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 Adfp<sup>A2-3/A2-3</sup> than in Adfp<sup>+/+</sup> mice and that Adfp<sup>A2-3/A2-3</sup> 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 Adfp<sup>A2-3/A2-3</sup> mice, a problem caused by abnormal maintenance of RESTs in the dark-adapted state. Retinyl ester accumulation was also reduced in Adfp<sup>A2-3/A2-3</sup> mice, a problem caused by abnormal maintenance of RESTs in the dark-adapted state. Retinyl ester accumulation was also reduced in Adfp<sup>A2-3/A2-3</sup> mice, a problem caused by abnormal maintenance of RESTs in the dark-adapted state. Retinyl ester accumulation was also reduced in Adfp<sup>A2-3/A2-3</sup> mice, a problem caused by abnormal maintenance of RESTs in the dark-adapted state.
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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-retinol, 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—Adfp2–3/2–3 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′-CTGAGGATCCGACAGAGCCCATGGTAGC-3′) and N7 (5′-ATGTTGCGATTTTCATAGCCTGAAG-3′), designed to read from the selection cassette into the long homology arm and the short homology arm, respectively. T7 (5′-ATTTAGGATCCACTATAGAACCT-3′) and P6 (5′-ATTATGCTGAGTGATATCCCTCT-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 129 Sv/Eti TL1 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′-CTATGAAGGATCCGACAGAGCCCATGGTAGC-3′) and N1 (5′-CTGAGGATCCGACAGAGCCCATGGTAGC-3′). A1(5′-CTATGAAGGATCCGACAGAGCCCATGGTAGC-3′)/AT2(5′-TGGCCTTGGTACACATAGCACATG-3′), and AT1(5′-TGGAGAGTCTAGTAGTGGACAT-3′)/N1(5′-TGGAGAGTCTAGTAGTGGACAT-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—Adfp2–3/2–3 mice were back-crossed four times with mice having either BALB/c or C57BL/6 backgrounds. Adfp2–3/2–3 mice with a BALB/c background were primarily used for this work. Adfp2–3/2–3 mice were genotyped by use of tail-snip genomic DNA, and the genotyping primers were as follows: Intron1F2, 5′-GTCAGCTGGGCTTTAGACA-3′; Exon3R, 5′-ACAGGCTCAATACTACGGG-3′, and NeoR, 5′-GGATCTCTGTCTATCTACACCCT-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 Adfp2–3 allele.

Adfp2–3/2–3Rpe65+/− Double Mutant Mice—Adfp2–3/2–3Rpe65+/− double knock-out mice were established by cross-breeding Adfp2–3/2–3 mice with Rpe65+/− mice (18). For two-photon imaging, we used albino lines of Rpe65+/− and Adfp2–3/2–3Rpe65+/− mice obtained by cross-breeding them with tyrosinase mutant mice (Ty+−).

Immunoblotting—Mammary gland tissue from Adfp+/+ and Adfp2–3/2–3 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, 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′-CCGGAACCTCCAGTTTGGAA-3′ and mAdfp-R10 5′-GCCTGGAACCTCAGACCTACC-3′. Hybridization was accomplished at 62 °C for 2 h.

RT-PCR—Mouse total RNA was isolated by TRIzol (Invitrogen) according to the manufacturer’s protocol. RNA concentrations were determined by measuring 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
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Adfp were mAdfp-F1 (5’-GACATGCATGCAAGACGACGAGCACGCTAGTGGGA-3’) and mAdfp-R1 (5’-CCCGAAGCTTTCTGAGCTTTTACCTCGACT-3’). Primers for GAPDH were 5GAPDH2 (5’-CCATCACACATCTTCCAGGAG-3’) and 3GAPDH2 (5’-CATCCACATCTTCTGGGTG-3’).

**Histology**—Eyecups for light microscopy were fixed in 2% glutaraldehyde, 2% paraformaldehyde 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. Specimens 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% osmium tetroxide and 3% potassium ferricyanide. After rinsing with distilled water, specimens were soaked overnight in an 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 Biotechnology, 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 Immunotechnology, Inc.), anti-MLDP (a generous gift from Dr. Takashi Osumi), or Alexa488-conjugated secondary antibody (Invitrogen). Sections were analyzed with a Leica TCS SP2 confocal microscope (Leica).

