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A novel LIM and SH3 protein (lasp-2) highly expressing in chicken brain☆

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Abstract

From eluates of F-actin affinity chromatography of chicken brain, we identified a novel actin-binding protein (lasp-2) whose gene was predicted in silico. We cloned cDNA of chicken lasp-2 and analyzed its structure, expression, activity, and localization with lasp-1 (LIM and SH3 protein 1), a previously identified actin-binding protein closely related to lasp-2. Chicken lasp-2 showed high homology to mammalian putative lasp-2. Both chicken lasp-1 and chicken lasp-2 have N-terminal LIM domains, C-terminal SH3 domains, and internal nebulin repeats. However, lasp-2 is greatly different from lasp-1 in the sequence between the second nebulin repeat and a SH3 domain, and the region is conserved in chicken, mouse, and human. As expected from its structural similarity to lasp-1, lasp-2 possessed actin-binding activity and localized with actin filament in filopodia of neuroblastoma. In contrast to lasp-1, which is widely distributed in non-muscle tissues, lasp-2 was highly expressed in brain.

Keywords: Brain; Chicken; Actin-binding proteins; F-actin column chromatography; Mass spectrometry; LIM and SH3 protein; Co-precipitation; Neuroblastoma; Filopodia

The complex process of nervous development is dependent on the actin cytoskeleton [1]. The motility machinery common to migrating cells and neuronal processes requires the organized polymerization of actin filaments. Lamellipodia and filopodia at growth cones are produced by the polymerization of filamentous actin underneath the cell membrane. Cortical neurons innervate many of their targets by collateral axon branching, which requires local reorganization of the cytoskeleton. Most of the structures containing actin cytoskeleton are temporally formed, and their integrities are regulated by actin-binding proteins (ABPs) and signal transduction pathways [2].

F-actin affinity column was designed to collect ABPs directly from extract of *Drosophila* embryo [3] and novel ABPs have been identified by the method. We used this approach to analyze proteins associated with the actin cytoskeleton of chicken brain and more than 30 proteins specifically bound to the ligand. From 34 kDa protein band, we identified a novel type of LIM and SH3 protein (lasp-2). A gene of lasp-2 has been identified in silico with similarity to lasp-1 (LIM and SH3 protein 1) [4]. Lasp-2 cDNAs have been reported in mouse and human and human lasp-1 and lasp-2 have been predicted to be derived from different genes by using bioinformatics [4].

☆ *Abbreviations:* BSA, bovine serum albumin; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CBB, Coomassie brilliant blue R-250; DTT, dithiothreitol; EGTA, ethylene glycol bis (1-aminoethyl ether)-*N*,*N*′-tetraacetic acid; GST, glutathione *S*-transferase; Hepes, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; NBT, nitroblue tetrazolium; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylydene difluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TAME, *N*-*p*-tosyl-L-arginine methyl ester; TCA, trichloroacetic acid; TBS, Tris-buffered saline; Tween 20, polyoxyethylene sorbitan monolaurat.

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Here we report the domain structure, tissue distribution, activity to actin, and cellular localization of chicken lasp-2. Compared with lasp-1, lasp-2 has unique sequences conserved in chicken, mouse, and human. Lasp-2 was highly expressed in brain, whereas lasp-1 was widely distributed in non-muscle tissues. The two closely related proteins may have overlapping and specific functions in the actin cytoskeleton.

Materials and methods

Preparation of brain extract. Adult chicken brain extract was prepared with low salt alkaline (LSA) buffer developed by Feramisco and Burridge [5]. Fresh brains were homogenized in 5 volumes of PBS (20 mM Na-PO_4, 0.15 M NaCl, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin, pH 7.2) with a homogenizer and then centrifuged at 7000*g* for 10 min. The pellet was resuspended in PBS again and washed three times. The insoluble pellet was then washed with distilled water, resuspended into LSA (2 mM Tris, 1 mM EDTA, and 0.1 mM PMSF, pH 9.2), and incubated at 4 °C overnight. The suspension was centrifuged at 12,000*g* for 30 min and the supernatant was stored at −80 °C. The stored extract was dialyzed against A-buffer (50 mM Hepes, 2 mM DTT, 0.5 mM EGTA/EDTA, 10% glycerol, 0.05% NP-40, 2 mM TAME, 0.1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin, pH 7.5) and centrifuged at 100,000*g* for 2 h. The resultant supernatant was applied to the affinity column.

