



Synapsin and synaptic vesicle protein expression during embryonic and post-natal lens fiber cell differentiation

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Purpose: Reorganization of cytoskeleton and membrane biogenesis are dynamically coordinated during lens fiber cell differentiation and development to produce an organ with precise dimensions and optical properties. Cargo vesicle trafficking is fundamental to cell elongation and has also been implicated in degenerative disease mechanisms. Alzheimer precursor protein (A β PP) acts with kinesin, synapsin, and synaptic vesicle proteins to mediate cargo vesicle transport and membrane fusion in neurons. In our previous studies we demonstrated that A β PP is also a key element in lens fiber cell formation, and in early-onset cataract that occurs along with early-onset Alzheimer disease in Down syndrome. In the present study we examine lens expression and regulation of a complement of genes associated with cargo and synaptic vesicle transport in neurons.

Methods: RT-PCR, immunoblot, and immunohistochemical methods were used to characterize expression of A β PP and kinesin associated motor proteins, synapsins, and synaptic vesicle proteins in mouse and rat embryonic, post-natal, and adult lenses. Phospho-specific anti-synapsin antibodies were used to determine the distributions of site-1 phosphorylated and dephosphorylated synapsin protein.

Results: We demonstrate that a substantial complement of cargo and synaptic vesicle proteins involved in A β PP mediated vesicle transport are expressed in lenses along the anterior-posterior axis of fiber cells in embryonic and adult lenses, consistent with vesicles, actin filaments, and neuron-like arrangement of microtubules in lenses shown by others. We identify temporal regulation of synapsins I, II, and III during embryonic and post-natal lens development consistent with their roles in neurons. Regulation of vesicle cytoskeleton attachment, actin polymerization, and the capacity to stimulate cell differentiation by synapsins are governed in large part by phosphorylation at a conserved Ser₉ residue (site-1). We demonstrate discrete distributions of Ser₉ phospho- and dephospho-synapsins along the axial length of rapidly elongating embryonic lens fiber cells, and decreased levels of site-1 phosphorylated synapsins in adult lenses.

Conclusions: The present findings demonstrate several fundamental parallels between lens and neuron vesicle trafficking cell biology and development, and suggest that more extensive A β PP related vesicle trafficking disease mechanisms may be shared by lens and brain.

Lens formation requires coordinated fiber cell differentiation to produce an organ of specific size and shape. Lens fiber cells extend from the anterior to posterior surfaces and elongate in a concerted manner to form its convex shape with optical properties that coordinate with the cornea to produce focused images on the retina. In adult humans, lens fibers approach 1 cm in length. In addition, plasticity of fiber cell elongation is required to maintain visual acuity during the proportional enlargement of the eye after birth [1,2].

A fundamental requirement for cell elongation involves the transport of membrane and protein constituents to cell surfaces distal from the cell nucleus and surrounding organelles. This process, intensively studied in neurons, involves Alzheimer precursor protein (A β PP) and Alzheimer precursor-like proteins (APLP1 and APLP2) that interact with kinesin proteins to tether vesicles to the cytoskeleton for transport [3-

7]. Our previous studies in lens demonstrated that A β PP has a key role in normal fiber cell development and in cataractogenesis [8,9], consistent with the demonstration of microtubules and vesicles in lens by Lo et al. [10]. In our examination of mice expressing A β PP from a complete copy of the human A β PP gene locus from chromosome 21 [11], in addition to the native mouse A β PP gene, we identified disrupted fiber cell organization and intracellular vesicle formation.

Additional proteins present in vesicles transported by A β PP and kinesins that have fundamental roles in cell development and in Alzheimer pathophysiology include Notch receptors, receptor tyrosine kinases, and secretases (proteases), which are also present in lens [12,13]. Secretases not only release receptor C-terminal domains that regulate processes in the cytoplasm and in the cell nucleus to control gene expression, but also have well-characterized roles in cleaving A β PP to release A β peptides, which have the capacity to bind metals and contribute to oxidative stress [14].

In lens, we demonstrated that A β PP and A β increase in response to oxidative stress in intact cultured lenses [15] and

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others showed A β mediated protein oxidation in lenses [16], which have well-characterized contributions to cataractogenesis [17]. Using an in vivo model of systemic oxidative stress that also produces Alzheimer related pathology in brain we demonstrated Alzheimer protein accumulation in regions of lens pathology [9].

Additional links between cargo vesicle transport and oxidative stress come from studies demonstrating that Jun N-terminal kinase (JNK) interacting protein (JIP1b) is required to facilitate A β PP-kinesin interactions to tether membrane vesicles to the cytoskeleton for transport [7]. However, JIP1b has a second critical function by providing a scaffold for nucleating MAP kinase and JNK proteins [18,19] that have fundamental roles in cell proliferation and in cell signaling for responses to oxidative stress [18-21] in the brain and lens [20,22].

It has become increasingly recognized that cargo vesicle trafficking defects contribute to disease [23,24]. Inappropriate ratios of A β PP and kinesins lead to aborted vesicle transport and accumulation, or "traffic jams", and also disrupt transport of other vesicle types [23-26]. Thus, it appears that defects in A β PP-JIP1b-kinesin vesicle transport can act synergistically with A β PP mechanisms involving A β and stress responses to produce disease.

A variety of synapsin and synaptic vesicle associated proteins also have critical roles in vesicle-cytoskeleton attachment and transport, as well as for vesicle docking and recycling at target cell surface membranes. Synapsin proteins coat synaptic vesicle surfaces, representing 9% of total vesicle protein, and regulate vesicle-cytoskeleton attachment in large part via phosphorylation at a highly conserved Ser₉ (site-1) residue [27-30]. Interestingly, synapsins also have the capacity to promote actin polymerization [31,32] and to stimulate cell differentiation [33-36], and these functions are also governed by site-1 phosphorylation. Cyclic AMP dependent Protein Kinase A (PKA) strongly regulates synapsin site-1 phosphorylation, with synapsins providing the most abundant PKA substrate in neurons [27-30].

Individual synapsin gene functions are indicated by separate temporal expression patterns during neuronal differentiation. Synapsin III is required for initial cell elongation and elaboration of neuronal processes [34-37]. As neurons mature, synapsin III expression decreases and synapsins I and II increase. Consistent with these observations, synapsin III is enriched in neurogenic brain regions, and also provides a marker of axonal growth cones [38]. Synapsins are also distinguished from one another by distinct ATP binding properties in the presence of calcium [39].

In addition to A β PP, kinesins, and synapsins, cargo vesicles in neurons also contain a variety of other synaptic vesicle proteins. Among these are synaptotagmins, synaptophysin, and Rab GTP binding proteins that regulate vesicle fusion and recycling at cell surface membranes [40-42]. In neurons, synaptotagmin I has an important role in sensing calcium levels and regulating interactions with Rab and other proteins that catalyze vesicle membrane fusion. Synaptophysin has a key role in the biogenesis of membrane vesicles at the cell surface during vesicle recycling in neu-

rons. These functions are utilized for cell differentiation and plasticity, as well as for neurotransmitter release.