**ERG**—Full-field ERG recordings, flicker ERGs, and single-flash recordings after intense constant illumination were performed 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 microscopy 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, separated 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 photomultiplier tube detector (R 9624, Hamamatsu). The objective lens was kept at 37 °C by an objective lens heater (PeCon, Germany). A temperature-controlled microscope stage (PeCon, Germany) maintained the reaction at 37 °C. For ex vivo imaging, mouse eyes or eyecups were located at the center of a glass-bottomed 35-mm dish (MatTek Corp.) and perfused with oxygenated (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 temperature-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 sclera 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 sclera while the emission fluorescence 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 Incorporated, San Jose, CA). Only linear adjustments were applied to the images.

**RESULTS**

**Generation of Adfp-deficient Mice**—We generated Adfp-deficient 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 neomycin 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 Adfp2–3/2–3 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+/2–3, and Adfp2–3/2–3 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+/+ mice, a 3.5-kb fragment from Adfp2–3/2–3 mice, and a mixture of both fragments in Adfp+/2–3 mice. To confirm the lack of Adfp expression in Adfp2–3/2–3 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 Adfp2–3/2–3 mice (Fig. 1C, top). Recently, an Adfp2–3/2–3 mouse line generated by another group (12) was shown to express a short isoform of Adfp in the lactating mammary gland (22). We found that the same short Adfp mRNA, which contains all the exons except exons 2 and 3, was still expressed in the RPE of Adfp2–3/2–3 mice (supplemental 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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FIGURE 1. Characteristics of Adfp^22–3/22–3 mice. A, Adfp gene knock-out construct. The ~11.5-kb region used to construct the targeting vector was first subcloned from a positively identified BAC clone. The region was designed such that the short homology arm extended ~1–1.5 kb 3' to exon 2. 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 with the ATG start codon in exon 2.

