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A Role for Lengsin, a Recruited Enzyme, in Terminal Differentiation in the Vertebrate Lens*

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Abstract

Lengsin is an eye lens-specific member of the glutamine synthetase (GS) superfamily. Lengsin has no GS activity, suggesting that it has a structural rather than catalytic role in lens. *In situ* hybridization and immunofluorescence showed that lengsin is expressed in terminally differentiating secondary lens fiber cells. Yeast two-hybrid (Y2H) and recombinant protein experiments showed that full-length lengsin can bind the 2B filament region of vimentin. In affinity chromatography, lengsin also bound the equivalent region of CP49 (BFSP2; phakinin), a related intermediate filament protein specific to the lens. Both the vimentin and CP49 2B fragments bound lengsin in surface plasmon resonance spectroscopy with fast association and slow dissociation kinetics. Lengsin expression correlates with a transition zone in maturing lens fiber cells in which cytoskeleton is reorganized. Lengsin and lens intermediate filament proteins co-localize at the plasma membrane in maturing fiber cells. This suggests that lengsin may act as a component of the cytoskeleton itself or as a chaperone for the reorganization of intermediate filament proteins during terminal differentiation in the lens.

The optical properties of the vertebrate eye lens depend upon hierarchies of organization from the cellular to the molecular level. A population of relatively undifferentiated epithelial cells proliferates and elongates under the control of growth factors from other parts of the eye to produce concentric layers of extremely elongated fiber cells (1–3). As new layers of fiber cells are added, the older cells pack more tightly, forming complex interdigitations between and within layers (4,5). In addition, the fiber cells undergo a characteristic terminal differentiation, with some interesting similarities to apoptosis, in which lens nuclei and other organelles are lost, and the cytoskeleton is reorganized (6–9). It is believed that this contributes to optical clarity through the center of the lens (10). At the molecular level, this program involves developmentally and spatially regulated expression of crystallins; to produce the gradients of refractive index necessary to diffract and focus light and to minimize spherical and chromatic aberrations; and of tissue-specific cytoskeletal and membrane proteins that organize the structure and junctions of the epithelial and fiber cells (1,2,11).

Lengsin, for lens glutamine synthetase-like (also known as glutamate-ammonia ligase (glutamine synthetase) domain containing 1, GLULD1), was discovered through the NEIBank project for ocular genomics (12–16). It is a survivor of an ancient group of class I glutamine synthetases but has lost enzyme activity (15). Here we show that lengsin is lens-specific and, furthermore is tightly regulated for expression specifically at the onset of

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terminal differentiation of secondary lens fiber cells. Furthermore, lengsin is capable of interacting with major intermediate filament proteins of the lens, and its expression correlates with the reorganization of lens fiber cell cytoskeleton. This suggests that lengsin arose from origins in an enzyme superfamily to a highly specialized, highly conserved role in vertebrate lens development and cellular differentiation.

Experimental Procedures

Northern and Western Blots

A mouse multitissue Northern blot was purchased from Seegene (Seoul, Korea). RNA from rat eye tissues was prepared and used to make a Northern blot by standard methods. A full-length pSport1 (Invitrogen, Carlsbad CA) cDNA clone for mouse lengsin was selected from an NEIBank mouse whole eye library (17). 5 μg was linearized at an internal PstI site to and used to generate an antisense RNA probe (~600 bp) labeled with psoralen biotin using the Ambion Strip EZ Sp6 kit (Ambion, Austin TX). Blots were hybridized at 68 °C overnight; washed for 20 min with low stringency buffer (Ambion) at room temperature, then twice with high stringency buffer at 68 °C for 20 min. Blots were visualized using the Ambion Biodetect kit (CDP star) and exposure to film. Western blots of mouse lens epithelium and fiber cells were performed using rabbit antibody to recombinant mouse lengsin as described previously (15).

In Situ Hybridization

ISH[2] was performed at Molecular Histology (Gaithersburg, MD). The mouse lengsin cDNA in pSport1 was linearized with either Mlu I or NotI to generate ~2kb sense and antisense probes from Sp6 and T7 promoters. Probes were radiolabeled with ^{35}S, fragmented by mild alkali hydrolysis, and used to hybridize mouse whole eye sections (18).

Antibodies

Anti-mouse lengsin antibody was raised against recombinant protein as described (15). Commercially available antibodies were purchased for vimentin: V9 mouse monoclonal (ICN, Costa Mesa, CA), mouse polyclonal 7752 (Abcam, Cambridge UK), chicken polyclonal ab24525 to rat vimentin (Abcam); and for CP49/phakinin: guinea pig polyclonal (03-GP63) to calf phakinin (ARP, Belmont MA) (includes reactivity to filensin).

Immunofluorescence Localization

10-μm frozen sections from adult mouse eye were used for immunofluorescence staining. Sections on Superfrost/Plus slides (Daigger, Wheeling, IL) were dried at room temperature, fixed in 4% paraformaldehyde in PBS for 10 min, washed, and blocked for 1 h at room temperature in ICC buffer (0.5% bovine serum albumin, 0.2% Tween 20 in PBS, pH 7.3) plus 5% goat serum. Slides were incubated with rabbit anti-lengsin, 1:200 dilution in ICC buffer, plus either chicken anti-vimentin (Abcam, 1:200 dilution) or guinea pig anti-phakinin (American Research Products, 1:50) as primary antibodies for 1 h at room temperature, washed with ICC buffer, and incubated with goat anti-rabbit Alexa Fluor 488 antibody (Invitrogen, 1:300 dilution) plus goat anti-guinea pig Cy3 antibody (Jackson ImmunoResearch, West Grove, PA, 1:100 dilution) or goat anti-chicken Cy3 antibodies (Jackson ImmunoResearch, 1:200 dilution) for 1 h at room temperature. After washing with ICC buffer, slides were incubated with DAPI (Invitrogen) 1 μg/ml)) for 10 min, washed, coverslipped, and sealed. Samples were examined under Leica SP2 confocal microscope

[2]The abbreviations used are: ISH, *in situ* hybridization; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; DTT, dithiothreitol; HA, hemagglutinin; GS, glutamine synthetase; Y2H, yeast two-hybrid; SPR, surface plasmon resonance.

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(Leica Microsystems, Exton, PA). Primary antibodies were omitted from negative control slides.

Yeast Two-hybrid

Poly(A) + RNA from adult C57Bl/6 mouse lenses was used to make a yeast two-hybrid library in *Saccharomyces cerevisiae* strain AH109 using the Match Maker System at Clontech (Palo Alto, CA). Bait constructs of mouse lengsin were cloned into NdeI and XhoI sites of the pGBKT7 bait vector and transformed into yeast strain Y187. Lengsin expression in yeast transformed with bait clones was determined by Western blot using anti-lengsin and GAL4 DNA-BD monoclonal (Clontech) antibodies. Bait and library yeast were mated overnight at 30 °C according to the manufacturer's protocols. Yeast zygotes were rinsed with 0.5× YPD medium (Clontech), spread onto plates containing 4-DO yeast medium (-Ade/-His/-Leu/-Trp) (Clontech) and incubated at 30 °C for 3–5 days.

