The Expression of the Leber Congenital Amaurosis Protein AIPL1 Coincides with Rod and Cone Photoreceptor Development

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Purpose. The Leber congenital amaurosis (LCA) protein AIPL1 is present only in the rod photoreceptors of the adult human retina and is excluded from the cone photoreceptors. LCA, however, is characterized by an absence of both rod and cone function at birth or shortly thereafter. Therefore, this study was conducted to determine whether AIPL1 is present in the rod and cone photoreceptors of the developing human retina. In addition, the expression of NUB1, a putative AIPL1-interacting partner, was examined.

Methods. A comprehensive spatiotemporal examination of AIPL1 distribution during development was performed by immunohistochemistry, using a previously characterized AIPL1 anti-serum. Immunofluorescence confocal microscopy was used to examine the coexpression of AIPL1 with the long/medium (L/M) and short (S) wavelength–sensitive cone photoreceptors in the developing human retina. The spatiotemporal distribution of NUB1 was also examined by immunohistochemistry, using a newly developed anti-serum to the C terminus of NUB1.

Results. AIPL1 protein was detected by 11.8 fetal weeks in the central fetal human retina. With continued development, AIPL1 expression spread gradually toward peripheral retina. AIPL1 was expressed in the L/M and S cone photoreceptors in addition to the rods of the developing human retina. NUB1 was localized in cell nuclei throughout the human fetal and adult eye at all time points.

Conclusions. The pattern of AIPL1 expression closely follows the centroperipheral gradient in photoreceptor development. The data suggest that AIPL1 is essential for the normal development of both rod and cone photoreceptor cells and that mutations in the AIPL1 gene cause the death or dysfunction of photoreceptors early in development resulting in blindness or severely impaired vision at birth. (*Invest Ophthalmol Vis Sci.* 2003;44:5396–5403) DOI:10.1167/iovs.03-0686

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Leber congenital amaurosis (LCA) is the most severe congenital retinopathy and is characterized by an absence or attenuation of detectable rod and cone function at birth or shortly thereafter, as measured by absent or poorly recordable electroretinographic responses. LCA is a complex and clinically heterogeneous condition with substantial variation between families, although intrafamilial similarities exist. Clinically, patients affected by LCA present with infantile nystagmus, sluggish papillary responses, and occasionally a paradoxical pupil response. Additional features include symmetric midfacial hypoplasia with enophthalmos and hypermetropic refractive errors (On-line Mendelian Inheritance in Man [OMIM] http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=204000). LCA is inherited in an autosomal recessive fashion and accounts for approximately 5% of all inherited retinal dystrophies.[1] LCA is genetically heterogeneous in that mutations in six different genes to date have been found to cause it (RetNet: www.sph.uth.tmc.edu/RetNet/ provided in the public domain by the University of Texas Houston Health Science Center, Houston, TX). The protein products of the LCA genes are fundamental in phototransduction (retGC-1 [GUCY2D] and RPE65), photoreceptor development, and differentiation (CRX), photoreceptor cell polarity (CRB1), and photoreceptor subcellular protein translocation (RPGRIP1).[2] The fourth LCA-associated gene to be identified encodes a 384-amino-acid protein termed the aryl hydrocarbon receptor interacting protein-like 1 (AIPL1).[3] Recently, we performed the first characterization of AIPL1 distribution in the adult human retina and photoreceptor cells.[4] AIPL1 was localized exclusively in the rod photoreceptors of adult human retina and was excluded from the cone photoreceptors. This finding was significant, because there is no detectable electroretinogram (ERG) derived from either rod or cone photoreceptors in patients with LCA, suggesting the early and severe impairment of both the rod and cone photoreceptor systems.

AIPL1 contains three consecutive tetratricopeptide repeat (TPR) motifs that mediate protein interactions during multiprotein complex assembly and translocation.[5] The C terminus of AIPL1 in humans contains a highly flexible 56-amino-acid polyproline-rich sequence, which may participate in the regulation of rapid protein exchange or recruitment in multiprotein complexes.[6] AIPL1 shares 49% identity with the aryl hydrocarbon receptor-interacting protein (AIP),[7] also termed the aryl-hydrocarbon receptor-activated protein (ARA9)[8] or the X-associated protein (XAP2).[9] AIP facilitates the transactivation of the aryl hydrocarbon receptor (AhR) by regulating the nuclear translocation of the receptor and stabilizing it to ubiquitination.[7–10] Collectively, the data suggest that AIPL1 may be involved in protein maturation or translocation in multiprotein complexes.

Recently, yeast two-hybrid analysis using a bovine retinal cDNA library identified a putative interaction between bovine AIPL1 and a protein termed NUB1 (NEDD8 ultimate buster 1).[11] Unlike AIPL1, which is photoreceptor specific,[3,4] NUB1 protein has been detected in several different human cell lines,

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TABLE 1. Detection of AIPL1 Expression in Developing ONL

Age (Fetal Week)	Eyes Examined (*n*)	Intensity of AIPL1 Staining in the ONL*		
		Central Retina	Midperipheral Retina	Peripheral Retina
11.8	5	+++	−	−
12.0	2	+++	−	−
15.0	1	+++	+	−
16.0	1	+++	+	−
17.0	2	+++	++	−
18.0	3	+++	++	−
19.0	2	+++	++	+
20.0	1	+++	++	+
21.0	2	+++	++	+
22.0	3	+++	++	+
24.0	3	+++	++	+
26.0	1	+++	++	+
29.0	1	+++	++	+
30.0	1	+++	++	+
32.0	1	+++	++	+
33.0	1	+++	++	++
35.0	1	+++	+++	++
36.0	1	+++	+++	++
38.0	1	+++	+++	++
40.0	1	+++	+++	+++

