avidity of the FLNa-FilGAP complex. Moreover, FLNa hinge 2 (32aa residues) does not contribute to valency but defines the configurations that favor FilGAP binding (Figure 8). Electron micrographs of purified FLNa-FilGAP complex are consistent with the model.

Although no posttranslational modifications have been found so far in the FLNa-FilGAP binding sites, their higher order structure shown in Figure 8 suggests a possible mechanism for the regulation of this interaction by mechanical force. Our recent studies demonstrated that FLNa crosslinks F-actin using the conserved srABD and the second ABD that locates in rod segment 1 (rs1ABD), whereas the rod segment 2 is free from F-actin and accommodates the FilGAP interaction [11]. In addition, the geometry of IgFLNa23 defines its binding strength to FilGAP, suggesting that stretching or pushing of FLNa subunits attached to an F-actin network that is subjected to external or internal forces in vivo changes the geometry of two IgFLNa23 in FLNa dimer, hence affecting the avidity of the complex. The low stoichiometry of FLNa-FilGAP interaction determined in vitro and in cells (~0.02, assessed from ref. [11,12]) is in agreement with this idea. This mechanism may be in part responsible for FLNa’s reported role in cellular mechanoprotection [19].

Figure 6. FilGAP specifically interacts with FLNa isoform. (A) Full-length FLNa, but not FLNb, pull down FilGAP. Increasing amounts of either FLNa or FLNb were incubated with FilGAP and immunoprecipitated with mAbs to FLNa or FLNb. Bound FilGAP was detected by immunoblotting using rabbit pAbs to FilGAP. (B) MBP-FilGAP649-748 specifically binds the C-terminal of FLNa, but not FLNb or FLNc. Equal amounts of repeats 23–24 of FLNa, b, or c (0.2 μM) were pulled down with increasing amounts of MBP-FilGAP659-748. Proteins were visualized by CBB staining (top and bottom). The top CBB-stained gel was destained and restained with silver (middle). (C) Sequence alignment of the C–E strands of the IgFLN23 isoforms. FLNa A2461T, M2474E and Y2483H point mutants do not interact with FilGAP as shown in Figures 4A, 5A and 6E. (D) Model of the IgFLNa23-FilGAP complex. Residues mutated in this study and some critical residues for their interaction are indicated. The purple dotted line shows the possible stabilizing hydrogen-bond between Tyr2483 and Thr733. (E) A point mutation of FLNa at Ala2461 to Thr or Tyr2483 to His are sufficient to abolish the complexing of FLNa and FilGAP. Full-length FilGAP (input: 10 nM constant) was pulled down with increasing amount of GST-IgFLNa20-24 immobilized on glutathione beads in a dose-dependent fashion. Mutations corresponding to A2461T or Y2483H in FLNa, but not D2467E, disrupt FilGAP binding. Bound FilGAP was detected by immunoblotting using rabbit pAbs to FilGAP. GST-FLNa constructs were detected by CBB staining. doi:10.1371/journal.pone.0004928.g006
Effects of FLNa-FilGAP interaction on cell spreading

Overexpression of wild-type FilGAP in cells has been shown to abolish integrin-mediated cell spreading, and a mutant FilGAP lacking its C-terminal 100 residues does not colocalize with FLNa in lamellae [12]. In this study we generated a FilGAP(V734Y) point mutant sufficient to disrupt FLNa-binding while keeping the coiled-coil domain intact. Consistent with a previous report, wild-type FilGAP suppressed lamella formation and cell spreading. Co-expression of EGFP-FLNa with wild-type FilGAP but not with the mutant FilGAP(V734Y) reduced the suppression effect, presumably because the mutant FilGAP unable to bind FLNa diffuses throughout cell and suppresses Rac-activity broadly. These results suggest that the binding of FilGAP to FLNa is important for proper spatiotemporal control of FilGAP functions.

FilGAP is the first FLNa specific interaction partner

A mouse model and human developmental anomalies associated with FLNa mutations suggest that FLN isoforms have both redundant and distinct functions in cells and in situ due to their overlapping and unique distributions and partnering function [9,20–24]. Here we found that FilGAP specifically interacts with FLNa, whereas FLNb and FLNc sequences are quite similar to FLNa and some binding partners interact with multiple filamin isoforms [25]. Specificity is conferred by single substitutions of amino acids at Ala2461 to Thr and Tyr2483 to His in FLNb and c, respectively.