Expression Profiles of Six PAT Domain Proteins in the Retinas—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 Adfp^22–3/22–3 mice (Fig. 2A 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 Adfp^22–3/22–3 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 data base (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 Adfp^22–3/22–3 and Adfp^+/+ mice (Fig. 3A). Immunofluorescence microscopy failed to reveal perilipin, S3-12, and MLDP in the RPE of Adfp^22–3/22–3 mice. 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 Adfp^22–3/22–3 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 Adfp^22–3/22–3 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 Adfp^22–3/22–3 mice than in Adfp^+/+ mice (Fig. 4A), indicating that Adfp^22–3/22–3 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). Adfp^22–3/22–3 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<sup>Δ2–3Δ2–3</sup> mice, we confirmed the Rpe65 genotypes of our Adfp<sup>+/+</sup> and Adfp<sup>Δ2–3Δ2–3</sup> 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<sup>+/+</sup> and Adfp<sup>Δ2–3Δ2–3</sup> 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<sup>Δ2–3Δ2–3</sup> and Adfp<sup>+/+</sup> mice (Fig. 4, top row). But in 1-year-old Adfp<sup>Δ2–3Δ2–3</sup> 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<sup>Δ2–3Δ2–3</sup> and Adfp<sup>+/+</sup> mice (Fig. 4F). The thickness of the outer nuclear layer and the number of nuclei were similar in both Adfp<sup>Δ2–3Δ2–3</sup> and Adfp<sup>+/+</sup> mice (Fig. 4G), indicating the absence of photoreceptor degeneration in Adfp<sup>Δ2–3Δ2–3</sup> 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⁻¹ m⁻¹) every 5 min for 60 min. The recovery rate was significantly attenuated in Adfp⁻²⁻⁻⁻⁻⁻⁻ mice (*, p < 0.01 compared with Adfp⁺⁺⁺⁺⁺⁺ mice; n = 5 each). B, ERG responses recorded from 8- to 10-week-old mice. Adfp⁻²⁻⁻⁻⁻⁻⁻ 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⁻¹ m⁻¹) 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⁻⁻⁻⁻⁻⁻ mice than in Adfp⁺⁺⁺⁺⁺⁺ mice (*, p < 0.01 versus Adfp⁺⁺⁺⁺⁺⁺ mice; n = 5 each). D, ERG responses recorded from Adfp⁻²⁻⁻⁻⁻⁻⁻ 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⁻²⁻⁻⁻⁻⁻⁻ (left) and Adfp⁺⁺⁺⁺⁺⁺ mouse (right). Sections were located on the superior side and −1.0 mm from the optic nerve head. F, lengths (μm) of ROS were compared between Adfp⁺⁺⁺⁺⁺⁺ (open circles) and Adfp⁻²⁻⁻⁻⁻⁻⁻ (closed circles) mice at the age of 1 year. x axis indicates the distance from the optic nerve head in mm. No significant differences were observed between Adfp⁺⁺⁺⁺⁺⁺ and Adfp⁻²⁻⁻⁻⁻⁻⁻ mice. G, thicknesses (μm) of outer nuclear layers were compared (n = 4). Inter- and intralayer thicknesses were compared for Adfp⁻²⁻⁻⁻⁻⁻⁻ (open circles) and Adfp⁺⁺⁺⁺⁺⁺ (closed circles) mice. x axis indicates the distance from the optic nerve head in mm. No significant differences were observed between Adfp⁺⁺⁺⁺⁺⁺ and Adfp⁻²⁻⁻⁻⁻⁻⁻ mouse data. Data are presented as means ± S.D. OS, photoreceptor outer segments; ONL, outer nuclear layer; IS, inner segment; OPL, outer plexiform 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⁻⁻⁻⁻⁻⁻ 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 isomerase 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 (9). All-trans-retinyl esters are substrates for Rpe65, the isomerase 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 (9).
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Absence of Adfp results in delayed processing and transport of all-trans-retinoids. A-D, quantities of retinoids in the visual retinoid cycle (all-trans-retinyl esters, 11-cis-retinal, all-trans-retinal, and all-trans-retinol) were examined in 2-month-old Adfp+/− (open circles) and Adfp2−/− (closed circles) mice by normal phase HPLC after flash. Amounts of retinoid per one eye are shown on the y axes. On the x axes, BF indicates “Before flash” and AF indicates “Immediately after the flash.” Other numbers indicate time (min) after flash. A, amounts of all-trans-retinyl esters (in pmol) were significantly lower (*) in Adfp2−/− mice than Adfp+/− mice. B, no significant differences were observed in amounts of 11-cis-retinal (in pmol) during recovery after the flash between Adfp2−/− and Adfp+/− mice undergoing dark adaptation. C, clearance of all-trans-retinyl esters (in pmol) was significantly delayed in Adfp2−/− mice at 0 and 15 min after the flash (*, p < 0.05 versus Adfp+/− mice). D, clearance of all-trans-retinol (in pmol) was significantly delayed in Adfp2−/− mice at 15–45 min after the flash (*, p < 0.01 versus Adfp+/− mice). Six mice were tested from 0 to 45 min; three mice were tested from 60 to 180 min. Data are expressed as means ± S.E. Please note that error bars at several data points are not visible because the experimental errors associated with those data points were too small.

