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Cellular Retinol Binding Protein 1 Modulates Photoreceptor Outer Segment Folding In the Isolated Eye

XiaoFei Wang¹, Yiai Tong², Francesco Giorgianni³, Sarka Beranova-Giorgianni⁴, John S. Penn⁵, and Monica M. Jablonski^{1,*}

¹Department of Ophthalmology, University of Tennessee Health Science Center, Memphis, TN

²Department of Anatomy and Neurobiology, University of Tennessee Health Science Center, Memphis, TN

³Department of Neurology, University of Tennessee Health Science Center, Memphis, TN

⁴Department of Pharmaceutical Sciences, University of Tennessee Health Science Center, Memphis, TN

⁵Department of Ophthalmology and Visual Sciences, Vanderbilt University, Nashville, TN

Abstract

In a previous study, we used differential proteomics to identify retinal proteins whose steady-state levels were altered in an experimental system in which photoreceptor outer segments were improperly folded. We determined that the steady-state level of cellular retinol binding protein 1 (CRBP1) was downregulated in eyes lacking organized outer segments. The purpose of this study was to determine if CRBP1 is a plausible candidate for regulating outer segment assembly. We used Morpholinos to directly test the hypothesis that a decreased level of CRBP1 protein was associated with the misfolding of outer segments. Results from these studies indicate that downregulation of CRBP1 protein resulted in aberrant assembly of outer segments. Because CRBP1 plays a dual role in the retina – retinal recycling and generation of retinoic acid – we evaluated both possibilities. Our data demonstrate that outer segment folding was not modified by 11-cis retinal supplementation, suggesting that CRBP1 influences outer segment assembly through a mechanism unrelated to rhodopsin regeneration. In contrast, retinoic acid is required for the proper organization of nascent outer segment membranes. The localization of CRBP1 within Muller cells and the RPE and its demonstrated role in modulating the proper folding of nascent outer segment membranes through retinoic acid further elucidates the role of these cells in directly influencing photoreceptor physiology.

Keywords

photoreceptor; Müller cell; retinal pigment epithelium; mass spectrometry; cell interaction

INTRODUCTION

Retinal photoreceptors are highly polarized and ultrastructurally unique neurons. While several of the events that lead to formation and organization of outer segments have been revealed, the influence of neighboring cells is not fully elucidated. Numerous studies have

*Corresponding author: Dr. Monica M. Jablonski, Department of Ophthalmology, Hamilton Eye Institute, The University of Tennessee Health Science Center, 930 Madison Avenue, Suite 731, Memphis, TN 38163 (901) 448-7572 (voice), (901) 448-5028 (fax), mjablonski@uthsc.edu.

documented the importance of the retinal pigment epithelium (RPE) and Müller cells, on photoreceptor health, function and outer segment integrity (Robinson and Dreher, 1990; Gaur et al., 1992; Reichenbach et al., 1993; Newman and Reichenbach, 1996; Cao et al., 1997; Sheedlo et al., 1998; Jablonski and Iannaccone, 2000; Ullian et al., 2001). Specifically, pigment epithelium derived factor and beta fibroblast growth factor secreted by the RPE and Müller cells, respectively, support photoreceptor health and structural integrity (Harada et al., 2000; Jablonski et al., 2000; Harada et al., 2002). It is highly probable that other molecules produced by neighboring cells also play a role in modulating photoreceptor integrity.

In a recent study, we demonstrated using 2D DIGE methodology that photoreceptors, Müller glial cells, and the RPE have multiple proteins that are differentially expressed when outer segment structure is severely compromised (Wang et al., 2009). One such protein, CRBP1 (cellular retinol binding pin vivo binding partner in the retina is all-trans retinol (Saari, 1982).

In vision, CRBP1 plays a role in retinoid recycling in which 11-cis retinal coupled to opsin within photoreceptor outer segment disc membranes is isomerized to all-trans retinal upon absorption of a photon of light. In order to re-couple with opsin, the retinoid chromophore must be regenerated through a series of reactions known to take place within the outer segments and the RPE (reviewed in (Saari, 2000)). In this capacity, CRBP1 is localized to the RPE where it functions as a chaperone in the presentation of all-trans retinol to lecithin retinol acyltransferase. CRBP1 is also localized to Müller cells (Bok et al., 1984; Eisenfeld et al., 1985) where it functions in the synthesis of retinoic acid from all-trans retinol (Edwards et al., 1992), thus enabling it to bind to retinoic acid receptors in neighboring cells. In the retina, retinoic acid has a documented role in determining cell fate and the early development of photoreceptors (Kelley et al., 1999; Prabhudesai et al., 2005). It also has a role in regulating the expression of NRL (neural retina leucine zipper), a photoreceptor transcription factor (Khanna et al., 2006). Interestingly, rod outer segment structure is markedly compromised in *Nrl* knockout mice (Mears et al., 2001). Taken together these data suggest a possible role for CRBP1 in the modulation of photoreceptor folding via retinoic acid.