F-actin affinity column chromatography. Columns for affinity chromatography were prepared according to the method of Miller and Alberts [3]. Rabbit back muscle actin was prepared by the method of Spudich and Watt [6] and further purified through a Sephadex G-100 column (Amersham Biosciences, Piscataway, NJ, USA). F-actin polymerized in 50 mM Hepes, 0.1 M KCl, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 0.2 mM ATP, pH 7.5, was stabilized with 10 μg/ml phalloidin, and then coupled to Affi-Gel 10 (BIO-RAD, Hercules, CA, USA) mixed with Sepharose CL-6B (Amersham Biosciences).

The F-actin column was equilibrated with A-buffer. The extract was applied to the column at a flow rate of 1 column volume/h. The column was rinsed with A-buffer at a flow rate of 3 column volumes/h. Proteins adsorbed to the column were then eluted successively with A-buffer containing 0.1 M KCl and A-buffer containing 1.0 M KCl, 1.0 mM ATP, and 3 mM $MgCl_2$.

SDS–PAGE and immunoblotting. SDS–PAGE was performed according to the method of Laemmli [7], using 10% or 3–15% linear-gradient acrylamide gels. The gels were stained with CBB or Silver Stain Plus kit (BIO-RAD).

Immunoblotting analysis was performed according to the procedure developed by Towbin et al. [8] with modifications. Electrophoresed proteins were transferred onto PVDF membranes and the membranes were blocked with 5% skimmed milk in TBS (20 mM Tris–HCl and 0.5 M NaCl, pH 7.6). They were incubated with primary antibodies in TBS containing 0.05% Tween 20 (TTBS) for 2 h, then incubated with alkaline phosphatase-labeled secondary antibody (MP Biomedicals, Aurora, OH, USA) for 1 h, and developed with BCIP and NBT.

Liquid chromatography/mass spectrometry. The F-actin column fractions were concentrated by precipitation with TCA and separated by SDS–PAGE. Gel pieces were excised and digested with 12.5 ng/μl trypsin (Promega, Madison, WI, USA) as described elsewhere [9]. The resulting tryptic peptide mixture was separated and analyzed by electrospray ionization mass spectroscopy with liquid chromatography (LCQ, Thermo Finnigan, San Jose, CA, USA).

MS/MS data were compared to the theoretical peptide masses of all available proteins from Chordata in NCBInr and chicken tentative consensus sequence database (TIGR *Gallus gallus* Gene Index) using the MASCOT search program (Matrix Science, London, UK).

Isolation of cDNA clones of chicken lasp-1 and lasp-2. Full-length cDNA clones of chicken lasp-1 and lasp-2 were obtained by RT-PCR of adult chicken brain mRNA. For lasp-1 cloning, cDNAs were amplified with primers derived from the nucleotide sequence of chicken EST clone (pgp1n.pk013.e3: GenBank Accession No. BI394039) already reported as a homologue of mammalian lasp-1 [10]. For lasp-2 cloning, cDNAs were amplified with primers derived from the nucleotide sequence of chicken EST clones (ChEST486f16: GenBank Accession No. BU273155 and ChEST11k19: GenBank Accession No. BU115256). The PCR products were subcloned into a pBluescript vector and sequenced using a 310 DNA sequencer (Applied Biosystems).

Antibodies. Antibodies against recombinant chicken lasp-1 and lasp-2 were affinity-purified from rabbit serum. Full-length cDNA clones of chicken lasp-1 and lasp-2 were subcloned into pRSET vectors (Invitrogen Japan K.K., Tokyo, Japan). Recombinant proteins were purified with Ni–NTA beads (QIAGEN K.K., Tokyo, Japan) and immunized to rabbits separately. Each clone was also subcloned into a pGEX vector (Amersham Biosciences). GST-tagged proteins were purified with glutathione–Sepharose 4B (Amersham Biosciences) and stabilized on Affi-gel 10 (BIO-RAD). Antisera against His-tagged proteins were affinity-purified using the columns of GST-tagged proteins. Affinity-purified anti-lasp-2 antibody was adsorbed using the GST-lasp-1 column to avoid cross-reactivity to lasp-1. Anti-lasp-1 antibody was adsorbed using the same method.

