Retinyl Ester Homeostasis in the Adipose Differentiation-related Protein-deficient Retina

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The retinal pigmented epithelium (RPE) plays an essential role in vision, including storing and converting retinyl esters of the visual chromophore, 11-cis-retinal. Retinyl ester storage structures (RESTs), specialized lipid droplets within the RPE, take up retinyl esters synthesized in the endoplasmic reticulum. Here we report studies of mice lacking exons 2 and 3 of the gene encoding adipose differentiation-related protein (Adfp), a structural component of RESTs. We found that dark adaptation was slower in Adfp2−3/2−3 than in Adfp+/+ mice and that Adfp2−3/2−3 mice had consistently delayed clearances of all-trans-retinal and all-trans-retinol from rod photoreceptor cells. Two-photon microscopy revealed aberrant trafficking of all-trans-retinyl esters in the RPE of Adfp2−3/2−3 mice, a problem caused by abnormal maintenance of RESTs in the dark-adapted state. Retinyl ester accumulation was also reduced in Adfp2−3/2−3 as compared with Adfp+/+ mice. These observations suggest that Adfp plays a unique role in vision by maintaining proper storage and trafficking of retinoids within the eye.

Lipid droplets are intracellular organelles specialized in storing lipid esters, such as triacylglycerol (TAG),1 cholesteryl esters, and retinyl esters (1, 2). Proteins associated with lipid droplets are potentially involved in their metabolism, as well as in membrane trafficking and signaling (3). The best characterized proteins of these droplets are the PAT domain proteins, named after perilipin, adipose differentiation-related protein (Adfp), and tail-interacting protein of 47 kDa (Tip47). PAT domain proteins serve as structural constituents by coating the surface of lipid droplets (4). Although perilipin expression is more limited to adipose and steriodogenic cells (5, 6), Adfp is expressed throughout the body, mainly in nonadipogenic tissues such as liver, lung, muscle, testes, and eye (7–9). Overexpression of Adfp can promote the accumulation of lipid in cultured cells (10, 11). Consistent with the proposed lipid storage function of Adfp in nonadipogenic tissues, Adfp-deficient mice have a reduced TAG content in the liver but fail to exhibit a pathological phenotype under normal conditions (12). Currently, the in vivo functions of nonadipogenic lipid droplets are poorly understood, and those of Adfp remain unknown. A recent cell culture study suggests that Adfp plays a role in modulating the turnover rate of lipid esters (13), but the physiological consequences of this effect require further investigation.

In the eye, the retinal pigmented epithelium (RPE) serves as the major storage site for retinyl esters, precursors of the visual chromophore 11-cis-retinal (14). After transfer to the RPE from the circulation or photoreceptors, all-trans-retinol is converted into all-trans-retinyl esters by lecithin-retinol acyltransferase (Lrat) (15, 16). These esters are key substrates for Rpe65 that catalyzes their hydrolysis and isomerization to the 11-cis configuration (reviewed in Refs. 15, 16). Thus, these esters are essential for generation of the visual chromophore, 11-cis-retinal, that links to opsin-forming rhodopsin and cone visual pigment (14). Retinyl esters are synthesized in the endoplasmic reticulum (ER) of the RPE where Lrat is localized (17) and then are transported to and temporarily stored in retinyl ester storage structures (RESTs or retinosomes), a type of lipid droplet unique to the RPE. Mechanisms by which RESTs located proximal to the plasma membrane take up newly synthesized retinyl esters have yet to be characterized (9). For the RPE to maintain proper retinoid homeostasis in the visual cycle, trafficking and storage of all-trans-retinyl esters must be well balanced to support the rapid turnover of retinyl esters needed for continuous replenishment of visual chromophores. In previous studies we gained some insight into how the quantity and quality of retinyl esters in the RESTs are regulated by enzymes involved in the visual cycle. RESTs overaccumulate in Rpe65−/− mice but are absent in Lrat−/− mice (9). Moreover, Adfp specifically localizes to RESTs in the RPE (9) where its expression level correlates with the amount of retinyl esters (9). These observations suggest a specific role for Adfp in retinoid storage and metabolism within the eye. However, they do not explain the mechanisms underlying retinyl ester uptake by the RESTs.
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Insight into the roles of REST proteins should increase understanding of overall REST function as well. Here we investigated whether Adfp contributes to the storage and transport of visual cycle retinoids. Our observations not only reveal an essential role for Adfp in the transport and storage of visual retinoids, they also suggest that Adfp promotes the accumulation of retinyl esters by serving as a transient storage site. This Adfp-mediated binding is unusual in that it does not limit the turnover of all-trans-retinyl esters and regeneration of 11-cis-retinal, a property required for rapid visual pigment regeneration. Our studies support the hypothesis that Adfp is a novel retinyl ester-binding protein functioning in vitamin A homeostasis of the retina.

EXPERIMENTAL PROCEDURES

Animals—Mice were maintained under complete darkness or in a 12-h light/12-h dark environment. Animal procedures and experiments were approved by the Case Western Reserve University Animal Care Committees and conformed to both the recommendations of the American Veterinary Medical Association Panel on Euthanasia and the Association of Research for Vision and Ophthalmology.

