A New Member of the LIM Protein Family Binds to Filamin B and Localizes at Stress Fibers*

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Toshiro Takafta†‡§, Mari Saeki†‡§, Tetsuro-Takahiro Fujimoto†, Kingo Fujimurai†, and Sandor S. Shapiro†‡¶

From the †Department of Medicine, Cardeza Foundation for Hematologic Research, Jefferson Medical College, Philadelphia, Pennsylvania 19104; the ‡Department of Clinical and Laboratory Medicine, Yamanashi Medical University, Yamanashi 498-3898, Japan; and the §Department of Clinical Pharmaceutical Science, Graduate School of Medicine, Hiroshima University, Hiroshima 734-8551, Japan

Human filamins are 280-kDa proteins containing an N-terminal actin-binding domain followed by 24 characteristic repeats. They also interact with a number of other cellular proteins. All of those identified to date, with the exception of actin, bind to the C-terminal third of a filamin. In a yeast two-hybrid search of a human placental library, using as bait repeats 10–18 of filamin B, we isolated a cDNA coding for a novel 374 amino acid protein containing a proline-rich domain near its N terminus and two LIM domains at its C terminus. We term this protein filamin-binding LIM protein-1, FBLP-1. Yeast two-hybrid studies with deletion mutants localized the areas of interaction in FBLP-1 to its N-terminal domain and in filamin B to repeats 10–13. FBLP-1 mRNA was detected in a variety of tissues and cells including platelets and endothelial cells. We also have identified two FBLP-1 variants. Both contain three C-terminal LIM domains, but one lacks the N-terminal proline-rich domain. Transfection of FBLP-1 into 293A cells promoted stress fiber formation, and both FBLP-1 and filamin B localized to stress fibers in the transfected cells. The association between filamin B and FBLP-1 may play a hitherto unknown role in cytoskeletal function, cell adhesion, and cell motility.

The human filamin (FLN) family consists of three ~280-kDa actin-binding paralogs (FLNs A, B, and C), each containing an N-terminal actin-binding domain followed by 24 characteristic ~96 residue β-sheet pleated sheet homologous repeats (1–3). In addition, each FLN contains two interleaved 24–36 residue “hinge” regions, the first between repeats 15 and 16 and the second between repeats 23 and 24. FLNs self-associate to form homodimers, an interaction that requires the presence of repeat 24.

Current data suggest that FLNs may have multiple functions. 1) They are involved in the organization of the cytoskeleton (4, 5) and appear to be necessary for normal cell adhesion and motility (6). 2) They bind to the cytoplasmic tails of a number of membrane proteins including several β-integrins (7, 8), Gplb (9), and tissue factor (10), suggesting that they play a role in cytoskeletal-membrane interactions. 3) They associate with several cytoplasmic components of signaling pathways including Rho GTPases (11), TRAF2 (12), Smads (13), and SEK-1 (14) and thus may serve as a scaffolding or point of convergence for signals between the cell membrane and the cell interior. Nevertheless, a clear mechanistic explanation for their importance is still lacking.

Most interactions with FLNs have been identified as a result of yeast two-hybrid studies using as bait a variety of membrane or cytoplasmic proteins. In this manner, one or another filamin has been identified as an interactant with some 25 proteins (2, 15–19). In every case where localization studies have been performed, the interaction site on the filamin has been in the C-terminal third of the molecule. Because the libraries that were screened tend to be relatively enriched in 3’-sequences, it seemed possible that interactions with the more N-terminal portions of the filamins might have been missed. Accordingly, we embarked on a yeast two-hybrid search using as bait FLNB repeats 10–18. We report here the identification and characterization of a new member of the LIM protein superfamily, filamin-binding LIM protein-1 (FBLP-1), that interacts with FLNB proximal to the binding site of all of the other known filamin interactants with the exception of actin. The interaction between FLNB and FBLP-1 may play an important role in actin cytoskeleton organization.