In the present study we examine lens expression and regulation of a number of additional proteins that act with A β PP in cargo vesicle transport. We also identify neuron-like temporal expression of synapsin genes, and provide evidence of synapsin site-1 phospho-regulation in lens. The present study strongly suggests that cargo and synaptic vesicle trafficking mechanisms strikingly similar to those required for neuronal development and synaptic plasticity are utilized for the coordinated elongation of lens fiber cells. These data further suggest that A β PP related vesicle transport defects may also contribute to lens disease.

METHODS

RT-PCR analysis: Wild-type Sprague Dawley rats and C57Bl/6 mice (Taconic, Germantown, NY) were used according to guidelines prescribed by the US Public Health Service Policy on humane care and use of laboratory animals. Males only were used in these initial studies of cargo vesicle proteins in lens to help obviate hormonal considerations. Lenses were

TABLE 1. OLIGONUCLEOTIDE PRIMERS USED FOR RT-PCR AMPLIFICATION OF SPECIFIC GENE TRANSCRIPTS

Gene	Accession number	Sequence	Exon
A β PP	MUSABPPA	1553-TGCTCTACAATGTCCTCGCGG-1573	13
		2033-ACCATGAGTCCGATGATGGCG-2013	17
APLPI	BC021877	372-GTACTGCAGACAGATGTACC-391	1
		894-CTCTTCTTCTTCAGCCTGAG-875	7
APLPII	NM009691	176-TGGCTGGCTACATAGAGGCT-195	1
		466-TGTGACTCTTGCACTGCCTT-447	3
Tau	BC014748	555-ATCCCTACCAACACCG-570	9*
		850-GCTTTGAGACTATTTGCACA-831	11*
JIP1b	AF054611	1355-AGTGACGAAGCGGACT-1350	
		1809-CGCTGTTTTTGGCAAGG-1793	
KLCI	BK000675	1005-GTACGGTAAGCGAGGG-1020	
		1519-GCCCTCGGTAAGGTGC-1504	
KLCII	AF055666	758-TTCACAAGGCCGTATG-774	
		1177-CTCCGGTAGTAGTATTCAC-1158	
Fe65	BC048395	905-GGACTTTGCCTACTG-920	2*
		1348-AGGAGAGAACCAGC-1333	3*
KIF5A	BC058396	1396-CATTGTGGTACGCATCG-1412	
		1682-GGTCGTAGTTGACCCG-1667	
KIF5B	NM008448	2246-GGTGAGGAGCTAGTCC-2261	
		2648-GCTTACGCAGGTTGTG-2633	
PKA	M12303	743-GACAGACTTCCGTTTTCG-760	7
		1168-AGTTACTCGTGTCCCA-1152	10
CamKI	NM144817	471-CTGCGGTGAATACCTT-487	
		881-ACCGTTCGTTTGGGTC-866	
Syn I	BC022954	1686-CAGGGTCAAGCCGCCAGTC-1705	
		1958-CACATCCTGGCTGGGTTCTG-1938	
Syn II	NM013681	903-AGGGGAGAAATCCAC-919	
		1252-CCCAGACTTGTACCCG-1237	
Syn III	NM_013722	1566-CCAACAGCAGCTCTCC-1581	
		1903-GGTTGCGGATTGTCTC-1888	
Syt I	BC042519	873-ATACTCGGAATTAGGTGGC-891	
		1281-TTGCTCGAACGGAACT-1266	
SypI	BC014823	154-TGCCAACAAGACGGAG-169	3*
		463-GGCGGATGAGCTAACT-448	4*
Rab5	D86066	605-TTCTGTCTGATGCC-620	
		908-CTGGTCGTTGGCCTTC-893	
Rab3A	NM019400	564-TGTCACACTTGTTC-547	4*
		197-CCAGAACTTCGACTATATGTTTC-218	2*
GAPDH	BC020407	134-GCTTAGAGAGGTAAAGATGG-153	2*
		433-GCGCCTCGGTAATAGG-418	3*
		582-TCCACCACCCTGTTGCT-603	
		1032-CCACAGTCCATGCCATCATGC-1016	

Genbank accession numbers and exon position are indicated. (the asterisk indicates exon number inferred from human or rat gene homologues).

removed for analysis of total RNA and extracted in Trizol reagent (Invitrogen, Carlsbad, CA). RNase free DNase (Invitrogen) was added to aid in removal of contaminating DNA. Total lens RNA was used to produce cDNA template with random primers (Invitrogen) and Superscript reverse transcriptase (RT, Invitrogen) for PCR amplification. Gene specific PCR primers, listed in Table 1, have been designed using sequence information from neighboring exons where the gene organization is known.

Control cDNA synthesis reactions with RT omitted did not produce cDNA products in parallel reactions, and all cDNA products were sequenced to verify their identity. Three lenses were used in parallel reactions. For semi-quantitative assays,

equal aliquots of random-primed cDNA template from a single lens were amplified with synapsin and GAPDH gene specific primers for 28, 32, and 36 cycles. The resulting cDNA products were resolved on agarose gels stained with ethidium bromide for comparison of products with expected molecular weight at increasing amplification cycles, using a Typhoon gel documentation system (Amersham Biosciences, Piscataway, NJ).

Immunoblot detection of proteins: Lens and brain tissues were removed and placed in "M-per" extraction buffer with DTT (Pierce, Rockford, IL). Samples were homogenized with a pestle and SDS sample buffer with DTT added. In some cases separate crude soluble and insoluble fractions were ob-