Antibodies against chicken p34-Arc were prepared as described elsewhere [11].

Co-sedimentation of lasp-1 and lasp-2 with F-actin. Purified GST-lasp-1 and GST-lasp-2 were dialyzed against 20 mM Na-PO_4 and 0.1 M KCl, pH 7.2. After centrifugation at 100,000*g* for 1 h, each of the supernatants was mixed with G-actin (0.2 mg/ml G-actin, 20 mM Na-PO_4, 0.1 M KCl, 5 mM $MgCl_2$, and 0.2 mM ATP, pH 7.2, at the final concentration). The samples were incubated for 2 h at room temperature and then centrifuged at 100,000*g* for 1 h. The supernatants and the pellets were analyzed by SDS–PAGE.

Motif search. Domain search was performed using the Motif Scan in Protein Sequence available on Hits web site (http://hits.isb-sib.ch/). Phosphorylation site analyses were performed using the NetPhos of the Center for Biological Sequence Analysis web site (http://www.cbs.dtu.dk/services/NetPhos/).

Cell culture and transfection. Neuroblastoma NG108-15 cells were cultured as previously described [12]. cDNAs of full-length lasp-1 and lasp-2 were inserted into the *Bam*HI site of pEGFP-C1 and C2 (Clontech, Palo Alto, CA), respectively. These constructs were transfected with SuperFect (QIAGEN K.K.) into NG108-15 cells according to the manufacturer's protocol.

Fluorescence microscopy. Living cells transfected with EGFP-lasp-1 and EGFP-lasp-2 were observed under a confocal laser scanning microscope (LSM510, Zeiss, Japan) as previously described [13]. For observation of actin filament, cells were fixed and stained with rhodamine–phalloidin (Sigma–Aldrich Japan K.K., Tokyo, Japan).

Results

Thirty-four kiloDaltons protein identified as lasp-2

Fig. 1 shows the SDS–PAGE profile from the F-actin columns loaded with brain extract. In case 10 ml F-actin column (0.5–0.8 mg F-actin/ml resin) was loaded with extract containing 150 mg total protein, more than 30 proteins bound to F-actin column (shown with

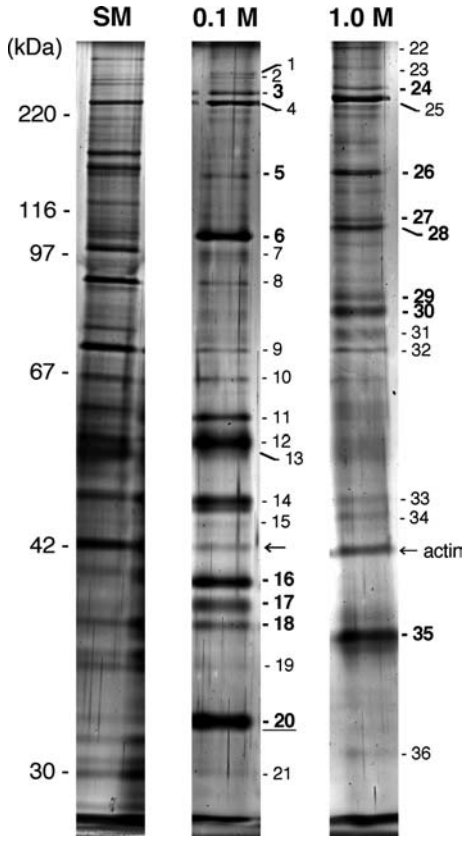

Fig. 1. Elution profiles of F-actin affinity chromatography. Extract and eluates of F-actin column were run in 10% gel and silver-stained. Bars with numbers indicate binding proteins. Bands in bold letters are proteins concentrated in the eluates. #20 indicates the 34 kDa protein band analyzed by LC/MS. Numbers on the left indicate the molecular weights $\times 10^3$ of marker proteins, chick gizzard myosin heavy chain, β-galactosidase, phosphorylase b, bovine serum albumin, rabbit muscle actin, carbonic anhydrase, and soybean trypsin inhibitor. SM, starting material; 0.1 M, 0.1 M KCl eluate from F-actin column; and 1.0 M, 1.0 M KCl eluate from F-actin column.

numbers). Amino acid sequencing and liquid chromatography/mass spectrometry (LC/MS) of 34 kDa band which seemed to be concentrated in column eluate (#20 in Fig. 1) detect p34-Arc of Arp2/3 complex (data not shown). Additionally, LC/MS data suggest that several peptides from the 34 kDa band had similar masses to those of theoretical peptides from mouse EST clone RIKEN cDNA 1200007O21 (GenBank Accession No. AK004645), which is predicted to be mouse lasp-2 [4].

