Identification of the Binding Partners for Flightless I, A Novel Protein Bridging the Leucine-rich Repeat and the Gelsolin Superfamilies*

(Received for publication, September 4, 1997, and in revised form, November 21, 1997)
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**Flightless-I** (fliI) is a novel member of the gelsolin family that is important for actin organization during Drosophila embryogenesis and myogenesis. Drosophila fliI and the human homolog FLI both contain the classic gelsolin 6-fold segmental repeats and an amino-terminal extension of 16 tandem leucine-rich repeats (LRR). LRR repeats form amphipathic $\beta$-$\alpha$ structural units that mediate protein-protein interactions. Although there are close to 100 known LRR domain-containing proteins, only a few binding pairs have been identified. In this paper, we used biochemical and genetic approaches to identify proteins that interact with human FLI. *In vitro* synthesized FLI bound to actin-Sepharose and binding was reduced by competition with excess soluble actin. Actin binding was mediated through the gelsolin-like domain and not the LRR domain. Although the FLI LRR module is most closely related to the LRR domains of Ras-interactive proteins, FLI does not associate with Ras, selected Ras effectors, or other Ras-related small GTPases. Two-hybrid screens using FLI LRR as bait identified a novel LRR binding partner. The 0.65-kilobase pair (kb) clone from the screen survived additional rounds of stringent two-hybrid pairwise assays, establishing a specific interaction. Binding to FLI LRR was corroborated by co-immunoprecipitation with FLI LRR. The translated sequence of the FLI LRR associated protein (FLAP) encodes a novel protein not represented in the data base. Northern blot analyses revealed four FLAP messages of approximately 2.7, 2.9, 3.3, and 5.1 kb, which are differentially expressed in the tissues tested. Skeletal and cardiac muscles are particularly rich in the 3.3-kb FLAP message, and the FLI message as well. Full-length FLAP clones were isolated from a mouse skeletal muscle cDNA library. They have an open reading frame which encodes for a protein containing 626 amino acids. Sequence analyses predict that the FLAP protein is rich in $\alpha$-helices and contains stretches of dimeric coiled coil in its middle region and COOH terminus. The identification of actin and FLAP as the binding ligands for the gelsolin-like domain and the LRR domain, respectively, suggests that FLI may link the actin cytoskeleton to other modules implicated in intermolecular recognition and structural organization.

Gelsolin is a Ca$^{2+}$- and phosphatidylinositol 4,5-biphosphate-regulated actin-binding protein (1). It has been implicated in the regulation of the actin cytoskeleton and the modulation of membrane-cytoskeletal cross-talks (2–4). Many gelsolin-like proteins have been identified and they appear to have evolved from an ancestral single segment gene that has duplicated multiple times to form proteins with 3- or 6-fold repeats (5). Recent three-dimensional structure analyses show that the segments within gelsolin (6), as well as segments from different gelsolin family members, have a similar core structure (7–9). Nevertheless, the gelsolin family of proteins have distinct actin binding characteristics and intracellular localizations. For example, unlike most gelsolin members, CapG is a nuclear as well as cytoplasmic protein (10) and it does not sever filaments (11). Therefore, the conserved residues in each protein appear to maintain the basic folds of the repeated segments, while actin binding *per se* involves residues customized for each segment and for each protein.

Flightless I (fliI) is a recently identified member of the gelsolin family (12). It was discovered as a mutation in Drosophila melanogaster that leads to flightlessness. This phenotype is accompanied by disorganization of the indirect flight muscle myofibrils (13). Other more severe filI mutations lead to late larval or pupal death. Eggs lacking maternally supplied filI show incomplete cellularization, abnormal furrow formation, and impaired gastrulation. Defective cellularization of the syncytiot blastoderm is associated with a disorganized cortical actin cytoskeleton (14). The flightless and cellularization phenotypes suggest that filI is required for actin organization during myogenesis and embryogenesis, respectively. The human flightless I (FLI) locus has been mapped to a region deleted in the Smith-Magenis syndrome (15), which is associated with a spectrum of developmental and behavioral abnormalities. The COOH-terminal half of human FLI has 31% identity and 52% similarity to human gelsolin (12), and has the same 6-fold segmental repeat typical of many gelsolin family members (Fig. 1A). Since FLI is more divergent from gelsolin than other gelsolin family members such as CapG, adseverin, and villin (1), it probably arose from the prototypical ancestral protein very early during phylogeny and evolved independently (16). Therefore, it is necessary to determine whether FLI is an actin-binding protein, and how it interacts with actin.

The NH$_2$-terminal half of FLI is distinct from that of the other previously identified gelsolin members. It contains 16 tandem 23-amino acid leucine-rich repeat motif (LRR) (12) (Fig. 1A) found in an emerging collection of proteins (17). Close

*This work was supported by National Institutes of Health Grant R01 GM51112 and a Welch Foundation grant (to H. L. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF045573.