Targeting Constructs and Transgenesis—Adfp$^{Δ2-3/Δ2-3}$ mice were generated by standard procedures (Ingenious Targeting, Inc., Rochester, NY). An ∼11.5-kb region used to construct the targeting vector was cloned from a positively identified BAC clone. The region was designed such that the short homology arm extended ∼1.5 kb 3′ to exon 3. The long homology arm started at the 5′ side of exon 2 and was 7.6 kb long. The neo cassette replaced 2.1 kb of the gene, including exons 2 and 3 that contained the ATG start codon in exon 2. The targeting vector was confirmed by restriction analysis after each modification step and by sequencing with the primers N1 (5′-TGCGAGGCCAGAGGCATTTGTGTAGC-3′) and N7 (5′-ATGTGTCAGTTCTAGGCTAC-3′), designed to read from the selection cassette into the homology arm and the short homology arm, respectively. T7 (5′-ATTTAGGGTTCGACTATAGAATCT-3′) and P6 (5′-ATTATGCTGAGTGTACATGATATCCCTCT-3′) primers were annealed to the vector sequence and read into the 5′- and 3′-ends of the BAC subclone. Ten μg of the targeting vector was linearized by NotI and transfected by electroporation into 129SvEviTIL embryonic stem cells. After selection in medium containing G418 and neomycin, surviving colonies were expanded, and PCR analysis was performed to identify clones that had undergone homologous recombination. Recombinant clones were identified by the 2.0-kb amplified PCR band using primer pairs A1 (5′-CATTAGGGAAGGATTTGCAAGTGTGAGG-3′) and N1 (5′-TGCGAGGCCAGAGGCACCTTGTGTAGC-3′), A1(5′-CATTAGGGAAGGATTTGCAAGTGTGAGG-3′)/AT2(5′-TTGCCTTTGTTACAAATCGAGATC-3′), and AT11(5′-TGAGAGCTTCTAGTGTCCCTC-3′)/N1(5′-TGCGAGGCCAGAGGCACCTTGTGTAGC-3′) were used as internal controls with expected size fragments of 1.5 and 1.8 kb, respectively. Correctly targeted embryonic stem cell lines were microinjected into C57BL/6 mouse blastocysts. A total of six male chimeras (90–100% agouti) and seven female chimeras (100% agouti) were produced from these injections. Chimeric mice were then mated to effect germ line transmission.

Genotyping—Adfp$^{Δ2-3/Δ2-3}$ mice were back-crossed four times with mice having either BALB/c or C57BL/6 backgrounds. Adfp$^{Δ2-3/Δ2-3}$ mice with a BALB/c background were primarily used for this work. Adfp$^{Δ2-3/Δ2-3}$ mice were genotyped by use of tail-snip genomic DNA, and the genotyping primers were as follows: Intron1F2, 5′-GTCAGCCTGGGCTTTAGACAAA-3′; Exon3R, 5′-ACACACGATCTCAGATACGGG-3′, and NeoR, 5′-GGATCTTGATCTCATCACCTT-3′. PCR conditions were 94 °C for 2 min and then 40 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min. After these reactions, samples were kept at 4 °C. Intron1F2 + Exon3R amplified a product of ∼700 bp from the Adfp+ allele, whereas intron 1F2 + NeoR amplified a product of ∼1.5 kb from the Adfp$^{Δ2-3}$ allele.

Adfp$^{Δ2-3/Δ2-3}$Rpe65−/− Double Mutant Mice—Adfp$^{Δ2-3/Δ2-3}$ Rpe65−/− double knock-out mice were established by cross-breeding Adfp$^{Δ2-3/Δ2-3}$ mice with Rpe65−/− mice (18). For two-photon imaging, we used albino lines of Rpe65−/− and Adfp$^{Δ2-3/Δ2-3}$Rpe65−/− mice obtained by cross-breeding them with tyrosinase mutant mice (Tyro−/−).

Immunoblotting—Mammary gland tissue from Adfp$^{+/+}$ and Adfp$^{Δ2-3/Δ2-3}$ female mice with 1-day-old pups was collected and homogenized in 136 mM NaCl, 11.4 mM sodium phosphate, pH 7.4, containing protease inhibitor with a Dounce homogenizer. Protein concentrations were determined by the Bradford assay. Each sample (15 μg of protein) was separated by a 10% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and probed with rabbit anti-Adfp C-terminal antibody (a kind gift from Dr. McManaman) followed by horseradish peroxidase-conjugated secondary antibody. The signal was visualized by applying SuperSignal West Pico chemiluminescent substrate (Pierce) and subsequent photography. Heart and white adipose tissue were collected and homogenized as above, separated by 10% SDS-polyacrylamide gel, and transferred. Rabbit anti-Tip47 N-terminal antibody (Novus Biologicals, Littleton, CO) was used to probe Tip47 followed by alkaline phosphatase-conjugated secondary antibody and developed by incubating with Western Blue stabilized substrate (Promega Corp., Madison, WI). β-Tubulin was detected by E7 antibody (Developmental Studies Hybridoma Bank at Iowa University) as internal control.

Genomic Southern Blotting—Ten μg of genomic DNA was digested with EcoRI overnight, run on a 0.8% agarose gel, and subsequently transferred to a Hybond N+ nitrocellulose membrane (GE Healthcare). The Adfp probe was generated by using ADS-F2 + mAdfp-R10 primers, i.e., ADS-F2 5′-CCGAACTTCCAGTTTTGAAA-3′ and mAdfp-R10 5′-GCCTGGAACCTACCAGCCTACCC-3′. Hybridization was accomplished at 62 °C for 2 h.

RT-PCR—Mouse total RNA was isolated from TRlZol (Invitrogen) according to the manufacturer’s protocol. RNA concentrations were determined by performing absorption at 260 nm, and 260 nm/280 nm ratios were used for quality control. The same amounts of total RNA were employed for first strand cDNA synthesis by using the cDNA synthesis kit from Invitrogen. GAPDH was amplified as an internal control. Primers for
Adfp were mAdfp-F1 (5'-GACATGACATGCTGACAGAGCAG-
CAGTAGTGGA-3') and mAdfp-R1 (5'-GCCGAACGTCTC-
GAGCTTTGACCTCAGACT-3'). Primers for GAPDH were
5GAPDH2 (5'-CCATCACATCTTCCAGGAG-3') and
3GAPDH2 (5'-CATCCACATCTTCGGTGTT-3').