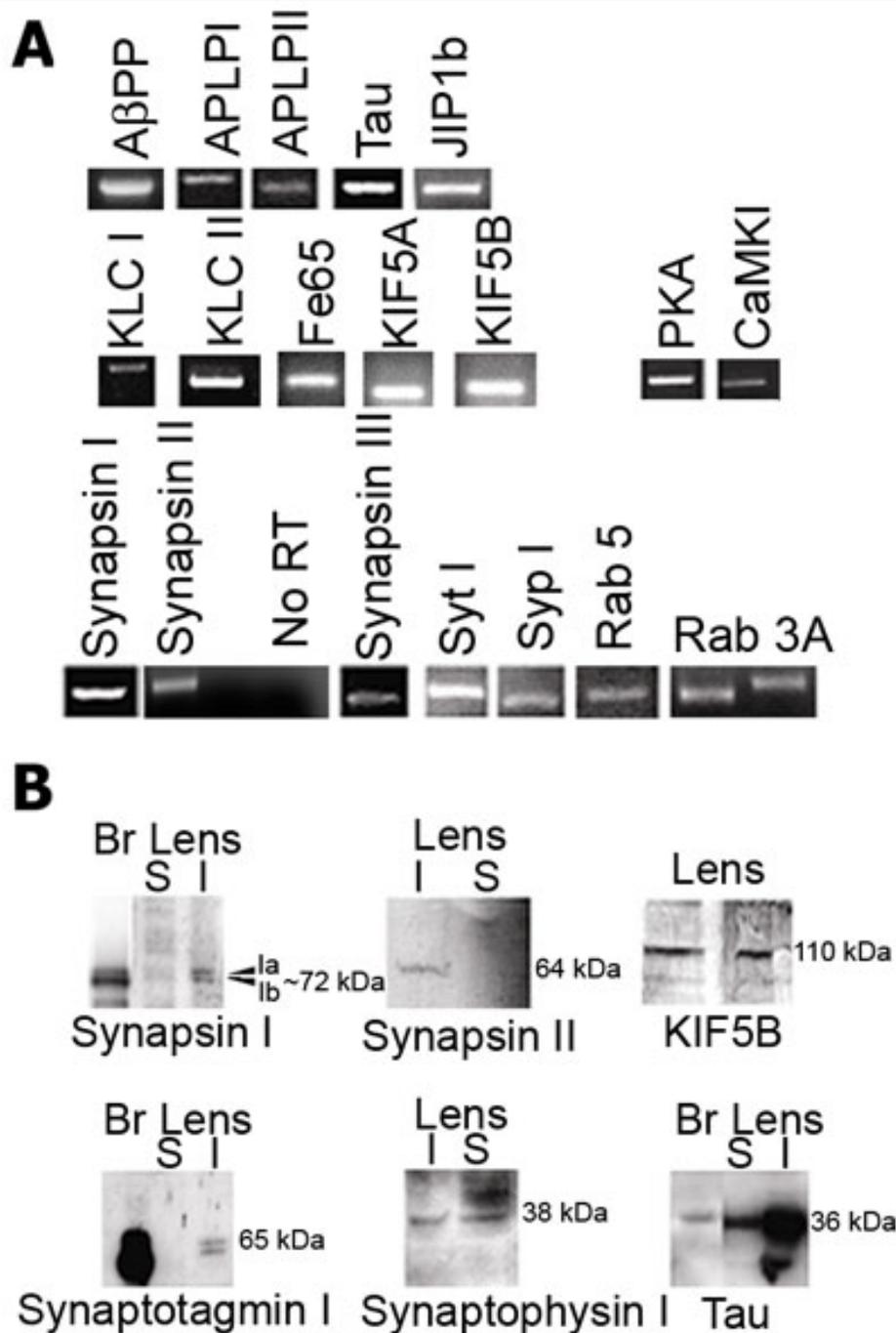


Figure 1. RT-PCR and immunoblot analysis of genes involved in AβPP mediated cargo and synaptic vesicle trafficking. **A:** RT-PCR analysis of lens expression of transcripts associated with AβPP mediated vesicle transport. Random primed lens RNA was amplified using gene specific primers listed in Table 1. Top row: cDNA products representative of three lenses corresponding to AβPP, Alzheimer precursor-like proteins (APLPI, APLPII), microtubule associated Tau protein and Jun N-terminal Kinase Interacting Protein (JIP1b). Middle row: Kinesin Light Chains (KLCI, KLCII), AβPP binding protein Fe65, and Kinesin Heavy Chains (KIF5A, KIF5B), and cDNAs corresponding to cAMP dependent Protein Kinase A (PKA) and Calcium/Calmodulin dependent Kinase I (CaMKI). Bottom row: synapsins I, II, III, synaptotagmin I (syt I), synaptophysin I (syp I), and GTP binding proteins Rab5 and Rab3A (two primer sets). All cDNAs were sequenced to confirm their identity. A representative control (no RT) is present in the lower panel. **B:** Western blot detection of synapsin I, synapsin II, KIF5B, tau, synaptotagmin I, and synaptophysin I in lens protein samples. Crude soluble and insoluble fractions preparation are described in Methods and total brain protein samples have been included for comparison. Diagnostic molecular weights are indicated.

tained by centrifuging samples at 12,000x g at 4 °C for 30 min. Equal amounts of protein in sample buffer with DTT were resolved on 10% or 12% SDS-PAGE gels and blotted to