In this study, we have examined the possibility that CRBP1, a protein expressed in both the RPE and Müller cells, modulates the proper folding of nascent photoreceptor outer segment membranes. Our data demonstrate that CRBP1 strongly influences outer segment membrane organization through a mechanism unrelated to retinoid recycling and rhodopsin regeneration. Rather, it modulates the assembly of membranes through a retinoic acid-dependent pathway.

MATERIALS AND METHODS

Removal of *Xenopus* retinas and culture protocol

The use of animals in this study were used in compliance with the Guiding Principles in the Care and Use of Animals (DHEW Publication NIH 80-23) and was approved by the Animal Care and Use review board of the University of Tennessee Health Science Center. Our culture methodology has been previously published (Wang et al., 2009). Briefly, eye rudiments were removed from stage 33/34 *Xenopus laevis* tadpoles. At this developmental

stage, photoreceptor outer segments are just beginning to be elaborated, thus all membranes are produced *in vitro* under tightly controlled conditions. Also at this stage, the RPE is the outermost cell layer. In RPE-supported retinas, the RPE was left juxtaposed to the neural retina. In RPE-deprived retinas, the RPE layer was nicked with finely polished forceps so that it retracted from the surface of the neuroepithelium yet it remained present at the edge of the retina at the ora serrata (Stiemke and Hollyfield, 1994; Wang et al., 2009). Groups of individual eyes were cultured in Niu-Twitty media (Jacobson, 1967) at 23°C for three days. Four biological replicates were analyzed.

Identification of CRBP1 as a differentially regulated protein

Our 2D-DIGE protocol has been previously described (Wang et al., 2009). Briefly, proteins were extracted from tadpole eyes, labeled with CyDye DIGE fluors (GE Healthcare), and multiplexed to eliminate potential dye bias. An internal standard containing protein samples from all eight experimental conditions was included on each gel. Samples were separated using IPG strips of pH 4–7 (GE Healthcare) and run on an Ettan DALTwelve system. Each 2D gel was imaged and analyzed using DeCyder software (GE Healthcare). Differentially expressed proteins were determined using Student's t-test and ANOVA analysis. Spot 22 (Wang et al., 2009) was excised from the gels and processed for in-gel protease digestion. The eluted peptides were analyzed online with a nanoESI-quadrupole ion-trap mass spectrometer (LCQ^{Deca}) (ThermoFinnigan) and TurboSEQUEST was the peaklist-generating and search engine software used to analyze the data using search parameters as described previously (Wang et al., 2009).

Immunohistochemistry

Immunohistochemistry was performed to locate the CRBP1 protein and retinoic acid in tadpole eyes. Intact eyes were cultured as above described and fixed in 10% neutralized formalin for two hours. After washing in phosphate buffer, the eyes were sectioned, blocked with 5% goat serum and permeabilized with 0.1% Triton. For CRBP1 protein localization, sections were processed for double labeling using anti-CRBP1 (1:50; Santa Cruz) and anti-glutamine synthetase (1:300; Millipore) antibodies. For retinoic acid localization, sections were incubated in anti-BSA conjugated retinoic acid (1:1000; Cellsciences). The appropriate Alexa fluor-tagged secondary antibodies (1:200; Invitrogen) and ToPro III Iodide (1:4000; Invitrogen) were used to illustrate the presence of the antigens of interest and nuclei, respectively. Sections were viewed and images were obtained using a Nikon C1 confocal microscope. All microscope settings, including laser levels and gain, were unmodified to allow for relative comparisons of signal intensity within an experiment. Primary antibodies were omitted in negative control conditions.

In situ hybridization

Stage 40 embryos were fixed in 4% paraformaldehyde for 4 hours at 4°C. 5 µm thick cryosections were obtained using a HM500OM cryostat (Carl Zeiss). The pCMV.SPORT6 plasmid vector containing dual SP6 and T7 promoters and the full-length *Xenopus laevis* hypothetical protein MGC81232 cDNA clone were obtained (Open Biosystems). A digoxigenin-labeled cRNA antisense probe was generated using T7 RNA polymerase. SP6 RNA polymerase was used to generate a digoxigenin-labeled sense control. Approximately 300 ng of cRNA probe was dissolved in one ml of hybridization buffer of 50% formamide (Fisher Scientific), 4 × SSC, 200 µg/ml denatured salmon testis DNA (Invitrogen), 250 µg/ml yeast tRNA (Invitrogen), 1× Denhard's solution, 10% dextran sulfate (Fisher Scientific) and 10 mM dithiothreitol (Invitrogen). The incubation was carried out in a humid chamber for 16 hrs at 55°C. After hybridization, the slides were washed at 55°C in salt buffer gradients ranging from 4X to 0.5X SSC and treated with RNase A (10 µg/ml) for 10 min at 37°C. An anti-digoxigenin mouse IgG (Jackson ImmunoResearch Laboratories) and HRP-