Histology—Eycups for light microscopy were fixed in 2%
glutaraldehyde, 2% paformaldehyde for 18 h, infiltrated
with 20% sucrose in 0.1 M sodium phosphate, pH 7.4, and then
embedded in 33% OCT compound (Miles) diluted with 20%
sucrose in 0.1 M sodium phosphate, pH 7.4. Thin sections were
cut at 5 μm.

Electron Microscopy—Mouse eyeballs were fixed with triple
aldehyde/DMSO fixative for 2 h at room temperature. Speci-
mens were thoroughly rinsed in 0.1 M phosphate buffer, pH 7.4,
and then postfixed for 2 h in an unbuffered 1:1 mixture of 2%
omium tetroxide and 3% potassium ferricyanide. After rinsing
with distilled water, specimens were soaked overnight in an
acidified solution of 0.25% uranyl acetate. After another rinse in
distilled water, they were dehydrated with increasing concen-
trations of ethanol, passed through propylene oxide, and
distilled water, they were dehydrated with increasing concen-
trations of ethanol, passed through propylene oxide, and embedded in an Epon mixture. Thin sections (80 nm) were cut
with an RMC MT 6000-XL ultramicrotome. Sections were
embedded in an Epon mixture. Thin sections (80 nm) were cut
with an RMC MT 6000-XL ultramicrotome. Sections were
sequentially stained with acidified methanolic uranyl acetate
and lead tartrate and examined with a JEOL 1200EX electron
microscope.

Immunocytochemistry—All procedures used have been
reported previously (1, 19). Cross-sections of mouse eyecups were incubated with anti-Adfp antibody (a generous gift from
Dr. James L. McManaman), anti-Tip47 (Santa Cruz Biotech-
nology, Inc.), anti-MLDP (a generous gift from Dr. Takashi
Osumi), anti-perilipin (Progen), or anti-S3-12 (a generous gift
from Dr. Perry E. Bickel). Signals were detected with either
Cy3-conjugated secondary antibody (Jackson Immuno-
Research, West Grove, PA,) or Alexa488-conjugated secondary
antibody (Invitrogen). Sections were analyzed with a Leica TCS
SP2 confocal microscope (Leica).

ERG—Full-field ERG recordings, flier ERGs, and single-
flash recordings after intense constant illumination were per-
formed by previously published methods (21).

Retinoid and A2E Analyses—Experimental procedures
involving extraction, derivatization, and separation of retinoids
from dissected mouse eyes have been described (21).

Two-photon Microscopy—Two-photon excitation micro-
copy was performed with a Leica TCS SP2 scanning head
(Leica) attached to a DM IRBE2 inverted microscope stand.
LCS three-dimensional software (Leica) was used for data
acquisition. Laser pulses from a mode-locked Ti:Sapphire laser
(Chameleon™-XR, Coherent, Mountain View, CA) were
focused on the sample by an HCX PL APO 40× oil immersion
objective lens (NA = 1.25, Leica). Autofluorescence from the
sample (385–545 nm) was collected by the objective lens, se-
arated from the excitation light by a dichroic mirror, filtered
through custom-made filters (HQ 465/160, from Chroma
Technology Corp., Rockingham, VT), and directed to a photo-
multiplier tube detector (R 9624, Hamamatsu). The objective
lens was kept at 37 °C by an objective lens heater (PeCon, Ger-
many). A temperature-controlled microscope stage (PeCon,
Germany) maintained the reaction at 37 °C. For ex vivo imag-
ing, mouse eyes or eyecups were located at the center of a glass-
bottomed 35-mm dish (MatTek Corp.) and perfused with oxy-
genated (95% O2, 5% CO2) artificial cerebral spinal fluid (119
mM NaCl, 2.5 mM KCl, 1.3 mM MgCl2, 2.5 mM CaCl2, 1.0 mM
NaHPO4, 11 mM glucose, 22.6 mM NaHCO3) at 37 °C. For in 
vivo observations, an anesthetized mouse was laid on the tem-
perature-controlled microscope stage, and the right side of the
eye was located on the cover glass of the microscope (44-mm
diameter, 0.16-mm thickness; Carl Zeiss MicroImaging, Inc.).
A small amount of cyanoacrylate glue was applied between the
scera and cover glass to minimize lateral movement of the eyes.
In this configuration, the retina was imaged at the periphery by
the laser penetrating through the scera while the emission flu-
orscence was collected coming back into the microscopic
objective lens. In case of a slight movement of the RPE cell layer,
the same area of the retina was traced using the unique texture
of the RPE cell layer formed by the randomly arranged single-
and dual-nucleated RPE. In most experiments, thoroughly
dark-adapted mice were exposed to intense 10-ms flashes that
bleached 60% of the visual pigment.

Image Analyses—Image software (National Institutes of
Health) was used to track the motion of retinyl ester aggregates
in the RPE. The contrast and brightness of the images were
adjusted by the Adobe Photoshop CS2 (Adobe Systems Incor-
porated, San Jose, CA). Only linear adjustments were applied to
the images.