Yeast colonies that appeared on 4-DO medium were replated on 4-DO plates, supplemented with 20 μg/ml X-Alpha-Gal (Clontech). The plates were incubated for 12–18 h at 30 °C and checked for the appearance of blue colonies. Blue colonies were inoculated into -Leu 1-DO medium (Clontech) and grown for 1–2 days. Prey plasmid DNA in pGADT7-Rec was isolated using Lyticase (Sigma) 100 units/ml, heat/thaw treatment and Wizard Plus SV Miniprep DNA purification system (Promega, Madison WI) and were then transformed into *Escherichia coli* XL-2 Blue (Stratagene) and plated on LB/Amp. Purified prey and bait plasmids were tested for self activation by transformation into AH109 and Y187 yeast with either the empty bait vector or empty prey vector, plated on 4-DO medium, and compared with Clontech positive controls following the manufacturer's protocols.

Specific prey clones for mouse CP49 and filensin were also prepared by PCR from cDNA clones and cloning into NdeI/XhoI sites of the prey vector pGADT7-Rec. These were introduced into yeast hosts, examined for expression of prey protein by Western blot using Gal4-AD monoclonal antibody (Clontech) and tested for interaction with the lengsin bait construct.

Expression, Co-expression, and Purification of Vimentin and Lengsin

Full-length mouse lengsin and the Vim2B fragment of mouse vimentin were cloned separately into pET31b (Novagen, EMD Biosystems San Diego, CA) for individual expression and were also cloned together into pCOLADuet-1 (Novagen) for co-expression in *E. coli*, BL-21 codon plus RIL (Stratagene, La Jolla, CA). For lengsin and co-expressed lengsin/Vin 2B, soluble whole cell lysates were dialyzed against 25 mM malonic acid pH 6.0/1 mM DTT then loaded onto an SP-fast flow, 26/60, cationic exchange column (Amersham Biosciences/GE Biosciences, Piscataway NJ) run on an AKTA Explorer 100 (Amersham Biosciences). Samples were eluted using a salt gradient in 25 mM malonic acid, pH 6, 1 mM DTT, 0.3–1 M NaCl. For Vim2B alone, whole cell lysate was dialyzed against 50 mM Tris pH 8.5, 1 mM DTT and separated using a Q-Sepharose, fast flow column (Amersham Biosciences) using a salt gradient of 0–1.0 M NaCl.

Co-expressed lengsin and Vim2B, either from *E. coli* lysate or from ion-exchange purified material, were loaded on a Sephacryl, S-400 column (1 CV = 880 ml, XK 50) (Amersham Biosciences) in 25 mM malonic acid, pH 6.0, 1 mM DTT, and eluted at 0.5 ml/min. The fractions were collected and evaluated using SDS-PAGE, Western blot and Voyager-DE (Applied Biosystems, Foster City CA) MALDI-TOF mass spectrometry in reflector mode.

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Expression of Recombinant HA-CP492B

The region of mouse CP49 equivalent to the Vim2B filament region was amplified from the pGADT7-Rec construct, incorporating the HA tag from the vector as an NheI/XhoI fragment. The HA-CP492B fragment was then cloned into pET17-b and expressed. The HA-tagged protein was isolated using an HA affinity column (Roche Applied Science, Indianapolis, IN).

Immunoprecipitation

Immunoprecipitation experiments were conducted using recombinant mouse lengsin and hamster vimentin (Cytoskeleton, Denver, CO) following the methods of Yabe *et al.* (19). 1 mg of soluble protein was incubated in 60 mM Tris, pH 7.4, 0.2% SDS, 0.1 mM DTT, 1 mM EDTA, and 1% Tween 20 with 1:10 dilution antibodies against vimentin overnight at 4 C with constant mixing. Immobilized IgM (Sigma, A4540) or IgG1 (Sigma A6715) secondary antibody slurries were added to reactions and incubated at room temperature using constant agitation for 3 h. Negative controls were prepared by adding immobilized secondary antibodies in the absence of primary antibodies. The beads were then collected by centrifugation, washed three times with the binding reaction buffer for 5 min using constant agitation. The protein/antibody complex was then eluted by adding 1% SDS, 100 mM DTT, and 50 mM Tris, ph 7.5 heated at 90 C for 5 min. The eluted fractions were then analyzed by Western blot analysis using the ECL Western blotting Analysis System (Amersham Biosciences).

Affinity Chromatography

An affinity column for CP492B was produced by binding HA-CP492B to an HA affinity column (Roche Applied Science). Mouse lengsin was applied to the column, washed, and then eluted using HA peptide, following the manufacturer's protocols. Flow-through, wash, and elute fractions were checked by Western blot for both lengsin and HA-CP492B.

Surface Plasmon Resonance Spectroscopy

The interactions of mouse lengsin with Vim2B and Ha-CP492B fragments were analyzed using surface plasmon resonance (Biacore 3000, Biacore). Antibody to lengsin was bound in analysis and reference cells of a CM5 sensor chip, following the manufacturer's protocols to reach 11,000 resonance units (RU). Recombinant mouse lengsin, (0.6 mg/ml in PBS), was manually injected into one flow cell, 10 μl/min and captured to reach 2000 resonance units. Following capture, lengsin was covalently bound using 50 μl of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 0.05 M *N*-hydroxy-sulfosuccinimide at a flow rate of 50 μl/min for 30 s. Activated amine groups in both cells were blocked with ethanolamine.

Association kinetics were studied by injecting increasing concentrations of purified Vim2B or HA-CP492B in PBS over both cells. Protein solutions were injected for 150 s during the association phase at a constant flow rate of 20 μl/min. The dissociation was followed for 200 s at the same flow rate. In all experiments, cell regeneration was performed by injecting NaCl, 2 M for 1 min. BiaEvalution 3.0 software was used to analyze and determine rate affinity constants.

Results

Localization in Lens

A cDNA probe for mouse lengsin was used to probe an adult mouse multitissue Northern blot using colorimetric methods (Fig. 1A). A positive signal was seen only in whole eye, just above the size of 18 S rRNA. The same probe was used in a Northern blot of newborn and

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adult rat lens and retina (Fig. 1B). Clear expression of a 2-kb mRNA was detected in adult lens, but not in retina or in newborn lens. This specificity is consistent with the observation of ESTs for lengsin, which, in libraries made from dissected tissues, are restricted to lens in all species examined. A Western blot of dissected mouse epithelial and fiber cells showed the doublet of lengsin bands at 62 and 51 kDa that correspond to full-length protein and to the loss of the N-terminal domain (15) only in fiber cells (Fig. 1C).

Detailed expression of lengsin mRNA in the mouse eye was examined by *in situ* hybridization (ISH) (Fig. 2). Consistent with the results of Northern blotting, no signal was observed in newborn or embryonic eye sections (not shown). However, in adult eye sections an intense, discrete ring of hybridization was observed in the region of terminal differentiation of secondary fiber cells. Staining patterns showed that while cell nuclei were present in cells expressing lengsin, no nuclei were present below these layers of secondary fiber cells.

This result was confirmed by immunofluorescence using the protein A-purified polyclonal antiserum to mouse lengsin (Fig. 3). No signal was seen in the central region of the lens (the lens nucleus), which contains primary fibers that formed in the embryonic lens. Staining was seen throughout the terminally differentiated secondary fibers, with expression first appearing in the ring of cells corresponding to the region of loss of cell nuclei. Although immunoreactivity was distributed through the cell cytoplasm, intense staining was also occasionally observed in nuclei immediately above the layers of nuclear breakdown. Occasionally, some lens sections also showed apparent immunoreactivity in a subpopulation of lens epithelial cells (as can be seen in Fig. 3), however this was not reproducible. Because neither ISH nor Western blotting detected lengsin in lens epithelium, this staining probably results from a sectioning artifact.