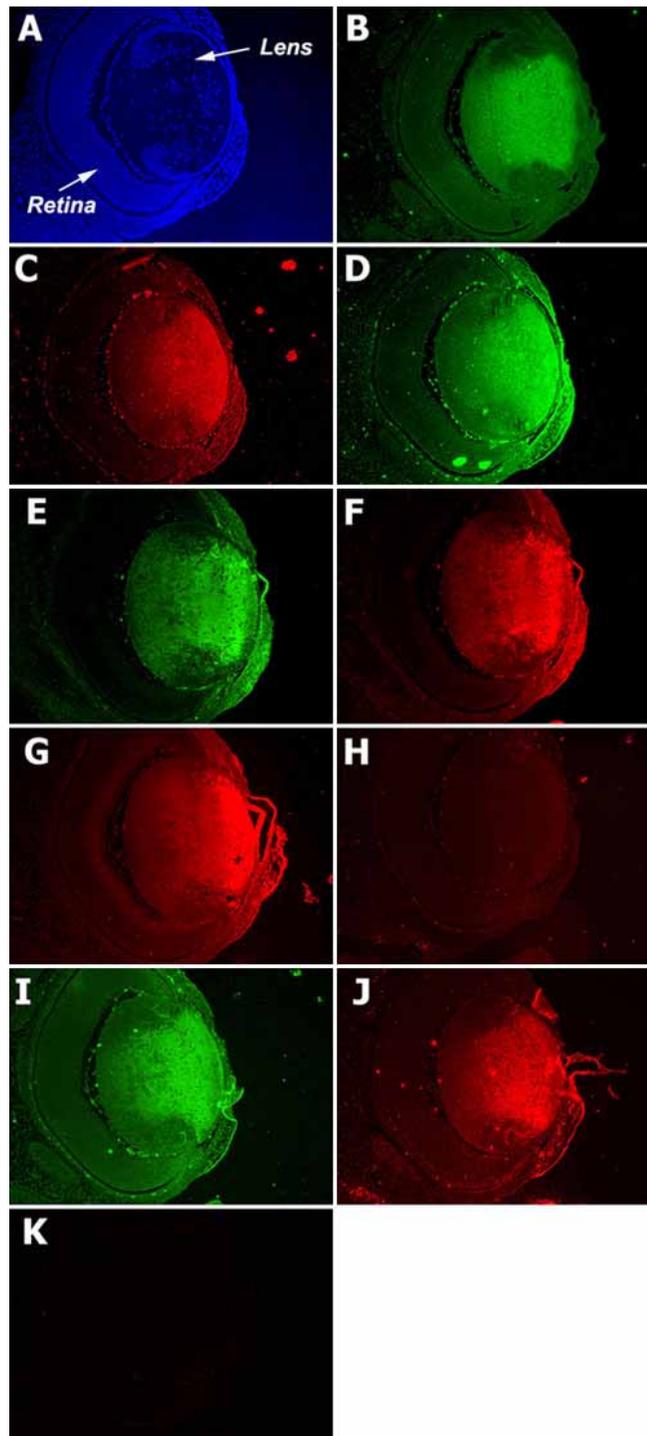


Figure 2. Immunolocalization of cargo vesicle associated proteins and synapsins in embryonic rat lenses. Immunofluorescence detection of proteins in embryonic day 17 rat lenses (equivalent to mouse stage about E15.5). Lenses are about 0.6 mm in diameter. **A:** DAPI nuclear stain. **B:** A β PP. **C:** JIP1b. **D:** Kinesin heavy chain KIF5B. **E:** Synaptotagmin I. **F:** Tau microtubule associated protein. **G:** Synaptophysin I. **H:** Synapsin I. **I:** Synapsin II. **J:** Synapsin III. **K:** Primary antibody omitted.

filters (described in previous studies [8,13]). Filters blocked in PBS (pH 7.4) with 2% horse serum were probed with antibodies raised against synapsin 1 (catalog number S193; Sigma, St Louis, MO), synapsin IIa (catalog number 8293; Santa Cruz, Santa Cruz, CA), synaptotagmin I specific (catalog number S2177; Sigma), synaptophysin I (catalog number S5768; Sigma), and Tau microtubule associated protein (catalog number T6402; Sigma). In addition, anti-Kinesin heavy chain protein KIF5B and kinesin light chain antibodies were provided by L. Goldstein (UCSD, San Diego, CA). Immune complexes were visualized with ABC secondary antibody and VIP detection kits (Vector, Burlingame, CA) or by chemiluminescence (Perkin Elmer, Shelton, CT).

Immunofluorescence detection of protein in histological sections: Eyes and staged embryos were prepared for histology by fixation in 4% paraformaldehyde in PBS (pH 7.4) for 24 h before embedding in paraffin for sectioning. To detect proteins in situ, deparaffinized and re-hydrated sections were incubated in antigen unmasking solution (Vector) for 20 min at 60 °C, and blocked in 2% horse serum in PBS. Sections were probed with antibodies listed above and with antibodies raised against JIP1b (gift from K. Verhey, University of Michigan, Ann Arbor; MI) [7], kinesin heavy and light Chains (gift from L. Goldstein UCSD) [6], Synapsin IIIa (sc-8292; Santa Cruz), and A β PP (22C11; Roche, Indianapolis, IN). Synapsin site-1 specific phospho-synapsin and dephospho-synapsin antibodies (HT Kao, NYU, New York City, NY) [36] for 4 h at room temperature, and visualized with Alexa fluor conjugated secondary antibodies (Molecular Probes, Eugene, OR). Fluorescence photomicrographs were digitally recorded with a liquid cooled SPOT II CCD camera (Diagnostic Instruments, Sterling Heights, MI).

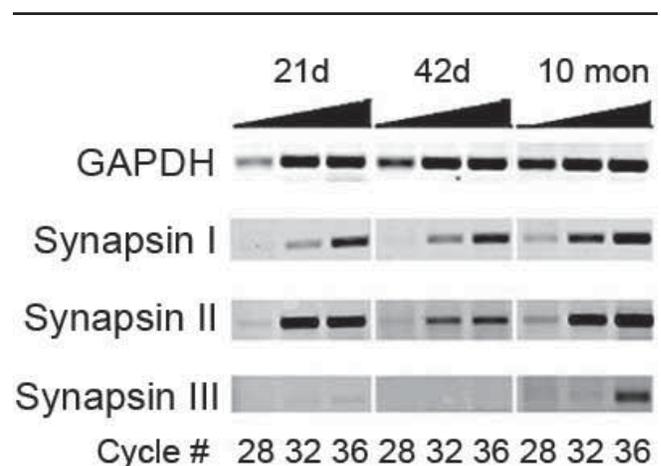


Figure 3. RT-PCR analysis of synapsin gene expression profiles in lenses. RT-PCR analysis of synapsin I, II, III, and GAPDH transcripts present in total RNA from post-natal day 21, day 42, and 10 month old mouse lenses. Data is representative of three eyes. Equal aliquots of random primed cDNA template from a single lens were amplified in separate reactions with gene specific primers. cDNA products generated after 28, 32, and 36 cycles of PCR amplification are resolved on agarose gels stained with EtBr for comparison.

RESULTS

Expression of A β PP-kinesin associated protein and synaptic vesicle proteins: To explore further a role for A β PP cargo vesicle transport biology in lens fiber cell formation and disease we examined lenses for expression of a substantial complement of genes that function together with A β PP to mediate membrane vesicle transport. We analyzed 6 week

mouse lenses by RT-PCR amplification of random primed lens RNA (Figure 1A) using gene specific primers listed in Table 1. In our previous studies we characterized the expression and alternative splicing of human and mouse A β PP transcripts [8]. Results shown in Figure 1 are representative of three lenses and demonstrate lens expression of A β PP, as well as expression of Alzheimer precursor-like proteins APLPI and APLPII.