RESULTS

Generation of Adfp-deficient Mice—We generated Adfp-de-
ficient mice to investigate the effects of its ablation on the visual
cycle. The targeting construct used for transgenesis consisted
of a 5'-long arm starting from the 5' end of exon 2, the neom-
cin selection (Neo) cassette, and a 3' short arm extending from
the 3' end of exon 3 (Fig. 1A). By homologous recombination,
the Neo cassette replaced exon 2 containing the ATG initiation
site and exon 3, and therefore mice deficient in exon 2 and 3 of
the Adfp-gene are referred as Adfp2−/−, Adfp−/−, and
Adfp−/− mice. Appropriate recombination was confirmed by a PCR-based method and
Southern blotting analysis (Fig. 1B, top and bottom). Genomic
Southern blotting analysis of Adfp−/−, Adfp−/−, and
Adfp−/− mouse genomes revealed the expected sizes of
EcoRI-digested DNA fragments probed by 32P-labeled Adfp
cDNA, i.e. a 7.5-kb fragment from Adfp−/−, a 3.5-kb
fragment from Adfp−/−, and a mixture of both fragments in
Adfp−/− mice. To confirm the lack of Adfp expres-
sion in Adfp−/− mice, we used RT-PCR, immunoblotting,
and immunofluorescence techniques to characterize Adfp gene
products in several tissues (Figs. 1 and 2). Expression of the
full-length Adfp was abolished at the RNA level in both the RPE
and white adipose tissue of
Adfp−/− mice (supple-
mental Fig. 1). To understand the expression of the Adfp gene
product in the RPE, we employed an antibody directed against
the C-terminal region of Adfp (22). We confirmed that this
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C-terminal specific antibody recognized both the full-length and the short isoforms of Adfp in the mammary glands (Fig. 1C, bottom). As reported previously (9), Adfp+/− mice expressed Adfp in RPE cells (Fig. 1, C and D) that we found to be located specifically to lipid droplet–like structures (Fig. 1D, left, red signals indicated by arrows). In the RPE of Adfp+/−/Δ2−3 mice, the C-terminal specific antibody showed a faint diffused signal in the cytoplasmic area (Fig. 1D). Faint staining also was observed in the inner retina of Adfp+/− and AdfpΔ2−3/Δ2−3 mice. These faint signals are possibly derived from N-terminally truncated Adfp suggesting that the short isoform of Adfp fails to bind to RESTs and is unstable in the RPE of AdfpΔ2−3/Δ2−3 mice.

Expression Profiles of Six PAT Domain Proteins in the RPE—The tissue distribution of Adfp was tested by RT-PCR with primer pairs that specifically amplify Adfp from its start to stop codons. In addition to the expected size product, we also amplified a short DNA fragment from testicular tissue of Adfp+/− and AdfpΔ2−3/Δ2−3 mice (Fig. 2, A and B). The full coding region of this cDNA then was cloned by 5′- and 3′-rapid amplification of cDNA ends PCR. Sequence analysis revealed that this cDNA encodes a novel PAT family protein with a predicted amino acid sequence comprising 159 residues (supplemental Fig. 2A). Because this coding region showed great similarity to that of Adfp (77.4% sequence homology for amino acids and 90% for nucleotides) (supplemental Fig. 2B), we named the protein Adfp2. Adfp2 is encoded by a gene located 27 kb downstream of the Adfp gene on the minus strand of chromosome 4C4 (Fig. 2C) suggesting that Adfp2 diverged from Adfp by recent gene duplication events. Adfp2 was expressed in the testis of AdfpΔ2−3/Δ2−3 and Adfp+/− mice (Fig. 2) implying that removal of exons 2 and 3 from the Adfp gene had no effect on Adfp2 expression (Fig. 2C).

Based on our analysis of the expressed sequence tag database (www.ncbi.nlm.nih.gov), Adfp2 also is expressed in the skin (GenBank accession number AA727312). Adfp2 seems to be absent from the human genome. With Adfp2 and the previously reported five other members (23–27), mice have six members of the PAT domain protein family in their genomes. Because Adfp2 is not expressed in the eye (Fig. 2B), we did not investigate it further.

We then studied the expression of PAT domain family members in the eye (Fig. 3). Previously it was shown that Tip47 is expressed in mouse RPE (28). Here we found that this protein is mainly localized to the cytoplasm of the RPE, both in AdfpΔ2−3/Δ2−3 and Adfp+/− mice (Fig. 3A). Immunofluorescence microscopy failed to reveal perilipin, S3-12, and MLDP in the RPE of AdfpΔ2−3/Δ2−3 and Adfp+/− mice (Fig. 3, B–D). Moreover, S3-12 and MLDP were not detectable in the RPE by immunoblotting analysis, and the perilipin mRNA level was about 0.1% that in white adipose tissue based on quantitative PCR analysis (data not shown). Thus the RPE expresses only a limited repertoire of PAT family proteins, namely Adfp and Tip47. Importantly, there was no apparent up-regulation of Tip47 in the absence of Adfp (Fig. 3E).

Delayed Dark Adaptation of AdfpΔ2−3/Δ2−3 Mice—Adfp was restricted to the RPE (Fig. 1D), where it co-localized with retinyl esters (9). We characterized the rate of dark adaptation in AdfpΔ2−3/Δ2−3 mice to determine whether Adfp is involved in the visual cycle. Two-month-old Adfp mice were exposed to intense light activation (500 cd m−2) for 3 min, a light stimulus that photoactivates about 80–90% of rhodopsin. After activation, recovery of the photoreceptor response was assessed by following a-wave amplitudes. a-wave amplitudes recovered more slowly in AdfpΔ2−3/Δ2−3 mice than in Adfp+/− mice (Fig. 4A), indicating that AdfpΔ2−3/Δ2−3 mice are deficient in photoreceptor dark adaptation. A more prominent delay in recovery was observed for these same BALB/c background mice at the age of 1 year (Fig. 4C). AdfpΔ2−3/Δ2−3 mice with a C57BL/6
background also showed a similar delay in dark adaptation (supplemental Fig. 3). It is well established that polymorphisms of the Rpe65 gene affect the rate of dark adaptation (29). To exclude a possible contribution of Rpe65 gene polymorphisms to the delayed dark adaptation noted in Adfp^{A2–3}/H9004^{A2–3} mice, we confirmed the Rpe65 genotypes of our Adfp^{H11001}/H11001 and Adfp^{A2–3}/H9004^{A2–3} mice. Regardless of Adfp genotype, all mice with BALB/c backgrounds retained the Leu residue at position 450, whereas mice with a C57BL/6 background had a Met residue at this position. Therefore, the observed difference in dark adaptation between Adfp^{+/+} and Adfp^{A2–3/A2–3} mice was not because of reported Rpe65 polymorphisms.