Yeast Two-hybrid Analysis

Full-length mouse lengsin was cloned as bait in the BD Clontech Matchmaker Y2H system and was used to screen a random-primed, adult mouse lens cDNA expression library (Fig. 4A). This produced a limited number of positive targets all of which corresponded either to lengsin itself or to the lens cytoskeleton intermediate filament protein vimentin (7). The vimentin clones obtained were identical and corresponded to partial sequence from the helix 2B region of the vimentin filament domain (Vim2B) (20) (Fig. 4, B and C).

Vimentin is a major component of the cytoskeleton in lens epithelium and in immature lens fiber cells. Previous studies have shown a marked decrease in vimentin levels close to the region of lens fiber cell terminal differentiation (7) and hence to the region of lengsin expression. The vertebrate lens also contains two highly abundant, lens-specific intermediate filament proteins BFSP1(CP94;filensin) and BFSP2 (CP49;phakinin) (here we refer to these two proteins as filensin and CP49), which are structurally related to vimentin (21). These two proteins also appear to undergo reorganization at a generally similar stage to the onset of lengsin expression (7). Although CP49 has some sequence similarity to vimentin in the helix 2B region, filensin is more distantly related to vimentin and has very little sequence similarity in the helical regions (Fig. 4B). Glial fibrillary acidic protein (GFAP), another filament protein that has significant sequence similarity to vimentin, is also expressed in lens but at much lower abundance and is restricted to epithelial cells (22,23). Other cytokeratin-like filament proteins may also be present in lens at much lower levels than vimentin, GFAP, and the beaded filament proteins (24).

To see if lengsin is also capable of interaction with the other major lens filament proteins expressed in lengsin-positive cells, the regions equivalent to the Vim2B regions in both CP49 and filensin (Fig. 4B) were cloned into the prey vector and used in pair-wise Y2H

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experiments with the lengsin bait vector. In Y2H neither CP49 nor filensin preys produced positive Y2H interactions with the lengsin bait. However, examination of the bait and prey fusion proteins by Western blot for the Gal4 activation domain showed that although full sized products for the vimentin and filensin fusions were expressed, the CP49 prey fusion was consistently too small (not shown). DNA sequence was verified for these clones, and the Y2H experiment repeated, yielding the same result. This suggested that modification of the CP49 fusion protein, perhaps by proteolysis, occurs in yeast. Thus Y2H provides evidence that lengsin can interact with the helical region of vimentin but not with the equivalent region of filensin. However interaction between CP49 and lengsin could not be examined by this technique.

Recombinant Proteins

To verify the vimentin interaction seen in Y2H and to further examine the possibility of an interaction between lengsin and the helical region of CP49, recombinant proteins were used. The Vim2B construct expressed at high levels in the soluble fraction in *E. coli* and was co-expressed with lengsin. When the total soluble bacterial cell extract was subjected to size exclusion chromatography, both lengsin and Vim2B, which could be visualized by SDS-PAGE, were found to co-purify in the same size range, while Vim2B expressed in the absence of lengsin ran in later fractions corresponding to smaller size aggregates (Fig. 5A). Co-expressed lengsin and Vim2 also co-purified on cation exchange chromatography (Fig. 5B). These results suggest that full-length lengsin and Vim2B associate in both gel filtration and ion-exchange chromatography.

Recombinant lengsin was mixed with full-length recombinant hamster vimentin and used in co-immunoprecipitation (co-IP) experiments (Fig. 6). Two different anti-vimentin antibodies, ab7752 and abV9, were linked to secondary antibody-agarose carrier beads and used to immunoprecipitate the mixture. Proteins bound to the beads were eluted and examined by Western blotting. Both antibodies immunoprecipitated vimentin but only abV9 also precipitated lengsin. Beads with no antibody did not IP lengsin. This suggests that vimentin-lengsin complexes can form in solution and can be immunoprecipitated in an antibody-specific manner. The lack of co-IP with ab7752 may reflect epitope masking in the complex so that lengsin/vimentin complex does not bind antibody.

The region of CP49 equivalent to the Vim2B fragment was cloned in a similar way but gave only very low levels of expression in *E. coli*. To provide a means for detection and purification, CP492B was re-expressed in *E. coli* with the addition of an N-terminal HA tag. While expression levels of this clone were still low, using an HA affinity column sufficient HA-CP492B protein was obtained for binding experiments. HA-CP492B was bound to the HA antibody column to create a CP49 affinity column. When mouse lengsin was applied to the HA-CP492B charged column a fraction was retained during washing and was only released by elution with HA peptide (Fig. 7). In contrast, no lengsin was retained on an HA column in the absence of HA-CP492B, indicating a specific interaction between lengsin and HA-CP492B.

SPR

As a more direct measurement of lengsin binding, both Vim2B and HA-CP492B were tested using surface plasmon resonance (SPR) spectroscopy (Fig. 8). Antibody to lengsin was immobilized on a Biacore chip and used to capture mouse lengsin, while blocked anti-lengsin antibody alone was used in the reference cell. To prevent dissociation of lengsin subunits during the experiment, the lengsin/antibody complex was stabilized by mild cross-linking. Vim2B or HA-CP492B in solution was then applied as analyte using a range of concentrations to allow modeling of the interaction kinetics. Both the filament protein

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fragments showed binding to the immobilized lengsin and exhibited similar binding characteristics with fast association and slow dissociation phases (Fig. 8). Overall, HA-CP492B showed higher affinity for lengsin.

The slow dissociation kinetics for both interactions can be explained by the existence of multiple binding sites on lengsin, allowing easy rebinding of dissociated analyte. This would be consistent with the multimeric (dodecameric) structure of lengsin. The analyte proteins are also likely to have multiple binding sites. Both vimentin and CP49 are expected to form coiled-coiled dimers and x-ray analysis has shown that helical region 2B of vimentin can associate into trimers of dimers (20). Indeed, size exclusion chromatography of Vim2B (not shown) was consistent with a 50–60kDa native size.

Colocalization of Lengsin and Intermediate Filament Proteins in Lens

The lens intermediate filament proteins, vimentin, filensin, and CP49 undergo reorganization as lens fiber cells mature (7). Immunofluorescence of adult mouse lens was used to compare the expression patterns of these proteins with that for lengsin. Vimentin showed intense signal throughout the epithelial cells and the nucleated secondary fiber cells of the lens. As previously described, the intensity of signal for vimentin diminished sharply close to the region of lengsin expression (Fig. 9, *panel a*). Immunofluorescence, using a commercial antibody which recognizes both CP49 and filensin, showed that the beaded filament proteins were also located in epithelial and nucleated fiber cells. However, staining for these proteins persisted in deeper layers of mature fiber cells than vimentin (Fig. 9, *panel b*) and showed considerable overlap with lengsin. Higher magnification confocal imaging of mouse lens sectioned transversely across fiber cells was used to examine the localization of these proteins within the cells (Fig. 9, *panels c* and *d*). Lengsin (*green*) appeared to be localized around the margins of the fiber cells, apparently lining the plasma membrane. Strong staining for beaded filaments proteins (Fig. 9, *panel d; red*) was also seen, with the same plasma membrane localization as lengsin. Vimentin (Fig. 9, *panel c; red*) gave the same pattern but with a weaker overall signal. This suggests that lengsin is associated with the lens fiber cell cytoskeleton and may be particularly associated with the lens-specific beaded filaments.

Discussion

The terminal differentiation of lens fiber cells is one of the most characteristic and unusual features of the vertebrate eye lens. Fully mature fiber cells become tightly packed and lose their nuclei and other cellular organelles, presumably to improve clarity along the optical axis of the lens (1,2,4,10). Nuclear breakdown involves condensation and degradation of chromatin in a process similar to apoptosis, following which nuclear fragments disperse. Lens cytoskeleton also undergoes reorganization during maturation (7,25).

