AIPL1, a protein implicated in Leber's congenital amaurosis, interacts with and aids in processing of farnesylated proteins

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The most common form of blindness at birth, Leber's congenital amaurosis (LCA), is inherited in an autosomal recessive fashion. Mutations in six different retina-specific genes, including a recently discovered gene, *AIPL1*, have been linked to LCA in humans. To understand the molecular basis of LCA caused by aryl hydrocarbon receptor-interacting protein-like 1 (AIPL1) mutations, and to elucidate the normal function of AIPL1, we performed a yeast two-hybrid screen using AIPL1 as bait. The screen demonstrated that AIPL1 interacts specifically with farnesylated proteins. Mutations in AIPL1 linked to LCA compromise this activity. These findings suggest that the essential function of AIPL1 within photoreceptors requires interactions with farnesylated proteins. Analysis of isoprenylation in cultured human cells shows that AIPL1 enhances the processing of farnesylated proteins. Based on these findings, we propose that AIPL1 interacts with farnesylated proteins and plays an essential role in processing of farnesylated proteins in retina.

Sohocki *et al.* (1) have reported the identification of a gene on chromosome 17 as a candidate for Leber's congenital amaurosis (LCA), a severe form of inherited retinal dystrophy resulting in complete blindness or severely impaired vision at birth. The protein encoded by this gene is 70% similar to the aryl hydrocarbon receptor-interacting protein (AIP), therefore it was named AIP-like 1 (AIPL1). Association of several mutations in AIPL1 with LCA shows that AIPL1 has a function that is essential for the retina. Consistent with this finding, AIPL1 is expressed only in retina (2).

Both AIPL1 and AIP contain three tetratricopeptide repeat (TPR) domains (3). This structure is present in a variety of proteins that participate in diverse biological functions, ranging from protein folding to protein translocation. TPR domains are sites of protein–protein interaction; in particular, they are thought to interact with the C termini of binding partners. For example, the TPR1 and TPR2A domains of p60/Hop, a heat shock protein (Hsp)70/Hsp90 adapter protein, bind highly conserved short peptides corresponding to the EEVD sequences at the C termini of Hsp70/90 proteins (4). The presence of TPR domains in AIPL1 suggests that it may also interact with the C termini of specific proteins within the retina.

To investigate the role of AIPL1 in retina, we performed a yeast two-hybrid screen to identify proteins that interact with AIPL1. Our results show that AIPL1 specifically binds to farnesylated proteins. Farnesylation, a type of prenylation, is a posttranslational modification that occurs at the C termini of many proteins. Approximately 2% of all proteins are prenylated (5). Prenylation enhances protein–membrane interactions and protein–protein interactions (6). Hypoprenylation of Rabs due to mutations in Rab escort protein-1 (REP-1) has been associated with choroideremia (CHM), a retinal degeneration (7). Protein prenylation is also required for maintenance of retinal cytoarchitecture and photoreceptor structure (8). Collectively, these studies indicate that protein modification by prenylation is essential in vision. Our study shows that AIPL1 interacts with and aids in processing of farnesylated proteins.

Methods

Strains and Plasmids. Full-length AIPL1 and AIPL1 lacking the last 56 amino acids were amplified from a λgt10 human retinal cDNA library (gift from J. Nathans). Primers used for amplification of AIPL1 introduced an *Eco*RI site at the N terminus and a *Sal*I site at the C terminus. After digesting with *Eco*RI and *Sal*I, the PCR products were cloned into *Eco*RI and *Sal*I sites of pOBD2 (gift from R. Hughes, University of Washington, Seattle) in-frame with a sequence encoding the DNA-binding domain of GAL4. Full-length AIPL1 was also amplified by using primers that contained a *Sma*I site at the N terminus and *Hin*dIII at the C terminus. After digestion with *Sma*I and *Hin*dIII, the PCR products were cloned into the pTRIEX4 expression vector (Novagen) to create a His-tagged version of full-length AIPL1. Full-length human AIP and human DNAJA2 was amplified from a λgt10 human retinal cDNA library. AIP was subcloned into GAL4 fusion vectors, pOBD2, in a similar fashion as AIPL1. The coding sequence of the aryl hydrocarbon receptor (AhR) was amplified from the plasmid pL66 (gift from C. Bradfield, University of Wisconsin Medical School, Madison) containing the full-length AhR. After digestion with *Nco*I/*Xho*I, the PCR product was cloned into pACT2 (Clontech) in-frame with the GAL4 transcriptional activation domain. DNAJA2 was cloned into pACT2 in a similar fashion as AhR. pCMV-hemagglutinin (HA)-tagged full-length human DNAJA2 and mutants were created by transferring the coding regions from their respective pACT2 clones to pCMV-HA vector (Clontech). All plasmid constructs were confirmed by sequencing.