Despite the observed delay in dark adaptation, scotopic and photopic ERG responses were comparable in 2-month-old Adfp^{A2–3/A2–3} and Adfp^{+/+} mice (Fig. 4, top row). But in 1-year-old Adfp^{A2–3/A2–3} mice, attenuations in ERG responses were noted (Fig. 4D). These affected both a-wave and b-wave amplitudes under scotopic conditions and b-wave amplitudes under photopic conditions. Disruption of Adfp in mice did not affect the global histology of the retina up to the age of 1 year (Fig. 4E). No differences in the lengths of photoreceptor outer segments were discerned between Adfp^{+/+} and Adfp^{A2–3/A2–3} mice (Fig. 4F). The thickness of the outer nuclear layer and the number of nuclei were similar in both Adfp^{+/+} and Adfp^{A2–3/A2–3} mice (Fig. 4G), indicating the absence of photoreceptor degeneration in Adfp^{A2–3/A2–3} mice. Thus, it is unlikely that the observed delay in dark adaptation was caused by global structural changes in photoreceptor neurons or the photoreceptor-RPE interface. Neither was the delay in dark adaptation caused by a defect in phototransduction machinery because
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FIGURE 4. Responses of Adfp-deficient mouse retina. A and B, ERG responses of 8–10-week-old mice. A, recovery of a-wave amplitudes after constant light activation. Dark-adapted mice were exposed to intense illumination (500 cd m$^{-2}$) for 3 min, and recovery of a-wave amplitudes was monitored with single-flash ERG (−0.2 log cd s m$^{-2}$) every 5 min for 60 min. The recovery rate was significantly attenuated in Adfp$^{2–3/2–3}$ mice (*, p < 0.01 compared with Adfp$^{+/+}$ mice; n = 5 each). B, ERG responses recorded from 8–10-week-old mice. Adfp$^{2–3/2–3}$ and Adfp$^{+/+}$ mice under scotopic (left) and photopic (right) conditions. Both a- and b-wave amplitudes are plotted as a function of light intensity (n = 5). C and D, ERG responses of 1-year-old mice. C, recovery of a-wave amplitudes after constant light activation. Dark-adapted mice were exposed to intense illumination (500 cd m$^{-2}$) for 3 min, and recovery of a-wave amplitudes was monitored with single-flash ERG (−0.2 log cd s m$^{-2}$) every 5 min for 60 min. Further delay in dark adaptation was noted in aged animals than in young animals (see A for comparison), and the delay was more pronounced in Adfp$^{2–3/2–3}$ mice than in Adfp$^{+/+}$ mice (*, p < 0.01 versus Adfp$^{+/+}$ mice; n = 5 each). D, ERG responses recorded from Adfp$^{2–3/2–3}$ and Adfp$^{+/+}$ mice under scotopic (left) and photopic (right) conditions. The a- and b-wave amplitudes are plotted as a function of light intensity (n = 5 each). Attenuations in ERG responses were noted for both a-wave and b-wave amplitudes under scotopic conditions and b-wave amplitudes under photopic conditions. E, cross-section of eyecups from 1-year-old Adfp$^{+/+}$ (left) and Adfp$^{2–3/2–3}$ mice (right). Sections were located on the superior side and −1.0 mm from the optic nerve head. F, thicknesses (μm) of RPE were compared between Adfp$^{+/+}$ (open circles) and Adfp$^{2–3/2–3}$ (closed circles) mice at the age of 1 year. x axis indicates the distance from the optic nerve head in μm. No significant differences were observed between Adfp$^{+/+}$ and Adfp$^{2–3/2–3}$ (open circles) and Adfp$^{2–3/2–3}$ (closed circles) mice. G, thicknesses (μm) of outer nuclear layers were compared (n = 4) between 1-year-old Adfp$^{+/+}$ (open circles) and Adfp$^{2–3/2–3}$ (closed circles) mice. x axis indicates the distance from the optic nerve head in μm. No significant differences were observed between Adfp$^{+/+}$ and Adfp$^{2–3/2–3}$ (closed circles) mice. Data are presented as means ± S.D. OS, photoreceptor outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

scotopic and photopic ERG responses were normal in both sets of 2-month-old mice. Nonetheless, a deficiency or variation in visual cycle components similar to that seen in Adfp-deficient mice often results in delayed dark adaptation of photoreceptor cells (21, 29–32). Our results are consistent with a proposed role of Adfp in the visual cycle.