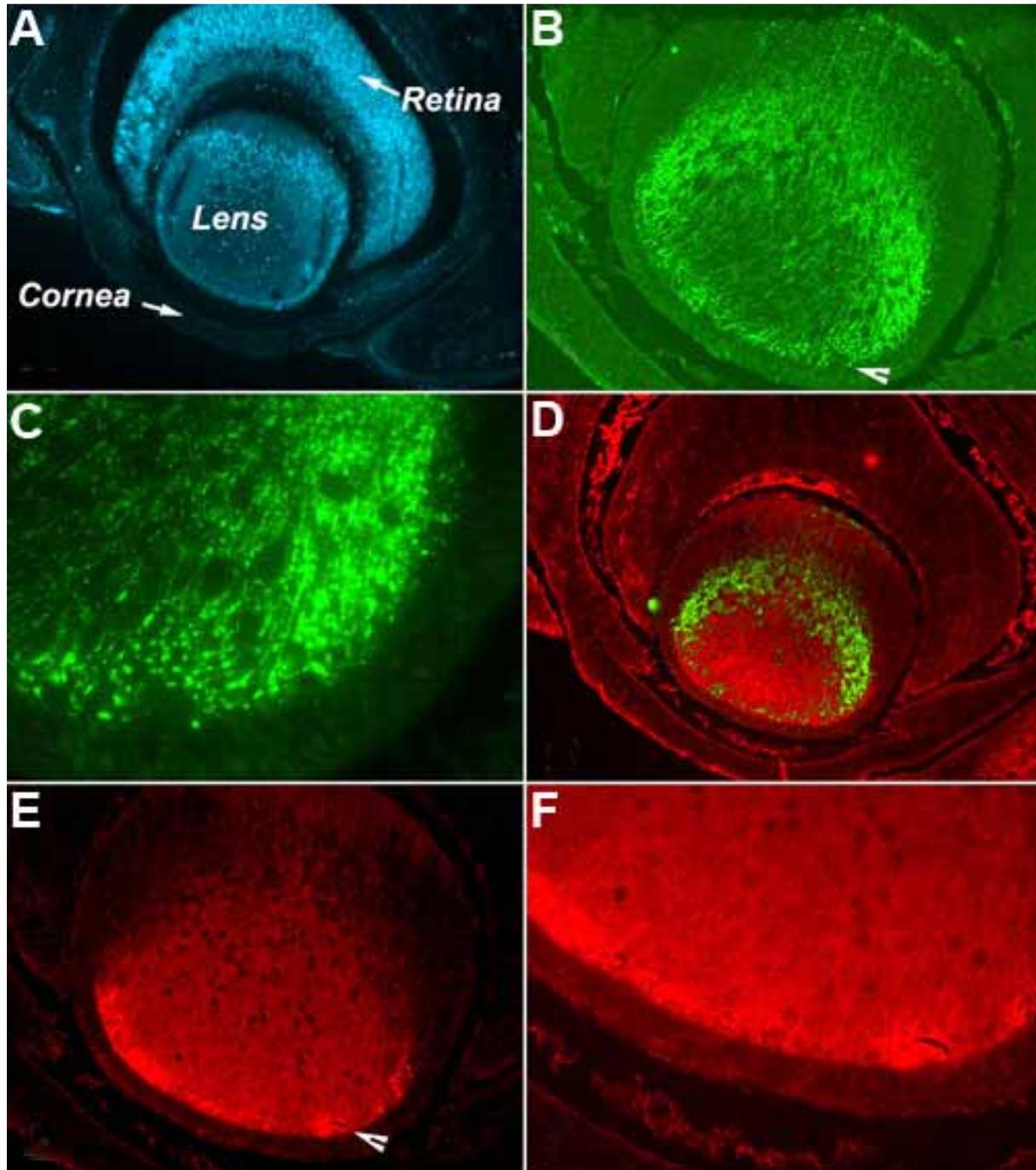


Figure 4. Immunolocalization of site 1 phosphorylated synapsin proteins in embryonic mouse lens. Distribution of site-1 phosphorylated synapsin and site-1 dephosphorylated synapsin protein in mouse E15.5 lenses. **A:** DAPI nuclear stain. **B,C:** anti-site-1 phosphosynapsin specific antibodies. **E,F:** anti-site-1 dephosphosynapsin specific antibodies. **D:** overlay of phosphorylated and dephosphorylated synapsins in mouse E15 lenses using Photoshop version 7.01 (Adobe Systems Inc., San Jose, CA). Arrows in **B** and **E** identify equivalent positions in **C** and **F**, respectively.

In addition, JIP1b, neuron specific kinesin heavy chain KIF5A, and the ubiquitously expressed KIF5B [43] are also expressed in lenses. We also identified expression of Kinesin light chains KLC I and KLC II in mouse lenses. In addition, the A β PP binding partner FE65, implicated in regulation of actin-based membrane motility, and dynamic neurite growth and synapse modification in neurons [44], is expressed in lenses. We also identified Tau microtubule associated protein expression in lenses, consistent with demonstrations of microtubules with unidirectional polarity, and vesicles in lens fibers by Lo et al. [10].

We next examined expression of a number of genes encoding synapsins and synaptic vesicle proteins in lenses. Figure 1A demonstrates expression of synapsin proteins in mouse lenses, as well as synaptotagmin I (involved in vesicle docking), and synaptophysin I (involved in vesicle release at synaptic terminals) [40,41]. In addition, we identify in lenses Rab5 and Rab3A GTP binding proteins that target specific vesicle

types to the membrane for fusion in neurons [42]. We also detected expression of protein kinase A and Calcium/Calmodulin dependent kinase I, involved in the regulation of each of the diverse functions identified for synapsins.

In the following experiments we examined expression at the protein level on immunoblot for Synapsin I, Synapsin IIa, synaptotagmin I, synaptophysin I, Tau, and KIF5B in lenses (Figure 1B). For comparison, co-migrating bands are present in mouse brain samples in parallel lanes. Synapsin, synaptotagmin I, and synaptophysin proteins were more readily detected in crude insoluble fractions, suggesting associations with membrane vesicles and cytoskeleton similar to neurons, and experiments are underway to investigate these possibilities.

To examine further the role of vesicle transport proteins in lenses, we began by examining the temporal and spatial expression patterns of A β PP and associated vesicle transport proteins in embryonic lenses. Fiber cell elongation begins at