Predicted structure of chicken lasp-1 and lasp-2

Several clones homologous to RIKEN cDNA 1200007O21 were deposited in two chicken EST databases (University of Delaware chicken EST database and BBSRC chick EST database). According to their sequences, a cDNA of 822 bp encoding 273 amino acids was obtained from chicken brain mRNA (the sequence was deposited as GenBank Accession No. AB114207). Predicted molecular mass is 31,434 Da and all peptides identified with LC/MS were included in the clone (data not shown). The clone showed 96% amino acid identity both with the mouse lasp-2 and human putative lasp-2 analyzed from two EST clones (GenBank Accession Nos. AK096540 and BF99808) in silico [4], so the clone was identified as chicken lasp-2. Chicken lasp-2 showed 64% amino acid identity to chicken lasp-1 (GenBank Accession No. BI394039) which was reported as a homologue of mammalian lasp-1 [10]. Both chicken lasp-1 and lasp-2 have N-terminal LIM domains, C-terminal SH3 domains, and internal nebulin repeats, but the sequence between the second nebulin repeat and the SH3 domain in lasp-2 is greatly different from that in lasp-1 (Fig. 2C). This sequence ("specific region" in Fig. 2A) is conserved in lasp-2 proteins of chicken, mouse, and human (Fig. 2B). Two of the phosphorylation serine residues (S61 and S99) analyzed in lasp-1 [10,14] were found in lasp-2, but another (S146) was not (Figs. 2B and C, arrowheads). Predicted isoelectric points of lasp-1 and lasp-2 are 7.15 and 8.31, respectively.

Tissue distribution of chicken lasp-1 and lasp-2

Immunoblotting of GST-lasp-1 and GST-lasp-2 showed that antibodies against lasp-1 and lasp-2 after adsorption were not cross-reacted (Fig. 3A, arrows). The apparent molecular mass of recombinant lasp-2 is lower than that of lasp-1 although chicken lasp-2 has 273 amino acids compared to chicken lasp-1 with 258 amino acids. However, their relative mobilities were similar to the result that antibodies against lasp-1 and lasp-2 reacted with 38 and 34 kDa bands in chicken brain, respectively (Fig. 3B, arrows). Anti-lasp-2 antibody reacted with 34 kDa band from the 0.1 M KCl eluate of the F-actin column from which peptides of lasp-2 were detected (Fig. 3C). The 34 kDa band was also reacted with anti-p34-Arc antibody (Fig. 3C) and these results were consistent with the LC/MS data.

Whole protein samples of various tissues were reacted with anti-lasp-1 and anti-lasp-2 antibodies. Anti-lasp-1 antibody reacted with all non-muscle tissues to the same content, but anti-lasp-2 antibody heavily reacted with brain (Fig. 4).

Actin-binding activity of recombinant lasp-1 and lasp-2

In the absence of actin, a small part of GST-lasp-1 and GST-lasp-2 precipitated but most of them remained in the supernatants. When actin was added, most of the GST-lasp-1 and GST-lasp-2 co-precipitated with F-actin but control GST did not (Fig. 5).