* Relative intensity within section; +++, high intensity; ++, medium intensity; +, low intensity; −, no staining detected.

and NUB1 mRNA has been detected in several different human tissues.[12] The expression and localization of endogenous NUB1 protein in human retina has not been reported, however. NUB1 interacts with NEDD8, a highly conserved 81-amino-acid protein that shares 60% identity and 80% homology with ubiquitin.[12] The conjugation of NEDD8 to target proteins is catalyzed in a manner analogous to ubiquitination and sentrinization.[13] All known targets of NEDD8 conjugation in mammalian cells are members of the cullin (Cul) family. The NEDD8 conjugation of Cul-1 is necessary for the ubiquitin ligase activity of the SCF complex, of which Cul-1 is a major component.[14,15] The ubiquitin ligase activity of the Cul-1 containing SCF complex catalyzes the ubiquitination of IκBα, p27 (Kip1), p21 (CIP1/WAF1), cyclin D proteins, and β-catenin.[16–20] Cul-2 and -3 are assembled into an SCF-like complex that targets the hypoxia-inducible factor (HIF)-1α and cyclin E, respectively, for ubiquitination.[21,22] Hence, the NEDD8 conjugation system is involved in several important biological functions, including the regulation of cell cycle transition and cell growth. Both the conjugated and unconjugated levels of NEDD8 protein are downregulated by NUB1 by means of a posttranscriptional mechanism involving the NUB1-directed recruitment of NEDD8 and the NEDD8 conjugates to the proteasome for degradation.[12,23]

The expression of AIPL1 exclusively in the rod photoreceptors of human adult retina is intriguing in light of the fact that LCA is thought to be the consequence of either the impaired function, impaired development, or extremely early degeneration of both the rod and cone photoreceptor systems. To solve this paradox and investigate the pathogenesis of LCA caused by mutations in AIPL1, it was important to establish whether the dysfunction of AIPL1 in developing rods is sufficient to disrupt cone cell function as well, or whether AIPL1 is present in both rod and cone photoreceptors during development. Therefore, we investigated the spatiotemporal expression of AIPL1 in the developing human retina, as well as that of NUB1, to gain insight into the significance of the AIPL1–NUB1 interaction in the pathologic course of LCA.

MATERIALS AND METHODS

Adult and Fetal Eyeball and Tissue Preparation

A panel of human eyes from fetal week (Fwk) 11.8-40 and one eye from an adult 65 years of age were used in the study (Table 1). All samples were provided with informed consent, institutional review board approval was obtained, and the tenets of the Declaration of Helsinki were followed. The adult human eye was fixed by immersion in formal saline within 2 minutes of enucleation, for at least 24 hours. The eye was dehydrated in a graded series of industrial methylated spirits (IMS), equilibrated in xylene, and embedded in paraffin wax. The paraffin-embedded eye was sectioned at 6 μm along the horizontal meridian through the macula and optic disc and mounted on polylysine microscope slides (VWR International Ltd., Lutterworth, UK). The fetal eyes were selected from fetal autopsy specimens at Seoul National University Children's Hospital. The chosen eyes were of fetuses that had no history of ophthalmologic congenital anomaly. The gestational age was determined from the last menstrual period (LMP) and fetal measurements. Before enucleation, the temporal limbus was marked and a short, linear incision made along the mark. After enucleation, the eyeballs were fixed by immersion in Carnoy's solution for 2 hours at room temperature. Each eye was dehydrated in a graded ethanol series and embedded in paraffin by standard techniques. Before embedding in paraffin, each eye was cut in half along the horizontal meridian through the developing macula and optic disc. The paraffin-embedded eyes were sectioned at 4 μm and mounted on slides coated with 0.5% white glue (Elmer's; Borden, Columbus, OH) for immunohistochemistry.

Immunohistochemistry

Immunohistochemistry in the adult and fetal sections was performed as described previously, using 3′,3′-diaminobenzidine (DAB; Sigma-Aldrich, Poole, UK) and 0.03% hydrogen peroxide in Tris-buffered saline (TBS; 100 mM Tris-HCl [pH 7.6]; 150 mM NaCl) to visualize the immunoreaction.[4] Retrieval of the AIPL1 antigen in adult and fetal sections and of the NUB1 antigen in adult sections was accomplished by microwaving the sections four times for 2.5 minutes each at 800 W in TBS containing 5% urea. Retrieval of the NUB1 antigen in fetal sections was accomplished by microwaving the sections four times for

2.5 minutes each at 800 W in McIlvane's citrate buffer (0.1 M citric acid; 0.2 M $Na_2HPO_4.2H_20$). The adult sections were incubated with Ab-hAIPL1 anti-serum (1:500) or Ab-hNUB1 anti-serum (1:1200) in antibody buffer (TBS containing 0.1% BSA) overnight at 4°C. The fetal sections were incubated with Ab-hAIPL1 anti-serum (1:500) or Ab-hNUB1 anti-serum (1:1000) in antibody buffer overnight at 4°C. To verify the specificity of the immunostaining, retinal sections were also stained with AIPL1 and NUB1 rabbit preimmune serum, and Ab-hAIPL1 or Ab-hNUB1 anti-serum, preadsorbed with the respective peptides (30 μg/mL) against which each antibody was raised. The sections were visualized with a light microscope (BX50; Olympus,) using bright field and differential interference contrast (Nomarski) optics. The sections were photographed using an integral digital camera (DP10; Olympus, Tokyo, Japan). Central retinal images were taken temporal to the optic disc within the macula and as close to the developing fovea as possible. Peripheral retinal images were taken as near as possible to the developing ora serrata, and midperipheral retinal images were taken approximately midway between the central and peripheral retina.