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‡ The abbreviations used are: filI, Drosophila flightless-I gene; filI, Drosophila flightless-I protein; FLI, human flightless-I protein; LRR, leucine-rich repeat, FLAP, FLI LRR associated protein; HC, IgG heavy chain; GST, glutathione S-transferase; kb, kilobase pair(s).
to 100 proteins containing this motif have been identified thus far. Proteins in this LRR superfamily have diverse cellular localizations (extracellular, cytoplasmic, transmembrane, and nuclear) and functions (receptor ligand binding, signal transduction, cell adhesion, development, bacterial virulence, DNA repair, and RNA processing). The unifying theme among these diverse functions is molecular recognition. The LRR motif contributes to protein-protein interactions, either directly as the ligand binding module, or as a regulator to enhance affinity and/or specificity of binding to a separate ligand-binding site.

In a few cases, the LRR binding partners have been identified. The ligand:LRR protein pairs include glycoprotein hormones: G-protein coupled receptors (18), collagen:matrix proteoglycans (such as decorin) (19), protein phosphatase-1:ds22 (required for the completion of mitosis) (20), neurotrophins:trk receptors (21), Ras:yeast adenylate cyclase (Cyr1) (22, 23), and pancreatic RNases:RNase inhibitor (24).

Among the LRR modules identified thus far, the FLI LRR 23-amino acid repeats fit the consensus for a LRR subgroup consisting of proteins which can potentially interact with Ras-like ligands (17, 25). This group includes Rsp-1 (also known as Rsu-1), which binds Raf-1 (26) and the yeast adenylate cyclase (Cyr1). Rsp-1 has 35% identity and 53% similarity to FLI. It binds Raf-1 (26) and suppresses the transformation activity of v-Ras (27). Cyr1 is regulated by Ras, and its LRR motif is required for membrane association and Ras binding (22). The similarity in LRR motifs raises the intriguing possibility that FLI may mediate interactions with Ras homologs (25). This is plausible because Ras transformation disrupts the actin cytoskeleton, and Rac1, a small G-protein which drives membrane ruffling (28), has been implicated in Ras transformation. Gelsolin severing and capping may be regulated by Rac1 (29), and gelsolin suppresses Ras-induced transformation in foci assays (30). We therefore used a variety of approaches to determine if FLI binds Ras or its downstream effectors. In addition, we tried to identify other FLI-LRR binding partners, to begin a molecular characterization of this novel member of the gelsolin and LRR superfamilies.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The human FLI cDNA which was inserted into pBluescript SK+ vector (Stratagene) through the EcoRI site, was kindly provided by H. D. Campbell (The Australian National University). This plasmid was used for the cloning of the FLI-LRR domain construct which contains an initiation codon and spans FLI between the LRR- and gelsolin-like domains (Fig. 1). 105 plaques were screened. The inserts in the positive plaques were sequenced by the Ready-To-Go DNA labeling kit (Pharmacia). Full-length FLI, H186 FLAP, and β-actin cDNAs were used as templates. The premade polyaRNA human tissue blot, purchased from OriGene (Rockville, MD) was hybridized in buffer containing 0.2% SDS, 5 × SSPE, 5 × Denhardt’s solution, 100 μg/ml denatured salmon sperm DNA, 50% formamide, 10% dextran sulfate at 42 °C for 14–16 h. The membrane was washed with 2 × SSC, 0.1% SDS for 5 min at room temperature 3 times, and with 0.25 × SSC, 0.1% SDS at 65 °C for 30 min 2 times. The membrane was exposed to X-ray film for 1 to 4 days. Membrane was stripped between probes.

cDNA Cloning of FLI LRR-associated Protein (FLAP)—A mouse I.M.A.G.E. consortium (LLNL) EST clone (ID 532888) whose 5’ sequence (GenBank accession number A068950) (36) is homologous to the two-hybrid human FLAP cDNA insert, was purchased from the ATCC. It was β3-P labeled and used to screen a mouse skeletal muscle 3T3 Stretch Plus αgt11 cDNA library (CLONTECH). Approximately 8 × 104 plaques were screened. α-gt11 inserts in the library were amplified in the Expand Long Template polymerase chain reaction system (Boehringer Mannheim), using the αgt11 LD-insert screening amplifier set (CLONTECH). The polymerase chain reaction products were cloned into the pGEM-T vector (Promega), and nucleotide sequences were determined by manual and automatic sequencing using external and internal primers.

Expression of Recombinant LRR-binding Partner and Antibody Production—The H186 FLAP cDNA was released from the HeLa matchmaker vector pGAD GH with SpeI and XhoI. The insert was ligated to the bacterial expression vector pGEX-KG (37) digested with XhoI and Xhol. The resultant plasmid was transfected into DH5α (Life Technologies) or BLR (Novagen). Bacteria were cultured to an OD600 of 0.5 and GST-H186 FLAP fusion protein (GST-H186 FLAP) synthesis was induced with 0.5 μM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. The bacteria were lysed and fusion protein was purified with glutathione-Sepharose. Eluted proteins were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by staining with 0.3 μ cupric chloride without fixation. The GST-H186 FLAP band was excised and used for rabbit immunization. The antibody from an early bleed, which recognizes H186 FLAP but not GST, was used for Western blotting.