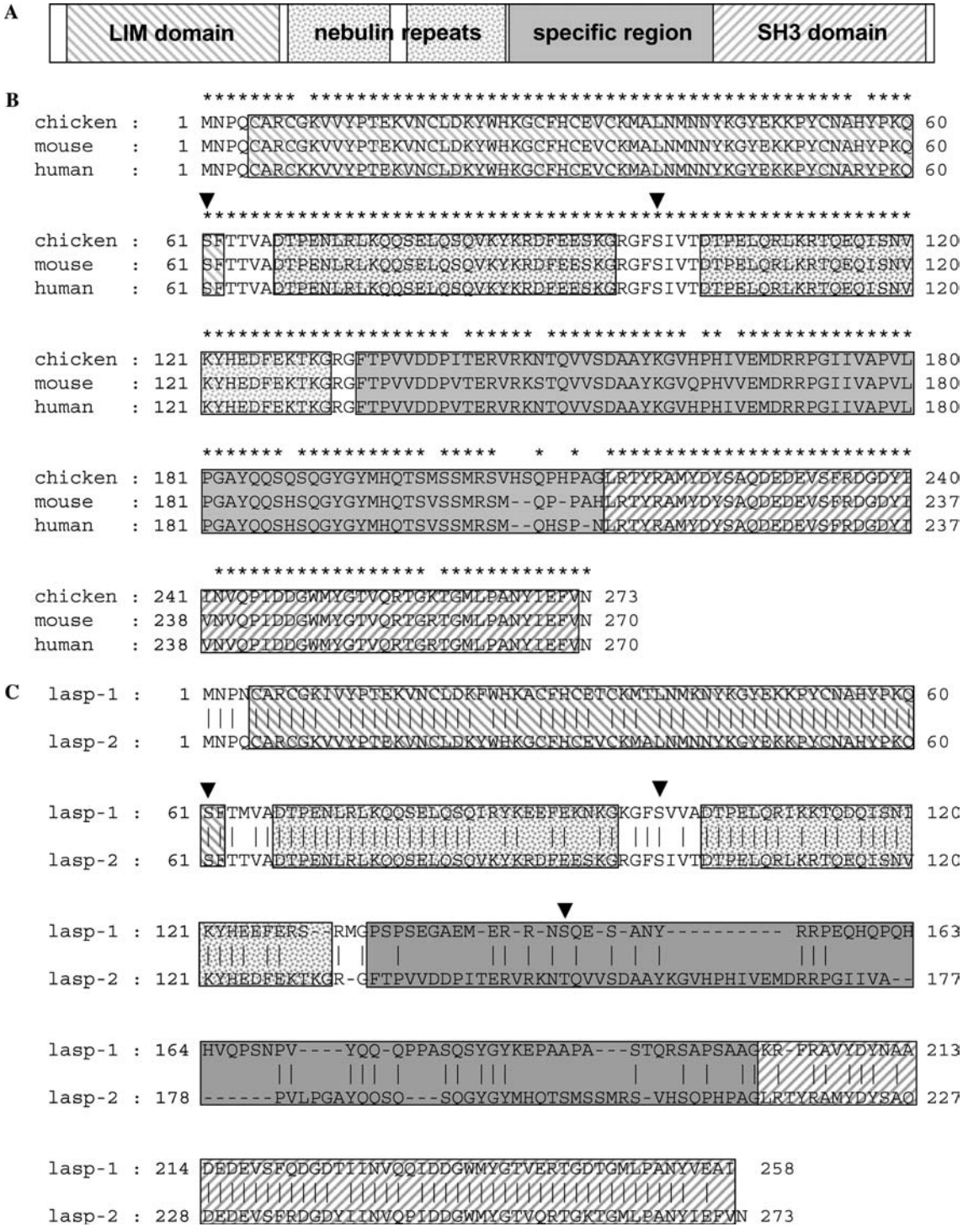

Fig. 2. Structures of lasp proteins. (A) Schematic structures of lasp proteins. (B) Deduced amino acid sequences of lasp-2 of chicken, mouse, and human. (C) Comparison of chicken lasp-1 and lasp-2. Shaded boxes indicate a LIM domain, two nebulin repeats, specific region, and a SH3 domain. Asterisk, identical amino acids between three species. Arrowheads, phosphorylated serine residues reported in lasp-1. The chicken lasp-2 clone was deposited in the DDBJ nucleotide sequence database with Accession No. AB114207.

Localization of lasp-1 and lasp-2 in neuroblastoma

In living NG108-15 cells, both EGFP-lasp-1 and lasp-2 showed liner thick filamentous structures in lamellipodia (Figs. 6A and B). To compare the distribution of lasp-1 and lasp-2 with actin filament, the transfected cells were stained with rhodamine–phalloidin. Both lasp-1 and lasp-2 were localized to actin filament bundles extended from lamellipodia into filopodia (Figs. 6C and F).