Visual Cycle in Adfp$^{2–3/2–3}$ Mice—Adfp specifically binds to RESTs, and the amount of this protein is well correlated with the accumulation of retinyl esters in the RPE (9). All-trans-retinyl esters are substrates for Rpe65, the isomerohydrolase responsible for generation of visual chromophore (33–35). Therefore, we studied how an Adfp gene deficiency might affect the metabolism and storage of retinoids by quantifying retinoids in the eye by normal phase HPLC. Under dark-adapted conditions, the amount of all-trans-retinyl esters in Adfp$^{2–3/2–3}$ mice was −25% that found in Adfp$^{+/+}$ animals (Fig. 5A). Photoactivation of rhodopsin (~90% of total amount present in the retina) led to rapid isomerization of 11-cis-retinal to all-trans-retinal in both...
rhodopsin resulted in a time-dependent increase of retinal ester fluorescence in pre-existing RESTs under ex vivo conditions (Fig. 6A, top row). But in dark-adapted Adfp<sup>2–3</sup>/Δ2–3 mice, retinyl esters were diffusely localized within the cells (Fig. 6A, bottom row). After light exposure of Adfp<sup>2–3</sup>/Δ2–3 mouse eyes, a gradual increase in the number of these retinyl ester aggregates was observed in the same intracellular area of the RPE occupied by the smooth ER. At 30 min after light exposure, the number of retinyl ester aggregates (or RESTs) in Adfp<sup>2–3</sup>/Δ2–3 mouse was −1.5 times greater than that in Adfp<sup>+/+</sup> mouse (supplemental Fig. 4). However, the amount of retinyl esters per each aggregate is lower in Adfp<sup>2–3</sup>/Δ2–3 mouse than in Adfp<sup>+/+</sup> mouse (supplemental Fig. 4). To understand the ultrastructural relationship between these newly formed aggregates and the ER, we analyzed RPE from Adfp<sup>+/+</sup> and Adfp<sup>2–3</sup>/Δ2–3 mice by transmission electron microscopy. Mice were exposed to an intense light flash, and eyes were fixed 30 min later. Under this light condition, Adfp<sup>2–3</sup>/Δ2–3 mice had significant amounts of retinyl esters diffusely localized within the cells (Fig. 6B, left arrows) was seen with a distribution similar to that of retinyl ester aggregates observed by two-photon microscopy. Only a small number of such structures were derivatives in RPE cells (reviewed in Refs. 15, 16). At 1 year of age, amounts of A2E and iso-A2E in Adfp<sup>2–3</sup>/Δ2–3 mice were 6.94 ± 0.51 and 2.43 ± 0.40 pmol/eye, similar to levels in Adfp<sup>+/+</sup> mice (7.36 ± 0.91, 2.81 ± 0.35 pmol/eye, mean ± S.D.). Thus, there was no significant difference in the accumulation of A2E and iso-A2E between Adfp<sup>2–3</sup>/Δ2–3 and Adfp<sup>+/+</sup> mice. This observation suggests that the degree of all-trans-retinal accumulation does not correlate quantitatively with the amount of A2E and iso-A2E in the RPE cells.

Aggregation of Newly Synthesized All-trans-retinyl Esters in Adfp<sup>2–3</sup>/Δ2–3 Mice—All-trans-retinol generated by photoreceptors is transported to the RPE where it is esterified to form all-trans-retinyl esters (36). Because Adfp is localized to RESTs in the RPE, we tested whether the observed delay in retinoid processing stems from dysfunctional handling of retinyl esters by the RPE. Two-photon microscopy (see Ref. 37) allowed us to monitor the changes in retinyl ester fluorescence at subcellular resolution and to determine the effect of Adfp on this process. As reported previously (9), photoactivation of

Adfp<sup>+/+</sup> and Adfp<sup>2–3</sup>/Δ2–3 mice (Fig. 5, B and C). However, the clearance of all-trans-retinal was slower in Adfp<sup>2–3</sup>/Δ2–3 than in Adfp<sup>+/+</sup> animals. The amount of all-trans-retinal in Adfp<sup>2–3</sup>/Δ2–3 mice was about 20% greater than in Adfp<sup>+/+</sup> mice immediately after light illumination, and about 40% greater 15 min later (Fig. 5C, *p < 0.01). Delayed clearance of all-trans-retinol was observed as well in Adfp<sup>2–3</sup>/Δ2–3 mice under similar conditions, i.e. the amount of all-trans-retinol in Adfp<sup>2–3</sup>/Δ2–3 mice eyes was greater than that in Adfp<sup>+/+</sup> mice by 17% at 15 min and by 70% at 45 min (Fig. 5D, *p < 0.01). Consistent with delayed processing of all-trans-retinol, amounts of all-trans-retinyl esters were lower in Adfp<sup>2–3</sup>/Δ2–3 than in Adfp<sup>+/+</sup> mice eyes from 0 to 45 min after light exposure (Fig. 5A, *p < 0.01 from 0 to 30 min). Thus Adfp gene deficiency slows the flow of all-trans-retinoids generated in photoreceptor outer segments. In contrast, the rate of 11-cis-retinol synthesis was not affected in Adfp<sup>2–3</sup>/Δ2–3 mice (Fig. 5B). Because previous studies indicate that 11-cis-retinol is synthesized from all-trans-retinyl esters (33–35), this result shows that the lack of Adfp in our mutant mice did not significantly affect the hydrolysis of all-trans-retinyl esters.

Delays in all-trans-retinal clearance are often accompanied by increased accumulation of A2E and iso-A2E, toxic retinoid
FIGURE 6. *Adfp* deficiency compromises intracellular transport of retinyl esters. **A**, ex vivo two-photon imaging of the RPE. Images were captured every 10 min after photoactivation of rhodopsin. *Top row*, RPE of 2-month-old *Adfp*<sup>+/+</sup> mouse. After the flash, a gradual increase in the fluorescence intensity of preexisting retinyl ester storage structures is shown. *Bottom row*, RPE of 2-month-old *Adfp*<sup>Δ2−3/Δ2−3</sup> mouse. After the flash, a gradual increase in the number of small retinyl ester aggregates is shown. **B**, RPE from 2-month-old *Adfp*<sup>Δ2−3/Δ2−3</sup> and *Adfp*<sup>+/+</sup> mice imaged by electron microscopy. Mice were exposed to an intense light flash, and eyes were fixed 30 min later. *Left*, number of small vacuole-like structures (arrows) are detectable in the cytoplasm of the RPE from *Adfp*<sup>Δ2−3/Δ2−3</sup> mice. *Inset* is a high magnification view of small vacuole-like structures. *Right*, in *Adfp*<sup>+/+</sup> mice, vacuole-like structures are observed in a distribution similar to RESTs (9). *N* indicates nucleus. **C**, movement of retinyl ester aggregates was tracked for 60 min in the RPE of *Adfp*<sup>+/+</sup> (left) and *Adfp*<sup>Δ2−3/Δ2−3</sup> (right) 1-month-old mice. Trajectories of each fluorescent particle are shown in various colors. **D**, actograms showing the movement of each fluorescent particle (*n* = 10) in *Adfp*<sup>+/+</sup> (left) and *Adfp*<sup>Δ2−3/Δ2−3</sup> (right) 1-month-old mice. *Y* axis indicates the movements of particles (µm) observed in 1 min. Particles exhibited substantially more movement in *Adfp*<sup>Δ2−3/Δ2−3</sup> mice.
Adfp Function in the Retina