Lengsin is highly specific for the lens and is well conserved throughout vertebrate evolution (15). As shown here, lengsin has a uniquely restricted pattern of expression during lens development. Expression is triggered in a specific layer of maturing lens secondary fiber cells as they approach the final stages of terminal differentiation. Lengsin mRNA is apparently tightly regulated and disappears rapidly in cells in which the nuclei have gone. A similar onset of lengsin expression is seen by immunofluorescence. Lengsin protein is seen throughout the ring of fiber cells undergoing loss of nuclei, but the protein is retained in deeper, older layers of mature fiber cells. In some sections, nuclei in the degradation zone also stained intensively for lengsin. While this could have functional significance for the mechanisms of nuclear breakdown it may simply reflect “leakiness” in the nuclei heading for condensation and breakdown that allows lengsin or antibodies to enter. A fascinating

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question for future studies is the nature of the signal that triggers lengsin expression in nuclei on the verge of breakdown.

The observation of lengsin as a fiber cell marker is consistent with results comparing gene expression in regenerating mouse lens (lacking mature fiber cells) with control lens. Along with several crystallins and other lens fiber markers, lengsin (incorrectly identified as glutamate-ammonia ligase, rather than glutamate-ammonia ligase (glutamine synthetase) domain containing 1), is one of the genes with the greatest decrease in mRNA levels compared with control lens (26).

Despite its place in the GS superfamily, lengsin exhibits no evidence of enzyme activity (15,16). Indeed, structural studies show major deletions and loss of functionally important residues in the catalytic and binding sites of lengsin compared with bacterial GS enzymes (15). It is also difficult to imagine a GS-related enzymatic role that would explain the localized, tissue-specific expression of lengsin. Rather, lengsin may a have a structural or binding role the lens. This would have parallels with the independent recruitment of several different enzymes as novel crystallins during vertebrate evolution (27–29). In these cases, enzyme activity or cofactor binding are not centrally important to the new crystallin role (although they may confer additional benefits on the lens). Indeed in some cases, notably *δ*1-crystallin, derived from argininosuccinate lyase (30,31), ancestral enzyme activity has been completely lost. Thus enzyme crystallins have principally structural rather than metabolic roles in the lens. Lengsin, derived from an ancient enzyme, seems to have acquired a specialized role in the lens in a similar way, although, in contrast to the major taxon-specific enzyme crystallins, lengsin is expressed throughout the vertebrates, suggesting a more ancient origin (15). Indeed, recent genome sequencing for the Sea Urchin (*Strongylocentrotus purpuratus*) has revealed the presence of multiple lengsin-like genes (15), suggesting that the lengsin family may have had wide use in early metazoans, although in vertebrates this function has only survived in the specialized context of the lens.

When yeast two-hybrid analysis was used to search for binding partners of lengsin in adult mouse lengsin, two proteins were detected. The first was lengsin itself. Full-length lengsin bait interacted with full-length lengsin prey, consistent with the multimeric structure of lengsin (15). The second prey for lengsin consisted of part of the helical domain of vimentin, essentially the 2B region (20). While sequence analysis showed that other clones containing different parts of vimentin were also present in the prey library, only clones for the 2B region were detected by Y2H. The interaction between lengsin and the vimentin 2B fragment (Vim2B) was confirmed by co-expression of recombinant proteins, by co-immunoprecipitation and by SPR.

Lens also expresses two other intermediate filament proteins related to vimentin, CP49 and filensin. These proteins are highly lens-specific and are also found throughout the vertebrates, matching well with the occurrence of lengsin. The regions of these two proteins equivalent to Vim2B were also examined for any ability to interact with lengsin. In this region CP49 has some sequence identity with vimentin, while filensin is more distantly related. The 2B region of filensin expressed well in yeast but gave no evidence for interaction with lengsin in Y2H experiments. However the 2B region of CP49 consistently failed to express correctly in yeast (a truncated fusion protein was always detected) and so could not be evaluated.

As an alternative, the recombinant CP492B region was expressed as an HA-tagged recombinant protein. Using affinity chromatography it was possible to demonstrate binding of lengsin to HA-CP492B. Furthermore, SPR spectroscopy confirmed that both Vim2B and HA-CP492B bound to mouse lengsin and demonstrated similar patterns of rapid association

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and slow dissociation, consistent with the existence of multiple binding sites. The SPR results are also consistent with a higher affinity of lengsin for CP49 than for vimentin. These results suggest that lengsin has the ability to bind both of these important lens intermediate filament proteins, although there may be preferred interaction between lengsin and CP49, both of which are highly specialized, tissue-specific lens proteins.

Expression of lengsin in mouse lens is closely coordinated with major changes in the levels and distribution of both the major intermediate filaments of the lens cytoskeleton. Filament proteins are prone to aggregation and formation of aggregates or plaques during the reorganization or breakdown of cytoskeleton in the highly organized, protein dense environment of lens fiber lens could have serious consequences for lens transparency. One role for lengsin as a lens-specific, intermediate filament-binding protein could be to act as a "chaperone" for both vimentin and CP49 as the lens fiber cell cytoskeleton is rearranged. Lengsin could also play a role in the organization of cytoskeleton during the formation of the specialized interdigitations that lock the mature lens fiber cells together and which also form at this stage (32). Indeed, localization of lengsin, vimentin and CP49/filensin in the region of lengsin expression in mouse lens shows that they all appear to have a principally plasmas membrane association. While signal for vimentin is relatively weak in this zone, consistent with its observed loss in maturing fiber cell, both lengsin and CP49 show strong immunofluorescence signals outlining the lens fiber cells. It has recently been shown that CP49 and filensin interact with MIP, the major membrane protein of the fiber cell plasma membrane (33). The similar localization of lengsin and beaded filament proteins suggests that lengsin itself could be a component of the cytoskeleton in mature secondary fiber cells. Such an interaction would certainly be consistent with the tissue specificity of lengsin and the beaded filaments proteins.

It is interesting that, in the mouse lens, lengsin seems to be specific for the maturation of secondary fibers. Lengsin has not been detected in embryonic or new born mouse lens, and there is no evidence for its expression in primary lens fibers even though primary fiber cells also undergo a terminal differentiation with loss of cell nuclei (1). This suggests that there may be different mechanisms for maturation in the two types of fiber cells. Indeed, the origins of the two populations of fiber cells are quite different. Primary fibers arise from the elongation of posterior cells in the embryonic lens vesicle and these essentially form parallel bundles of cells that fill the lens vesicle with no formation of fiber to fiber junctions or sutures. By itself, this process would produce a primitive, size-limited cellular lens. Secondary fibers are derived from anterior epithelial cells that elongate at the lens equator and wrap around the core of the lens (the so-called lens nucleus) that is formed from the primary fibers. The layers of secondary fibers meet up anteriorly and posteriorly and form sutures (4). Secondary fiber cells may thus be the result of an elaboration of lens development to produce larger, more sophisticated lenses during the evolution of the vertebrate eye, and lengsin may have been recruited to serve as part of their cellular machinery.