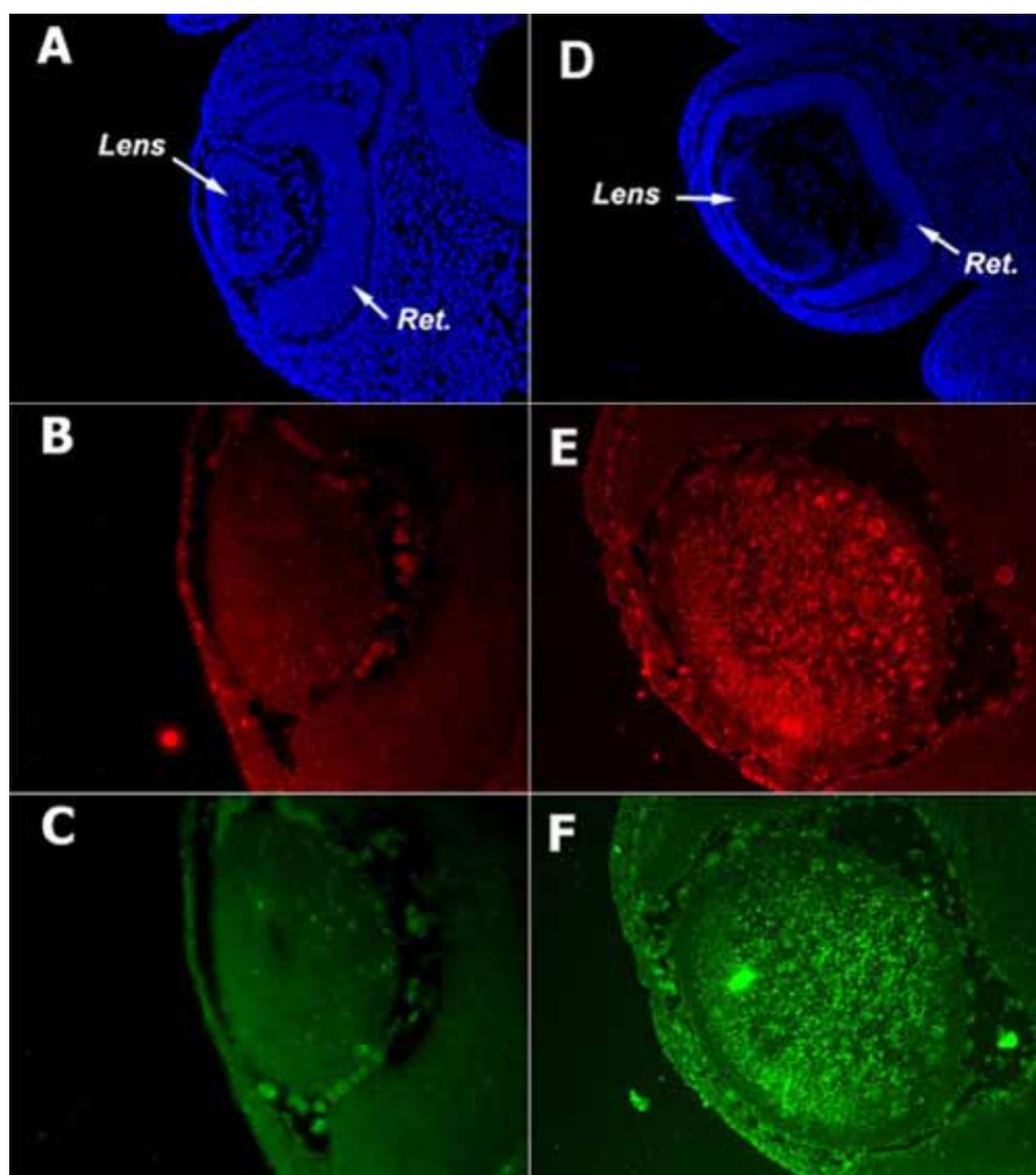


Figure 5. Immuno-localization of site 1 phosphorylated synapsin proteins during early embryonic fiber cell differentiation. Distribution of phosphorylated and dephosphorylated site-1 (Ser₆) synapsin proteins in rat E13 (A-C) and rat E15 (D-F) lenses. A,D: DAPI nuclear stain. B,E: anti-site-1 dephosphosynapsin specific antibodies. C,F: anti-site-1 phosphosynapsin specific antibodies. Lens and retina (Ret.) are labeled.

embryonic day about E13 in rats (equivalent to about E11.5 in mouse embryonic development). By day E17 rat lens fibers are about 0.5 mm long. At stage E17 (about E15.5 in mouse) the lens anterior surface is covered with cuboidal epithelial cells that begin elongating at the lateral margins of the lens in a process that continues in peripheral coritcal fiber cells of adult lenses.

Rat E17 embryonic sections were probed with anti-A β PP, (Figure 2B), anti-KIF5B, (Figure 2D), and anti-JIP1b, (Fig-

ure 2C), specific antibodies. Each antibody identifies overlapping expression along the anterior/posterior axis of actively differentiating fibers in the interior region of E17 rat lenses, and equivalent day E15.5 mouse lenses (not shown), consistent with interactions required to tether vesicles to the cytoskeleton for transport. Stronger signal is detected closer to the cell nuclei, and may also reflect association with additional organelles surrounding cell nuclei. The DAPI stained lens in Figure 2A identifies cell nuclei in interior elongating fiber cells