Construction of Yeast Farnesyl Transferase (RAM1) Deletion. The RAM1 deletion from yeast strain BY4743 (gift from C. Tucker, University of Washington, Seattle) was transferred into the yeast two-hybrid host PJ694A/α (gift from T. R. Hazbun, University of Washington, Seattle) by using standard PCR techniques (9). The diploid PJ694A/α was then sporulated to select for the haploid RAM1 deletion strain. As reported, yeast strains harboring the RAM1 deletion were temperature-sensitive (10). All two-hybrid analysis in yeast lacking farnesyltransferase was done at room temperature.

Mutagenesis. The pTRIEX4-hAIPL1 plasmid was used as template for generation of point mutants by using a PCR-based primer-directed mutagenesis. For yeast two-hybrid analysis, all mutant AIPL1 fragments were transferred to pOBD2. For generation of various mutations in the prenylation motif, similar

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Abbreviations: LCA, Leber's congenital amaurosis; AhR, aryl hydrocarbon receptor; AIP, AhR-interacting protein; AIPL1, AIP-like 1; TPR, tetratricopeptide repeat; hsp, heat shock protein; CHM, choroideremia; REP, Rab escort protein; HRP, horseradish peroxidase; HA, hemagglutinin; FTase, farnesyltransferase; HEK, human embryonic kidney.

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methods of mutagenesis were used. The pACT2 clones containing various preys were used as templates for mutagenesis. All mutant clones were sequenced by using the Big Dye Terminator (Perkin–Elmer) ready reaction sequencing kit in order confirm the presence of mutations and to avoid unintentional changes.

Yeast Two-Hybrid Screening. The bait plasmid pOBD2-hAIPL1 (amino acids 1–327) was transformed into yeast strain PJ69-4A. The final 56 amino acids in the proline-rich C-terminal region of human AIPL1 were excluded from the bait to avoid spurious interactions. A bovine retinal cDNA library fused to the GAL4 transcriptional activation domain (gift from Ching-Hwa Sung, Cornell University, Ithaca, NY) was cotransformed into PJ69-4A containing the bait plasmid. The transformation procedure was based on a modified lithium acetate method (Clontech), according to the manufacturer's instructions. Colonies that grew on a synthetic media lacking uracil, leucine, and histidine, but containing 2 mM 3-aminotriazole, were further tested for growth on more stringent growth conditions in media lacking adenine. The interaction was further confirmed by using a qualitative β-galactosidase assay (Clontech), according to the manufacturer's instructions. Plasmids containing the prey were isolated from the primary positives and transformed into yeast strain PJ69-4α. The interactions were confirmed by mating the PJ69-4α containing the prey plasmids, or various controls with PJ69-4A containing either pOBD2-hAIPL1 (amino acids 1–329) or full-length hAIPL1 or various controls. All interactions were confirmed as described in the text.

Expression in Human Embryonic Kidney (HEK)-293 or COS-7 Cells. One microgram of the pTRIEX-4 constructs expressing native or various mutant AIPL1 was transiently transfected into 50–60% confluent HEK-293 or COS-7 cells by using FuGENE-6 reagent (Roche Molecular Biochemicals). For coexpression studies, 0.5 μg of each construct was transfected. The cells were grown in a DMEM/nutrient mixture F-12 (50/50; Invitrogen), 10% heat-treated FCS, 10 mM Hepes, pH 7.3, in six-well tissue culture dishes. Cells were harvested after 72 h, washed with PBS, and removed from the dish by rocking for 10 min at room temperature in PBS containing 0.2% EDTA. Cells were pelleted by centrifugation and then lysed by M-PER (Pierce) for 10 min at room temperature. The total lysates were spun at 28,000 rpm for 15 min in a Beckman TLA-55 rotor in a tabletop Beckman optima ultracentrifuge to obtain the soluble fraction. Protein content was measured with the Bio-Rad protein reagent by using BSA as a standard.

Preparation of Anti-AIPL1 Antibodies. The His-tagged version of native AIPL1 was expressed at room temperature in BL21 (DE3)pLysS *Escherichia coli* containing pTRIEX-hAIPL1. His-tagged fusions were found in the soluble fraction of *E. coli* extracts and were purified according to manufacturer's guidelines (Novagen). A polyclonal antibody against full-length human AIPL1 was raised in rabbit using purified His-tagged native AIPL1 protein as the immunogen. AIPL1 antibody was affinity-purified by using a column made of GST-AIPL1 protein crosslinked to *N*-hydroxysuccinimide-activated Sepharose 4B beads (Amersham Biosciences).

Tissue Preparation. Retinas were dissected from 4-week-old C57/Bl6 mice and fixed for 1 h in 4% paraformaldehyde. Retinas were then cryoprotected in 30% sucrose/PBS overnight at 4°C, and then in a 1:1 solution of 30% sucrose/PBS and optimal cutting temperature compound (OCT) (Sakura Finetek, Torrance, CA) for 3 h. Retinas were quick-frozen in OCT, sectioned at 12 μm, and mounted on Superfrost Plus slides (VWR Scientific).