Why would a GS enzyme acquire a role involving interaction with cytoskeleton? Many familiar enzymes have been shown to interact with cytoskeletal elements in the cell (34–36). This may allow anchoring of enzyme complexes, enhancing the flow of metabolites by proximity rather than by free diffusion in the cytoplasm. During evolution the enzymes and the cytoskeletal proteins would be expected to co-adapt, allowing enzymes to participate in the structure and function of the cytoskeleton itself. Perhaps a class I GS acquired a role of this kind in a distant ancestor of multicellular organisms, a role that expanded in some metazoan lineages (such as that which includes Sea urchins), but in vertebrates was preserved only in the lens. While lengsin is lens-specific, its acquisition of a structural role associated with cytoskeleton illustrates more generally the evolutionary flexibility of

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proteins and suggests that other proteins identified with particular roles, such as enzymes, may also be playing important structural roles in cell biology.

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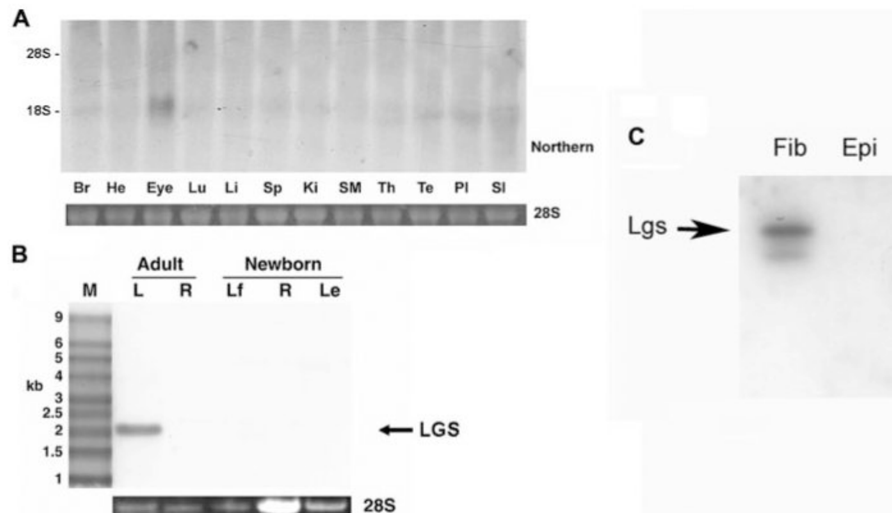

FIGURE 1. Lengsin is lens-specific

A, multitissue Northern blot of mouse tissues probed with biotinylated mouse lengsin cDNA and visualized by ECL. A strong signal is seen only in eye, just above the size of 18 S rRNA (over 2 kb). *B*, Northern blot of rat lens and retina tissues probed with mouse lengsin cDNA. Lengsin signal is seen in adult lens but not in retina or in newborn lens epithelium (*Le*) or lens fibers (*Lf*). *C*, Western blot detects lengsin in fiber cells but not in epithelial cells in adult mouse lens.

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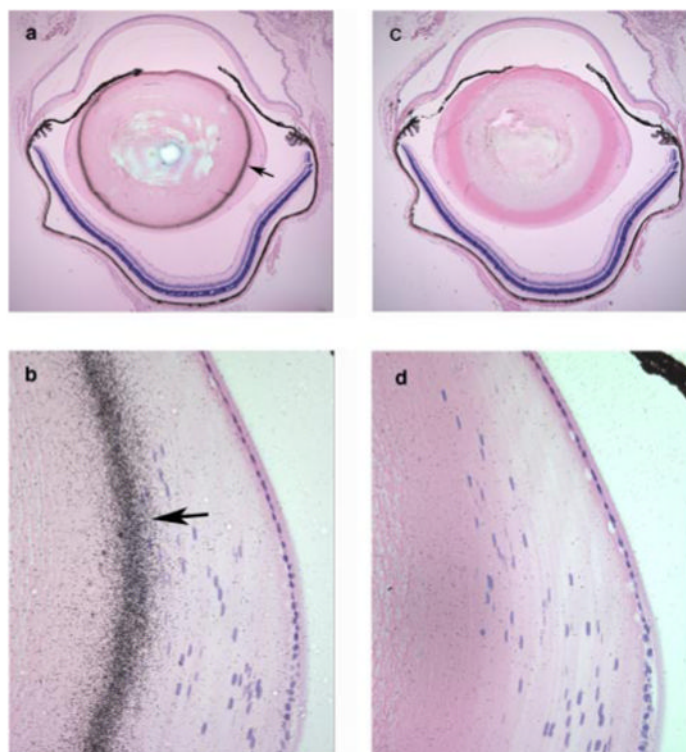

FIGURE 2. Lengsin mRNA is specific to maturing lens fiber cells

ISH of adult mouse eye, using radiolabeled antisense and sense probes to mouse lengsin. *Panel a*, whole eye. A ring of silver grains marks lengsin expression in lens (*arrow*). *Panel b*, detailed view shows that ISH signal is restricted to the layers of cells immediately preceding loss of nuclei. *Panel c*, sense probe version of *panel a. Panel d*, sense probe version of *panel b*.

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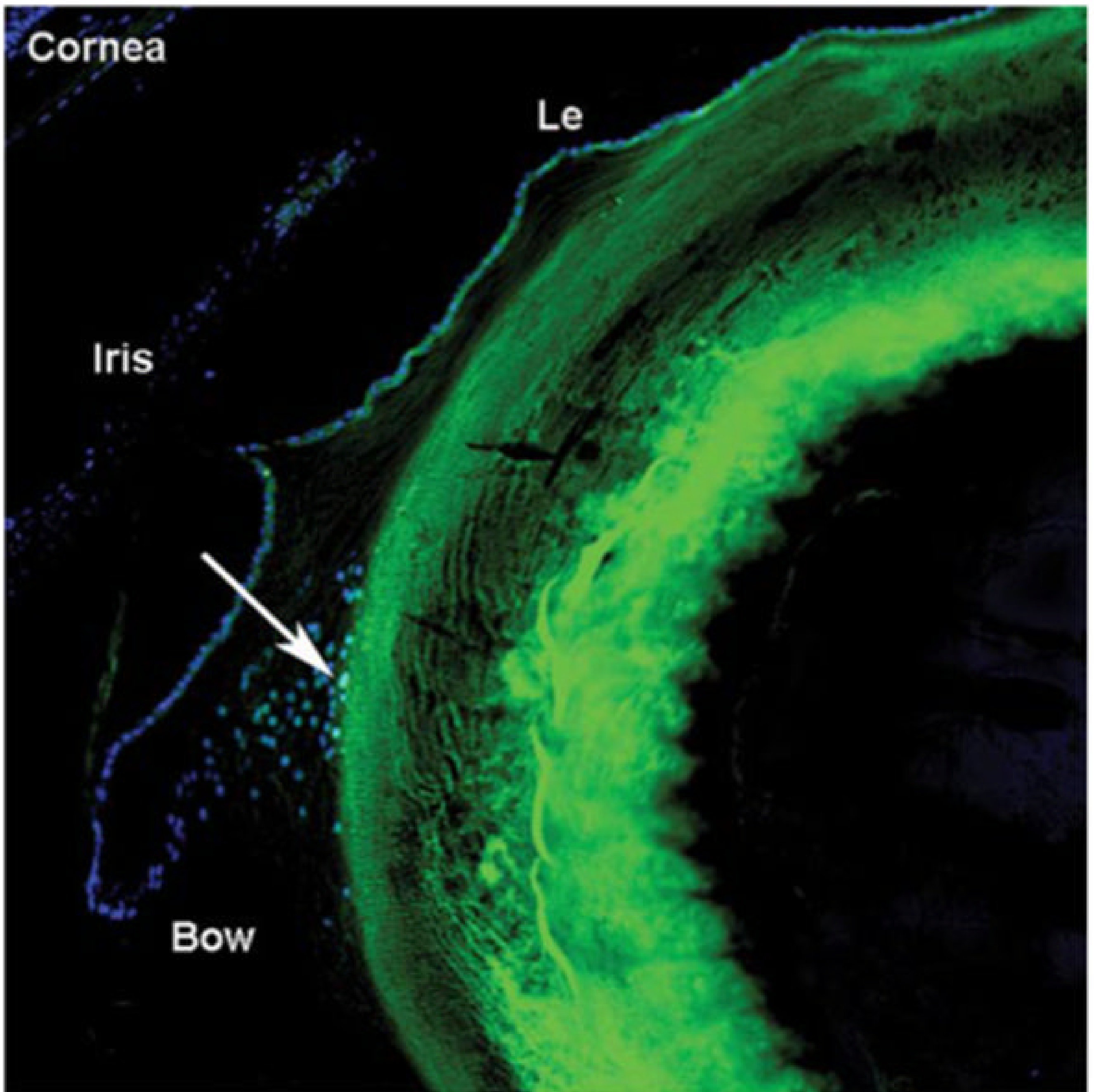