Fluorescence Scanning Confocal Microscopy

The adult and fetal sections were immersed in three changes of xylene and taken to water over a graded series of IMS. The sections were incubated in 0.1% sodium borohydride in TBS for 30 minutes at room temperature, before retrieval of the AIPL1 antigen, as described earlier. The sections were blocked with 10% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories, West Grove, PA) and 2% BSA in TBS for 45 minutes at room temperature. The sections were double labeled by incubation with Ab-hAIPL1 anti-serum (1:500) and hybridoma supernatant COS-1 (1:100) or ascites fluid OS-2 (1:10,000) in antibody buffer overnight at 4°C. The sections were incubated with CY3-conjugated donkey anti-rabbit and CY2-conjugated donkey anti-mouse secondary antibodies (Molecular Probes, Eugene, OR) in antibody buffer for 45 minutes at room temperature. 4′6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was included in the final TBS wash, the sections were mounted in fluorescent mounting medium containing 15 mM sodium azide (Dako, Ely, UK) and visualized with a laser scanning confocal microscope (LSM510; Carl Zeiss Meditec, Oberkochen, Germany). The sections were also stained with the AIPL1 rabbit preimmune serum and Ab-hAIPL1 anti-serum preadsorbed with the AIPL1 peptide (30 μg/mL) to confirm the specificity of the immunostain.

Antibody and Western Blot Analysis

The NUB1 cDNA was amplified from human retinal cDNA, using NUB1 specific primers NUB1-forward (5′-AGATCTGGGATGGCACAAAA-GAAATATCTTCAAGC) and NUB1-reverse (5′-GGTACCTTAGTTTT-TCTTTGTTGCTGACTTCC). A rabbit polyclonal anti-serum, Ab-hNUB1, was raised against peptide LSYVENRKSATKKN (amino acid residues 587-601) conjugated to keyhole limpet hemocyanin (KLH; Genosys Biotechnologies, Cambridge, UK). Total protein extracts were prepared by homogenization of human retina and cell lines in tissue resuspension buffer (20 mM Tris-HCl [pH 7.5], 500 mM NaCl, 12.5 mM KCl, 1 mM EDTA, 1 mM DTT, protease inhibitor cocktail for mammalian cell extracts [Sigma-Aldrich]). The Bradford microassay (Bio-Rad Laboratories, Hercules, CA) was used to determine total protein concentrations to ensure even loading on gels. Protein samples were prepared by the addition of an equal volume of 2× sample buffer (0.0625 M Tris-HCl [pH 6.8], 2.5% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.25% bromophenol blue), resolved by SDS-PAGE on 12% gels and electroblotted onto chemiluminescence nitrocellulose (ECL; Amersham, Little Chalfont, UK). Western blot analysis was performed as described previously.[4] The rabbit preimmune serum and Ab-hNUB1 anti-serum were used at a titer of 1:20,000. For the peptide competition assay, the Ab-hNUB1 anti-serum (1:20,000) was preadsorbed by the NUB1 peptide (1 μg/mL). The mouse anti-rabbit secondary antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL) was used at a titer of 1:40,000. Bands were visualized using chemiluminescence (ECL; Amersham).

Y79 Subcellular Fractionation

Y79 cells were collected by centrifugation for 5 minutes at 4°C and 600*g*. The cells were washed three times with ice-cold 1× phosphate-buffered saline (PBS) and resuspended in lysis buffer (PBS; 0.5% Nonidet-P40; Sigma-Aldrich). The cells were incubated on ice for 3 minutes with gentle agitation. Isolation of nuclei was confirmed by phase-contrast microscopy. The nuclei were pelleted by centrifugation for 3 minutes at 4°C and 1000*g* and the supernatant removed as a cytoplasmic fraction. Equal volumes of the cytoplasmic and nuclear fractions were resolved by SDS-PAGE and transferred to nitrocellulose membrane, and Western blot analysis was performed as described previously with both the AIPL1 and NUB1 antisera.

Results

The Spatiotemporal Expression of AIPL1 in Human Fetal Retina

Specific immunolabeling with the Ab-hAIPL1 anti-serum[4] was detected only in the developing retina, and no staining was observed in any other region of the fetal eyes (Figs. 1, 2). No reaction product was detected when immunolabeling was performed with the rabbit preimmune serum, with the Ab-hAIPL1 anti-serum in the presence of competing peptide (30 μg/mL), or when the primary antibody was omitted. The distribution of AIPL1 in retina was compared at a range of ages from fetal week (Fwk) 11.8 to 40 (Table 1). The observations made in the central retina are illustrated at Fwk 11.8, 16, 19, 21, 29, 36, and 40 (Fig. 1), and expression patterns in central versus midperipheral and peripheral retina are illustrated at Fwk 11.8, 19, and 40 (Fig. 2).

At Fwk 11.8, AIPL1 was first detected in the outermost cells of central retina (Fig. 1A). The AIPL1-positive cells were regularly shaped and aligned in a single layer that corresponds to the presumptive photoreceptors of the developing outer nuclear layer[24–27] (ONL; Fig. 1A). AIPL1 was not detected in the outer plexiform layer (OPL); the inwardly migrating layers of horizontal (h), bipolar (b), and amacrine (a) cells of the immature inner nuclear layer (INL); the inner plexiform layer (IPL) or in the ganglion cell layer (GCL; Fig. 1A). During development the differentiation of the sensory retina into a laminated structure progresses from the central retina gradually toward the periphery, such that peripheral retina is the last to differentiate[24–28] (Fig. 2). At Fwk 11.8, the central retina was well differentiated and had all of its distinctive laminae (Fig. 2A, central). In midperipheral retina, the outer neuroblastic layer (OBL) and inner neuroblastic layer (IBL) were evident (Fig. 2A, midperipheral), whereas the outer nuclear zone (ONZ) of the peripheral retina was without distinct layers (Fig. 2A, peripheral). AIPL1 was detected only in the presumptive photoreceptors of the central retina (Fig. 2A, central) at Fwk 11.8. AIPL1 was not detected either in the midperipheral or peripheral retina (Fig. 2A, midperipheral and peripheral).