Disease-related FLNa mutations

Biological and rheological data suggest that two human disease-causing mutations (Δ7 and Δ3) impact on both signal transduction and mechanical deformation of cells. Since several binding partners have been reported to interact with the C-terminus of FLNa molecule, these mutations may perturb their interaction as well [1,26]. However, our data demonstrate that these mutations completely disrupt FLNa interaction with FilGAP, suggesting that the Δ7 and Δ3 mutations at least perturb downstream of Rac, thereby affecting cell spreading. The F-actin-FLNa networks stiffen as they are strained [27], preventing large deformations that could threaten cellular integrity. Indeed, substantial mechanical stress induces apoptosis of FLNa-null cells [19]. The reduction of
The structure explains how disease-causing mutations affect the complexing and mechanical properties of FLNa-actin networks, and suggests a novel mechanism of the regulation of FLNa-FilGAP interaction. Although we focused on the structure of the FLNa-FilGAP complex among over 50 binding partners identified thus far, this study reports first functional validation of FLNa-partner interactions in diseases using specific reagents informed by structural analysis.

Materials and Methods

Protein expression and purification
FLAG-tagged full-length FLNa was expressed using a Baculovirus Expression System (Invitrogen) in Sf9 insect cells and purified as previously described [11]. GST-His-FilGAP or MBP-His-FilGAP constructs were made by inserting PCR products of FilGAP domains to pGEX-4T-3-HT or pMALc-HT vectors [11], expressed in E. coli, and purified using glutathione, amylose, or Ni-NTA affinity columns as previously described [11]. Human full-length FilGAP fused to His-tag was expressed in Sf9 cells and purified using Ni-NTA affinity column. Tag-free FilGAP was prepared from His-FilGAP using TEV protease. His-tag FilGAP lacking 649–725 was expressed in E. coli (BL21(DE3)) using pET25-HTa vector [11] and purified by Ni-NTA affinity and Superose 6 gel filtration chromatography. GST-FLNa20-24 was expressed in E. coli using pGEX-4T-1 vector and purified by glutathione-Sepharose affinity chromatography. All the point or deletion mutants were generated using the QuickChange site-directed mutagenesis kit (Stratagene). His-IgFLNa23-24 was expressed in Sf9 cells and purified using Ni-NTA affinity column. After TEV cleavage, IgFLNb23-24 was gel filtered on Superdex200 10/300 column (GE healthcare). IgFLNa23-24 was prepared as previously described [28]. 15N-IgFLNa23 and 13C15N-IgFLNa23 and 15N-IgFLNa23 mutants were produced in E. coli as previously described [14]. For NMR studies the protein samples were buffered to pH 6.8 with 20 mM sodium phosphate. The samples also contained 150 mM NaCl, 1 mM DTT, 2 mM NaN3 and 7% (vol/vol) D2O.

Structure determination
NMR experiments for structure determination were recorded on Varian INOVA 500 MHz, 600 MHz and 800 MHz spectrometers equipped with 5 mm inverse z-gradient triple resonance probe heads at 25°C. The structure determination was performed with 1 mM 13C15N-labeled IgFLNa23 sample. VNMR 6.1C software was used in spectrum acquisition and processing (Varian Inc., Palo Alto, CA). The triple-resonance experiments recorded for backbone resonance assignment were ihHNC and ihHNCA, HNCA, HN(CO)CA, HNCACB, HN(CO)CACB and HNCO [29–31]. The side-chain resonance assignment for aliphatic residues was done using CC(CO)NH and HCCH-COSY spectra. The aromatic side-chain resonances were assigned using the distance information from 13C-edited three-dimensional NOESY spectrum. Structural restraints were extracted from 13C- and 15N-edited three-dimensional NOESY spectra. The structure visualization software Sparky 3.110 was used in spectrum analysis [32]. Structure determination was done with the automatic NOE assignment mode of CYANA 2.1 software [33]. The set of 20 best structures selected after torsion angle dynamics calculation were refined with molecular dynamics in AMBER 8.0 using generalized Born implicit solvent model [34]. The quality of the refined structures was analyzed by using the WHAT_CHECK [35] and PROCHECK [36]_NMR programs. Ramachandran plot statistics for the ensemble of 20 refined structures: most favored, 91.1%;
allowed, 8.0%; generously allowed, 0.6%; disallowed, 0.4%. The figures representing the protein structure were generated with MOLMOL [37] and PyMOL [38].