FIGURE 3. Lengsin protein localization in adult mouse lens by immunofluorescence
Signal for anti-mouse lengsin is *green*; nuclei are stained with DAPI (*blue*). Lens epithelium (*Le*) and bow region (zone of fiber cell elongation) are indicated. Strong lengsin immunoreactivity is seen throughout maturing and mature secondary fiber cells. *Arrow* indicates an intensely stained cell nucleus. Some signal is visible in regions of epithelium, but this probably a sectioning artifact. The unstained region in the center of the lens corresponds to the lens nucleus, consisting largely of primary lens fiber cells, which is intact in this section.

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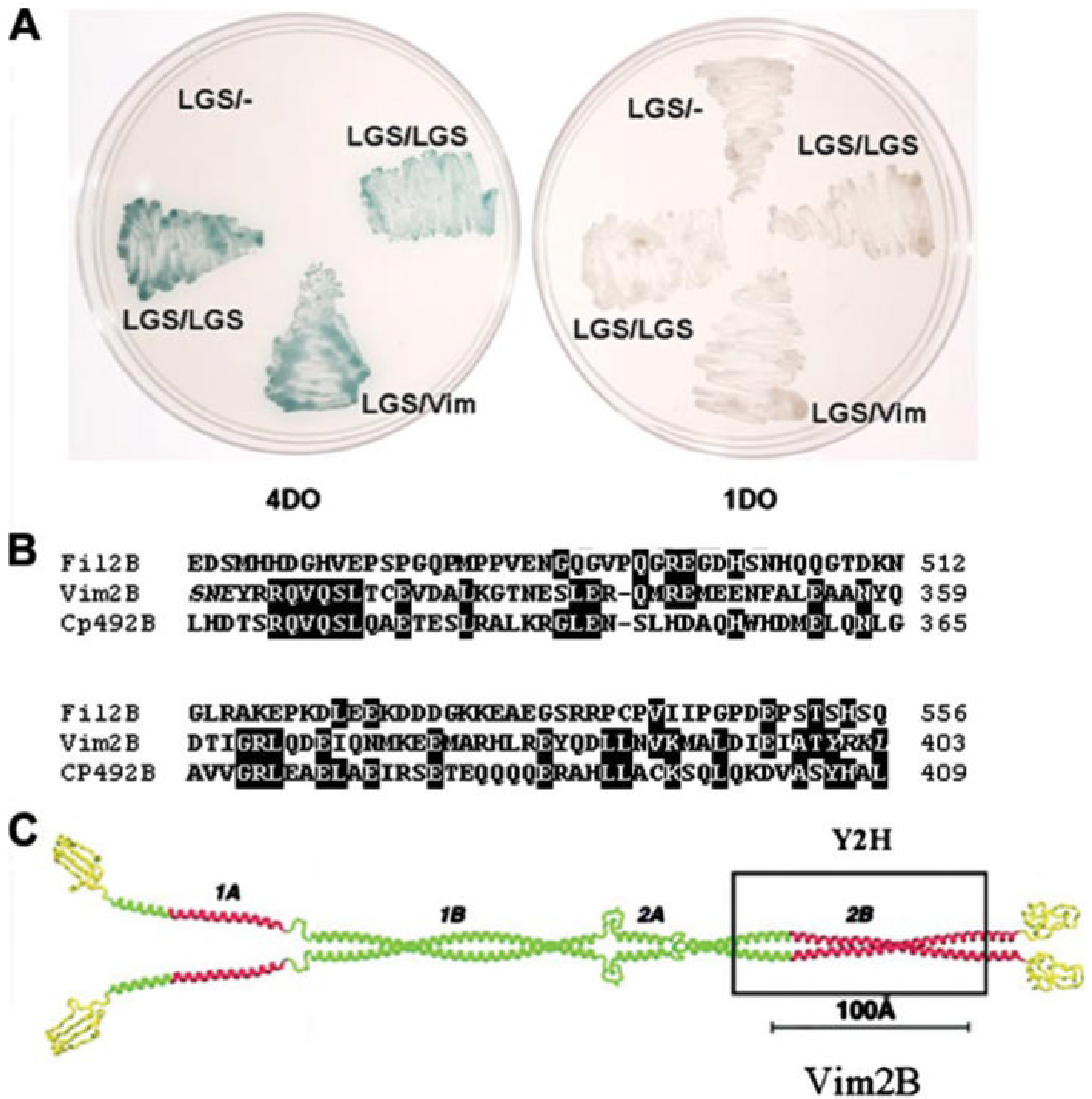

FIGURE 4. Y2H screening for lengsin binding partners

A, lengsin/lengsin and lengsin/vimentin bait/prey combinations on 4DO (-Ade, -His, -Leu, -Trp) and 1DO (-Trp) selective plates. Growth of yeast on 4DO medium and activation of *β*-galactosidase indicates interaction of bait and prey in the yeast. *B*, sequence of the mouse vimentin prey domain from Y2H, aligned with equivalent regions of mouse CP49 and filensin. For recombinant protein production, the sequence derived from Y2H was extended slightly at both ends (*italics*) to complete heptad repeats in the filament protein. Sequence numbers for mouse proteins are shown. Identical residues are *boxed*. *C*, schematic of the position of the Vim2B domain in a full-length model of a vimentin coiled-coiled dimer (adapted from Strelkov *et al.*, 20).