At Fwk 16, AIPL1 was detected in the cells of the ONL, which appeared thicker due to the increased proliferation and differentiation of the presumptive photoreceptors[24–27] (Fig. 1B). By Fwk 19 and 21, photoreceptor differentiation involves primarily the cone photoreceptors, as rod photoreceptor differentiation lags considerably behind that of the cones[24–27] (Figs. 1C, 1D). The cone nuclei are arranged in a row adjacent to the now distinct outer limiting membrane (OLM) and the rod nuclei are located more internally[24–27] (Figs. 1C, 1D). AIPL1 was detected at various intensities in the photoreceptor nuclei, but there was no clear distinction between presumptive rods and cones. AIPL1 was also detected in the immature inner segments of the photoreceptors protruding through the OLM and in the presumptive outer segment precursors, the emergence of which characterizes photoreceptor differentia-

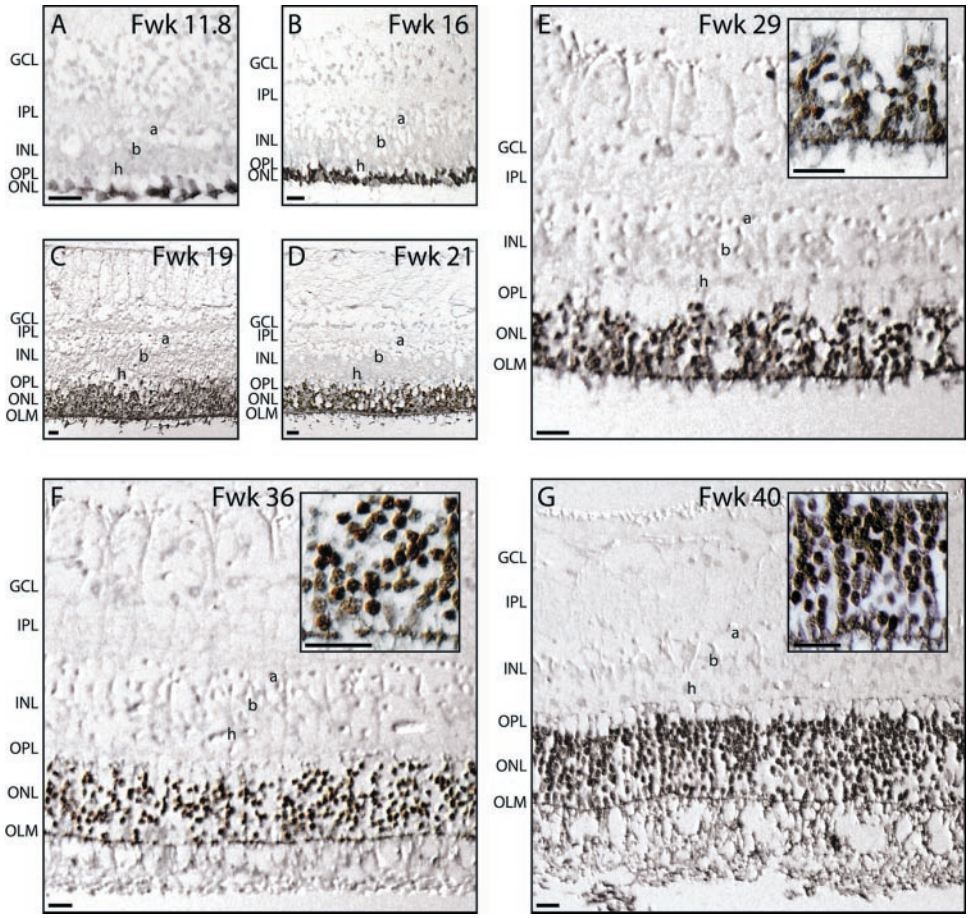

FIGURE 1. Immunohistochemistry in the central region of the developing human retina using the Ab-hAIPL1 anti-serum at Fwk 11.8 (**A**), 16 (**B**), 19 (**C**), 21 (**D**), 29 (**E**), 36 (**F**), and 40 (**G**). Scale bars, 20 μm.

