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Human Lymphocyte-Specific Protein 1, the Protein Overexpressed in Neutrophil Actin Dysfunction with 47-kDa and 89-kDa Protein Abnormalities (NAD 47/89), Has Multiple F-Actin Binding Domains

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Human lymphocyte-specific protein 1 (LSP1) is an F-actin binding protein, which has an acidic N-terminal half and a basic C-terminal half. In the basic C-terminal half, there are amino acid sequences highly homologous to the actin-binding domains of two known F-actin binding proteins: caldesmon and the villin headpieces (CI, CII, VI, VII). However, the exact numbers and locations of the F-actin binding domains within LSP1 are not clearly defined. In this report, we utilized 125I-labeled F-actin ligand blotting and high-speed F-actin cosedimentation assays to analyze the F-actin binding properties of truncated LSP1 peptides and to define the F-actin binding domains. Results show that LSP1 has at least three and potentially a fourth F-actin binding domain. All F-actin binding domains are located in the basic C-terminal half and correspond to the caldesmon and villin headpiece homologous regions. LSP1 181–245 and LSP1 246–295, containing sequences homologous to caldesmon F-actin binding site I and II, respectively (CI, CII), binds F-actin; similarly, LSP1 306–339 can bind F-actin and contains two inseparable villin headpiece-like F-actin binding domains (VI, VII). Although LSP1 1–305, which does not contain VI and VII regions, retains F-actin binding activity, its binding affinity for F-actin is much weaker than that of full-length LSP1. Site-directed mutagenesis of the basic amino acids in the KRYK (VI) or KYEK (VII) sequences to acidic amino acids create mutants that bind F-actin with lower affinity than full-length wild-type LSP1. High KCl concentrations decrease full-length LSP1 binding to F-actin, suggesting the affinity between LSP1 and F-actin is mainly through electrostatic interaction.


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2 Address correspondence and reprint requests to Dr. Thomas H. Howard, Division of Hematology-Oncology, Department of Pediatrics, University of Alabama, 1600 7th Avenue South, Birmingham, AL 35233. E-mail address: thaward@peds.uab.edu

3 Abbreviations used in this paper: PMN, polymorphonuclear neutrophil; NAD, neutrophil actin dysfunction; LSP1, lymphocyte-specific protein 1; IPTG, isopropyl β-D-thiogalactopyranoside; 125I-F-actin, 125I-labeled F-actin.
Materials and Methods

Reagents

Restriction enzymes BamHI and HindIII, T4 DNA ligase, and in vitro mutagenesis kit were purchased from Promega (Madison, WI). QIAEX II gel extract kit was from Qiagen (Santa Clarita, CA). Proband resin was obtained from Invitrogen (San Diego, CA). PCR primers were synthesized at the Department of Biochemistry and Molecular Genetics at the University of Alabama (Birmingham, AL.). 125I-labeled G-actin was prepared in the University of Alabama cancer center. Prokaryotic expression plasmid pET21C was from Novagen (Madison, WI). Isopropyl β-D-thiogalactopy- ranoside ( IPTG), ampicillin, gelsolin and phallolidin were purchased from Sigma (St. Louis, MO). Tag DNA polymerase was purchased from Perkin-Elmer (Norwalk, CT). Bovine brain tubulin was purchased from Cytoskeleton (Denver, CO).

Rabbit skeletal muscle G-actin preparation

Rabbit skeletal muscle G-actin was prepared according to the method of Spudich and Watt (11). G-actin dialyzed against G-buffer (5 mM Tris-HCl (pH 8.0), 0.2 mM ATP, 0.2 mM DTT, 0.2 mM CaCl2, and 1 mM NaN3) was further purified by passage over a Sephadex G-75 column. Before use, the G-actin was centrifuged at 100,000 × g for 30 min in the Beckman (Palo Alto, CA) Optima TL Ultracentrifuge.

Construction of truncated LSP1 peptides

The strategy for creating truncated LSP1 peptides involved preparing PCR primers which place a BamHI restriction site in front of the 5′ primer, a stop codon, and a HindIII restriction site behind the 3′ primer (the LSP1 cDNA contains neither BamHI nor HindIII restriction sites). The PCR products were excised using BamHI and HindIII and purified by QIAEX II gel extract kit. The purified PCR products were ligated into prokaryotic expression vector pET21C, which had been cut with BamHI and HindIII and purified. When correctly ligated, this vector added six histidine residues to the C terminus of the peptide, which was then used to purify the peptides by nickel affinity chromatography.