conjugated goat-anti-mouse IgG (Molecular Probe, Eugene, OR) were used as primary and secondary antibodies, respectively. Tyramide working solution (Invitrogen) was applied to the sections and incubated for 10 minutes according manufacturer's instructions. Propidium iodide was used as a nuclear marker. Sections were viewed and images were obtained using a Nikon C1 confocal microscope.

Functional analysis of CRBP1 using Morpholinos

CRBP1 Morpholino antisense oligos, standard control Morpholinos and endoporter were obtained from Gene Tools, LLC. The antisense morpholino sequence used was: 5'-GTA CCC GTT GAA ATT AGT AGA CAT G 3', which complements the sequence from -1 through 24 relative to the initiation codon of *Xenopus laevis rbp1* mRNA. The standard control Morpholino had the sequence: 5'-CCT CTT ACC TCA GTT ACA ATT TAT A 3'. This sequence has no *Xenopus* target and no significant biological activity (<http://www.gene-tools.com/node/23#positiveantisensetestsystem>). Endoporter (1:166 dilution per manufacturer instructions), included as a negative control, is a peptide with weak-base amphiphilic characteristics that safely delivers Morpholinos to the cytosolic compartment of cells. RPE-supported retinas were removed from embryos at stage 33/34, as described above and incubated in Niu-Twitty medium or medium supplemented with 10 µM, 20 µM, or 30 µM antisense Morpholino. Control Morpholino was used at 30 µM. Eyes were cultured in the presence of Morpholino for 24 hours, after which time the medium was switched to un-supplemented Niu-Twitty and eyes were cultured for another 48 hours. Transfection efficiency was estimated by visualization of 3'-carboxyfluorescein tagged control Morpholino using confocal microscopy. Eyes were either harvested to determine the expression level of CRBP1 or fixed in for morphological assessment of outer segment structure. RPE-supported and RPE-deprived retinas cultured in non-supplemented Niu-Twitty medium were used as controls. To determine if the function of CRBP1 could at least be partially regained, eyes exposed to 30 µM Morpholino were supplemented with 11 cis-retinal or retinoic acid, as described in subsequent sections.

Steady-state protein levels of CRBP1 in eyes cultured in Morpholino were determined using semi-quantitative Western blots were performed as previously described (Nookala et al., 2009). Briefly, identical amounts of protein from RPE-supported and RPE-deprived tadpole eyes were separated under reduced conditions using pre-cast 12% Bis-Tris gel and MOPS buffer (Invitrogen). After separation, proteins were transferred to PVDF membrane (Invitrogen) and analyses were performed using the Dylight 680/800 Western blotting kit (Pierce) following manufacturer's specifications. Anti-CRBP1 (Santa Cruz) and anti-actin (Millipore) were used as primary antibodies. Membranes were scanned using the 700 nm and 800 nm channels of the LI-COR Odyssey Infrared imager. The signal from CRBP1 was normalized to the actin levels to allow for determination of relative steady-state protein levels.

For morphological assessment and evaluation of outer segment membrane assembly, tadpole eyes were fixed in Tucker fixative followed by ethanol dehydration and embedding in Araldite/Embed 812 (Electron Microscopy Sciences), as described previously (Jablonski et al., 2000). One-micron thick sections were cut, stained with Toluidine blue and viewed on a Nikon 800 Eclipse microscope equipped with a color camera (Photometrics). Because *Xenopus* retinas mature most rapidly in the posterior pole, assessment of outer segment structure was performed on sections taken exclusively from this area (Stiemke et al., 1994). The grading of nascent photoreceptor outer segments was performed in a masked fashion as described previously (Jablonski et al., 2001). Using this system, a grade of 4 represents proper folding of photoreceptor outer segment membranes, while a grade of 0 represents complete disorganization of membranes. Eight contiguous photoreceptors from three individual retinas were evaluated. Data were statistically analyzed by one-way analysis of

variance (ANOVA) using Prism 4 for Macintosh (GraphPad Software, Inc.). Differences between groups were determined using Bonferroni's Multiple Comparison Test.