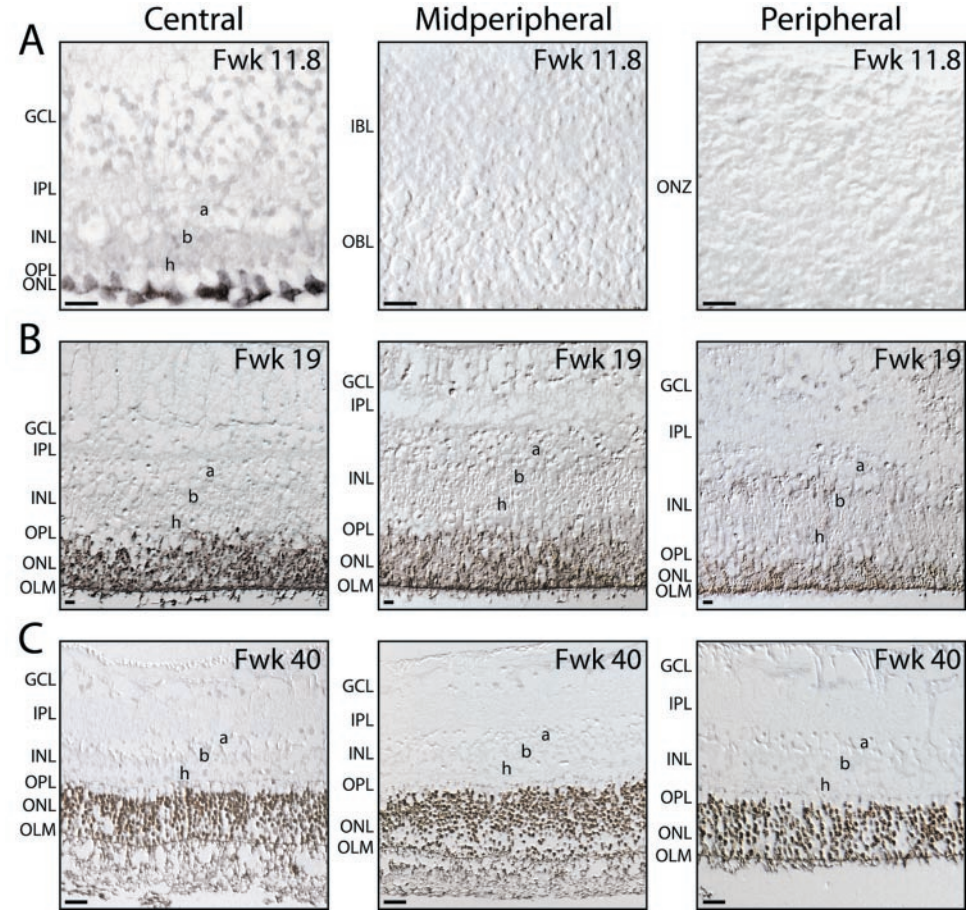

FIGURE 2. Immunohistochemistry in the central, midperipheral, and peripheral regions of the developing human retina using the Ab-hAIPL1 anti-serum at Fwk 11.8 (**A**), 19 (**B**), and 40 (**C**). Scale bars, 20 μm.

tion at this stage[24–27] (Figs. 1C, 1D). In the midperipheral and peripheral parts of the sensory retina at Fwk 19, the ONL and INL are immature, and the inward migration of the horizontal, bipolar, and amacrine cells is less evident (Fig. 2B, midperipheral and peripheral). Relative to the central retina, the intensity of AIPL1 immunostaining decreased in the immature ONL of the midperipheral retina and decreased still further in the immature ONL of the peripheral retina at Fwk 19 (Fig. 2B, midperipheral and peripheral).

By Fwk 29, the differentiation of the rod photoreceptors was well advanced, and AIPL1 was detected in the photoreceptor cell bodies of the ONL in central retina as well as the developing photoreceptor inner segments protruding through the OLM[24–27] (Fig. 1E). At Fwk 36 and 40, the Ab-hAIPL1 anti-serum labeled the photoreceptor cell bodies in the ONL, which are arranged more regularly in columns. In addition, AIPL1 was detected in the photoreceptor inner and outer segments, which have acquired a mature appearance[24–27] (Figs. 1F, 1G). The AIPL1 immunostain detected in the nuclei of the ONL in central retina at Fwk 29 was of uneven intensity (Fig. 1E, inset), and, by Fwk 36 and 40, some nuclei appeared to have less or no detectable AIPL1 (Figs. 1F, 1G, insets). By Fwk 28 to 30, the laminae characteristic of adult retina are present throughout from central to peripheral retina[24–27]. At Fwk 40, AIPL1 was detected with equal intensity in the ONL of the central, midperipheral, and peripheral parts of the sensory retina (Fig 2C, central, midperipheral, and peripheral).

Therefore, at all ages examined, AIPL1 was detected exclusively in the ONL of the developing retina and was not detected in any other nuclear or plexiform layers. Furthermore, the intensity of AIPL1 staining proceeded in a centroperipheral direction, closely following the centroperipheral gradient of photoreceptor differentiation.

Coexpression of AIPL1 and Cone Opsins in Adult and Fetal Human Retina

The pattern of AIPL1 expression in the developing retina suggests that AIPL1 could be expressed in both developing rod and cone cells. Therefore, sections from adult and fetal retinas were processed for immunofluorescence confocal microscopy with the Ab-hAIPL1 anti-serum in combination with the long/medium wavelength–sensitive (L/M)-cone opsin antibody COS-1, or the short wavelength-sensitive (S)-cone opsin antibody, OS-2.[29] In adult retinal sections, the AIPL1 immunostain was the same as that shown previously.[4] AIPL1 (Figs. 3A, 3B, red) was detected only in the rod photoreceptors, from the inner segments to the rod spherules in the OPL and was not detected in either the L/M or S cone (green) photoreceptors.

In sections of central retina at Fwk 29, AIPL1 (red) was detected in the differentiating photoreceptors of the ONL, with different degrees of intensity in the nuclei and cytoplasm of the cell bodies and was granular or punctate in appearance (Figs. 3C, 3D). AIPL1 was also detected in the presumptive inner and outer segments of the photoreceptors. The antibodies COS-1 and OS-2 (green) detected the small and immature cone outer segments of L/M and S cone photoreceptors respectively at Fwk 29 (Figs. 3C, 3D). The Ab-hAIPL1 immunolabel colocalized with both the COS-1 and OS-2 immunosignal in the presumptive photoreceptor outer segments at Fwk 29 (Figs. 3C, 3D). Clearly, the AIPL1 immunolabel in the presumptive cone outer segments could be traced back to AIPL1-positive cell bodies and nuclei in the ONL. Hence, AIPL1 is present in the nucleus, cell bodies, and inner and outer segments of both the L/M and S cone photoreceptors during human development, but is absent from all cone photoreceptors in adult human retina.