MATERIALS AND METHODS

Two-hybrid Library Screening and Full-length cDNA Cloning—During the cloning of full-length FLNB, we had previously isolated from a human placental cDNA library a cDNA fragment (Fig. 5, construct 1) designated FLNB-(10–18), which codes for repeats 10–18 (residues 1128–1856) of FLNB but lacks the first hinge domain (residues 1704–1727) because of alternative splicing. FLNB-(10–18) was directionally cloned into the yeast expression vector pGBK77 (Clontech, Palo Alto, CA). This construct expresses a fusion protein consisting of repeats 10–18 of FLNB and the yeast GAL4 DNA-binding domain.

A yeast two-hybrid human placental cDNA library (in pACT2, Clontech) was screened. Yeast strain AH109 was transformed sequentially with the FLNB-(10–18) vector and the library vectors. Transformants were plated on SD/Tep/Leu/His/Ade plates and incubated at 30 °C until colonies appeared. These colonies were re-streaked on plates containing X-gal to determine α-galactosidase activity of individual colonies. To exclude false positives, positive clones were re-introduced into yeast together with either the FLNB-(10–18) vector or pLAM5; a vector encoding a human lamin cDNA in the two-hybrid DNA-binding domain vector pGBK77.

To obtain the remaining 5’ end of the cDNA, double-stranded cDNA
was synthesized from human placental poly(A)⁺ RNA and subjected to 5' RACE using a Marathon cDNA amplification kit (Clontech). After ligating the Marathon adaptor to the 5' ends of the double-stranded cDNA, two-step (nested) PCR amplification was performed using two adaptor primers and two gene-specific primers: FP4, 5'-ACAAACTGCCTGCCTCAC-3', and FP4-in, 5'-ACTTCCTCCGCCACGGCCACATC-3'. A 600-bp PCR product was extracted from agarose gel, transferred to pT7BLUE (Novagen, Madison, WI), and sequenced. Isolated cDNA

**FBLP-1**

![Image of FBLP-1](image1)

**FBLP-1A, 1B**

![Image of FBLP-1A, 1B](image2)

Fig. 1. Nucleotide and deduced amino acid sequences of human FBLP-1 cDNA. Nucleotide numbers and amino acid numbers are indicated above the sequence (nucleotide/amino acid). The in-frame stop codon in the 5'-upstream sequence is underlined. Two LPPPP amino acid repeats are boxed. The nuclear export signal sequence is indicated by a double underline. The sequence of FBLP-1 3' to the open arrowhead is spliced out and connected to the sequence shown below in both FBLP-1A and FBLP-1B. The sequence demarcated by the two black arrows is spliced out in FBLP-1B.
IgG was purified by passage over a column of fusion protein coupled to ECL detecting system (Amersham Biosciences). Repeats 14 were detached by 0.5M EDTA-PBS, washed with PBS, and lysed in radioimmune precipitation assay buffer (1% Triton X-100, 0.5% deoxyribonuclease, 0.1M sodium fluoride, 10 µg/ml leupeptin, pH 7.5) on ice for 15 min. After centrifugation at 15,000 for 10 min, lysates were pre-cleaned by incubation with protein G-agarose (Pierce) at room temperature for 1 h.

Antibodies—A glutathione S-transferase-FNLB fusion protein expression vector was constructed by subcloning a FNLB cDNA coding repeats 14–24 (residues 1519–2602), including the first hinge, into pGEX-5X-3 (Amersham Biosciences). A rabbit polyclonal antibody was prepared by immunizing with the fusion protein. IgG was purified by protein G-Sepharose, the anti-glutathione S-transferase fraction was removed on a glutathione S-transferase-Sepharose column, and specific IgG was purified by passage over a column of fusion protein coupled to Sepharose.