The folding state of IgFLNa23 mutants L2439M and M2474E was confirmed using $^{15}$N-HSQC spectra. The protein concentrations of the IgFLNa23-L2439M and IgFLNa23-M2474E NMR samples were 0.1 mM and 1 mM, respectively. The $^{15}$N-HSQC spectrum of IgFLNa23-M2474E was recorded on Varian INOVA 600 MHz spectrometer at 25°C. The $^{15}$N-HSQC spectrum of IgFLNa23-L2439M was recorded on Varian INOVA 600 MHz spectrometer equipped with 5 mm cryo probe at 25°C.

FilGAP titration

The NMR titration experiments were performed with 0.5 mM $^{13}$C$^{15}$N-labeled IgFLNa23 sample. The FilGAP peptide (FilGAP: sequence 723EQFFSTFGELTVEP736) was purchased from EZBiolab Inc. A concentrated solution (5 mM) of FilGAP peptide was prepared in protein buffer and pH was adjusted to 6.8 with 1 M NaOH. The titration experiments were performed on Varian INOVA 500 MHz spectrometer equipped with 5 mm reverse z-gradient triple resonance probe head at 25°C.

In silico modeling

The structure-based sequence alignment of IgFLNa23 and IgFLNa17 was made with Bodil [39]. The X-ray structure of IgFLNa17-GPbxz complex (pdb-code: 2BPS) [14] was used as a template structure to model the IgFLNa23 into peptide-binding conformation. The three-dimensional model of IgFLNa23-FilGAPC32 complex was constructed with Modeller 9v1 [40]. Coiled-coil domain structure was predicted with EMBnet COILS [http://www.ch.embnet.org/software/COILS_form.html]. Sequence alignment was generated using ClustalW program [http://www.ebi.ac.uk/Tools/clustalw2/index.html].

FLAG-FLNa pull-down assay

Various concentration of GST-His- or MBP-His-FilGAP constructs were incubated with 10 µl of FLAG-specific mAb M2 agarose (50% vol/vol slurry, Sigma) in the presence or absence of 25 nM FLAG-FLNa and in binding buffer (50 mM Tris-Cl, 150 mM NaCl, 0.1% (wt/vol) Triton X-100, 0.1 mM β-mercaptoethanol, 0.1 mM EDTA, pH 7.4, 400 µl) for 1 h at 25°C. The beads were sedimented and washed 3 times with binding buffer. Proteins bound to the beads were solubilized in SDS sample buffer and separated by 12.5% (wt/vol) SDS-PAGE followed by immunoblotting using rabbit polyclonal antibodies (pAbs) against GST (Sigma), MBP (New England Biolabs), or FilGAP [12] or mouse mAb against His conjugated with horse radish peroxidase.

In vitro immunoprecipitation

Purified FilGAP (50 nM) were incubated with increasing amounts of either FLNAs or FLNb in binding buffer and co-immunoprecipitated with mouse mAb to FLNa (5 µg each of 3-14 and 4-3) [11] or FLNb (10 µg of 1-11c) [14] immobilized on 20 µl of GammaBind G-Sepharose beads (GE healthcare) for overnight at 4°C. The beads were sedimented and washed 3 times with binding buffer. Proteins bound to the beads were solubilized in SDS sample buffer and separated by 12.5% (wt/vol) SDS-PAGE followed by immunoblotting using rabbit pAbs to FilGAP.

MBP-FilGAP pull-down assay

FLNa or its truncates were incubated with 30 µl of amylase resin (50% vol/vol slurry, New England Biolabs) coated with various concentration of MBP-FilGAP 649–748 in the binding buffer for 1 h at 25°C. The beads were sedimented and washed 3 times with binding buffer. Proteins bound to the beads were solubilized in SDS sample buffer and separated by 9% or 12.5% (wt/vol) SDS-PAGE. Polypeptides in the gels were visualized by Coomassie brilliant blue (CBB) staining or immunoblotting.