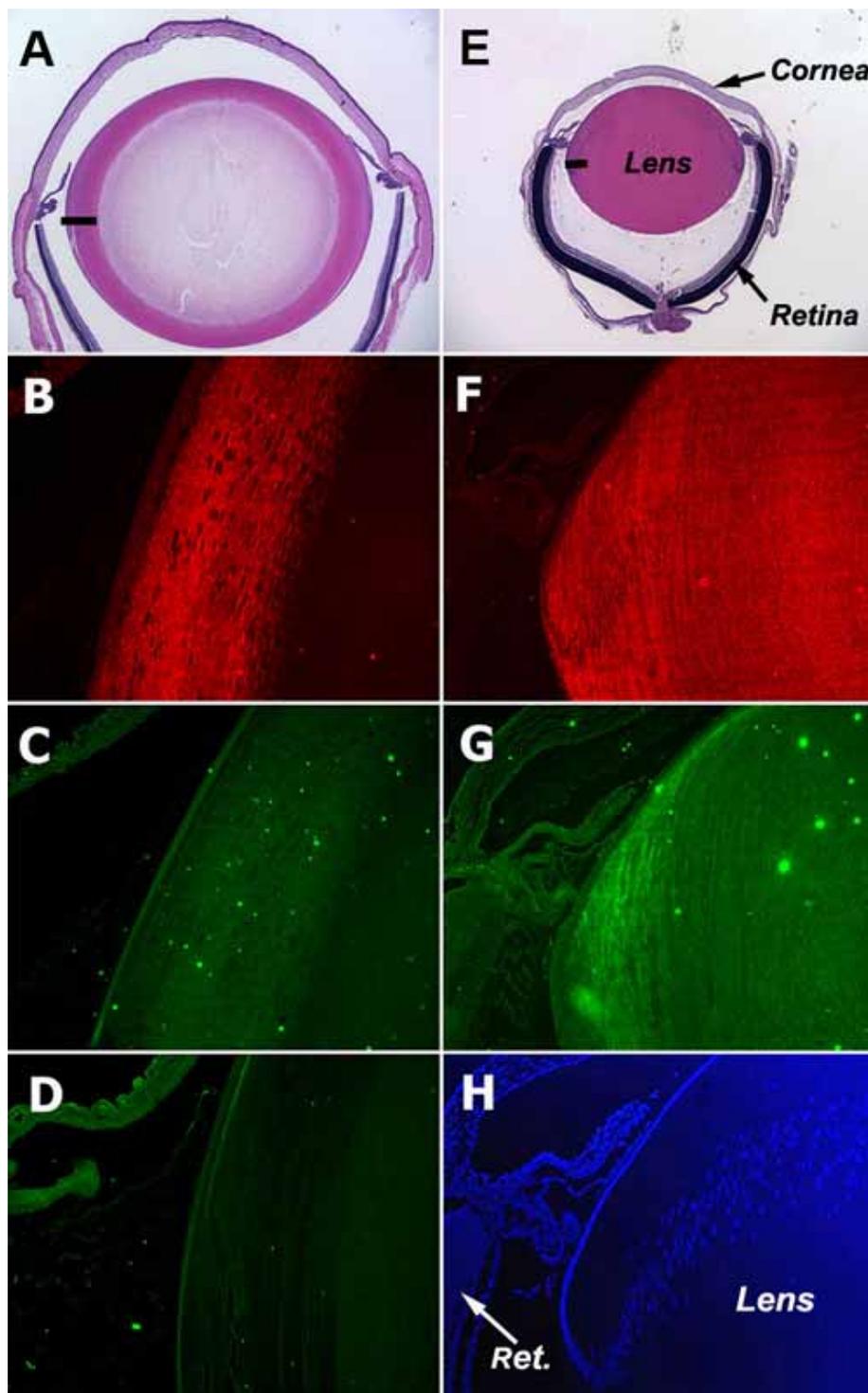


Figure 6. Analysis of site 1 phosphorylated synapsin protein distribution in post-natal and adult lenses. Analysis of site-1 phosphorylated synapsin and site-1 dephosphorylated synapsin protein in adult (A-D) and post-natal day 5 (E-H) rat lenses. A,E: Hematoxylin and Eosin histological stain. B,F: anti-site-1 dephosphosynapsin specific antibodies. C,G: anti-site-1 phosphosynapsin specific antibodies. D: primary antibody omitted. H: DAPI nuclear stain. Black horizontal bars in A and E indicate the general area shown in B-D and F-H, respectively.

aligned next to one another in adjacent cells across the width of the developing lens at this stage. In Figure 2E-G, Tau microtubule associated protein, synaptotagmin I, and synaptophysin I are also present along the anterior-posterior axis of elongating fiber cells in E17 rat lenses. Tau protein distribution is consistent with vesicles and microtubules present in elongating lens fiber cells [10].

We next examined the distribution of individual synapsin proteins in E17 lenses. Immunofluorescence analysis of lenses in Figure 2I-J identifies expression of synapsins IIa and IIIa along the axial length of elongating fiber cells in E17 rat lenses. In contrast, little or no synapsin I was detected in rapidly elongating fibers at this stage, Figure 2H appearing similar to the negative control in Figure 2K, however this antibody does identify Synapsin I protein in adult mouse lens and brain protein samples on immunoblots (Figure 1B).

To compare expression of individual synapsin genes in post-natal lenses we analyzed 21 day, 42 day, and 10 month old lenses by semi-quantitative PCR. In Figure 3, cDNA products generated after 28, 32, and 36 rounds of amplification of random-primed RNA from the same lens are resolved by molecular weight on agarose gels stained with ethidium bromide. Relative to synapsin I, synapsin II, and GAPDH transcripts, synapsin III is expressed at lower levels in 21 day and 42 day mouse lenses. Thus, it appears that similar to the regulation of synapsin expression in differentiating neurons [35], synapsin III expression is higher relative to synapsin I during rapid fiber cell elongation in embryonic development, and later as lens fibers mature synapsin III expression is decreased relative to synapsin I. However, in 10 month mouse lens we detected increased synapsin III expression that may be linked with stress during aging, and we are currently investigating this possibility. Finally, in contrast to the temporal order of synapsin gene expression during neuronal development, readily detectable synapsin II expression was present in both embryonic and post-natal lens fiber cells.

Synapsin site-1 (Ser₁) phosphorylation during lens development: We next used antibodies that specifically recognize phosphorylated or de-phosphorylated synapsin site-1 that is conserved in synapsins to examine their relative distributions in lens fibers. In Figure 4 phosphorylated and de-phosphorylated synapsins are again demonstrated along the axial length of E15.5 mouse lens fiber cells, consistent with the distributions of synapsins in E17 rat lens (Figure 2). Equivalent distributions of phosphorylated and dephosphorylated synapsin proteins were also present in E17 rat lenses (not shown). However, discrete patterns of phosphorylated and de-phosphorylated synapsin protein expression are present at this developmental stage. Site-1 de-phosphorylated synapsin is more strongly localized near apical ends of elongating fiber cells distal from the cell nuclei and surrounding organelles, whereas site-1 phosphorylated synapsin is more strongly localized near cell nuclei close to the center of fiber cells in these sections. In a higher magnification view in Figure 4C, site-1 phosphorylated synapsins appear in clusters in linear arrays and suggests a closer association with the fiber cell perimeter. In contrast, site-1 de-phosphorylated synapsin distribution in

Figure 4F appears more diffuse and more equally distributed across the width of lens fiber cells. In Figure 4C and Figure 4F, little synapsin is detected in the epithelial cell layer, again consistent with the presence of vesicles and microtubules in lens fiber cells [10].

Lo et al. [10] previously demonstrated a greater abundance of vesicles and microtubule arrays in the interior region of elongating lens fiber cells, and identified filamentous actin primarily along the perimeter of fiber cells [45]. In neurons, synaptic vesicles range from about 30-200 nm in diameter [46] and therefore would not be resolved by light microscopy. In light of these observations the present data are consistent with dephosphorylated synapsin proteins associated with vesicles linked to microtubules in the interior of lens fibers, as well as with vesicles associated with actin fibers near the fiber cells periphery. However, synapsin-vesicle associations in lenses similar to neurons have not yet been demonstrated. In contrast, phosphorylated synapsin is present in clustered distributions along the fiber cell perimeter, suggestive of focal regions where vesicles interact with the fiber cell surface. In addition, this distribution is also consistent with a role for synapsins in the polymerization of actin filaments that are present along the perimeter of fiber cells [45].

We next examined synapsin expression at earlier stages of development in E13 and E15 rat lenses. In Figure 5, little synapsin protein is detected in E13 lenses using either phosphorylation state specific antibodies. In contrast, relatively strong expression of both phosphorylated and dephosphorylated synapsin forms are present in E15 rat lenses where fiber cell elongation has begun, consistent with a role for synapsins in stimulating cell elongation, and a concomitant increase in vesicle transport. However, in E15 rat lenses we did not observe discrete distributions of phosphosynapsin and dephosphosynapsins along the axial length of fiber cells at this stage of development.

In the last experiments we examined synapsin protein distributions in post-natal rat lenses. In Figure 6, lower levels of site 1 phospho-synapsins relative to dephosphorylated synapsins are detected in P5 lenses. In addition, phospho-synapsin protein is relegated to peripheral elongating fiber cells that continue to differentiate throughout life. In adult lenses (Figure 6), in contrast to dephosphorylated synapsins, site-1 phospho-synapsin protein is nearly undetectable under these staining conditions, appearing similar to the negative control.

DISCUSSION

In our previous studies we identified A β PP as an important element in lens fiber cell development, and in early onset cataract that occurs in Down syndrome. Studies from a number of laboratories have demonstrated a fundamental role for A β PP in the cell biology of synaptic and cargo vesicle transport. Here, we demonstrate that in addition to A β PP, the lens also expresses a substantial complement of associated cargo and synaptic vesicle proteins, kinesin motor proteins, and A β PP interacting proteins beginning during embryonic fiber cell differentiation. These proteins are expressed along the anterior-posterior axis of rapidly elongating embryonic fiber cells, and

in adult cortical fiber cells that continue to differentiate throughout life, consistent with their known roles in membrane vesicle transport and regulating membrane vesicle release and fusion at distal cell surfaces.