Mammalian Transfection and Immunoprecipitation—As a transfection control, we used the GFP expression vector pEGFP-N1 (Clontech) without any insert. Transient transfection of human fibroblast 293 cells was performed using the calcium phosphate method. Transfected cells were detached by 0.5M EDTA-PBS, washed with PBS, and lysed in radioimmune precipitation assay buffer (1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 30 mM Tris-HCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, pH 7.5) on ice for 15 min. After centrifugation at 15,000 for 10 min, lysates were pre-cleaned by incubation with protein G-agarose (Pierce) at room temperature for 1 h. Polyclonal anti-FNLB-(14–24) or polyclonal anti-GFP (Clontech) was incubated with protein G-agarose for 90 min at room temperature and washed five times with Tris-buffered saline. The pre-cleaned lysates were mixed with the antibody-agarose complexes and incubated overnight at 4 °C. Immunoprecipitates were washed five times in Triton X-100 buffer and boiled in SDS-PAGE sample buffer. Each sample was separated on a 5 or 7.5% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and incubated with anti-FNLB-(14–24) or monoclonal anti-GFP (Medical & Biological Laboratories, Nagoya, Japan). Antibody binding was detected using peroxidase-conjugated secondary antibodies and was visualized with the ECL reagent (Amersham Biosciences).

Immunohistochemistry—The 293A cell line we used, a subclone of the 293 cell line, was kindly provided by Dr. Jeffrey Benovic (Kimmel Cancer Institute, Jefferson Medical College). This subclone has greater adhesive and spreading ability than the parental 293 cells. Transient transfection was performed using Effectene reagent (Qiagen). After transfection, fixed and permeabilized cells were incubated with 3% bovine serum albumin-PBS for 30 min at room temperature and then incubated with anti-FNLB-(14–24), anti-FNLB monoclonal antibodies Ti10 and FM6917 (Biosign, Saco, ME), or the anti-vinculin monoclonal antibody IV8-1 (Sigma). After washing with 0.1% bovine serum albumin-PBS and further incubation with 3% bovine serum albumin-PBS, the cells were incubated with Cy3-labeled goat anti-rabbit or anti-mouse IgG (Sigma). Other cells not incubated with antibodies were incubated with TRITC-labeled phallolidin (Sigma). Stained cells were visualized by inverted fluorescence microscopy (Olympus, Tokyo, Japan).

RESULTS

Isolation of FBLP-1—Using as bait FNLB-(10–18), we screened 2 × 10⁵ clones of a human placental two-hybrid library and isolated seven positives. To eliminate false-positives, we re-introduced these clones into yeast togetether with either the FNLB-(10–18) bait or pLAM5'. Six clones showed transactivation of HIS3, ADE2, and LacZ in the presence of the FLNB bait. However, five of the six also interacted with the lamin bait and were considered nnspecific. The sixth clone, FPC1–22, which reacted specifically with FLNB-(10–18), contained a 1.3-kilobase insert terminating in a poly(A) sequence and was sequenced.

As shown in Fig. 1, the sequence of FPC1–22 predicts a polypeptide consisting of 374 amino acids. Since upon sequencing, no in-frame termination codon was found upstream of the putative first ATG codon, we performed 5'-RACE, using human placental poly(A)+ RNA and identified an in-frame stop codon 72 bp upstream of this ATG. Thus, this ATG was designated as the initiation codon. We term this protein human FBLP-1 (filamin-binding LIM protein-1 (GenBank™ accession number AF459643)). FBLP-1 has a proline-rich domain near its N terminus, two LIM domains, and a unique ~70 amino acid sequence at its C terminus. As shown in Fig. 2, the structure of FBLP-1 is similar to that of the zyxin family of proteins (zyxin, LPP, and trip6) and more weakly homologous to other LIM family members (paxillin, hic-5, and leupaxin). FBLP-1 has two (F/L)PPP motifs in its proline-rich domain. In other LIM family members, these motifs serve as a ligand for a subset of Ena-VASP homology 1 domain proteins. FBLP-1 also contains a leucine-rich nuclear export signal as indicated in Fig. 1. This nuclear export signal motif (LXXLXXL) is also present in zyxin family members and is thought to play a role in nuclear-cytoplasmic trafficking.