Cell Culture and Transfection—The FLI clone containing the inserted initiation codon (as described above) was further modified by attaching a COOH-terminal HA epitope tag (YPYDVPDYA). The construct was subcloned into pcDNAs (Invitrogen) via the BamHI and XhoI sites in the multiple cloning region. H186 FLAP was cloned into

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In Vitro Transcription and Translation—Full-length and truncated FLI cDNA were cloned into the pTMI vector (31). 1 μg of cDNA was added to the T7 polymerase-coupled reticulocyte lysate system (TNT, Promega) in the presence of Trans-[35S]-label (ICN Biomedical, Inc.), and in vitro transcription and translation were carried out in a 50-μl volume according to the manufacturer’s protocol.

Actin-Sepharose Binding—2 and 4 μl of the in vitro transcribed and translated product was diluted to 80 μl with a buffer containing 2 mM Tris-HCl, 0.2 mM CaCl2, and 0.1% gelatin, pH 7.5, and added to 20 μl of packed actin-Sepharose beads (32). The samples were incubated for 30 min at room temperature, and the beads were then washed three times in the same buffer. Proteins bound to the beads were analyzed by SDS-polyacrylamide gel electrophoresis, and radioactive bands were detected by autoradiography. In some samples, 4 μl of 3 mg/ml actin or bovine serum albumin was added to determine if binding to actin-Sepharose was reduced by competition with soluble actin.

Binding to Small GTPases—GST-Ras, GST-RasV12, GST-RhoA, and GST-DCC42 (gifts of A. Hall, University College London and M. White, University of Texas Southwestern Medical Center) were expressed in BL21 and purified with glutathione-Sepharose (Pharmacia). They were charged with GTP or GDP. 35S-Labeled FLI and LRR produced by in vitro transcription and translation were added to the beads and binding was determined as described (33).

Yeast Two-hybrid Screening—The LexA based yeast two-hybrid system was used initially to identify candidate FLI LRR interactive clones. The FLI LRR domain was fused in-frame to the 3’-end of the sequence encoding the LexA DNA-binding domain of the yeast two-hybrid vector pBTM116. The construct (pLEX-LRR) was used as a bait to screen a GAL4 activation domain-based human HeLa matchmaker cDNA library (pGAD-GH vector, CLONTECH). The yeast L40 strain (MATa trp1 leu2 his3 lys2::lexA-His URA3::lexA-lacZ) was sequentially transformed, and potential interactors were identified as described previously (34). Approximately 1.6 million transformants were screened. Positive plasmids were isolated and tested against pLEX-lamin to rule out nonspecific interaction. Pairwise assays were also used to detect interaction with small GTPases (plasmids provided by M. White and J. Frost, University of Texas Southwestern Medical Center). The FLI LRR domain was fused in-frame to the 3’-end of the sequence encoding the LexA DNA-binding domain in the pAS2 vector to create pAS2-LRR. This construct was co-transformed with the previously identified LRR interactive plasmids into yeast Y190 (MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3-112 URA3::GAL-lacZ LYS2::GAL-HIS3 cyh2). Transformants were plated as patches on selective medium, and replica plated on Whatman No. 50 filter paper on agar plates for β-galactosidase assays.

Northern Blotting—32P-Labeled probes were synthesized with random primers by the Ready-To-Go DNA labeling kit (Pharmacia). Full-length FLI, H186 FLAP, and β-actin cDNAs were used as templates. The pmade polyaRNA human tissue blot, purchased from OriGene (Rockville, MD) was hybridized in buffer containing 0.2% SDS, 5 × SSPE, 5 × Denhardt’s solution, 100 μg/ml denatured salmon sperm DNA, 50% formamide, 10% dextran sulfate at 42 °C for 14–16 h. The membrane was washed with 2 × SSC, 0.1% SDS for 5 min at room temperature 3 times, and with 0.25 × SSC, 0.1% SDS at 65 °C for 30 min 2 times. The membrane was exposed to X-ray film for 1 to 4 days. Membrane was stripped between probes.
pCMV5 (38). The expressed protein contains a 12-residue Myc tag (MEQKLIISEEDELN) at the NH2 terminus, 218 amino acids from H186 FLAP, and 31 residues of pCMV5 sequence acquired because H186 does not contain its own stop codon. The FLAP-1 cDNA has its own translation start and stop codons and was cloned into pcDNA3. DNA was not contain its own stop codon. The FLAP-1 cDNA has its own trans-