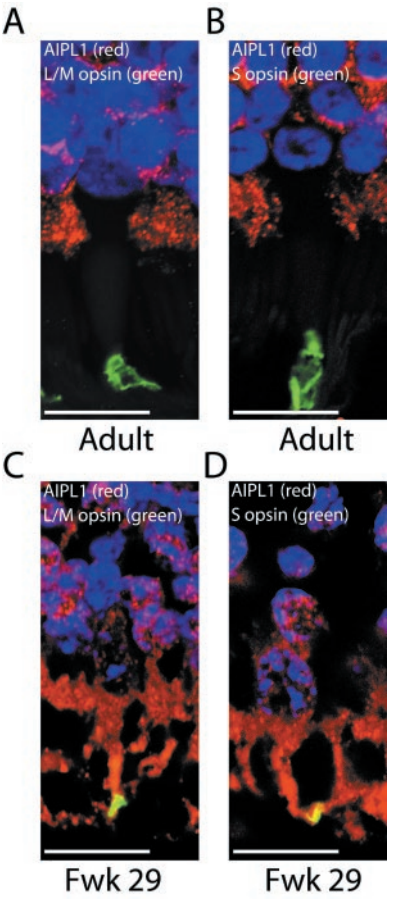

Figure 3. Double immunofluorescence confocal microscopy in the adult (**A**, **B**) and developing (**C**, **D**) human retina using the Ab-hAIPL1 anti-serum (AIPL1, in *red*) and the monoclonal antibody COS-1 (L/M opsin, in *green*; **A**, **C**) or the monoclonal antibody OS-2 (S opsin, in *green*; **B**, **D**). Scale bars, 20 μm.

NUB1 Expression and Subcellular Localization

To investigate the expression of the AIPL1-interacting partner NUB1 in the human retina, a rabbit polyclonal anti-serum, Ab-hNUB1, was raised against keyhole limpet hemocyanin (KLH)–conjugated peptide LSYVENRKSATKKN comprising amino acid residues 587-601 of the predicted protein sequence of human NUB1. The Ab-hNUB1 anti-serum detected a single protein of ~69 kDa in human retinal extracts under reducing conditions, corresponding to the predicted molecular weight of NUB1 (Fig. 4A). No reactivity with this protein was detected by the Ab-hNUB1 anti-serum in the presence of the NUB1 peptide (1 μg/mL) against which the antibody was raised or by the preimmune serum. Hence, the Ab-hNUB1 reactivity in human retina was specific.

The Ab-hNUB1 anti-serum was also characterized against a panel of human cell lines (Fig. 4B). The Ab-hNUB1 anti-serum detected a specific band of ~69 kDa in all cell lines examined, including the Y79 retinoblastoma cell line, which shares several photoreceptor characteristics. No reactivity with this protein species was detected by the preimmune serum or when the Ab-hNUB1 anti-serum was preadsorbed with the NUB1 peptide (1 μg/mL; data not shown). Hence, NUB1 protein is present in both human retina and Y79 retinoblastoma cells, and the results confirm previous suggestions of a ubiquitous distribution of the NUB1 protein in different human tissues and cell lines.[12]

The subcellular distribution of AIPL1 and NUB1 in Y79 cells was examined (Fig. 4C). Fractionation of Y79 cell extracts

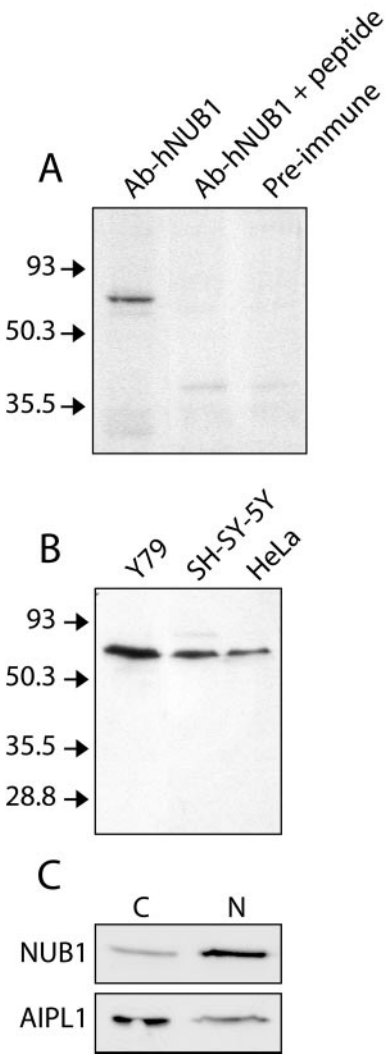

FIGURE 4. Immunoblot of human retinal proteins with the Ab-hNUB1 anti-serum, Ab-hNUB1 anti-serum preadsorbed with competing peptide and preimmune serum (**A**). Immunoblot of proteins from Y79, SH-SY-5Y and HeLa cells with the Ab-hNUB1 anti-serum (**B**). Immunoblot of equal volumes of Y79 cytoplasmic (C) and nuclear (N) fractions for NUB1 and AIPL1 (**C**). Molecular mass markers are indicated on the *left* in kilodaltons.

revealed that NUB1 was predominantly localized to the nuclear fraction, with only a small amount present in the cytoplasmic fraction. In contrast, AIPL1 was predominantly cytoplasmic.

Spatiotemporal Expression of NUB1 in the Developing and Adult Human Retina

The spatiotemporal distribution of NUB1 in the developing and adult human eye was examined. In adult and all fetal sections examined between Fwk 11.8 and 40, specific immunolabeling with the Ab-hNUB1 anti-serum was detected in all the different tissues of the eye including the cornea, choroid, sclera, ciliary fibers, iris and retina, as illustrated in Fwk 36 sections (Fig. 5A). The NUB1 immunolabel was detected predominantly in the cellular nuclei in all tissues examined (Fig 5A).