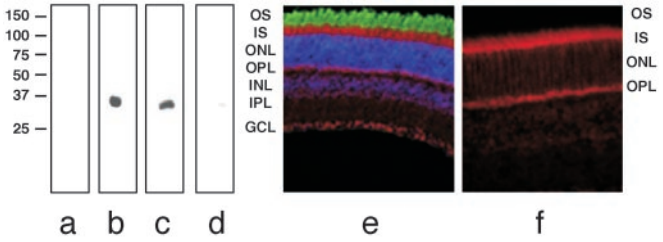

Fig. 1. Localization of AIPL1 in photoreceptor inner segments and synaptic termini in mouse retina is shown. Mouse retinal extracts (≈100 μg) were subjected to immunoblot analysis by using anti-AIPL1 (*b*). Control experiments, using preimmune sera (*a*), anti-AIPL1 blocked with excess purified GST-AIP (*c*), and anti-AIPL1 blocked with excess purified GST-AIPL1 (*d*) are also shown. Frozen retinal sections prepared from mouse adult retina were incubated with affinity-purified anti-AIPL1 polyclonal antibody, followed by Alexa 568-conjugated anti-rabbit secondary antibody (red). Rod outer segments (OS) were stained with rhodopsin monoclonal antibody (green). Cell nuclei were stained with DAPI (blue). (*e*) The photoreceptor inner segments (IS) and synaptic termini in the outer plexiform layer were specifically labeled with anti-AIPL1 (red). (*f*) Segments shown are the same as in *e*, but are shown at higher magnification. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

Immunohistochemistry. Slides were washed in PBS and incubated overnight in primary antibody (1:500 polyclonal AIPL1 blocked overnight with purified GST-AIP and 1:1,000 rhodopsin monoclonal (4D2; gift of R. Molday, University of British Columbia, Vancouver). After extensive washing in PBS, slides were incubated in secondary antibody (Alexa anti-mouse 488 and Alexa anti-rabbit 568 1:500; Molecular Probes) for 1.5 h. All incubations were done at room temperature in 5% goat serum/0.3% Triton X-100/PBS. DAPI (4′,6-diamidino-2-phenylindole) was used to visualize nuclei and slides were coverslipped with Fluoromount-G (Southern Biotechnology Associates). Sections were viewed on an Axioplan 2 microscope (Zeiss) and photographed with a SPOT 2.3.1 digital camera (Diagnostic Instruments, Sterling Heights, MI).

Western Blotting. Proteins were separated on SDS/PAGE and then transferred to Immobilon P membrane (Millipore). Membranes were blocked with 5% nonfat dry milk in TBST (10 mM Tris·HCl, pH 8.0/150 mM NaCl/0.05% Tween 20) and incubated with primary antibodies for 1 h at room temperature. After extensive washing with TBST, the membranes were incubated with horseradish peroxidase (HRP)-coupled secondary antibodies for 1 h at room temperature. After several washes with TBST, membranes were developed by using chemiluminescence (Amersham Biosciences). Yeast GAL4 DNA-binding domain fusions were detected by using HRP-linked Anti-GAL4 (RK5C1) (Santa Cruz Biotechnology), and His-tagged proteins were detected by HRP-linked INDIA His probe (Pierce). HA-tagged proteins were detected by using HRP-linked high-affinity (3F10) HA antibody (Roche Molecular Biochemicals).

Results

AIPL1 Is Present in Inner Segments and Synaptic Terminals. To determine the intracellular localization of AIPL1 protein within mouse adult retina, we performed immunofluorescence by using affinity-purified AIPL1 antibody. In agreement with the predicted size of the AIPL1 protein, the antibody specifically recognized a protein of ≈36 kDa on Western blot analysis of mouse retinal extracts (Fig. 1 *a–d*). To avoid any crossreactivity with the related protein, AIP, the antibody was preadsorbed with purified GST-AIP fusion protein. We observe a strong immunostaining in inner segments of photoreceptors and synaptic terminals (Fig. 1 *e* and *f*). Fainter immunolabeling was detected