Using the BLAST program to search the GenBank database, we identified two closely related full-length cDNAs (GenBank™ accession numbers AK055259 and AK027444) coding for hypothetical proteins (Fig. 3). Both predicted proteins have three C-terminal LIM domains, whereas AK027444 lacks a proline-rich domain. In this paper, we refer to AK055259 and AK027444 as FBLP-1A and FBLP-1B, respectively.

By searching the data base, we found a genomic DNA sequence (GenBank™ accession number AL450998) from bacterial contigs of human chromosome 1 that contains the sequence of FBLP-1. On the basis of this sequence, the exon-intron organization of the FBLP-1 gene can be depicted as shown in Fig. 3. The proline-rich domain of FBLP-1 is a product of exons 3 and 4, the first and second LIM domains are produced by exons 5 and 6a, and the C-terminal unique sequence is produced by exon 6b. A consensus sequence for alternative splicing is present at the junction of exons 6a and 6b. Exon 6b is spliced
out in FBLP-1A and FBLP-1B. The additional LIM domain of these two variants is coded by exon 7. Exons 3 and 4 are spliced out in FBLP-1B, resulting in the deletion of the proline-rich domain.

During this analysis, we observed that an STS marker, WI-14085, was part of the gene. This marker has been linked to microsatellite marker D1S3543 in 1p36.13. These data have been grouped as UNIGENE Hs.8728, UniSTS SHGC-13447, and LocusLink 54751.

Verification of the Interaction Between FLNB and FBLP-1—To confirm the interaction between FLNB and FBLP-1 at the protein level, we transiently co-transfected 293A cells with full-length FLNB and either GFP or GFP-FBLP-1. After 48 h, cells were lysed with radioimmune precipitation assay buffer and immunoprecipitated with anti-GFP IgG. Western blotting was performed with anti-GFP or anti-FLNB-(14–24) IgG (Fig. 4). The anti-GFP IgG precipitated equally GFP alone and GFP-FBLP-1; however, FLNB was detected only in precipitates from the GFP-FBLP-1 co-transfectants. A large amount of FLNB was still present in the supernatant after immunoprecipitation. We also performed the reverse immunoprecipitation using anti-FLNB IgG and detecting with anti-GFP IgG; however GFP-FBLP-1 was not detected in the immunoprecipitate (data not shown). These data suggest that only a small fraction of FLNB is associated with FBLP-1, although most of the FBLP-1 is associated with FLNB.

Localization of the Interacting Domains of FLNB and FBLP-1—To identify the domain interacting with FBLP-1, each of several subdomains of FLNB was introduced into yeast together with FPC1–22, and X-a-gal assays were performed. As shown in Fig. 5A, only constructs 1 and 2 interacted specifically with FPC1–22. This result indicates that FBLP-1 binds to FLNB between repeats 10 and 13.

To identify the domain of FBLP-1 interacting with FLNB, each of the FBLP-1 constructs shown in Fig. 5B was introduced into yeast together with FLNB-(10–18) in pGBK7. As shown in the figure, the N-terminal 70 amino acid sequence (before the proline-rich domain) contains the filamin-binding domain. Yeast transformed with FLNB-(10–18) and FBLP-1A or FBLP-1B were slow-growing and produced small colonies on
plates, although both were positive in the X-gal assay. As these transformed yeast grow slowly even on Trp/Leu plates, it is possible that the third LIM domain present in these two variants reduces the yeast growth rate.

**Tissue and Cellular mRNA Expression of FBLP-1 and Variants**—Using primers FP3 and FP7, a 206-bp fragment of FBLP-1 was amplified (Fig. 6, *upper panel*). Primers FP3 and FP8 amplified a 531-bp fragment of FBLP-1; however, some extra products were observed (data not shown). Primers FP3 and FP9 designed to amplify the common region of FBLP-1A and FBLP-1B amplified a 498-bp fragment from both FBLP-1A and FBLP-1B (Fig. 6, *lower panel*). FBLP-1 was detected in most tissues tested with the except of liver; however, FBLP-1A and FBLP-1B could not be detected in brain, liver, or skeletal muscle. FBLP-1 was detected both in platelets and the megakaryocyte-like cell line, CMK, although FBLP-1A and FBLP-1B were detected only in platelets.