**RESULTS**

**FLI Binding to Actin**—To determine if FLI is an actin-binding protein, FLI and the FLI LRR domain (Fig. 1A) were expressed by in vitro transcription and translation (Fig. 1B). In each case, a single in vitro transcribed product which migrated on a SDS-polyacrylamide gel with mobility consistent with the calculated molecular mass was generated (145 and 68 kDa, respectively, for FLI and LRR). The in vitro transcription products were incubated with actin-Sepharose in the presence and absence of excess soluble actin. FLI bound actin-Sepharose in a dose-dependent manner (Fig. 1C). The FLI domains. The NH2-terminal half has sixteen 23-amino acid leucine-rich repeats. The COOH-terminal half has six segmental repeats resembling that of the gelsolin family. GLD, gelsolin-like domains. a.a., amino acid residue number; nt#, nucleotide number. The LRR-HA construct used in this paper extends past the true LRR domain to residues 466. B, in vitro transcription and translation. 35S-Labeled FLI and LRR were synthesized in vitro, and the reaction products were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. 2, 4, and 6 μl of FLI or LRR reaction products were loaded on the left and right panels, respectively. C, actin-Sepharose binding. Lanes 1 and 3, 2 μl of in vitro synthesized FLI, with and without soluble actin, respectively. Lane 2, 4 μl in vitro synthesized FLI P, pelleted beads; S, supernatant. The entire pellet and one-fourth of the supernatant were analyzed. Densitometry scanning of the supernatants and pellets show that the combined amount of radioactivity recovered was 84, 204, and 92 units for lanes 1–3, respectively. The amounts of FLI bound to actin-Sepharose were 33, 20, and 5%, respectively, when expressed as percent of total input.

**Lack of Interaction between FLI-LRR and Ras and Other Small GTPases**—The close resemblance between FLI-LRR and those of Cyr1 and Ras-1 raises the possibility that FLI may also interact with Ras or its downstream effectors. This possibility was examined in two ways. First, two-hybrid pairwise assays were used to identify interactions in vivo. Second, recombinant Ras and RasVal-12, the constitutively active form of Ras, were expressed as fusion proteins with GST, and their binding in vitro transcribed and translated LRR were determined by sedimenting GST fusion proteins with glutathione beads. Neither assay was able to detect evidence for Ras interaction with LRR. In the two-hybrid assay, LRR did not interact with RasVal-12 or Ras (data not shown), although RasVal-12 interacted with its known effectors, Ral GDS (39), Raf (34), and Cyr1 (34), under identical conditions. In addition, there was no evidence for LRR binding to other small G proteins, such as Rac2, RhoA, or CDC42 (data not shown). Likewise, we did not detect specific binding of in vitro synthesized LRR to GST-RasVal-12, GST-Ras, GST-CDC42, or GST-RhoA, either in the presence of GTP or GDP with the GST bead pull down assay (data not shown).

**Identification of a Novel FLI LRR-binding Partner**—Two-hybrid screens using LRR fused with the LexA DNA-binding domain as bait yielded four interactive clones that did not bind to the negative control, lamin. All of these clones have similar DNA sequences, and one (H186) was selected for further analysis. H186 survived additional stringent tests for specific interactions (Table I). H186 interacted with LRR regardless of whether it was fused to the LexA DNA-binding domain or the transcriptional activation domain. It did not bind lamin, and it still bound LRR when fused with the GAL4 DNA-binding do-
main instead of the LexA domain (Table I). In contrast, H186 did not bind the FLI gelsolin-like domain. We will call this protein H186 FLI LRR-associated protein (H186 FLAP).

H186 FLAP association with LRR was corroborated by immunoprecipitation after coexpression in mammalian cells. HEK293 cells were transfected with H186 FLAP and HA-tagged LRR (LRR-HA) either individually or in combination, and the cells were metabolically labeled with Tran35S-label. Anti-HA was used to immunoprecipitate HA-tagged LRR and associated proteins. In cells transfected with LRR-HA alone, a single 68-kDa band was immunoprecipitated (Fig. 2A, left panel, lane 3). When cells were co-transfected with LRR-HA and H186 FLAP, an additional 32-kDa protein coprecipitated with the LRR (lane 4). This band was not immunoprecipitated in the absence of LRR-HA (Fig. 2A, lane 5), even though the autoradiogram of cell lysates shows that the 32-kDa band was expressed at a high level under both conditions (Fig. 2A, right panel, compare lanes 3 and 4). This band is only found in cells transfected with the H186 FLAP cDNA (Fig. 2A, right panel, lanes 4 and 5, but not lanes 1–3), and its size corresponds to that predicted from the nucleotide sequence of the H186 FLAP expression construct. These results indicate that H186 FLAP was captured in the anti-HA immunoprecipitate through binding to LRR-HA.