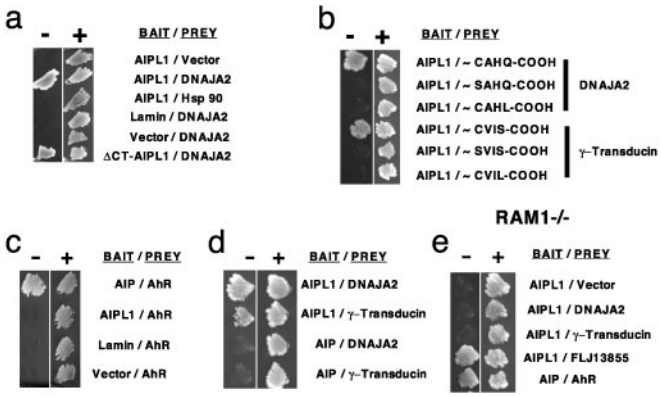

Fig. 2. Interaction of AIPL1 and AIPL1 mutants with farnesylated proteins *in vivo*. Yeast (PJ69-4) containing the indicated bait fused to the GAL4-activation domain and preys fused to GAL4-DNA-binding domain were tested for their ability to promote growth of yeast on synthetic media lacking leucine, tryptophan, histidine, and adenine (LTHA) (−). As a control, the same combination was grown in medium lacking leucine and tryptophan, but containing histidine and adenine (+). (*a*) Interaction between AIPL1 and DNAJA2 is specific. Yeast containing bait AIPL1 or AIPL1 lacking the C-terminal 56 amino acids (ΔCT-AIPL1) and prey DNAJA2 grow on LTHA media (−). (*b*) AIPL1 specifically interacts with proteins that contain a conserved C-terminal farnesylation signal. AIPL1 does not interact with farnesylation-defective mutant forms of DNAJA2 (–SAHQ) or γ-transducin (–SVIS). AIPL1 does not interact with geranylgeranylated forms of DNAJA2 (–CAHL) or γ-transducin (–CVIL) mutants. (*c*) AIPL1 does not interact with AhR. Yeast containing AIPL1 or AIP as bait and AhR as prey were tested for the ability to grow on LTHA media (−). (*d*) AIP does not interact with farnesylated proteins. (*e*) Farnesylation of target proteins is necessary for interaction with AIPL1 in yeast. Yeast (PJ69-4) lacking the farnesyltransferase gene (ΔRam1) were tested for their ability to support the interaction of AIPL1 with DNAJA2 and γ-transducin. As a control, interaction of AIPL1 with FLJ13855 and AIP with AhR was tested.

in outer and inner nuclei. No immunostaining was observed in control sections incubated with preimmune sera (data not shown). The staining was AIPL1-specific because preadsorption with GST-AIPL1 fusion protein completely abolished the labeling with the affinity-purified anti-AIPL1 antibody (data not shown). Collectively, these results show that bulk of AIPL1 protein is present in inner segments and synapses of photoreceptors.

AIPL1 Interacts Specifically with Farnesylated Proteins in Yeast. Although the essential biochemical role of AIPL1 in retina is unknown, the presence of three TPR domains suggests that AIPL1 may be part of a protein complex in retina. To identify potential binding partners of AIPL1, we used amino acids 1–329 of human AIPL1 as bait to screen a bovine retinal cDNA library (11) by yeast two-hybrid analysis. Of $\approx 2.5 \times 10^6$ colonies screened, 33 grew on selective medium and showed β-galactosidase activity in a colony filter lift assay. These 33 clones represent eight different proteins (see *Supporting Methods*, which is published as supporting information on the PNAS web site, www.pnas.org). Both the full-length AIPL1 and AIPL1 lacking the last 46 amino acids interact with all positive interactors. One of the interactors is DNAJA2 (GenBank accession no. AY324827), a chaperone belonging to the Hsp40 family (12, 13) (Fig. 2*a*). Similar to bovine DNAJA2 isolated from the two-hybrid screen, full-length human DNAJA2 also interacted with AIPL1 (data not shown). Hsp 90, which is known to interact with AIP, does not interact with AIPL1 (Fig. 2*a*). These results show that the interaction of AIPL1 with its protein targets is specific.

Six of the eight AIPL1 targets identified by the two-hybrid screen encode proteins with a C-terminal prenylation motif (see Table 1, which is published as supporting information on the PNAS web site). Prenylation is a posttranslational addition of either a C15-farnesyl or a C20-geranylgeranyl isoprenyl group to a cysteinyl residue at the C terminus of a protein. This protein modification is highly conserved and occurs in all eukaryotes, including yeast (14, 15). The consensus motif for prenylation is CAAX, where C is a highly conserved cysteine, and A is usually, but not necessarily, an aliphatic amino acid. The identity of the last amino acid (X) determines the type of isoprenoid to be attached to the protein. When X is serine, methionine, or glutamine, then the protein is farnesylated. When X is leucine or phenylalanine, the protein is geranylgeranylated (14). Each of the predicted prenylated proteins isolated in our yeast two-hybrid screen has a CAAX box that favors farnesylation. For example, DNAJA2 (12, 13) and γ-transducin (16, 17), which are known to be farnesylated *in vivo*, interact with AIPL1 (Fig. 2*b*). This result suggests that AIPL1 specifically recognizes farnesylated C termini.