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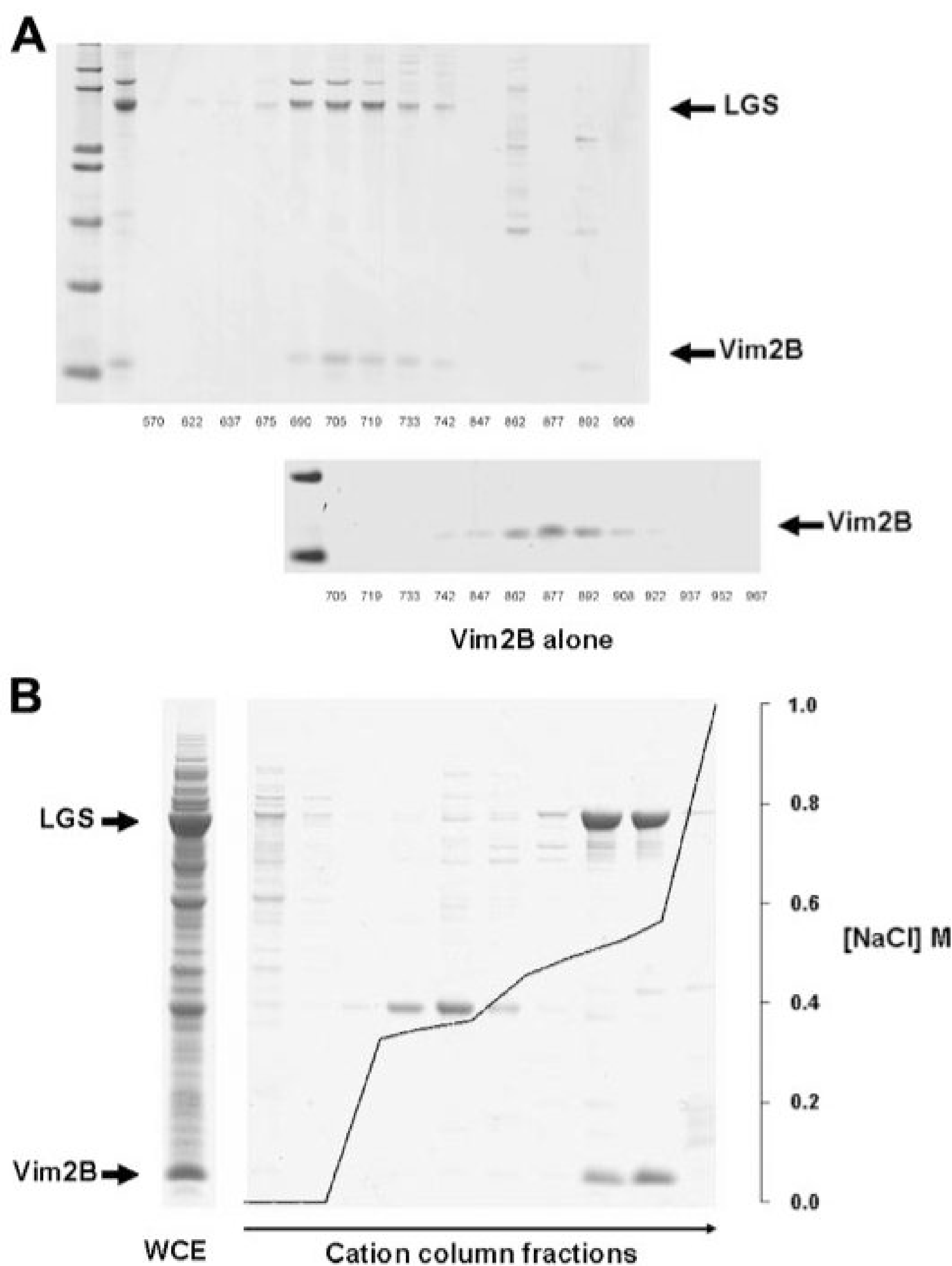

FIGURE 5. Association of recombinant lengsin and vimentin

A, co-purification of co-expressed lengsin and Vim2B domain by size exclusion chromatography. The figure shows Coomassie Blue-stained SDS-PAGE of protein fractions from the gel filtration column. The *lower panel* shows the elution profile of recombinant Vim2B alone, which eluted at a smaller apparent size under the same conditions. *B*, co-purification of co-expressed lengsin and Vim2B domain by cation exchange. Shown on the *left* is SDS-PAGE of whole cell extract of co-expressed lengsin and Vim2B in *E. coli*. The *main panel* shows SDS-PAGE of selected fractions from the cation exchange column. The *graph line* indicates the salt concentration at each fraction.

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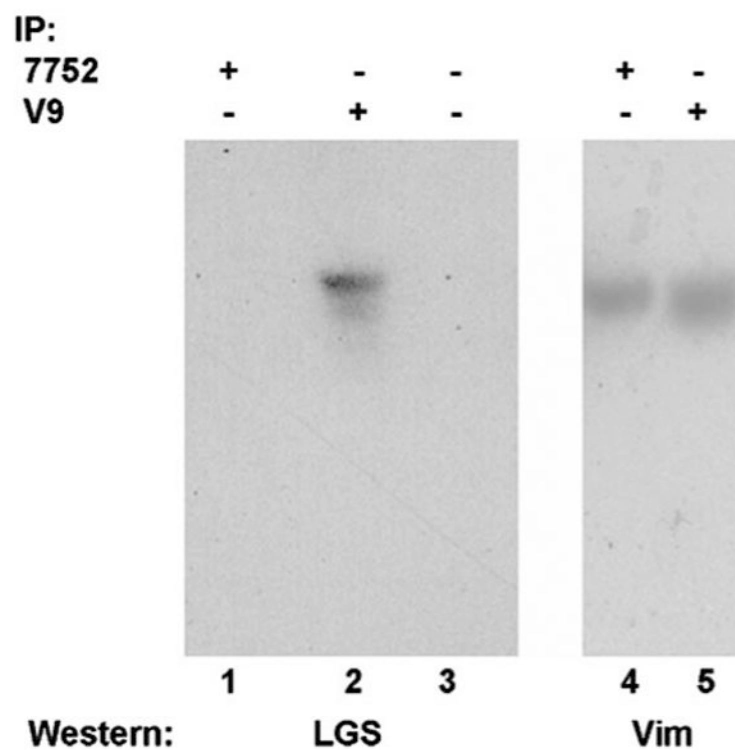

FIGURE 6. Co-immunoprecipitation of isolated lengsin and vimentin

A solution of lengsin and vimentin was treated with V9 or 7752 antibodies to vimentin as indicated. Immunoprecipitated (*IP*) proteins were then examined by Western blotting for lengsin (*left*) and vimentin (*right*). Both vimentin antibodies were able to immunoprecipitate vimentin (*right*). In addition, V9 coprecipitated lengsin (*left*).

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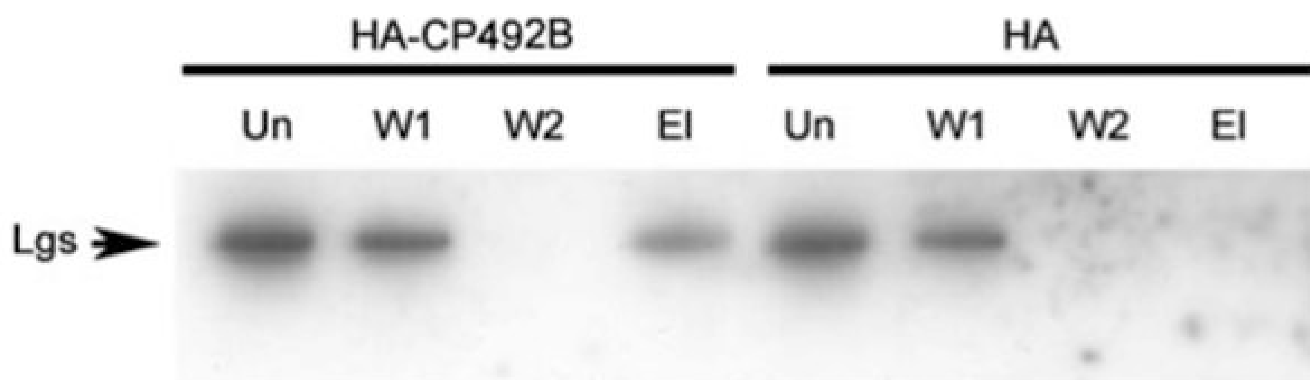

FIGURE 7. Lengsin interacts with HA-CP492B in affinity chromatography
HA-CP492B was pre-bound to an HA column to create a CP492B affinity column (*left*). An empty HA column was used as negative control (*right*). Recombinant lengsin was applied to both columns. The figure shows a Western blot of lengsin in unbound (*Un*), wash 1 (*W1*), wash 2 (*W2*), and elute (*El*) fractions. Only in the presence of the CP492B ligand was lengsin retained on the column and released by elution with HA peptide.