FLNa and FilGAP mammalian expression vectors

The Clal/Xbal FLNa fragment of pEGFP-FLNa [14] vector was cloned into pBlueScript II SK vector, mutated using the QuickChange site-directed mutagenesis kit, and then cloned back to pEGFP-FLNa or pREP4-FLNa[41] vectors. The pCDNA5-HA-FilGAP [12] was directly mutated using the QuickChange site-directed mutagenesis kit.

Cell culture and immunoprecipitation

Human melanoma FLNa-deficient cell lines (M2) were transfected with the pCDNA5-HA-FilGAP (wild-type or V734Y) and the pREP4-FLNa (wild-type or M2474E) using FuGENE 6 as described by the manufacturer’s instructions (Roche applied science). Forty-eight hours later, the cells were rinsed three times with PBS and lysed in 50 mM Tris-Cl, 150 mM NaCl, 1% (wt/vol) Triton X-100, 1 mM β-mercaptoethanol, 5 mM EGTA, 10 µg/ml leupeptin, pepstatinA, aprotinin, 2 mM PMSA, 0.1 mg/ml DNAseI. The cell lysates were pre-cleared and subjected to immunoprecipitation with antibodies to FLNa [42] to precipitate transfected FLNa. Bound protein was detected by western blotting using mouse mAbs to FLNa for FLNa and rat mAb to HA (Roche applied science) for FilGAP.

Cell spreading assay

M2 cell lines were transfected with the pCDNA5-HA-FilGAP (wild-type or V734Y) and/or pEGFP-FLNa [14] using FuGENE 6. After 48 h, cells were trypsinized and plated on coverslips coated with 10 µg/ml human fibronectin (Sigma) and fixed at indicated time with 3.7% (wt/vol) formalin in PBS for 30 min. The fixed cells were permeabilized with 0.5% (wt/vol) Triton X-100 in PBS for 5 min and blocked with 0.2% (wt/vol) BSA, 0.1% (wt/vol) sodium azide and 0.1% (wt/vol) Triton X-100 in PBS for 1 h. HA-FilGAP was stained with mouse mAb to HA (HA-7, Sigma) at 1:500 dilution and mouse IgG-specific goat pAbs conjugated with Alexa-488 (Invitrogen) at 1:400 dilution. Spread cells were examined as described previously [12].

Gel-filtration Analysis

Purified FLNa constructs were loaded on a Superose 6 10/300 GL column (GE healthcare) and eluted at a flow rate of 0.4 ml/min in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 mM EGTA, 0.5 mM β-mercaptoethanol at 4°C. Molecular size standards used were thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), and ovalbumin (43 kDa).

Rheology

To probe the mechanical properties of F-actin-/FLNa networks, we employed a stress-controlled mechanical shear rheometer (Bohlin, Malvern Instruments) equipped with a 40-mm parallel plate geometry and a gap of 100 µm as previously described [27]. Briefly, 12 µM monomeric actin was mixed with 0.06 µM wild-type or mutant FLNa and polymerization buffer (total volume 150 µl) and immediately placed between the stainless steel plates of the rheometer. The sample was allowed one hour to polymerize before measuring the dynamic viscoelastic moduli.
Gel points were measured as previously described [43].

Accession codes
The resonance assignments and the distance restraints used in IgFLNa23 NMR structure calculation have been deposited in BioMagResBank under accession number 15777. The coordinates of the structure ensemble have been deposited in Protein Data Bank under accession code 2k3t.

Supporting Information
Figure S1  Physical properties of the recombinant proteins. (A) Coomassie blue stain of 8–16% gradient Tris-Glycine SDS-PAGE of each purified recombinant protein (0.5 mg). (B) Gel filtration analysis of FilGAP constructs on Superose6 10/300 GL. Apparent molecular weight (Mr) were determined from elution volumes by comparing to those of throglobulin, ferritin, aldorase, conalbumin, ovalbumin, and ribonuclease A (left to right).

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Figure S2 Electron micrographs of the purified FLNa and its mutants. Structure of full-length FLNa and mutant FLAG-FLNa were determined by low angle rotary shadowing of molecules sprayed onto mica and dried under vacuum.