The spatiotemporal distribution of NUB1 in adult retina and in fetal retina from Fwk 11.8 to 40 was compared, and the observations made are illustrated in midperipheral retina at Fwk 12, 20, and 32 and in adult (Fig. 5B). At each age examined, NUB1 was detected in all the nuclear layers of the developing retina and was not restricted to the ONL. No centroperipheral gradient in NUB1 immunolabel was detected. At any age examined, NUB1 was detected in all the nuclear layers of central, midperipheral, and peripheral retina. Some nuclei within each retinal nuclear layer were labeled more intensely than others by the Ab-hNUB1 anti-serum; however, there was a decreasing gradient in the intensity of the NUB1 immunosignal from the inner retinal nuclear layers to the outer retinal nuclear layers. In midperipheral retina at Fwk 12, the inwardly migrating ganglion cells, the first to become postmitotic at any particular locus, were the most intensely labeled, whereas the outermost cells of the neuroblastic layer, the last to become postmitotic at a particular locus, were less intensely labeled by the Ab-hNUB1 anti-serum[24–27] (Fig. 5B). In midperipheral retina at Fwk 20, the NUB1 immunosignal was most intense in the cell nuclei of the distinct ganglion cell layer (GCL), followed by the cell nuclei of the inwardly migrating amacrine cells of the presumptive INL. The remaining cell nuclei of the immature INL and ONL were the least intensely labeled by the Ab-hNUB1 anti-serum (Fig. 5B). At Fwk 32, when distinct retinal nuclear layers were evident in the midperipheral retina, the Ab-hNUB1 immunosignal was least intense in the cell nuclei of the ONL. The intensity of the Ab-hNUB1 immunolabel was more intense in the horizontal, bipolar, and amacrine cell nuclei of the INL, and reached the greatest intensity in the ganglion cell nuclei (Fig. 5B). In adult retina, the inner to outer gradient in the intensity of the NUB1 immunolabel was less severe (Fig. 5B). Therefore, the immunosignal detected with the Ab-hNUB1 anti-serum appears to follow the inner to outer gradient of cellular differentiation within the human sensory retina.[24–27]

DISCUSSION

In our studies, the AIPL1 protein was present in both the rod and cone photoreceptor precursors of the presumptive ONL during development of the human sensory retina. AIPL1 was not present in any other layers of the developing retina or in any other tissues of the developing human eye. Furthermore, the spatiotemporal patterning of the AIPL1 protein in the developing sensory retina followed a centroperipheral gradient that corresponded to the differentiation of photoreceptors. The expression of AIPL1 in the outer retina coincided with rod and cone photoreceptor specification and structural maturation. Cone nuclei first appear at approximately 10 weeks of development in the central retina during the formation of the fovea.[30] Rod nuclei are not present until approximately 12 weeks of development.[24–27] AIPL1 was present in the central human retina at 11.8 weeks. Therefore, it is likely that the first cells to express AIPL1 in the developing human retina are the newly born central cone photoreceptors. The expression of AIPL1 in developing photoreceptors and the early onset of vision loss in patients with LCA suggests that AIPL1 function is critical during the period of rod and cone photoreceptor development.

The cone–rod homeobox protein CRX is critical for photoreceptor differentiation during retinal development and photoreceptor maintenance in the adult retina.[31–33] *Crx* expression in the developing human retina is first detected at 10.5 weeks of development by RT-PCR and at 13 weeks of development by in situ hybridization.[34] The CRX protein, however, is only detected in the human retina at 15 weeks of development.[34] The Maf-subfamily transcription factor NRL is an important regulator of rod photoreceptor differentiation.[35] The NRL protein is not detected in rod nuclei of the central retina until Fwk 17.4 and by Fwk 19 in peripheral retina.[36] Thus, AIPL1 protein is detectable in the developing human retina well before CRX and NRL, suggesting that the critical function of AIPL1 during rod and cone development and differentiation may precede CRX and NRL function.