Expression of truncated protein and protein purification

The constructed plasmids were transformed into Escherichia coli strain BL21 (DE3), the bacteria were induced with 1 mM IPTG for 3 h to express the proteins, the bacteria were lysed by three freeze-thaw cycles in liquid nitrogen and a 37°C water bath. After centrifugation at 100,000 × g for 1 h, the supernatants and pellets were analyzed by SDS-PAGE, and then the bands were visualized by Coomassie blue staining and quantified by a Bio-Rad Multiprobe (Palo Alto, CA) Optima TL Ultracentrifuge.

High-speed F-actin cosedimentation assay

Truncated and mutated LSP1 peptides in P-buffer (10 mM imidazole (pH 7.0), 75 mM KCl, 0.2 mM DTT, 0.2 mM EGTA, and 0.01% Nonidet P-40) were first spinned at 100,000 × g at Beckman centrifuge for 45 min to remove aggregates. Assays of F-actin cosedimentation were performed by mixing actin with LSP1 in P-buffer containing 2 mM MgCl2, and 0.1 mM ATP. After incubation for 60 min at room temperature, the samples were spun at 100,000 × g for 45 min. The pellets were washed twice with P-buffer. Both supernatants and pellets were analyzed by SDS-PAGE, and then the bands were visualized by Coomassie blue staining and quantified by a Bio-Rad (Hercules, CA) densitometric scanner.

125I-labeled G-actin labeling

Column-purified G-actin was labeled with 125I-Bolton Hunter reagent (New England Nuclear, Boston, MA). The labeled G-actin was separated from the free 125I by passing over G-75 gel filtration column. The concentration of the G-actin was measured by BCA method.

F-actin ligand blotting

125I-labeled G-actin at 1 mg/ml was polymerized to F-actin in the presence of 0.2 μM gelsolin (100:1 mol actin to gelsolin) in polymerizing buffer (20 mMPIPES (pH 7.0), 50 mM KC1, 2 mM MgCl2, and 50 mM CaCl2) for 10 min on ice, then phallolidin was added to a final concentration of 40 μM and polymerization was continued at room temperature for an additional 15 min. 125I-labeled F-actin (125I-F-actin) was diluted to a final concentration of 50 μg/ml with blocking buffer (10 mM Tris-HCl (pH 7.5), 90 mM NaCl, 0.5% (v/v) Tween 20, 5% nonfat milk, 1% BSA, and 0.25% gelatin). Truncated LSP1 peptides were expressed in E. coli, and equal OD600 total cells were lysed with Laemmli buffer and E. coli proteins separated on SDS-PAGE and transferred to nitrocellulose (NC) membrane. The NC membrane was first treated with blocking buffer at room temperature for 2 h. After incubation with 50 μg/ml 125I-F-actin at room temperature for 6 h during constant agitation, the blots were then washed extensively with TBST (10 mM Tris-HCl (pH 7.5), 0.5% (v/v) Tween 20, and 90 mM NaCl) five times (15–30 min/wash). Blots were air-dried, and F-actin binding proteins were identified by autoradiography (12, 13). For cold probe competition assays, the blots were incubated with either cold F-actin or microtubule at room temperature for 2 h before the blots were air-dried.

In vitro site-directed mutagenesis

In vitro site-directed mutagenesis was done according to the protocol described for the Promega GeneEditor in vitro site-directed mutagenesis system. Synthesized oligonucleotides containing the mutated bases were phosphorylated by T4 polynucleotide kinase, then the phosphorylated oligonucleotides were annealed with the plasmid which contained the LSP1 cDNA. The primers were extended by T4 DNA polymerase, and the gap was ligated by T4 DNA ligase, the mutants were selected by DNA sequencing. The mutated proteins were purified by inserting the mutated gene into prokaryotic expression vector pET21C and subsequently induced with IPTG. The expressed proteins were purified.