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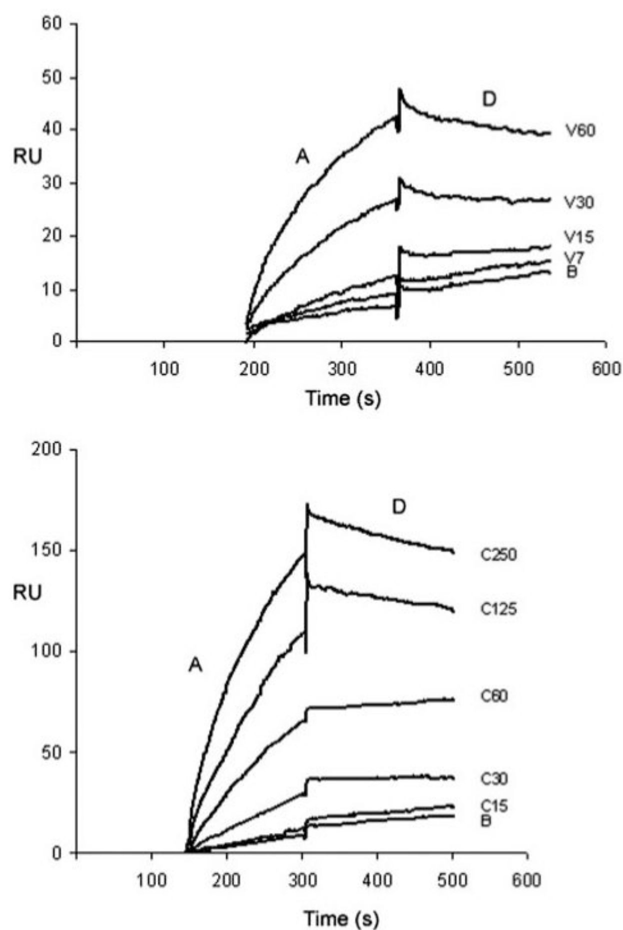

FIGURE 8. 2B filament regions of vimentin and CP49 interact with lengsin in SPR
Lengsin was bound to a CM5 sensor chip (Biacore). Vim2B (*upper panel*) and HA-CP492B (*lower panel*) solutions were used as analytes to measure binding activity. Panels show resonance units (*RU*) traces for several concentrations or either analyte (*V* or *C*) shown in μg/ml. *B* indicates blank. *A* and *D* indicate association (start to end of analyte injection) and dissociation phases.

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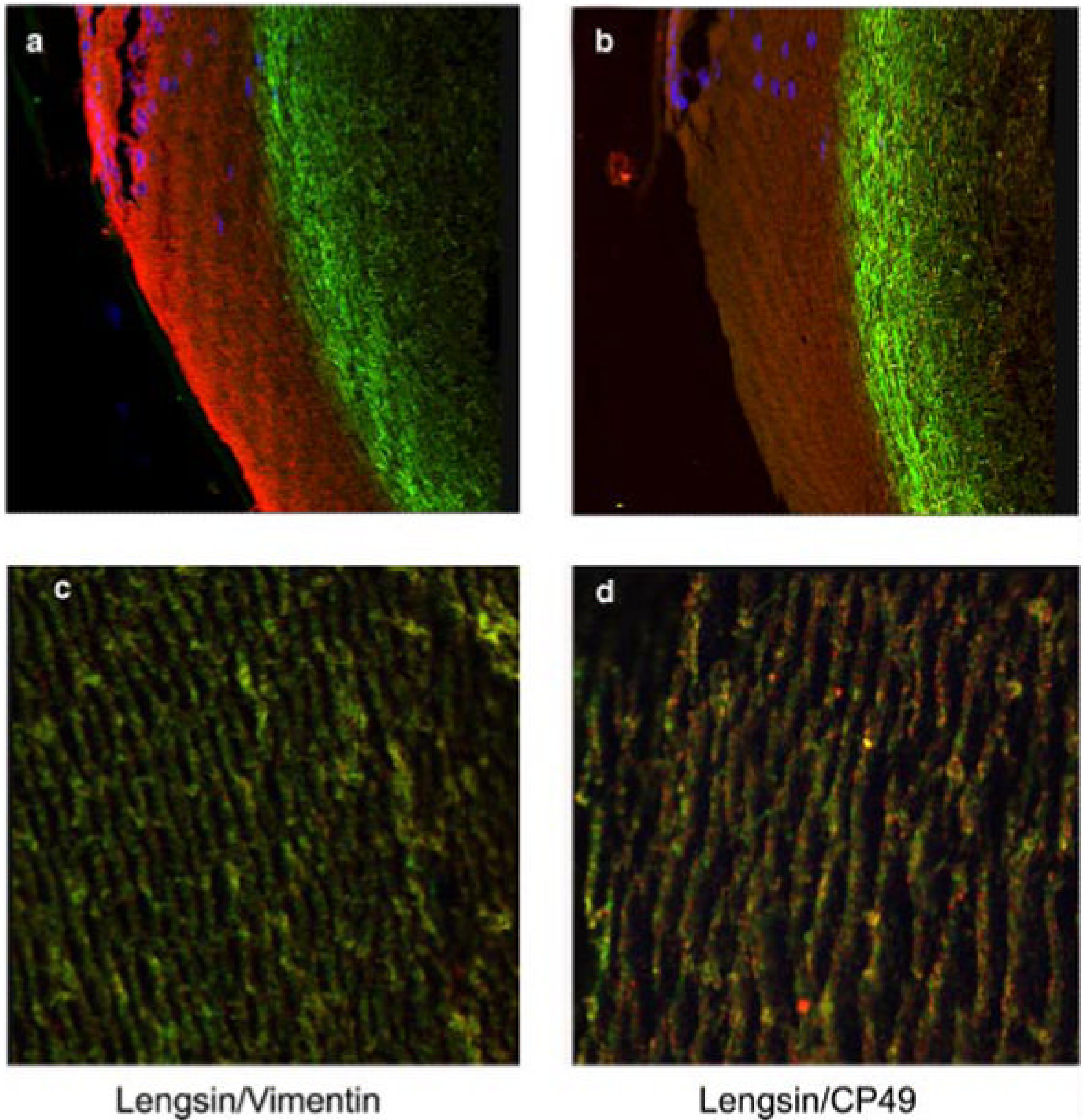

FIGURE 9. Localization of lengsin and major lens intermediate filament proteins in adult mouse lens

Panel a, immunofluorescence for vimentin and lengsin in adult mouse lens. *Red* shows vimentin, *green* shows lengsin, and *blue* is DAPI staining for nuclei. *Panel b*, immunofluorescence for beaded filament proteins and lengsin. *Red* shows beaded filaments (filensin and Cp49), *green* shows lengsin, and *blue* is DAPI staining for nuclei. *Panel c*, higher resolution image corresponding to *panel a*, showing localization of both vimentin and lengsin at the margins of lens fiber cells. *Panel d*, higher resolution image corresponding to *panel a*, showing localization of both beaded filament proteins and lengsin at the margins of lens fiber cells.