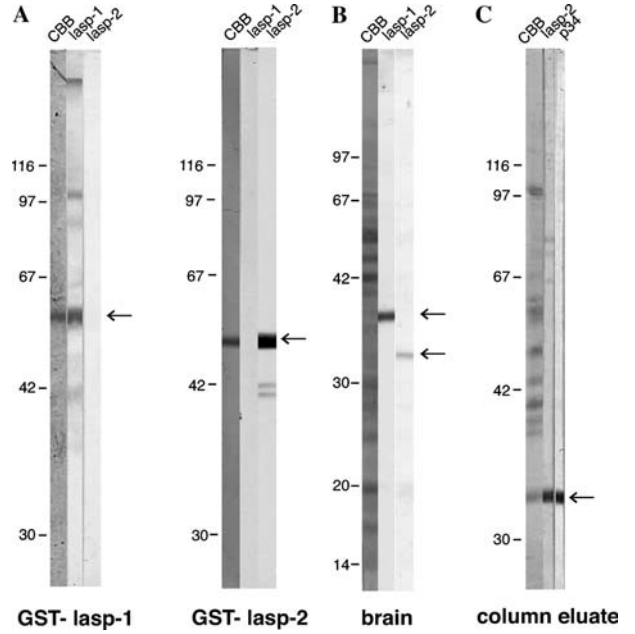

Fig. 3. Reactivity of antibodies against chicken lasp-1 and lasp-2. (A) Immunoblot of GST-fusion proteins with anti-lasp-1 and anti-lasp-2 antibodies. (B) Immunoblot of chicken brain with anti-lasp-1 and anti-lasp-2 antibodies. (C) Immunoblot of 0.1 M eluate of F-actin column with anti-lasp-2 and anti-p34-Arc antibodies. Samples of (A,C) were run in 10% gel and sample of (B) was run in 12.5% gel. CBB, CBB staining pattern; lasp-1, anti-lasp-1 antibody; lasp-2, anti-lasp-2 antibody; and p34, anti-p34-Arc antibody.

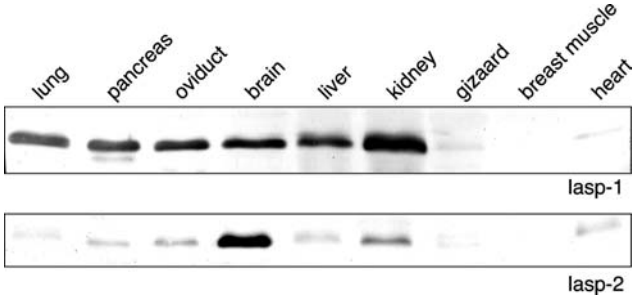

Fig. 4. Tissue distribution of lasp-1 and lasp-2. Whole samples of various tissues were run in the same 12.5% gels and reacted with anti-lasp-1 (upper panel) and anti-lasp-2 (lower panel) antibodies. Each sample contained 30 μg protein.

Discussion

Lasp-2 identified with the F-actin column and LC/MS analysis

The advent of proteomics techniques has been enthusiastically accepted in most areas of biology including brain research [15]. Mass-spectrometric approaches or microsequencing was approved to identify proteins. An additional advantage is that unknown proteins, so far only proposed from their nucleic acid structure, designated as hypothetical proteins can be identified.

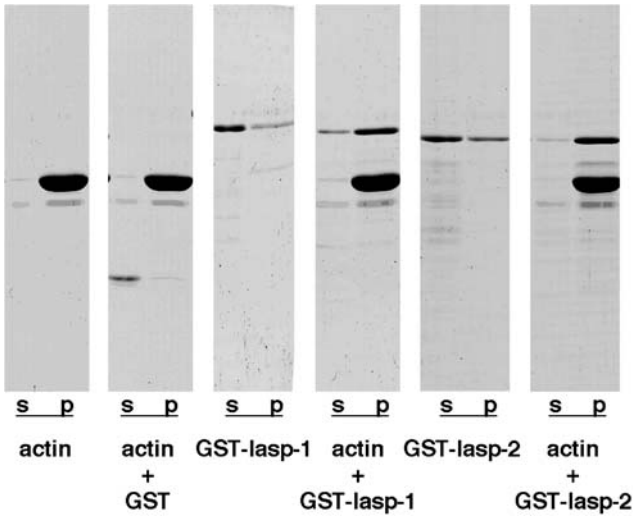

Fig. 5. Actin-binding activity of recombinant lasp-1 and lasp-2 in co-precipitation assay. As much as 0.2 mg/ml of G-actin from rabbit back muscle was polymerized under physiological condition in the presence of GST-lasp-1, GST-lasp-2, or control GST.