To confirm that farnesylation is required for DNAJA2 and γ-transducin to interact with AIPL1, the highly conserved cysteine residue was mutated to serine in both proteins. Substitution of serine at this site in CAAX boxes abolishes prenylation (14). Neither of the mutants interacts with AIPL1 in a yeast two-hybrid assay (Fig. 2*b*), confirming that prenylation is required for AIPL1 to interact with DNAJA2 and γ-transducin. In a separate experiment, the last amino acid of the CAAX prenylation motifs of DNAJA2 and γ-transducin were changed to leucine to promote geranylgeranylation, rather than farnesylation (18). These mutations also abolished the yeast two-hybrid interactions (Fig. 2*b*), confirming the specificity of the interaction between AIPL1 and farnesylated polypeptides. Immunoblotting with anti-HA antibodies confirmed that all DNAJA2 or γ-transducin mutants were expressed in equal amounts (data not shown).

To confirm the idea that the ability to interact with farnesylated peptides is unique to AIPL1, the related protein, AIP, was used as bait in the yeast two-hybrid assay. Although the interaction of AIP with AhR, was confirmed in this assay (Fig. 2*c*), AIP did not interact with any of the farnesylated proteins that interact with AIPL1 (Fig. 2*d* and data not shown). Furthermore, AIPL1 did not interact with AhR (Fig. 2*c*). These results further demonstrated that AIPL1 specifically interacts with farnesylated proteins. Even though AIPL1 and AIP are similar, they interact with different protein partners, and probably play very different functional roles.

Yeast Lacking Farnesyl Transferase Do Not Support the Interaction Between AIPL1 and Farnesylated Proteins. Farnesylation in *Saccharomyces cerevisiae* is catalyzed by farnesyltransferase (FTase), a heterodimeric enzyme composed of α- and β-subunits. The α-subunit is shared between FTase and geranylgeranyl transferase (GGTase I) and is essential for the growth of yeast. Previous studies (10) have shown that deletion of the yeast FTase β-subunit blocks farnesylation and results in temperature-sensitive growth. To further support our observation that AIPL1 interacts with farnesylated proteins, we tested the two-hybrid interaction in a yeast host that lacks RAM1, the gene that encodes for the FTase β-subunit. In the absence of farnesyltransferase, both DNAJA2 and γ-transducin did not interact with AIPL1 (Fig. 2*e*). The absence of interaction was not due to low expression of the target protein, because an immunoblot analysis showed normal levels of DNAJA2 or γ-transducin GAL4 fusion proteins (data not shown). As a control, we showed that the yeast strain lacking FTase does support the interaction between AIPL1 and FLJ13855 (Fig. 2*e*), which is a nonfarnesylated target protein identified in our yeast two-hybrid screen (see Table 1), and the interaction between AIP and AhR (Fig. 2*e*). These results indicate that the lack of interaction between AIPL1 and DNAJA2 or γ-transducin is specifically related to farnesylation.

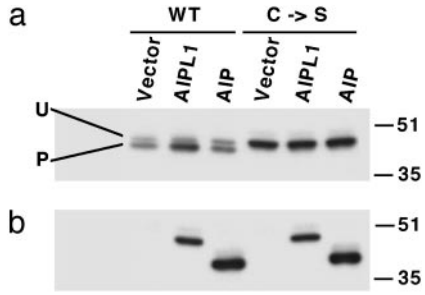

Fig. 3. AIPL1 enhances farnesylation of proteins *in vivo*. Western blot analysis of total cell extracts from HEK-293 cells are shown. The cells express His-tagged AIPL1, AIP, or vector control, along with HA-tagged WT DNAJA2, or the farnesylation-defective mutant DNAJA2 C → S. Equal amounts of protein (10 μg) from each extract were run on SDS/PAGE and were blotted. (*a*) Processing of DNAJA2. To detect the HA-tagged DNAJA2, the blot was probed with anti-HA crosslinked to HRP. The two forms of DNAJA2 corresponding to unprocessed (U) and processed (P) precursors were observed. (*b*) Coexpressed AIPL1 or AIP was detected with INDIA-probe HRP. Protein molecular mass markers (in kilodaltons) are indicated on the right.

AIPL1 Aids the Processing of Farnesylated Proteins *in Vivo*. The findings from our yeast two-hybrid analysis suggested that AIPL1 specifically recognizes farnesylated C termini. We next investigated the possibility that AIPL1 helps in the processing of farnesylated proteins. In eukaryotes, FTase modifies cytosolic proteins containing the CAAX box by adding an isoprenyl group. Isoprenylated proteins are further processed at the endoplasmic reticulum (ER), where they are proteolysed by membrane proteases to remove the last three amino acids, and are finally carboxymethylated at the newly exposed cysteine residues. These modifications usually result in modified proteins migrating faster on SDS/polyacrylamide gels. Previous studies show that this mobility shift readily occurs in DNAJA2 (12, 13, 19). Therefore, we used the faster mobility as an assay to evaluate the role of AIPL1 in farnesylation of DNAJA2. Both AIPL1 and HA-tagged full-length human DNAJA2 were expressed in HEK-293 cells. These cells do not express endogenous AIPL1 (data not shown). When coexpressed with DNAJA2, AIPL1 enhances farnesylation of DNAJA2 by 2.5 fold ($n = 3$; Fig. 3*a*, lane 2). AIP did not promote the farnesylation of DNAJA2 (Fig. 3*a*, lane 3). AIPL1 does not alter the mobility of mutant DNAJA2 (C-S) that cannot be farnesylated (Fig. 3*a*, lanes 4–6). To exclude the possibility that enhanced farnesylation is caused by an inefficient CAAX box in DNAJA2, we replaced the native DNAJA2 CAAX box (CAHQ) with a well characterized H-Ras protein CAAX box (CVIM). Similar to the native DNAJA2, DNAJA2 containing the Ras CAAX box was also processed more efficiently in the presence of AIPL1 (data not shown). These results suggest that AIPL1 plays a role in enhancing the protein farnesylation process.