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Figure S3 NMR titration of FilGAP peptide to IgFLNa23. (A) 15N-HSQC spectrum of IgFLNa23+660%FilGAP14 (blue) superimposed on 15N-HSQC spectrum of IgFLNa23 (red). (B) Chemical shift changes in the 15N-HSQC spectrum of IgFLNa23 upon addition of 6.6-fold excess of FilGAP14 as a function of sequence. Black = overlapping signal which could not be traced reliably. Light gray = signal has broadened beyond detection or it has divided into multiple peaks. The chemical shift difference was set to 0.22 ppm.

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Figure S4 Comparison of IgFLNa23, IgFLNa17 and IgFLNa17-Gplbαx complex. Based on the X-ray structure of IgFLNa17-Gplbαx complex (pink ribbon), it was possible to build a model for IgFLNa23-FilGAP C32 complex (not shown) where the IgFLNa23 is in such conformation that it can bind the FilGAP C32 peptide. When compared to the NMR structure of IgFLNa23 (gray ribbon) and IgFLNa17 (cyan ribbon), peptide binding seems to force the C and D strands further away from each other, especially between the N-terminus of C strand and C-terminus of D-strand. The binding of FilGAP peptide to IgFLNa23 reflects at the structural level particularly to the conformation of the Y2483 side-chain that is pushed aside to allow the binding of peptide binding. Chemical shift changes of Y2483 are seen in the FilGAP peptide NMR titration experiments (Figure 3 and Figure S3).

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Figure S5 Chemical shift changes induced to the 15N-HSQC spectrum of IgFLNa23 by M2474E mutation. (A) Superimposition of the 15N-HSQC spectra of IgFLNa23 (red) and M2474E IgFLNa23 (blue). (B) Chemical shift difference as a function of sequence. Black = mutated residue, light gray = signal has shifted too much to be identified without complete reassignment. The chemical shift difference was set to 0.4 ppm. (C) Chemical shift differences exceeding 0.1 ppm mapped on the structure of IgFLNa23. The mutated residue is indicated with stick model.

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Figure S6 Rheological properties of 12 μM F-actin networks cross-linked with 0.06 μM purified FLNa and its mutants. (A) The linear elastic moduli, G' (closed circles), and viscous moduli, G″ (open circles), as a function of frequency. (B) The nonlinear elastic moduli as a function of strain, measured at f=0.1 Hz. (C) The nonlinear elastic moduli as a function of applied stress, measured at f=0.1 Hz.

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Figure S7 Chemical shift changes induced to the 15N-HSQC spectrum of IgFLNa23 by L2439M mutation. (A) Superimposition of the 15N-HSQC spectra of IgFLNa23 (red) and L2439M IgFLNa23 (blue). (B) Chemical shift difference as a function of sequence. Black = mutated residue, light gray = signal has shifted too much to be identified without complete reassignment. The chemical shift difference was set to 0.4 ppm. (C) Chemical shift differences exceeding 0.1 ppm mapped on the structure of IgFLNa23. The mutated residue is indicated with stick model.

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Figure S8 Electron micrographs of the purified FLNa and FilGAP. Rotary shadowed images of purified FilGAP (top), FLNa (second from the top), the FLNa/FilGAP complex (third from the top), mutant FLNaM2474E mixed with FilGAP (the second from the bottom), and FLNaM2474E (bottom). FLNa and FilGAP were mixed at a 1FLNa:20FilGAP ratio for 1 h end-over-end at room temperature, diluted 1:4 in 67% glycerol to a final protein concentration of 25 μg/ml (50% glycerol), and sprayed onto mica as previously described. About 26% (26 / 100) of wild-type FLNa molecules were complexes with FilGAP, whereas FLNaM2474E mutant molecules were not.

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Table S1 NMR and refinement statistics for IgFLNa23.

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Author Contributions
Conceived and designed the experiments: FN OH OTP IK JY JHH TPS. Performed the experiments: FN OH OTP TMO KEK PP JY. Analyzed the data: FN OH OTP TMO KEK OK PP JY. Contributed reagents/materials/analysis tools: FN OH OTP DW IK JY JHH TPS. Wrote the paper: FN OH OTP TMO KEK PP JY JHH TPS.

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