This study is the first report on the identification of a novel type of LIM and SH3 protein (lasp-2) on protein basis. We obtained lasp-2 from F-actin affinity column eluates of chicken brain and cloned its cDNA based on LC/MS data. Chicken lasp-2 possesses a LIM domain, two nebulin repeats, and a SH3 domain similar to EST clones of mouse and human which are putative lasp-2 in silico [4] (Fig. 2B). Lasp-1, a protein with the same domains, has been previously reported [16], but peptides from 34 kDa band showed much higher identity to chicken lasp-2 than chicken lasp-1 in MASCOT search (data not shown). In addition, the specific antibody against chicken lasp-2 reacted with the 34 kDa bands of the whole brain sample and the column eluate (Figs. 3B and C). Thus, we concluded that chicken lasp-2 specifically bound to the F-actin column. Anti-lasp-1 antibody reacted with 38 kDa band in brain (Fig. 3B) and the molecular mass is similar to reports of lasp-1 in other vertebrates [17,18].

Domain structure of chicken lasp-1 and lasp-2

Lasp-1 (MLN50) was identified as a human gene product amplified in breast carcinoma [16,19]. Lasp-1 was also identified as pp40 whose phosphorylation increased in parietal cells by histamine stimulation [17]. An N-terminal LIM domain and a carboxyl-terminal SH3 domain are major protein-interacting motifs expected to interact with multiple-binding partners and two nebulin repeats are responsible for actin-binding activity [18]. Three serine phosphorylation sites have been reported and the sites were thought to affect actin-binding activity and localization [10,14].

Lasp-2 has the same domain structure as lasp-1, but the sequence between the second nebulin repeats, and