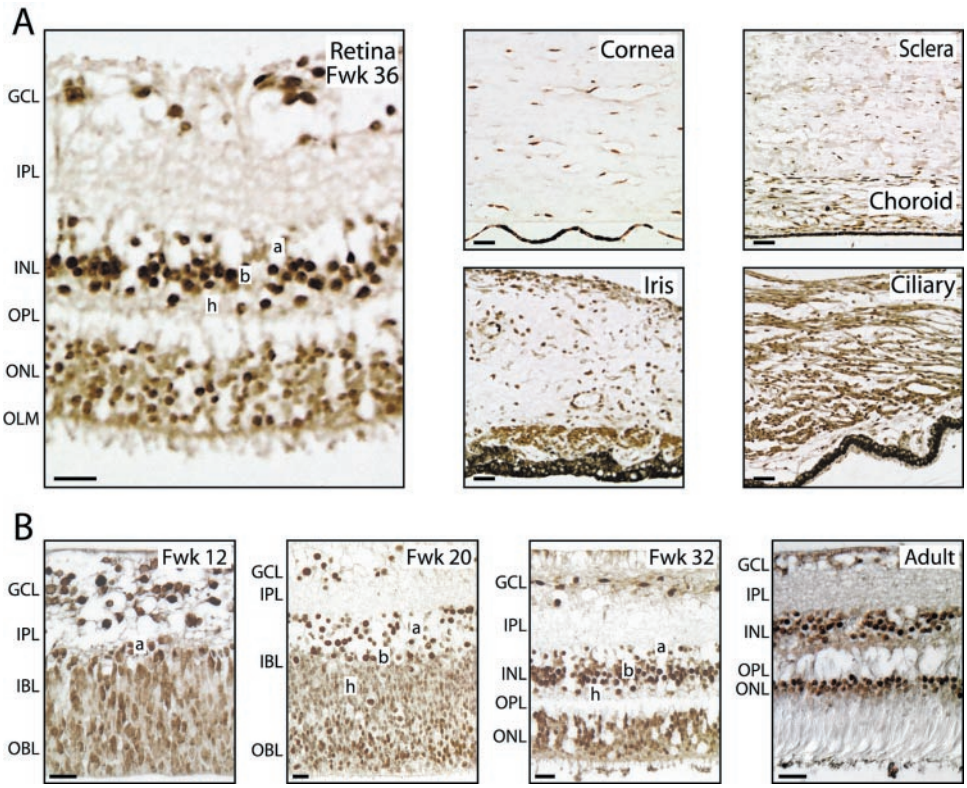

FIGURE 5. Immunohistochemistry in the retina, cornea, sclera, choroid, iris, and ciliary body of the developing human eye at Fwk 36 with Ab-hNUB1 anti-serum (**A**). Immunohistochemistry in the developing human midperipheral retina at Fwk 12, 20 and 32 and in the adult human retina with the Ab-hNUB1 anti-serum (**B**). Scale bars, 20 μm.

Our results confirm the previous finding that AIPL1 is not present in either the L/M or S cone photoreceptors of the adult human retina.[4] This conclusion is supported by a comprehensive serial analysis of photoreceptor gene expression (SAGE) in libraries from murine adult retina and microdissected ONL, which shows the specific expression of the *AIPL1* gene in rod photoreceptors in adult retina.[37] In addition, the expression of the *AIPL1* gene has been shown to increase during murine retinal development and attain the highest levels in the adult rod photoreceptors.[37] Our data show that at some time between 40 weeks of development and adulthood, *AIPL1* switches its expression from rod and cone to rod only. The protein is maintained in the rod photoreceptors but is significantly reduced in cone photoreceptors to below the limit of detection. This suggests that AIPL1 function, though still necessary in adult rod photoreceptors, is redundant in adult cone photoreceptors. The mechanism by which *AIPL1* expression is switched off in cone photoreceptor cells may involve both transcriptional regulation by local *cis*- or *trans*-acting factors, or translational regulation. Identification of the factors involved in AIPL1 differential regulation is important in determining the role of AIPL1 in the developing and mature retina.

Recently, yeast two-hybrid analysis of a bovine retinal cDNA library identified a putative interaction between bovine AIPL1 and NUB1.[11] AIPL1 and NUB1 localization overlapped in the developing photoreceptor nuclei; however, there were several differences in the distribution of the two proteins. Unlike AIPL1, NUB1 protein was present not only in the retina, but also in all the tissues of the developing and adult human eye. Within the developing and adult retina, NUB1 protein was present in all the retinal nuclear layers and was not restricted to the ONL as is the case with AIPL1, which is photoreceptor specific. NUB1 and AIPL1 also showed some differences in their subcellular distribution. The subcellular distribution of the NUB1 protein was predominantly nuclear. It has been shown that HA-tagged NUB1 transfected into HeLa cells is predominantly nuclear.[12] The nuclear localization of NUB1 is consistent with the presence of a nuclear localization signal (NLS) in NUB1 from amino acid residues 414-431 and with the proposed function of NUB1 in the regulation of the cell cycle progression and cellular proliferation. AIPL1 was present in the nuclei of the photoreceptors, but it was not restricted to the nucleus. For example, the protein was predominantly cytoplasmic in Y79 cells. In the developing rod and cone photoreceptors, AIPL1 was present in the presumptive outer and inner segments as well as the photoreceptor cell bodies. In the adult retina, AIPL1 was present in the inner segments, cell bodies, and spherules of the rod photoreceptors.

The human retina develops in an inner to outer gradient in addition to a centroperipheral gradient in that differentiation proceeds from the inner retinal layers (GCL) to the outer retinal layers (ONL) as well as from central to peripheral retina.[24–27] The spatiotemporal patterning of the NUB1 protein in the developing sensory retina followed the gradient of cellular differentiation from the inner retinal nuclear layers to the outer retinal nuclear layers. This distribution of NUB1 in the developing human retina is consistent with the proposed function of NUB1 in cell growth and cell cycle transition, in that the highest levels of NUB1 were present in the first cells to become postmitotic and differentiate, whereas the lowest levels of NUB1 were present in the last cells to become postmitotic and differentiate. There was no change in the centroperipheral distribution of the NUB1 protein. Consequently, NUB1 was present in midperipheral and peripheral regions of the developing human retina before AIPL1.

These data suggest that whereas NUB1 may perform a general function in the development or differentiation of all the retinal cell types during development, AIPL1 is critical only in the development of the photoreceptors. Critical functions of AIPL1 in photoreceptor development may be modulated by NUB1, by an as yet unknown mechanism. Recently, it has been shown that ubiquitin activating enzyme 3 (Uba3) interacts directly with steroid hormone receptors and suppresses receptor-mediated transactivation.[38] Uba3 is the catalytic subunit of the activating enzyme in the NEDD8 conjugation pathway, and neddylation activity was required for the Uba3-mediated sup-

pression of receptor transactivation. Furthermore, it has also recently been shown that NEDD8 itself interacts directly with the aryl hydrocarbon receptor and modulates its transcriptional activity.[39] Similarly, NUB1 and AIPL1 may function together to regulate the subcellular translocation and transactivation activity of an as yet unidentified retinal receptor that is important in photoreceptor development. Collectively, however, the data suggest that the AIPL1–NUB1 interaction may be transient and only occurs as part of the AIPL1 functional cycle. Further studies are thus necessary to determine the molecular basis of the AIPL1–NUB1 interaction and the functional significance of the interaction in retinal development and normal vision.

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