Here we show that Adfp, a specific component of the RESTs in the RPE, is both involved in the transport and storage of retinyl esters and required for normal dark adaptation of photoreceptors. Our data suggest a specific role of Adfp on the distribution and localization of RESTs that cannot be counterbalanced by other members of the PAT domain protein family. Thus, this study clearly demonstrates that RESTs function to sustain the normal rate of vision recovery after photoactivation of visual pigments by regulating the storage and transport of retinol/retinyl esters in the eye. As reported recently, generation of biologically active retinoic acid also is regulated by the sequestration of retinol as retinyl esters by Lrat during zebrafish development (38). Our studies show that compartmentalization by Adfp binding is an essential process for regulating the temporal and spatial distribution of biologically active vitamin A derivatives. Possibly PAT domain proteins can increase the cellular storage capacity of retinyl esters and regulate the equilibrium between retinyl esters and retinol in tissues other than the eyes.

Role of Adfp in Retinoid Compartmentation and Transport within the Eye—The RPE plays a unique role in the processing of retinoids and storage of retinyl esters (1). Lrat, the major enzyme responsible for retinyl ester formation in several tissues, including hepatic stellate cells and the RPE (19, 20), also is required for lipid droplet formation in hepatic stellate cells (20) and the RPE (supplemental Fig. 5). In the absence of Lrat, fluorescent RESTs did not form in the RPE and Adfp localized diffusively in the cytoplasm (9), but in the presence of Lrat, Adfp localized to RESTs often found proximal to the plasma membrane (9). Because of this unique retinyl ester-dependent translocation, we postulated that Adfp is involved in the transport of retinyl esters. Lrat predominantly localizes to the smooth ER where esterification of all-trans-retinol occurs (17). Because the subcellular sites of retinyl ester synthesis and storage are distinct, an uncharacterized mechanism must exist that facilitates the ER to REST translocation of retinyl esters essential for normal visual cycle functioning.

Direct imaging of retinyl esters in ex vivo and in vivo mouse eyes revealed that Adfp is essential for normal intracellular transport of retinyl esters in the RPE. Our current model of Adfp function in the visual cycle is depicted in Fig. 8. In dark-adapted Adfp<sup>−/−</sup> mice, RESTs are well maintained and capable of serving as the “destination” of newly synthesized retinyl esters (Fig. 8A). But in dark-adapted Adfp<sup>Δ2–3Δ2–3</sup> mice, these structures are poorly maintained such that newly synthesized retinyl esters form aggregates instead (Fig. 8B). Those retinyl ester aggregates increase in number after the release of all-trans-retinol from the rod outer segments (ROS); however, the
Adfp Function in the Retina

![Image](50x536 to 299x734)

FIGURE 8. Possible role of Adfp in the visual cycle. A, our results are consistent with the function of Adfp in increasing the capacity of RESTs to accumulate retinyl esters (REs). In the presence of Adfp, RESTs have sufficient capacity to accommodate newly synthesized all-trans-retinyl esters, which are transported from ER. In this model, rapid clearance of retinyl esters from the ER allows all-trans-retinol to enter quickly into the RPE for further esterification. B, in the absence of the full-length Adfp, RESTs are poorly maintained under dark adaptation. Newly synthesized retinyl esters cannot go to pre-existing RESTs, and as a result, retinyl esters aggregate in cytoplasmic area proximal to ER. Because of slow clearance of retinyl esters from the ER, all-trans-retinol enters the RPE slowly. Red, aggregate of retinyl esters; green, ER; blue, full-length Adfp.

retinyl ester content per aggregate is lower in AdfpΔ2–3/Δ2–3 mice than in Adfp+/+ mice (supplemental Fig. 4). These observations are in line with our model that Adfp increases the capacity of each aggregate to store retinyl esters. Considering the specific localization of Adfp to RESTs (9), these imaging results indicate that Adfp is required for the maintenance of RESTs during dark adaptation and that well maintained RESTs are essential for proper transport of retinyl esters in the RPE.

Retinyl esters were decreased in AdfpΔ2–3/Δ2–3 mice, consistent with an essential function of Adfp in the maintenance of RESTs. Several previous studies have linked Adfp to the accumulation of lipid, both in cultured cells and in vivo. Overexpression of Adfp led to accumulation of lipid droplets in hepatic stellate cells in vitro (10, 11). Also, diet-induced fatty liver was significantly reduced in Adfp-deficient mice (12) suggesting that Adfp promoted pathological accumulation of these lipid droplets. Exactly how Adfp achieves these effects in vivo remains unclear. Adfp was shown to reduce association of adipose triacylglycerol lipase (AGTL) with lipid droplets (13) leading to the hypothesis that reduced lipid ester hydrolysis in the presence of Adfp might directly contribute to the overall increase in TAG accumulation. However, this AGTL-related mechanism cannot directly account for the function of Adfp in the RPE, where Rpe65 is the only enzyme known to be responsible for the hydrolysis/isomerization of all-trans-retinyl esters (33–35). So if Adfp protects retinyl esters from Rpe65-catalyzed hydrolysis, the absence of Rpe65 in AdfpΔ2–3/Δ2–3 mice should not affect the amount of retinyl esters. But instead Adfp increased the amount of retinyl esters in the RPE of mice with Rpe65−/− backgrounds. Another remarkable observation was that the rate of 11-cis-retinal synthesis did not differ significantly between Adfp+/+ and AdfpΔ2–3/Δ2–3 mice. Because formation of 11-cis-retinoid and hydrolysis of all-trans-retinyl esters are coupled reactions catalyzed by Rpe65, our results suggest that Adfp-mediated storage of retinyl esters is independent of the enzymatic activity of Rpe65.