Results

Sequence comparison to known proteins reveals four potential actin-binding sequences in LSP1

Database searches of the LSP1 amino acid sequences to define regions homologous to the actin-binding domains of known actin-binding proteins revealed four regions of interest in the C-terminal half of the protein. Two sequences separated by 23 aa were more than 60% homologous to the F-actin binding sites of caldesmon (CI and CII, Fig. 1) (14–17). The CI region contains a 33-aa sequence (aa 205–237), where 18 of 33 aa are similar; the second region of homology, CII, spans 17 aa (aa 260–276), where 9 of 17 aa are similar. In addition, near the extreme C-terminal end of LSP1, there are also two short stretches of amino acids (VI and VII) that are almost identical to the KKEK sequences identified in the headpiece of villin as an F-actin binding domain. This headpiece is essential for the bundling activity and its morphologic effect(s) of the villin molecule on cells (18–20) (see Fig. 1). Furthermore, computer-based secondary structure analysis reveals a series of α-helices positioned between and following CI and CII. Also three β-sheeted sheets that include the VI and VII domains follow the helical structure and are similar to those found in other F-actin-binding proteins including spectrin and ABP280.

Truncated peptide analysis shows LSP1 has at least three F-actin binding domains

Our initial results showed human LSP1 purified from SO9 cells infected with LSP1 cDNA recombinant baculovirus, similar to mouse LSP1, can bind to F-actin. In addition, human LSP1 colocalizes with F-actin in A7 melanoma cells that stably express LSP1 protein from the pCEP4 vector (21). Because the basic C-terminal half of LSP1 contains regions highly homologous to the F-actin binding proteins caldesmon and villin headpiece, PCR was utilized to create an array of truncated LSP1 peptide constructs and were analyzed for F-actin binding activity. The constructs, designated LSP1 x-y where x is N-terminal and y is C-terminal amino acid, were designed to include and exclude the V and C homologous sequences in combinations and alone within unique peptides. Truncated LSP1 cDNA was cloned into the prokaryotic expression vector pET21C and transformed into E. coli strain BL21 (DE3) and induced to express truncated peptides by IPTG. Initially, the LSP1 truncates expressed in bacteria were screened for F-actin binding domains by 125I-F-actin ligand blot. Results of the 125I-F-actin ligand blot screen for F-actin binding domains are shown in Fig. 2. In whole bacterial lysates of E. coli expressing LSP1 peptides induced by IPTG, 125I-F-actin binds to several, but not all, LSP1 peptides, suggesting that LSP1 contain more than one F-actin domain. No actin-binding peptides are present in control
IPTG induced bacterial lysates, and no binding was observed with the nonspecific control, purified BSA protein. Gelsolin, a known F-actin binding protein on ligand blots (4), is included as a positive control (Fig. 2C). Specific screening results show that the full-length and C-terminal LSP1 (LSP1 181–339), but not the N-terminal LSP1 (LSP1 1–180), peptides bind F-actin in the ligand blot. Also, both the LSP1 1–305, which contains both CI and CII, and the LSP1 306–339, which contains both VI and VII regions, bind to F-actin on ligand blots (Fig. 2A). F-actin binding by ligand blot via CI and CII independently is also demonstrated with LSP1 181–245 and LSP1 246–295 peptides (Fig. 2B). The screening results suggest that LSP1 has at least three F-actin binding domains and that these binding domains may differ in their apparent affinity for F-actin.

To specifically define the F-actin binding domains in human LSP1, truncated LSP1 peptides purified from E. coli were analyzed for F-actin binding domains by cosedimentation with F-actin at high speeds (100,000 × g for 45 min). Results with the high-speed F-actin cosedimentation assay show that full-length LSP1 and the C-terminal half of LSP1 (LSP1 181–339) (Fig. 3A) but not the N-terminal half (LSP1 1–180) (Fig. 3B) cosediment with F-actin. None of these peptides alone sediment under these assay conditions. These results are consistent with the overlay data that F-actin binding domain(s) reside within the C-terminal half of the molecule.