A Common Mutation, W278X, Causes Complete Loss of AIPL1. Three classes of homozygous mutations in the *AIPL1* gene have been linked to retinal dysfunction in humans (20). These classes include missense mutations (M79T and C89R) in the N-terminal immunophilin domain (denoted as class 1 mutations in this study), missense mutations (A197P and C239R) in the TPR domains, or nonsense mutations (W88X, Q163X, and W278X) that lack one or more TPR domains (class 2 mutations), and deletion mutations (Δ336–337 and Δ351–354) in the C-terminal region that is unique to primates and humans (20) (class 3 mutations). Class 3 mutations have been linked to autosomal dominant juvenile retinitis pigmentosa and cone-rod dystrophy in a small group of families (20). Class 1 and class 2 mutations

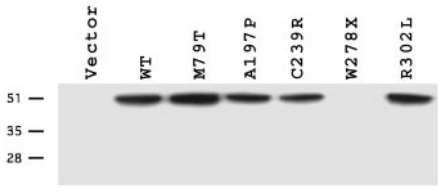

Fig. 4. A nonsense mutation (W278X) in AIPL1, the most common mutation in AIPL1 linked to LCA, causes complete absence of protein. Western blot analysis of soluble extracts from COS-7 cells overexpressing WT AIPL1 and various AIPL1 mutants associated with LCA is shown. Equal amounts of protein (10 μg) from each indicated extract were run on SDS/PAGE and were blotted onto Immobilon P membranes. The blot was probed with an affinity-purified rabbit anti-AIPL1 antibody at 1:5,000 dilution. Protein molecular mass markers (in kilodaltons) are indicated on the left.

have been linked to LCA and they were further characterized in this study.

To examine the effects of the LCA-associated mutations on the stability of AIPL1, we expressed WT AIPL1 and the LCA mutants in HEK-293 or COS-7 cells. These cells do not express AIPL1 endogenously (Fig. 4). Immunolocalization in COS-7 cells showed that the expressed WT AIPL1 is mainly in the cytosol and perinuclear region (data not shown). The mutant AIPL1 proteins localized to the same regions of the cell (data not shown). Whereas four of five mutants tested were as stable as WT AIPL1, the class 2 mutation, W278X, caused complete loss of protein (Fig. 4). W278X is the mutation in AIPL1 most frequently associated with LCA (22). The same result was obtained when blots were probed with the antibody against the N-terminal His tag present in each of the expressed AIPL1 proteins (data not shown). Even when 20 times more W278X extract than WT extract was loaded, very little immunoreactive protein was detected (data not shown). These results suggest that LCA patients with homozygous W278X mutations suffer from complete loss of AIPL1 protein.

Mutations in AIPL1 Linked to LCA Prevent AIPL1 from Interacting with Farnesylated Proteins. In the yeast two-hybrid assay, all class 1 and class 2 AIPL1 mutations completely abolished interaction with the farnesylated proteins DNAJA2 and γ-transducin (Fig. 5 *a*

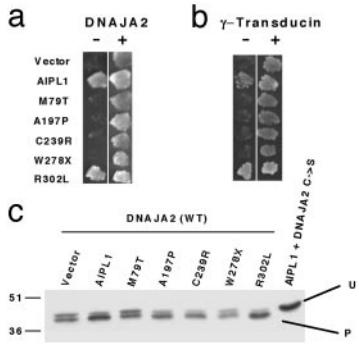

Fig. 5. AIPL1 mutants that are associated with LCA do not interact with farnesylated proteins. Yeast containing native AIPL1 or AIPL1 mutants were tested for the ability to interact with farnesylated partners, DNAJA2 (*a*) or γ-transducin (*b*), as assayed by their growth on −LTHA media. All AIPL1 mutants, except R302L, do not interact with DNAJA2 or γ-transducin. (*c*) AIPL1 mutants are defective in enhancing the farnesylation of DNAJA2 protein. Shown is Western blot analysis of extracts from HEK-293 cells coexpressing His-tagged native AIPL1 or AIPL1 mutants linked to LCA, along with HA-tagged WT DNAJA2. As a control, farnesylation-defective mutant DNAJA2 C → S was coexpressed with native AIPL1 (lane 8). HA-tagged DNAJA2 was detected with an anti-HA crosslinked to HRP.