We also identified temporally regulated expression of synapsin genes during fetal and post-natal lens development. Strong expression of synapsin III occurs during the period of rapid fiber cell elongation that characterizes fetal lens development, similar to the requirement for synapsin III during the initial elaboration of neuronal processes [34-38]. In post-natal lenses, synapsin III expression is decreased while synapsin I increases, again similar to neuronal maturation, and consistent with the expression of synapsin I in adult brain. However, in contrast to neuronal synapsin gene expression where synapsin II is associated with cell maturation in neurons, synapsin II is uniformly expressed throughout fetal and post-natal lens development. These expression patterns may reflect the opposing effects of calcium on Synapsin I and III and the refractory effects of calcium on synapsin II, with changes in calcium levels that occur during lens fiber cell differentiation [47].

The presence of kinesin heavy and light chain proteins, A β PP, JIP1b, synapsins, and synaptic vesicle proteins along the long axis of differentiating fiber cells suggests a role in cargo vesicle transport and regulated vesicle release at distal cell surfaces similar to what has been described in neurons. The present study also demonstrates overlapping distributions of these proteins with Tau microtubule associated protein along the anterior-posterior axis of fibers, where Lo et al. [10] demonstrated the presence of microtubules and membrane vesicles. Consistent with these observations, we also identified overlapping distributions of A β PP, JIP1b, and kinesins in guinea pig lenses, as well as in mouse and rat (not shown).

In addition to A β PP, we provide evidence that APLPI and APLPII, as well as Rab3A and Rab5 GTP binding proteins are also expressed in lens, suggesting that a similar array of cargo vesicle types are present in lens fiber cells and neurons. APLPs are thought to link different classes of cargo vesicles to the cytoskeleton [3,6], and Rab3A and Rab5 proteins also have been identified as targeting different vesicle types to the cytoplasmic membrane for fusion.

Each of the basic functions identified for synapsin proteins in neurons are strongly regulated by phosphorylation at site-1 (Ser₉), a site that is highly conserved in all known synapsin proteins. These functions include actin polymerization, initiation or stimulation of cell differentiation and cell elongation, and regulation of cargo and synaptic vesicle attachment to the cytoskeleton. A key mechanism for regulating site-1 phosphorylation involves cyclic AMP dependent PKA, which is also expressed in lenses (in this study and [48]).

In contrast to E13 rat lenses where little fiber cell elongation has occurred and little synapsin protein expression was detected, we demonstrated both phospho- and dephospho-synapsin proteins in elongating fiber cells in E15 rat lenses, consistent with a role for each of the known synapsin functions in early stages of fiber cell differentiation. In E15.5 mouse lenses (and equivalent E17 rat lenses) dephosphorylated site-

1 synapsin protein was more strongly detected near the apical ends of elongating fibers, suggesting synapsin coated vesicle accumulation may also occur near distal ends of fiber cells that is reminiscent of vesicles accumulating at presynaptic terminals ready for regulated release from the cytoskeleton and fusion with cell membranes. These observations are also supported by the presence of synaptophysin I and synaptotagmin I along the axial length of rapidly elongating lens fiber cells in fetal lens fibers. In neurons, synaptotagmins form homo- or hetero-multimers and regulate synaptic plasticity, and synaptophysins perform essential functions in synaptic plasticity, which do not necessarily involve neurotransmitter release [40]. The present data indicate that mechanisms required for synaptic vesicle transport, and for the subsequent fusion of vesicles with cell surface membranes in neurons are also utilized during lens fiber cell elongation.

The relative distributions of phosphorylated and dephosphorylated synapsin proteins in lens fiber cells is likely to be determined by a combination of roles site-1 phosphorylation has in regulating each of the known synapsin functions. Thus, the distributions of phosphorylated synapsin at different stages of fiber cell development may reflect a competition and/or a synergy of synapsin functions. Furthermore, at different stages of development specific synapsin functions may have a greater role in determining synapsin phosphorylation states and protein distributions.

Beginning in the 1970's a fundamental role for cAMP and PKA was demonstrated in cell-cycle regulation of lens epithelial cells and fibers, as well as for the coordination of fiber cell elongation [48-50]. Interestingly, in the 1980's investigations on cAMP dependent protein phosphorylation in peripheral lens fiber cells of calf lenses identified a few predominant phosphorylated proteins having molecular weights consistent with synapsins [51,52], however their identities were not confirmed at that time. Those studies, together with the present findings, suggest that cAMP dependent PKA could function in the lens via regulation of synapsins to modulate cargo vesicle transport, actin polymerization, and stimulation of cell differentiation, and we are now investigating these possible relationships.

A number of internal controls were used to confirm the existence of synaptic vesicle protein gene expression in lenses. First, similar results were obtained in mouse and rat lenses at various developmental stages. In addition, we demonstrated similar protein distributions in guinea pigs lenses (not shown), and we did not detect expression of synapsins, for example, in the corresponding synapsin triple knockout mouse (unpublished). In addition, the pattern of expression we observed for individual synapsin proteins was completely consistent with the distribution of phosphorylated and dephosphorylated synapsin proteins in lens fiber cells. Moreover, expression of specific synapsins and synaptic vesicle proteins was determined at the protein and mRNA level, as well as identifying proteins *in situ*.

Our observation that a large complement of synapsins, kinesin motor associated proteins, and synaptic vesicle proteins are expressed in lenses may be viewed as surprising and

unexpected. There have only been a few reports of non-neuronal expression of synapsins and other synaptic vesicle proteins. Very low levels of synapsin I have been reported in the pancreas [53] and liver [54]. Moreover, analysis of synapsin I and II promoters driving transgene expression demonstrated very highly neuron specific gene expression and was also present in the eye, however lenses were not examined [55,56]. To our knowledge there have been no previous reports describing synaptotagmin I, or synaptophysin I in non-neuronal tissues.

In summary, our findings demonstrate expression of a large complement of cargo vesicle transport proteins, synapsins, and synaptic vesicle proteins in lenses beginning early in fetal lens development. In addition, we demonstrate developmentally regulated expression of synapsin genes and phosphorylation of synapsin proteins that suggest a conservation of synapsin functions in lens and neuronal cell differentiation and elongation. Our findings expand the parallels between lens and brain Alzheimer related cell biology and vesicle trafficking mechanisms that have been linked with age related degenerative diseases. Finally, the apparent similarity in the cell biology of cargo vesicle transport in lens cells and neurons suggests that the lens may also provide discernable phenotypes related to synaptic vesicle protein gene defects, which are linked with additional neurological or psychiatric diseases.

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