Tissue Distribution of FLAP—H186 FLAP is 0.65 kb and contains an open reading frame which contains a potential translation initiation codon but no termination codon. The translated amino acid sequence in this frame, or in the other two frames (which are interrupted by multiple stop codons), has no strong homology to known proteins in the GenBank database. It matches mouse EST clone sequences AA068950 and AA106954 by BLAST search (40), except for the existence of two inserts in the latter sequence (Fig. 4C). These results raise the possibility that H186 FLAP is not full-length, and that there may be multiple splice variants or multiple FLAP genes.

To estimate the size and number of FLAP messages, H186 FLAP was used to probe a human tissue poly(A) RNA blot. H186 hybridized with at least four bands of 2.7, 2.9, 3.3, and 5.1 kb (Fig. 3B) which are present at varying ratios in the tissues examined. The 2.7-kb message is ubiquitous, and is least abundant in brain. Brain has a unique 2.9-kb message. Skeletal muscle and heart have a unique 3.5-kb FLAP message. The heart poly(A) RNA lane was apparently underloaded compared with the other lanes, because neither the cardiac muscle α-actin nor cytoplasmic actin message is visible at a low exposure of the Northern blot (Fig. 3C, top panel), while the actin message in other lanes is detectible at this exposure. A longer exposure showed that heart muscle has the two expected actin messages (Fig. 3C, bottom panel). Skeletal muscle contains predominantly the lower skeletal α-actin message (41). The fact that we can detect significant FLAP and FLI signals in the heart lane despite underloading suggests that they are abundant in the heart. Kidney, lung, and small intestine have the 5.1-kb FLAP mRNA predominantly. Overall, the Northern blot results are

| DNA-binding domain | Activation domain | Interaction |
|--------------------|------------------|------------|
| H186 FLAP          | LRR              | +          |
| LRR                | H186 FLAP        | +          |
| LRR                | LRR              | -          |
| GLD                | H186 FLAP        | -          |
| Lamin              | H186 FLAP        | -          |
| Lamin              | LRR              | -          |
| FLAP-1             | LRR              | +          |
| FLAP-1(R)          | LRR              | -          |
| LRR (GAL4)         | H186 FLAP        | +          |

Fig. 2. Co-immunoprecipitation of FLAP with FLI LRR.
HEK293 cells were transfected with expression vectors for HA epitope tagged-LRR (LRR-HA), FLI-HA, H186 FLAP, or FLAP-1, either singly or in combination. P, immunoprecipitate; S, supernatant after immunoprecipitation. A, FLAP binding to LRR-HA. Transfected cells were 35S-labeled, lysed, and HA-tagged proteins were immunoprecipitated with anti-HA, and analyzed on a 5–15% gradient gel. Labeled proteins were detected by autoradiography. One-tenth of the amount of cell lysates used for immunoprecipitation was loaded in the supernatant lanes. Only H186 FLAP was expressed at sufficiently high level to be detected by the eye in the supernatants (right panel, lanes 4 and 5). B, FLAP-1 binding to LRR-HA. LRR-HA was immunoprecipitated with anti-HA, and Western blotting with anti-HA and anti-FLAP was used to detect LRR-HA and FLAP-1, respectively. Lane 1, cells transfected with FLAP-1; lane 2, LRR-HA and FLAP-1; lane 3, LRR-HA, LC, IgG light chain. The secondary antibody used to detect α-HA also cross-reacted with the mouse IgG HC in the immunoprecipitate and LRR-HA comigrated with the IgG HC. The staining of IgG HC accounts for the appearance of a faint band in the lane 1 pellet, at a position overlapping with that of LRR-HA in lanes 2 and 3. C, Western blotting to show that FLAP-1 co-immunoprecipitated with FLI. Overexpressed proteins were immunoprecipitated with anti-HA or anti-FLAP, and the proteins in the immunoprecipitates were analyzed by gel electrophoresis in a 2.5% acrylamide gel. Immunoprecipitated proteins were identified by Western blotting. Lane 1, cells transfected with FLAP-1; lane 2, FLAP-1 and FLI-HA; lane 3, FLI-HA; lane 4, FLAP-1 and FLI-HA; lane 5, FLI-HA; lanes 6 and 7, supernatants of lanes 4 and 5, respectively.
in human tissues. A human tissue poly(A) RNA blot was probed with FLI (A), H186 FLAP (B), and β-actin (C) cDNAs sequentially. The blot was stripped between hybridization with each probe. Two exposures of the actin blot were shown. The size of RNA standards are indicated in kb.

consistent with the existence of multiple FLAP splice variants or multiple genes, and suggest that they are differentially expressed in a tissue-specific manner.