and *b*). The only substitution that did not affect the interaction of AIPL1 with farnesylated proteins was R302L, which lies just outside the TPR 3 domain. Similar results were observed with other farnesylated proteins identified in the yeast two-hybrid screen (data not shown). A recent report of an independent yeast two-hybrid screen with AIPL1 identified NUB1 (23), a protein implicated in regulation of proteolysis. We have confirmed that the C terminus of human NUB1 interacts with AIPL1 in our two-hybrid system (data not shown). However, both the A197P and C239R forms of AIPL1, which are unable to interact with farnesylated proteins, still interact with NUB1 (23). This finding confirms that these mutants are expressed and folded, but it also suggests that the biochemical defect caused by these mutations *in vivo* is likely to be loss of farnesyl interaction, rather than loss of NUB1 interaction.

To test the ability of class 1 and class 2 AIPL1 mutants to enhance the farnesylation of DNAJA2 *in vivo*, we coexpressed different AIPL1 mutants with HA-tagged DNAJA2. Except for W278X, all other AIPL1 mutants expressed well in HEK cells and were stable (Fig. 4). All mutants, except R302L, were defective at enhancing the processing of DNAJA2 (Fig. 5*c*). M79T and W278X were the two most severe mutations in AIPL1. They caused the most accumulation of DNAJA2-unprocessed precursors (U) and DNAJA2 processing in the presence of these mutants was similar to processing in the absence of AIPL1 (Vector control). A197P and C239R showed an intermediate defect. In agreement with the result of the yeast two-hybrid assay, R302L did not show any defect in DNAJA2 processing. Although the R302L mutation in AIPL1 was reported to be linked to LCA (20), mouse and rat AIPL1 genes normally encode leucine at position 302. These observations suggest that R302L may not be a disease-causing variant.

Limited Proteolytic Digestion of AIPL1 and Mutants Associated with LCA. To understand the structural basis for the loss of interaction between the mutant forms of AIPL1 and farnesylated proteins, we modeled the three AIPL1 TPR domains (TPR1, TPR2, and TPR3) by using the known structures of the TPR domains of FKBP51 (see Fig. 7, which is published as supporting information on the PNAS web site) (24). The model of AIPL1 amino acid residues 176–298 predicts that all three TPR motifs in AIPL1 are contiguous. Similar to TPR domains in other proteins, each TPR domain in AIPL1 is made up of a pair of antiparallel helices. The class 2 mutation sites, A197 (helix B of TPR1) and C239 (helix A of TPR2) occur in highly conserved position 20 of helix B of TPR-I, and position 10 of helix A of TPR 2, respectively (see Fig. 7), and these changes are likely to alter helix packing. The nonsense mutation at W278X eliminates the last helix of TPR 3. This occurrence is likely to destabilize TPR packing, which would account for the observed complete degradation of the protein.

To directly assess the effects of LCA mutations on the secondary or tertiary structure of AIPL1, native AIPL1 and the LCA mutant forms of AIPL1 were expressed in HEK-293 cells, and were subjected to limited proteolysis by trypsin. Both forms of AIPL1 that bind to farnesylated proteins (full-length and R302L) are stable to the digestion conditions for up to 10 min. A 32-kDa fragment then forms, which is stable for 60–120 min (Figs. 6 and 7). All of the mutants that do not interact with farnesylated proteins are stable to the digestion conditions for only 6 min and the 32-kDa fragment produced from these mutants is degraded rapidly (Figs. 6 and 7). Interpretation of the C239R mutant digestion seemed consistent with this possibility, but it is complicated by the creation of an additional trypsin site. Overall, these partial proteolysis studies show that the mutants that fail to interact with farnesylated proteins have different structures than do the native or R302L AIPL1 proteins.

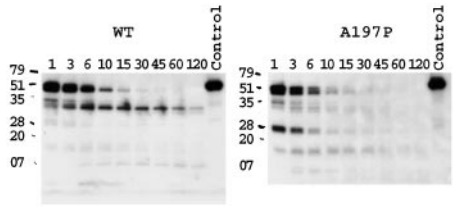

Fig. 6. The folding of the AIPL1 variant associated with LCA is analyzed by limited proteolytic digestion of expressed AIPL1 protein. Soluble extracts from COS-7 cells overexpressing WT AIPL1 and A197P mutant associated with LCA were subjected to trypsin digestion (Calbiochem; 60 ng/μl) at 37°C. At indicated times (min), the trypsin digestion was stopped by the addition of excess trypsin inhibitor (Calbiochem). Samples were run on SDS/PAGE, blotted, and probed with affinity-purified rabbit anti-AIPL1 antibody. The control lane contains extract incubated for 120 min at 37°C without trypsin. Protein molecular mass markers (in kilodaltons) are indicated on the left.