Our previous and current studies suggest that Adfp is a retinyl ester-binding protein and that their interaction results in mutual stabilization. Thus, less accumulation of retinyl esters occurred in the absence of Adfp (Figs. 5 and 7), whereas induced accumulation of retinyl esters led to up-regulation of Adfp (9). An interaction of Adfp with retinyl esters also is indicated by the localization of Adfp on the surface of the RESTs (9). Intriguingly, the quantity of Adfp is tightly regulated by the ubiquitin/proteasome pathway that actively degrades Adfp in the absence of fatty acids (39). Pharmacological inhibition of proteasome degradation led to increased accumulation of cellular lipids that correlated with increased Adfp levels (39). Possibly up-regulated Adfp promotes accumulation of retinyl esters in the RPE by a similar mechanism, as illustrated by more accumulation of retinyl esters in Adfp+/+Rpe65−/− mice as compared with AdfpΔ2–3/Δ2–3 mice. These genetic studies provide evidence that direct interactions between Adfp and retinyl esters facilitate compartmentalization of retinyl esters to the RESTs.

Transport and compartmentalization of retinyl esters confined to RESTs should reduce the concentrations of retinyl ester in the ER where retinyl ester biosynthesis occurs. In AdfpΔ2–3/Δ2–3 mice, increases of retinyl ester in the ER would reduce the rate of retinyl ester synthesis by Lrat via product inhibition. Such potent product inhibition was shown previously with the retinyl ester analog, all-trans-retinyl bromacetate (40).

Esterification of all-trans-retinol by Lrat is essential for transport of all-trans-retinol between photoreceptor cells and the RPE (9, 19) so inhibition of Lrat activity should have a negative impact on this process (Fig. 8). Consistent with this concept, we observed delayed transport of all-trans-retinol and all-trans-retinyl ester in AdfpΔ2–3/Δ2–3 mice. Reduction in Lrat activity also should cause less accumulation of retinyl esters, as observed in AdfpΔ2–3/Δ2–3 mice, but without affecting the processing of retinyl esters by Rpe65. Collectively, our findings are consistent with Adfp-mediated transport and compartmentalization in enhancing the storage of retinyl esters.

Another possible role for Adfp is its direct binding to hydrophobic substances to increase the rate of their uptake. Adfp expressed in COS7 cells enhanced the rate of long chain fatty acid uptake (41), and recombinant Adfp can bind to fatty acids in vitro (42). Adfp can be modified by an acyl group (43), and this hydrophobic residue may promote its interaction with other hydrophobic compounds such as retinyl esters and all-trans-retinol. It will be interesting to investigate the possible role of Adfp in the direct transport of all-trans-retinol.

Delayed Dark Adaptation and Retinoid Transport in AdfpΔ2–3/Δ2–3 Mice—Delayed dark adaptation is one of the common phenotypes in mice with a deficiency or variation in particular visual retinoid cycle components. For example, polymorphisms in the Rpe65 gene largely affect the rate of 11-cis-retinal regeneration (29). In other cases, delayed dark adapta-
tion can occur without any observable difference in the rate of 11-cis-retinal regeneration. In general, mouse models deficient in retinol dehydrogenases show delayed dark adaptation (21, 31, 32). Yet another example is the ABCR transporter-deficient mouse (30). Despite delayed processing of all-trans-retinal to retinol, the rate of 11-cis-retinal generation is not affected in all these mutant mice. But the degree of delay in dark adaptation is well correlated with the rate of all-trans-retinal clearance from ROS. All-trans-retinal may slow dark adaptation by binding to opsin and activating the transducin cascade (44, 45). All-trans-retinal in the nanomolar range can also bind to cGMP-gated channels, and this has been suggested to slow dark adaptation by inhibiting the reopening of these channels (46, 47). All these observations are consistent with the idea that delayed clearance of all-trans-retinal is the direct cause of the slow dark adaptation observed in AdfpΔ2–3/Δ2–3 mice.

We also noticed that the delay in dark adaptation was more prominent in 1-year-old than in 2-month-old AdfpΔ2–3/Δ2–3 mice (Fig. 4, A and C). From those observations, it is apparent that aging affects the visual performance of mice and that Adfp is required for the rapid restoration of photoresponses in old animals. Our recent studies suggest that clearance of all-trans-retinal and regeneration of 11-cis-retinal are delayed by aging in mice. We assume that the attenuation of photoresponses in 1-year-old AdfpΔ2–3/Δ2–3 mice (Fig. 4D) were caused by abnormalities in retinoid metabolism during the process of aging. Our experimental conditions for scotopic ERG can lead to subtle photoactivation of rhodopsin and generate free all-trans-retinal, which is more difficult to clear in older animals. Thus, the AdfpΔ2–3/Δ2–3 mouse could serve as a model to study age-dependent changes of dark adaptation and retinoid metabolism.

Possible Compensation for Adfp Deficiency by Other PAT Domain Family Members—This study (Fig. 2 and supplementary Fig. 2) and previous studies indicate that six members of the PAT domain family exist in the mouse genome (4, 26, 27, 48). Domain Family Members (Fig. 2) and previous studies indicate that six members of the PAT domain family exist in the mouse genome (4, 26, 27, 48).

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