The high abundance of FLAP message in skeletal muscle was matched by high level expression of FLI (Fig. 3A). The enrichment of FLI message in skeletal and heart muscles was reported recently by Campbell et al. (16).

cDNA Cloning of Mouse FLAP—To determine the sequence of full-length FLAP, a mouse skeletal muscle cDNA library was screened with a mouse embryonic carcinoma EST clone (GenBank sequence number AA068950) which has extensive homology to the human H186 FLAP clone (Fig. 4C). Phage clones with inserts ranging from 0.9 to 3 kb were identified. The 2.7-kb clone (called FLAP-1) contains an open reading frame which encodes for a protein of 628 amino acids (Fig. 4A). The shorter clone (FLAP-1a, 1.6 kb) overlaps with the first clone and extends the 5′-untranslated sequence by 11 base pairs (Fig. 4C). The 5′-untranslated sequence of the mouse cDNA and mouse embryonic carcinoma EST clones are identical to each other, but not to human H186 FLAP. In contrast, the coding sequences of the mouse skeletal muscle and human HeLa H186 FLAP clones are highly homologous (95% identity, 100% similarity at the amino acid level), except for the existence of a 148-amino acid insert (residues 52–199) and a 24-amino acid insert (residues 253–276) in the former. Since these inserts are found in two independently isolated muscle cDNA clones, they are unlikely to result from cloning artifacts. Furthermore, parts of the first insert and the entire second insert are found in another mouse embryonic carcinoma EST clone (sequence AA106954) and in a human skeletal muscle EST clone (sequence AA180174), respectively. The existence of several cDNA inserts and multiple mRNA strongly indicate that there are tissue and/or species-specific alternative splice variants.

FLAP-1 has a predicted molecular mass of 71 kDa. The translated sequence of FLAP-1 is not represented in the protein data base. Secondary structure analyses predict that it is very rich in α-helix at its COOH-terminal half. The Heidelberg PHD algorithm (42) predicts that the insert at the NH2-terminal region (residues 52–199) has 13.5% α-helix, 13.5% β-sheet, and 73% random coil, while the remainder of the molecule has 79% α-helix, 2.7% β-sheet, 18.3% random coil. The α-helices in the middle region and the COOH-terminal end of FLAP-1 show weak homology to the coiled coils of skeletal muscle myosin heavy chain (28% identity, 55% similarity in a 74-amino acid stretch). The PAIRCOIL algorithm (43) predicts that these regions in FLAP-1 form coiled coils (Fig. 4B) (Table II). There are four discontinuities in the coiled coils. The first two are due to the omission of three or four residues in the heptad repeats. Analyses of other coiled coil proteins show that these discontinuities, called stutters and stammers, respectively, have negative and positive effects on the extent of supercoiling (44). The remaining discontinuities are due to interruption by sequences with low coiled coil potentials. The existence of coiled coils is confirmed using the MULTICOIL algorithm (45) (Table II). The program also predicts that a large fraction of the coiled coils have a high tendency to dimerize. The FLAP-1 sequence has no obvious signal peptide or membrane spanning segments, and is therefore unlikely to be a secreted or transmembrane protein. There are multiple phosphorylation and myristylation consensus sites, suggesting that FLAP may be modified post-translationally.

Evidence for the Binding of Full-length FLAP-1 to FLI—FLAP-1 fused to the LexA DNA-binding domain interacted with FLI-LRR in the two-hybrid assay to an extent comparable to that of H186 FLAP (Fig. 5) (Table II). In contrast, FLAP-1 cloned in the antisense orientation was not positive.

Binding was also demonstrated by co-immunoprecipitation from cells transfected with both FLAP-1 and LRR-HA (Fig. 2, A and B). Anti-HA immunoprecipitated LRR-HA and at least two additional bands with molecular mass of approximately 85 kDa from cells co-transfected with both constructs (Fig. 2A, left panel, lane 2). The 85-kDa bands were not immunoprecipitated in the absence of LRR-HA (Fig. 2A, lane 1), confirming that they were captured as a result of binding to LRR-HA. These bands are larger than the 71-kDa size predicted from the FLAP-1 amino acid sequence, and can be resolved into even more bands on a 7.5% gel (see Fig. 2C). The reasons for the anomalous migration and the existence of multiple FLAP-1 bands are not known, but are consistent with FLAP-1 post-translational modifications. This possibility will be explored in future studies.