To define more precisely the location and the number of F-actin binding domains, additional truncated peptides that include or exclude the CI, CII, and VI, VII domains were created and analyzed. Both LSP1 181–295 that contains both CI and CII and LSP1 306–339 that contains both VI and VII can bind F-actin independently (Fig. 3C). Thus, LSP1 has at least two F-actin binding domains. Because the VI and VII domains are separated by only 7 aa, it was...
technically difficult to express and purify VI and VII and analyze them as independent peptides. In contrast, truncated peptides including CI or CII (LSP1 181–245 and LSP1 246–295) were sufficiently large and sufficiently spaced to allow independent analysis of CI and CII to further define F-actin binding domains of LSP1. The results show these peptides each retain an F-actin binding domain (Fig. 3D). In summary, these results show LSP1 has at least three, and possibly four, F-actin binding domains, CI, CII, and VI/VII. Given the sequence homologies, it is possible that VI and VII can also independently bind F-actin. These results, summarized in Fig. 4, support the contention that LSP1 has at least three F-actin binding domains.

Comparison of affinity of the F-actin binding domains in LSP1 truncates and LSP1 mutants

Quantitative analysis by Scatchard plot of murine LSP1 binding to F-actin by Jongstra-Bilen et al. (6) suggested that murine LSP1 might contain high- and low-affinity F-actin binding domain. However, these domains were not clearly characterized. Our quantitative analysis of F-actin binding by cosedimentation shows that LSP1 1–305 binding to F-actin is lower affinity than the binding of full-length LSP1 (Fig. 5A). This finding also suggests that LSP1 306–339 indeed contain another F-actin binding domain. It should be noted that at equal molar ratio of both LSP1 peptides, the quantity of LSP1 1–305 which cosediments with F-actin is quantitatively less (<20%)

FIGURE 3. Recombinant LSP1 peptide cosedimentation with F-actin defines three F-actin binding domains. Shown are SDS-PAGE of supernants (S) and pellets (P) from solutions of purified LSP1 peptides alone or LSP1 peptides mixed with F-actin sedimented at 100,000 × g for 45 min. Cosedimentation with F-actin of full-length LSP1 (LSP1 1–339), C-terminal one-half of LSP1 (LSP1 181–339), LSP1 181–295, LSP1 296–339, LSP1 306–339, LSP1 181–245, and LSP1 246–295 peptides but not N-terminal half of LSP1 (LSP1 1–180) indicate there are at least three F-actin binding domains in the C-terminal half of the LSP1 molecule.

FIGURE 4. Summary of F-actin binding sites within LSP1 molecule. At least three F-actin binding fragments were identified within LSP1 molecule: one that includes the CI region, the second contains the CII region, and at least one additional binding fragment is retained in the VI and VII region.

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by cosedimentation) than that of LSP1 1–339. The result indicates the CI, CII binding to F-actin is weaker than full length and suggests the V regions may represent strong binding domains while C regions may represent relatively weaker binding domains. It is also possible that VI and VII domains may cooperate with each other and with CI and CII to strengthen F-actin binding.

To exclude the possibility that the weaker binding of LSP1 1–305 to F-actin was due to the conformational change accompanying peptide truncation and to determine whether VI and VII can bind F-actin independently, full-length LSP1 mutants were created by site-directed mutagenesis to assay the contribution of VI and VII to F-actin binding independently. The results show both the VI and VII sequences are independently important for F-actin binding. In combination with analysis of LSP1 truncates these results demonstrate that LSP1 contains at least three, and likely four, independent F-actin binding domains in its C-terminal half.

**FIGURE 5.** Both the deletion mutation and point mutation in the villin headpiece-like regions weaken LSP1 binding to F-actin. Shown are the results of F-actin cosedimentation assay of LSP1 1–305 truncate (A) and LSP1 K326E mutant (B). Compared with full-length wild-type LSP1 protein, both LSP1 1–305 and LF K326E bind to F-actin weakly, though the amount of proteins and actin is similar in each assay. C, Saturation binding curves and $K_d$ of wild-type and mutated LSP1 to F-actin.