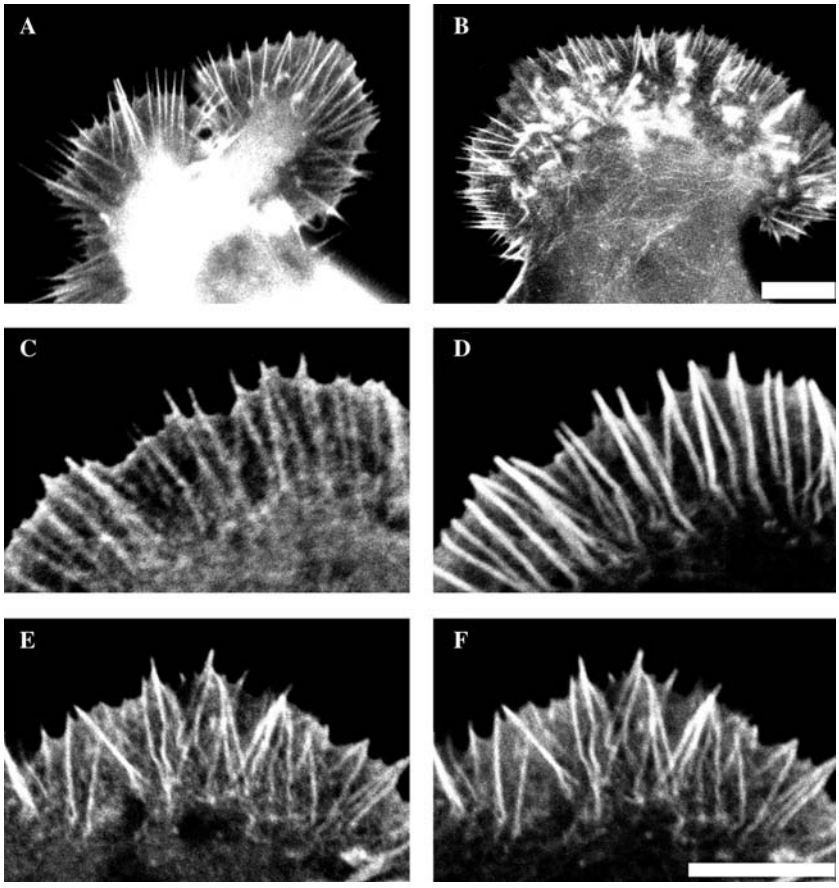

Fig. 6. Localization of lasp-1 and lasp-2 in NG108-15 cells. NG108-15 cells were transfected with EGFP-lasp-1 (A,C,D) or lasp-2 (B,E,F). (A–C and E) Fluorescence from EGFP; (D,F) rhodamine–phalloidin staining. In (C–F) the thickness of focal planes was set at 0.7 μm, however (A,B) were observed without a pinhole to obtain fluorescence in living cells. Bars, 10 μm.

the SH3 domain is lasp-2 specific ("specific region" in Fig. 2). Sequences of the LIM domain, nebulin repeats and SH3 domain of lasp-1 and lasp-2 are almost identical, so they are expected to interact with the same proteins in those domains. Dynamin, a lasp-1-binding partner, is thought to bind to the SH3 domain [20], and therefore, lasp-2 may also bind to dynamin and regulate vesicle transport. The specific region is considered to be essential for specific functions of lasp-1 and lasp-2 because they are well conserved between mammal and avian (Fig. 2B). Lasp-1 and lasp-2 are different from each other in content of charged amino acids in the specific region, so it is also possible that the region would have specific-binding partners, affect structures of whole molecules, or have additional activity. Two of the three phosphoserine sites (S61 and S99) analyzed in lasp-1 were conserved (Fig. 2, arrowheads), so phosphorylation would regulate lasp-2 activity in vivo.

Lasp-2 highly expressed in nerve tissue

Sequence similarity of lasp-1 and lasp-2 necessitated the immunoabsorption of the antibodies with each protein to generate specific antibodies (Fig. 3A). Immunoblot analysis with the antibodies revealed that expression of lasp-2 is prominent in brain while lasp-1 is widely distributed in non-muscle tissues.

Lasp-1 mRNA and its product were detected in various tissues [14,17,21,22] and lasp-1 is thought to be involved in many functions in various types of cells. Lasp-1 was first identified as a gene highly expressed in breast cancer, and intense cytoplasmic localization in malignant cells was thought to affect cytoskeletal modifications [18]. In the parietal cell, elevation of intracellular cAMP induces a partial translocation of lasp-1 to the apically directed F-actin-rich intracellular canaliculus, which is the site of active HCl secretion [10,22]. This suggests that lasp-1 may play a role in the regulation of vesicle trafficking via actin cytoskeleton plasticity. In platelets, lasp-1 is a substrate of cAMP protein kinase and cGMP protein kinase and thought to play roles in reorganization of the actin cytoskeleton [14].

Almost EST clones of chicken lasp-2 were from library of nerve tissue, except one clone obtained from muscle. Two lasp-2 clones of human were also derived from brain, but the mouse lasp-2 clone was from lung. So

expression of lasp-2 was not restricted to brain, but its relative abundance suggests specific functions in nerve tissues. It is interesting to note that two proteins of similar structure, lasp-1 and lasp-2, are co-expressing in brain.

Interactions between Lasp-2 and actin in vitro/vivo

Actin-binding activity of lasp-1 was demonstrated with GST-pull-down assay and co-precipitation assay [10,14,18]. Lasp-1 has been reported to localize in actin-rich subcellular regions, such as focal complex, cell–cell contact, leading edges, and tips of lamellipodia and the localization varied according to cell types [10,14,18,22]. Lasp-2 was also shown to bind F-actin in vitro (Fig. 5) and concentrated to actin filament bundles of filopodia with lasp-1 (Figs. 6A and B). Although several ABPs have been reported to play roles in the formation of filopodia [23], the regulatory mechanism how filopodial actin bundles emanate from lamellipodial actin filament meshwork has not been well documented. Since both lasp proteins have tandem nebulin repeats, they could contribute to the formation of filopodial actin bundles through their multiple acitn filament-binding activity. A reason why two closely related proteins are localized in the same region of the cells is unclear. Further analysis of localization in tissues/cells, phosphorylation status, and binding partners of lasp-1 and lasp-2 would elucidate their physiological roles.

Acknowledgments

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