To establish unequivocally that the bands which bound LRR-HA were FLAP-1, we generated a polyclonal antibody against recombinant H186 FLAP. It recognized a 85-kDa band(s) in the lysates of cells transfected with FLAP-1 (Fig. 2B, right panel, lanes 1 and 2), but not with cells transfected with LRR-HA (lane 3). Thus this antibody specifically recognized FLAP. The anti-FLAP antibody recognized the same cluster of bands which co-immunoprecipitated with LRR-HA (Fig. 2B, left panel, lane 2), establishing that FLAP bound LRR-HA. The secondary antibody used to detect anti-FLAP also recognized the IgG light chain, and to a lesser extent, the IgG heavy chain.
Western blotting with anti-HA confirmed that the 68-kDa band in the immunoprecipitates was LRR-HA (Fig. 2B, left panel, lanes 2 and 3). This band comigrated with the IgG HC, and the secondary antibody used to detect anti-HA in the Western blot also recognizes the HC, accounting for the presence of a faint band in the immunoprecipitates even in the absence of LRR-HA (Fig. 2B, left panel, lane 1). FLAP-1 also bound full-length FLI. This was established by using both anti-HA and anti-FLAP to pull down the FLAP-FLI-HA complexes. Transfection of FLAP-1 resulted in the overexpression of a cluster of bands of approximately 85 kDa which were detected with anti-FLAP (Fig. 2C, right panel, lane 4). The FLAP-1 bands were much better separated here compared with Fig. 2A because a non-gradient 7.5% gel was used instead of a 5–15% gradient gel. Immunoprecipitating FLI-HA with anti-HA brought down FLI-HA (Fig. 2C, left panel, lanes 1 and 2). This antibody did not immunoprecipitate the FLAP-1 bands in cells transfected with FLAP-1 alone (lane 3), but immunoprecipitated FLAP-1 in cells co-transfected with FLAP-1 and FLI-HA (lane 2). We consistently observed an enrichment of the fastest migrating FLAP-1 band in the anti-HA immunoprecipitates. We cannot explain why this is the case, and suspect that the fast migrating FLAP-1 may bind preferentially to FLI. Thus, FLAP-1 binding to FLI may be regulated post-translationally. This possibility will be investigated in future experiments. There is no enrichment of a particular FLAP-1 species in the anti-FLAP immunoprecipitate (lane 4), presumably because the anti-FLAP does not discriminate between the different FLAP-1. Our results show that the antibody to either FLI or FLAP-1 was able to bring down its putative partner in the complex, providing definitive evidence for the high affinity interaction of FLAP-1 and FLI in vivo.

**FIG. 4.** Sequence analyses of mouse skeletal muscle FLAP-1. A, predicted amino acid sequence. The predicted coiled coil regions are shown in bold. Inserts found in the mouse skeletal muscle cDNA clones but not in human H186 FLAP are underlined. The nucleotide sequence is deposited in GenBank (accession number AF045573). B, predicted location of the coiled coil regions, with the PAIRCOIL program (43). A score of above 0.5 indicates high probability of coiled coil structure. C, alignment of mouse skeletal muscle FLAP-1 cDNA clones with H186-FLAP and EST clones. FLAP-1 and FLAP-1a were isolated from a mouse skeletal muscle cDNA library. H186-FLAP is the human HeLa LRR-interactive plasmid identified by two-hybrid screens. Sequences AA06895 and AA108954 are derived from mouse embryonic carcinoma EST clones. Sequence AA180174 is derived from a human skeletal muscle EST clone. Solid bars, sequenced regions. *, initiation codon; ×, termination codon. Lines linking solid bars denote deleted regions compared with FLAP-1. Shaded bar, 5′-untranslated region of the human H186 FLAP which diverges from the mouse sequences.

**TABLE II**

| Position | PAIRCOIL scores | MULTICOIL scores |
|----------|-----------------|------------------|
|          | Coiled/coil probability | Initial register | No. heptad repeats | Coiled coil probability | Dimer probability |
| 253–270  | 0.7 f            | 2.6              |                  | 0.8                   | 0.6               |
| 271–347  | 1.0 f            | 11               |                  | 0.9                   | 0.6               |
| 348–390  | 1.0 c            | 6                |                  | 1.0                   | 0.9               |
| 472–501  | 0.5 b            | 4                |                  | 0.8                   | 0.8               |
| 528–616  | 1.0 f            | 12.7             |                  | 1.0                   | 0.8               |

The PAIRCOIL (43) and MULTICOIL (45) algorithms were used to assess the tendency of FLAP-1 to form coiled coils and the tendency of the coiled coils to dimerize, respectively. The scale is 0 to 1.0. A score of 0.5 or above indicates high probability. The amino acid in the initial register of the heptad repeat (abcdefg) is indicated.
FLI is unique among the members of the LRR and gelsolin superfamilies. Although many LRR proteins contain additional modules implicated in molecular recognition (17), such as epidermal growth factor repeats, immunoglobulin domains, G-protein coupled receptors, and leucine zippers, FLI is the first actin-binding LRR protein to be identified. FLI is also unusual in the gelsolin family, because it is the first example of the bridging of a motif with no known actin binding function to gelsolin proper. Villin is the only other gelsolin family protein with an extension identified thus far, but its extension binds actin and is much shorter (46). The novel juxtaposition of the LRR tandem repeats with the gelsolin 6-fold repeats suggests that FLI may link the actin cytoskeleton to other structures in the cell in a far more direct manner than has been envisioned for the more traditional gelsolin-like proteins. The membrane signaling systems are favored candidates for interaction with FLI, because many LRR proteins and actin regulatory proteins are involved in signal transduction and adhesion, and the FLI LRR repeats best fit the consensus found in several Ras interactive proteins (17, 25).