LSP1 binds to F-actin through electrostatic interaction

The actin-binding domains of many actin-binding proteins contain clustered basic amino acids. This observation seems to be a repeating theme in actin-binding proteins, which may reflect their evolutionary origin. Actin-binding domains at clustered basic amino acids in proteins include the KKGGKKKG sequence of myosin, DAIKKK sequence in actin depolymerizing factor, cofilin and tropomyosin, and KSKLKKT sequence in thymosin $\beta_4$ (22). Vandekerckhove (22) has suggested that the extreme N-terminal peptide Ac-DEDE of actin molecule is the binding site on actin that is the ligand for these positively charged sequences on actin-binding proteins. Several lines of evidences including chemical cross-linking are consistent with this suggestion (22, 23). Such a situation will inevitably lead to competition between different actin-binding proteins for actin in cells. Because the F-actin binding domains of LSP1 localize in the basic C-terminal half of the molecule, we reasoned that the interaction of LSP1 with F-actin may also be through electrostatic interaction and therefore that LSP1 may compete with other actin-binding proteins. To test the possibility that the LSP1 to actin interaction is controlled electrostatically, we increased the concentration of KCl in the F-actin cosedimentation assay to determine whether increased ionic strength would dissociate LSP1 from F-actin. The increase in KCl concentration did not affect the solubility of LSP1 protein (data not shown). Increasing KCl concentration did not affect the polymerization of actin, as shown in Fig. 6, which was consistent with the fact that actin-actin interaction is mainly hydrophobic (24). However, when the concentration of KCl was increased, the binding of LSP1 to F-actin decreased. This observation suggests that at least one of the three F-actin binding domains associate with F-actin.

**FIGURE 6.** LSP1 binds to F-actin through electrostatic interaction. LSP1 has greatly decreased binding to F-actin with increasing KCl concentration, whereas KCl concentration does not affect the polymerization of actin. A. SDS-PAGE analysis of LSP1 in the presence of increasing KCl concentrations (100–500 mM). B. The amount of actin and LSP1 in the pellet determined by scanning of Coomassie blue stained gels.
the molecule: aa 181–245, aa 246–295, and aa 306–339. Each domain contains amino acid sequences homologous to those in the F-actin binding proteins caldesmon or the villin headpiece. Two complementary methods were utilized to define the F-actin binding domains. The high-speed F-actin cosedimentation assay utilizes purified native proteins and excludes specific or nonspecific interference from other proteins. The F-actin ligand blotting utilizes $^{125}$I-radioactive labeled F-actin. This method is more useful in screening for potential F-actin binding domains including those peptides that have weak binding to F-actin and those peptides which might form homologous aggregates or easily precipitate under the conditions of F-actin cosedimentation assay condition. With the F-actin cosedimentation assay, it is difficult to determine whether homologous aggregates might explain cosedimentation with F-actin. In contrast, $^{125}$I-F-actin ligand blot screens independent of peptide aggregates. One limitation of the F-actin ligand blotting is that this method uses denatured and renatured proteins, resulting in the uncertainty that some proteins may not be properly renatured. Combining these two methods provides the most accurate and easily interpreted results.

Although domain mapping through creation of actin-binding protein truncates is a standard method for defining the functional domains of actin-binding proteins, it has its limitation. For example, truncates may have a significantly altered conformation resulting from deleting adjacent sequences, and therefore may provide little insight into the function of full-length actin-binding proteins. An example is the microtubule associated protein 2 (MAP2) and tau molecules binding to microtubule. It was shown that the extreme C-terminal hydrophobic $\alpha$-helix in these proteins is responsible for the microtubule bundling activity of MAP2 and tau by domain mapping (32). However, in subsequent experiments exploiting site-directed mutagenesis and deletion mutation experiments, it was shown that the regions identified by truncation are not directly responsible for microtubule bundling activity of these proteins (33). Therefore, caution should be taken in interpreting data from truncated peptides alone to map the functional domains of a molecule as the sole method for defining actin-binding domains. The same situation has been encountered in this study with analysis of LSP1 actin-binding domains. Clearly, the results show the truncated peptide LSP1 181–245 and peptide LSP1 246–295, which respectively contain CI and CII alone, can each bind to F-actin independently, and LSP1 1–295, which include these two fragments, can also bind to F-actin. However, LSP1 1–275, which include LSP1 181–245 and part of LSP1 246–295, does not bind to F-actin either in the F-actin cosedimentation assay or the F-actin ligand-blotting assay. This result suggests aa 275–295 may modify the conformation of the truncates and therefore affect the F-actin binding. Thus the three-dimensional structure of the molecule is as important as the primary amino acid sequences. To fully confirm the findings reported here, the crystal structure of the LSP1 would be required.

**Acknowledgments**

We thank Dr. William Nauseef for the completing initial screen for binding sites with the $^{125}$I-F-actin ligand blot assay.

**References**

LSP1 HAS AT LEAST THREE F-ACTIN BINDING DOMAINS