**Intracellular Localization of FBLP-1**—To evaluate the cellular localization of FBLP-1, 293A cells were transfected with GFP-FBLP-1 and plated on uncoated plastic culture dishes (Fig. 7). Although 293A cells spread more readily than the parental 293 cell line, 293A cells demonstrate limited numbers of stress fibers when grown on uncoated plastic culture dishes.

The overexpression of FBLP-1 markedly increased stress fiber formation (Fig. 7, *A*–*F* versus *I*). Furthermore, both FLNB and FBLP-1 are localized to stress fibers in the transfected cell (Fig. 7, *A*–*C*, *left*). In contrast, FLNB is distributed diffusely throughout the cytoplasm in the non-transfected cell (Fig. 7C, *right*). FBLP-1 co-localized with F-actin stained by phalloidin (Fig. 7H) on stress fibers. In the GFP-transfected control, GFP was present diffusely throughout the nucleus and cytoplasm (Fig. 7, *I* and *J*). These results strongly suggest that FBLP-1 may dynamically support the reorganization of stress fibers. FLNA also co-localized with FBLP-1 to stress fibers (Fig. 7, *D*–*F*). Although we have not examined directly the interaction of FBLP-1 and FLNA, this observation suggests that such an interaction occurs.

To compare the localization pattern of FBLP-1 with zyxin, 293A cells grown on gelatin-coated plates were transfected with GFP-FBLP-1. All of the images are of the same transfected cell. Lower images are higher magnification views of upper images. FBLP-1 was detected as GFP signal (*green*), and vinculin was detected by anti-vinculin antibody (*red*).
with GFP-FBLP-1 or GFP-zyxin. As shown in Fig. 8, GFP-FBLP-1 (green color) strongly localized to stress fibers (Fig. 8, A, C, and E) but not to filopodia (solid arrowheads) or lamellipodia (open arrowhead). On the other hand, GFP-zyxin localized at focal adhesions and at filopodia and lamellipodia (Fig. 8F, orange) but not on stress fibers (Fig. 8, B, D, and F). Because other members of the zyxin family have been reported to localize at focal adhesion plaques, GFP-FBLP-1-transfected cells were stained with anti-vinculin antibody to identify focal adhesion plaques (Fig. 9). Although weak signals were detected at focal adhesions, GFP-FBLP-1 localized preferentially to stress fibers. Vinculin, on the other hand, localized largely to focal adhesions in the transfected cells.

**DISCUSSION**

Filamins are represented in almost all living species, usually as a small family of paralogs. As already mentioned, filamins are involved in the organization of the cytoskeleton and appear to be necessary for normal cell adhesion and motility. Furthermore, they bind to the cytoplasmic tails of a growing number of membrane proteins, suggesting that they play a role in cytoskeletal-membrane interactions. In addition, they bind several cytoplasmic components of signaling pathways and may serve as a point of convergence for signals between the cell membrane and the cell interior.