Adfp+/− and Adfp2−/− mice (Fig. 5, B and C). However, the clearance of all-trans-retinal was slower in Adfp2−/− mice than in Adfp+/− animals. The amount of all-trans-retinal in Adfp2−/− mice also was about 20% greater than that in Adfp+/− 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 Adfp2−/− mice under similar conditions, i.e. the amount of all-trans-retinol in Adfp2−/− mice was greater than that in Adfp+/− mice by 17% at 15 min and by 70% at 45 min (Fig. 5D, *, p < 0.01). Consistent with delayed processing of all-trans-retinal, amounts of all-trans-retinyl esters were lower in Adfp2−/− mice than in Adfp+/− mouse 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-retinyl synthesis was not affected in Adfp2−/− mice (Fig. 5B). Because previous studies indicate that 11-cis-retinyl 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 rhodopsin resulted in a time-dependent increase of retinyl ester fluorescence in pre-existing RESTs under ex vivo conditions (Fig. 6A, top row). But in dark-adapted Adfp2−/− mice, retinyl esters were diffusely localized within the cells (Fig. 6A, bottom row). After light exposure of Adfp2−/− 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 Adfp2−/− mice was ~1.5 times greater than that in Adfp+/− mice (supplemental Fig. 4). However, the amount of retinyl esters per each aggregate is lower in Adfp2−/− mice than in Adfp+/− mice (supplemental Fig. 4). To understand the ultrastructural relationship between these newly formed aggregates and the ER, we analyzed RPE from Adfp+/− and Adfp2−/− mice by transmission electron microscopy. Mice were exposed to an intense light flash, and eyes were fixed 30 min later. Under this light condition, Adfp2−/− mice had significant amounts of retinyl esters (about 72% the amount of Adfp+/−) in their eyes (Fig. 5A). In Adfp2−/− mice, a population of vacuole-like structures (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 Adfp2−/− mice were 6.94 ± 0.51 and 2.43 ± 0.40 pmol/eye, similar to levels in Adfp+/− 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 Adfp2−/− and Adfp+/− mice. This observation suggests that the degree of all-trans-retinyl ester 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 Adfp2−/− Mice—All-trans-retinyl 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 retinal
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+/− 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 AdfpΔ2-3/Δ2-3 mouse. After the flash, a gradual increase in the number of small retinyl ester aggregates is shown. B, RPE from 2-month-old AdfpΔ2-3/Δ2-3 and Adfp+/− 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 AdfpΔ2-3/Δ2-3 mice. Inset is a high magnification view of small vacuole-like structures. Right, in Adfp+/− 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+/− (left) and AdfpΔ2-3/Δ2-3 (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+/− (left) and AdfpΔ2-3/Δ2-3 (right) 1-month-old mice. y axis indicates the movements of particles (µm) observed in 1 min. Particles exhibited substantially more movement in AdfpΔ2-3/Δ2-3 mice.
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in the RPE. This contribution of Adfp in Rpe65<sup>−/−</sup> background is in line with its function of in the maintenance of RESTs.

DISCUSSION

Here we show that Adfp, a specific component of the RESTs in the RPE, is 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>−/−</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
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![Diagram of Adfp Function in the Retina](image)

**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 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 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 promotes 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 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-retinoid synthesis did not differ significantly between Adfp+/+ and AdfpΔ2-3 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/Rpe65−/− 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 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 bromoacetate (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 in AdfpΔ2-3 mice. Reduction in Lrat activity also should cause less accumulation of retinyl esters, as observed in AdfpΔ2-3 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 mice—Delayed dark adaptation is one of the common phenotypes in mice with a deficiency or variation in Adfp. Late stages of rod outer segment differentiation are dependent on 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/Rpe65−/− mice. These genetic studies provide evidence that direct interactions between Adfp and retinyl esters facilitate compartmentalization of retinyl esters to the RESTs.
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 Adfp2–3/3 mice.

We also noticed that the delay in dark adaptation was more prominent in 1-year-old than in 2-month-old Adfp2–3/3 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.5 We assume that the attenuation of photoresponses in 1-year-old Adfp2–3/3 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 Adfp2–3/3 mice 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 supplemental Fig. 2) and previous studies indicate that six members of the PAT domain family exist in the mouse genome (4, 26, 27, 48). Among them, only Adfp and Tip47 are expressed in the RPE eye phenotype observed in Adfp2–3/3 mice. Future studies of the eye phenotype in either Tip47−/− or double mutant Adfp2–3/3–Tip47−/− mice should be revealing in this regard. The essential function of Tip47 in recycling mannose 6-phosphate receptor (53) could hamper the generation of live Tip47−/− mice, although a conditional knock-out of this gene in the eye would provide a feasible option to study the role of PAT proteins in the cell biology of lipid droplets.

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