Discussion

This study demonstrates an interaction between the retinal protein, AIPL1, and farnesylated proteins. It also shows that AIPL1 enhances protein farnesylation. In agreement with the localization of proteins involved in farnesylation, AIPL1 is present in inner segments of photoreceptors. Mutant forms of AIPL1 that are linked to LCA are defective in enhancing farnesylation. Another important finding of this study is that the most common AIPL1 mutation in patients with LCA (W278X) causes complete loss of AIPL1 protein.

Prenylation is a three-step process. The first step occurs in cytosol, where FTase catalyzes the covalent attachment of a farnesyl group to the cysteine residue of a CAAX box-containing protein. In step 2, farnesylated proteins are targeted to the ER, where they are proteolysed by membrane-bound proteases to remove the last three amino acid residues. In step 3, the proteins are carboxymethylated in the ER at the newly exposed farnesyl cysteine groups. Once the modifications are complete, farnesylated proteins are further transported to their target membranes (25). Our findings suggest that AIPL1 likely plays a role in at least one of these steps to enhance the farnesylation process.

We suggest that AIPL1 may interact with proteins containing farnesylation signal (CAAX), such as DNAJA2 or γ-transducin in presence of the substrate, farnesyl pyrophosphate, and FTase. This interaction would account for the requirement that a CAAX box protein and FTase needs to be present for the interaction to occur. In this way, AIPL1 could enhance farnesylation, and then could chaperone prenylated proteins to their target membranes. This model is very similar to the suggested role of CHM protein REP-1 in assisting geranylgeranylation, and targeting proteins to membranes. REP-1 has been proposed to bind with high affinity to a complex of geranylgeranyl pyrophosphate and RabGGTase to recruit Rab protein and mediate its prenylation (26). After prenylation, Rab protein is targeted to membranes by REP-1 (27).

Previous studies (25) suggested that even though the farnesylation of proteins is very efficient, the further processing of prenylated proteins is relatively inefficient. Pulse–chase analyses showed that only 10% of farnesylated Ras in the cytosol was further processed and targeted to membranes (25). The majority of the cytosolic pool of Ras, which is farnesylated, is degraded without further processing (25). Because AIPL1 enhances the efficiency of the farnesylation process, it is possible that it helps target farnesylated proteins to the ER for further processing. AIPL1 may also protect farnesylated proteins from proteolysis in the cytosol. Other studies (28) have shown that farnesylation of the γ-subunit of G proteins is required for targeting of the heterotrimeric complex, G$\alpha\beta\gamma$, to plasma membranes. Our

studies show AIPL1 and γ-transducin interact, suggesting that AIPL1 could play a role in transport of transducin complexes.

AIPL1 mutations linked to LCA are defective at enhancing the prenylation of proteins. Previous studies (7, 8) have suggested that prenylation is essential in retina. Retinal proteins, such as γ-transducin, rhodopsin kinase, and α-phosphodiesterase along with several small GTPases that belong to the Ras superfamily, are known to be prenylated (16, 29, 30). Defects in geranylgeranylation due to mutations in REP-1 protein cause CHM, a severe form of human retinal degeneration, resulting in loss of vision (7). Recent studies (31) have suggested that the rod photoreceptor cell is the site of the primary defect in CHM patients, which is consistent with the localization of REP-1 in the rod cytoplasm. In retinal degeneration caused by mutations in AIPL1, the primary defect also appears to be in rods (S. Dharmaraj and I. H. Maumenee, personal communication), which is consistent with the localization of AIPL1 in rods (2). In addition, inhibition of farnesylation or methylation of proteins in adult rat retina causes complete degeneration of photoreceptor inner and outer segments, and also morphological changes in retina (8). These results suggest that prenylation is required for maintaining the retinal cytoarchitecture and photoreceptor structure (8). The prenylated proteins that are responsible for promoting and maintaining the adult retinal structure are not known. Altogether, these findings demonstrate that prenylation is essential for rod photoreceptor function.

Based on the essential role of farnesylation in vision, and the ability of AIPL1 to enhance the farnesylation, we propose that mutations in AIPL1 disrupt the farnesylation of multiple proteins to cause the retinal phenotype in LCA patients. This result is similar to that of CHM patients, where the generalized effect of REP-1 mutations on prenylation of multiple Rabs is thought to account for the retinal phenotype (7). TPR proteins generally interact with multiple proteins to form functional complexes. For example, AIP, a close homologue of AIPL1, interacts with both hsp90 and AhR (21). Similarly, AIPL1 may interact with proteins such as NUB1 (23) or FLJ13855 (see Table 1), in addition to its interaction that promote the farnesylation of proteins. Further characterization of these interactions, and the role of AIPL1 in farnesylation by using *in vitro* and *in vivo* methods, are needed to identify the step at which AIPL1 enhances the farnesylation process.

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