Filamin A (ABP-280) and chicken gizzard filamin were the first members of the superfamily to be identified and were originally characterized as proteins that promote actin filament bundling in F-actin solutions. FLNA appears to promote orthogonal actin filament organization (5), whereas chicken gizzard filamin appears to induce parallel bundling of actin filaments (20). Flanagan et al. (21) demonstrated that in cells expressing FLNA, this protein plays an essential role in the stabilization of orthogonal actin networks required for locomotion. Recently, we reported (8) that a splice variant of FLNB interacts with the cytoplasmic domain of the integrins β1, A and β1, D at the tips of stress fibers, suggesting that filamins interact with these proteins and may organize actin filaments depending on the state of cell activation. However, a large body of recent literature concludes that the Arp2/3 complex has a predominant intracellular role in promoting the formation of orthogonal actin networks, at least at the leading edge of the cell (22), and that an actin filament network, which resembles closely that seen in cells, can be produced in cell-free systems in the absence of filamins (23). In Drosophila, the Arp2/3 complex is required for specific subsets of actin polymerization events (e.g., ring canal formation during oogenesis); however, the formation of parallel actin bundles apparently does not depend on Arp2/3 function (24). From study of the Drosophila cheerio mutant, the suggestion has been made that a branching F-actin network may be transformed into parallel bundles through the action of filamin, the product of the cheerio gene (25). Because both the Arp2/3 complex and filamins localize in sites of actin reorganization (22), it is possible that the Arp2/3 complex synergizes in some manner with one or more of the filamins.

Actin cytoskeleton organization is orchestrated both by polymerization of actin monomers and by cross-linking of the resultant actin filaments. The bacterial pathogen, *Listeria monocytogenes*, has been very instructive in studying several facets of these phenomena (26). This pathogen grows an F-actin “tail” or “comet” after cellular entry, a phenomenon dependent on a surface protein, ActA (27). ActA has an N-terminal domain that interacts with the Arp2/3 complex (28, 29) followed by a proline-rich domain that interacts with Ena/VASP family proteins (30). The Ena/VASP proteins, in turn, recruit actin monomers bound to profilin and accelerate actin polymerization (31). The organization of F-actin induced by ActA is strikingly similar to the actin cytoskeletal organization that occurs at the leading edge of lamellipodia in normal cells. Several human proteins have properties similar to ActA. The zyxin family proteins that include zyxin (32), LIM-containing lipoma-preferred partner (LPP) (33), and the trip6 protein (thyroid receptor-interacting protein-6) (34) have N-terminal proline-rich domains that contain the same (P/IVP)PPP motifs present in ActA, and these proteins can bind to Ena/VASP proteins. The VCA (Verprolin homology, Cofilin homology, Acidic) domain of the WASp (Wiscott-Aldrich syndrome protein) family is similar to the N-terminal domain of ActA and can interact with Arp2/3 (35). It was reported recently that VASP binds WASp directly in vitro, an interaction that may be essential in stimulating actin assembly and membrane protrusion at the leading edge (36). The zyxin, VASP, and WASp family proteins are all found at sites of cell adhesion and in lamellipodia.

LIM domains were first identified in three transcription factors, lin-11, jsl-1, and mec-3, but have since been identified in many other proteins including the paxillin and zyxin families, LIM kinases, and LIM-containing transcription factors (37, 38). The LIM domain is a cysteine- and histidine-rich, zinc-coordinating domain and functions in mammalian cells as a protein-protein interaction motif and probably as a DNA binding motif as well. Proteins containing LIM domains may play important roles in a variety of biological processes including cytoskeletal organization, cell development, and signaling (38). The LIM domains of paxillin bind to protein tyrosine phosphatase-PEST and support the function of this molecule at focal adhesions (39). Paxillin also binds to α-tubulin and may facilitate cross-talk between actin filaments and microtubules (40). Hic-5 has been reported to bind to DNA in a zinc-dependent manner through its LIM domains and may regulate transcription of several proteins (38).

In this study, we have identified a new FLNB-binding protein, FBLP-1, that contains both a proline-rich domain and LIM domains. Unlike several other LIM family members we have tested, FBLP-1 has the ability to bind to FLNB and to induce stress fiber formation. Thus, FBLP-1 may play a role in the dynamic organization of stress fibers and perhaps other aspects of the cytoskeleton. The role of its LIM domains in this activity remains to be explored. Furthermore, it is possible that FBLP-1 may interact with its proline-rich domain, FBLP-1 may interact with Ena/VASP family members and, thus, may provide a link between filamins and the Arp2/3 complex. Studies to examine this possibility are currently underway.

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