In this paper, we demonstrate that FLI binds actin, establishing it as a bona fide member of the gelsolin family. This is expected because of its homology to gelsolin, and is consistent with the effects of fil mutations on actin organization in Drosohila embryos and flight muscles. Nevertheless, interaction with actin cannot be assumed a priori because FLI is far more divergent than the other traditional members of the gelsolin family. Although a comparison of FLI and gelsolin suggests that residues essential for the structural integrity the S1 core are preserved in FLI S1 (7, 47), some of the other FLI segments have unique insertions (5, 12). FLI binding to actin was demonstrated by using in vitro synthesized FLI. This assay has been used to identify proteins which bind monomeric actin with high affinity. Most gelsolin family members bind actin-Sepharose (48), although CapG does not (11). Therefore, FLI is likely to be bind actin with high affinity. It will be important to determine whether FLI caps and severs actin filaments like other gelsolin family proteins. These issues cannot be addressed at present because we cannot isolate soluble recombinant FLI. Preliminary results suggest that endogenous FLI is a low abundance protein and we have not purified enough to allow functional characterizations.

Despite the resemblance of the FLI LRR to the LRR of known Ras effectors such as Cyr1 and Rsp-1, we were unable to demonstrate FLI LRR binding to Ras. FLI LRR also did not bind several Ras downstream effectors, and other Ras-related small G proteins. These proteins are, therefore, unlikely to be high affinity FLI interactive partners, although we cannot rule out low affinity binding or linkage through other interactive proteins. The negative result is not surprising, because the LRR motif is probably used to present a platform for interaction with other proteins, while binding specificity is dictated by the nonconsensus amino acids in the repeats. Furthermore, FLI does not have the same numbers of LRR repeats as Cyr1 and Rsp-1 (16, 24, and 7 repeats, respectively) and it is flanked by unique sequences as well.

We identified a novel FLI LRR interactive partner, FLAP, by two-hybrid screens, and confirmed its specific binding to FLI by immunoprecipitation. Northern blotting and cDNA analyses indicate that there are multiple FLAP messages, which are most likely generated through differential splicing. Skeletal and cardiac muscles have high level expression of the 3.3-kb FLAP mRNA and the FLI mRNA as well, lending further support to the possibility that FLAP and FLI are in vivo partners. FLAP is predicted to form coiled coils at its middle and COOH-terminal regions. α-Helical coiled coils are the most common assembly motif in proteins and provide the potential for homotypic or heterotypic interactions (44, 49). The FLAP coiled coils are predicted to have a high tendency for dimerization, in a manner similar to that of well characterized cytoskeletal proteins like tropomyosins, myosins, and kinesins. However, FLI has fewer heptads and may not assemble into a fibrous structure. On the other hand, short coiled coils have been identified in many other proteins and they participate in important heterotypic interactions. These include the cyclic GMP-dependent protein kinase, the βγ dimer of heterotrimeric G proteins, and the basic leucine zippers of certain transcription factors (reviewed in Ref. 49).

In analogy, we hypothesize that the FLAP coiled coils bind FLI LRR. While this has not been established directly, it is supported by the finding that both Fl186 FLAP and FLAP-1 bind LRR, even though the former has a large deletion in the NH2-terminal region upstream of the coiled coil domain. In this context, it should be noted that there is precedent for LRR protein interaction with α-helical structures. The best example is the binding of decorin, a small proteoglycan with eleven 24-amino acid LRR repeats, to the triple helix of collagen (50). How decorin affects collagen fibrillogenesis is predicted by molecular modeling based on the crystal structure of the RNase inhibitor (51, 52). The entire RNase inhibitor molecule is composed of 15 tandem alternating 28- and 29-residue LRR repeats with alternating short β-strands and α-helices parallel to a common axis. This symmetrical arrangement folds into an open, nonglobular protein with a horseshoe shape, which is lined by β-strands in its inner surface and α-helices on its outer surface. RNase inhibitor inhibits RNase, and the crystal structure of the complex shows that RNase binds to a broad region in the concave face (52). Decorin, which has fewer and shorter repeats than RNase inhibitor, is predicted to fold into an arch shape with an internal cavity just large enough to accommodate a single triple helical collagen molecule (50). Decorin therefore acts as a spacer to prevent lateral fusion of collagen fibrils and to guide collagen fibril assembly (50). In analogy, the FLAP coiled coil may insert into the concave FLI LRR binding surface.

The identification of FLI as an actin-binding protein and the discovery of a novel coiled coil ligand for the its LRR domain suggests that FLI is a linkage protein between the cytoskeleton and an as yet unidentified structure in the cell. Based on the intimate connections between the cytoskeleton and the plasma membrane, we favor the possibility that FLAP is part of the membrane cytoskeleton.

Acknowledgments—We thank our colleagues for providing various plasmids (acknowledged individually in text), and particularly Michael White for help with the yeast two-hybrid system. We also thank Ha Do for expert technical assistance.

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