Evaluation of the ability of 11-cis retinal to support outer segment assembly

11-cis retinal was a generously provided by Dr. Rosalie Crouch (Medical University of South Carolina, Charleston, SC). All handling and use of 11-cis retinal was performed in total darkness using infrared illumination. The retinoid was dissolved in 100% ethanol at a stock concentration of 100 mM and stored at -70.0°C. Delivery of 11-cis retinal was performed using the protocol of Li et al. (Li et al., 1999). Fatty acid free BSA was used to protect the retinoid in solution. The concentration of BSA that stabilized 11-cis retinal yet was not toxic to the tadpole eyes was empirically determined to be 0.5%. The retinoid stock solution was diluted to a final concentration of 100 µM in Niu-Twitty medium containing 0.1% ethanol and 0.5% BSA. Tadpole eyes were obtained as described above and cultured in six conditions detailed as follows: (1) RPE-supported eyes; (2) RPE-deprived eyes; (3) RPE-deprived eyes supplemented with 0.5% BSA and 0.1% ethanol; (4) RPE-deprived eyes supplemented with 11-cis retinal stock solution containing BSA and ethanol as in condition 3; (5) RPE-supported eyes incubated in 30 µM Morpholino supplemented with 11-cis retinal; and (6) RPE-deprived eyes supplemented with all-trans retinal containing BSA and ethanol as in condition 3. 11-cis retinal was converted to all-trans retinol by exposure to light. The structural stability of both retinoids was examined by UV/visible spectra with a spectral scan by µQuant spectrometer (BioTek Instruments Inc.). Eyes were cultured for three days and harvested for morphological assessment as described above.

Evaluation of the ability of retinoic acid to support outer segment assembly

Handling and use of retinoic acid (Sigma) was performed in total darkness using infrared illumination. The retinoic acid was dissolved in DMSO (Sigma) and serially diluted in Niu-Twitty medium to reach final concentrations of 0.01% DMSO and 0.01 µM, 0.1 µM, 0.5 µM, 1 µM, or 10 µM retinoic acid. RPE-deprived eyes were cultured *in vitro* for three days in the presence of various concentrations of retinoic acid and harvested for morphology assessment as described above. RPE-supported eyes incubated in 30 µM Morpholino was also supplemented with 0.5 µM retinoic acid *in vitro* for 3 days and harvested for morphological assessment. Controls included RPE-supported and RPE-deprived eyes, and RPE-deprived eyes incubated in 0.01% DMSO.

To determine if suppression of retinoic acid synthesis was able to negatively impact outer segment folding, RPE supported retinas were exposed to citral, a competitive inhibitor of alcohol dehydrogenase that converts retinaldehyde to retinoic acid (Hyatt et al., 1996). Citral (SAFC Supply Solutions) was dissolved in Niu-Twitty medium to final concentrations of 10 µM, 25 µM, or 50 µM. In parallel experiments, 0.5 µM of retinoic acid was added to the dilutions of citral. RPE-supported eyes were cultured in these media for 3 days and harvested for morphological assessment as described above. RPE-supported and RPE-deprived eyes were included as controls.

RESULTS

Identification of the protein in spot 22, a differentially regulated retinal protein

Using 2D DIGE we identified 27 spots that differed in the amount of protein when comparing extracts derived from RPE-supported retinas with organized outer segments and RPE-deprived retinas with disorganized outer segments (Wang et al., 2009). Spot 22, with a relative molecular weight of 16kD and pI of 5.4 (Figure 1A), was on average 1.36 fold less abundant in RPE-deprived retinas ($p=0.039$; Figure 1B). Following identification of the

protein in this spot, semi-quantitative Western blotting confirmed the trend in protein expression level (Figure 1C).

The chromatogram from the nanoLC-MS/MS analysis of spot 22 is shown in Figure 2A. The MS/MS spectra of the peptide ions (doubly-charged, precursor ion masses 567.25, 705.81, and 630.29) that matched to sequences NDQLVC#EQK, EFEEDLSGVDDR, and NYIM*EFDVGR, respectively, are shown in Figure 2B–D. Based upon the peptide sequences, the best match for the protein is *Xenopus laevis* hypothetical protein MGC81232. Illustrated in Figure 3A is the sequence of the full-length *Xenopus laevis* hypothetical protein MGC81232 (black text) and those peptide matches that were obtained by our MS/MS analysis (grey text). Our peptide sequences cover 24% of the total protein sequence. A BLAST search of the protein sequence of MGC81232 demonstrated that this protein has 77.6% identity with mouse cellular retinol binding protein 1 (CRBP1; Figure 3B). The high level of sequence homology demonstrates that the hypothetical protein MGC81232 is *Xenopus laevis* CRBP1.

Localization of CRBP1 gene products

Immunohistochemistry was used to demonstrate that the CRBP1 protein was abundant in the RPE and Müller cells of RPE-supported retinas (Figure 4A and 4B). The intensity of signal appeared to be reduced in the Müller cells of RPE-deprived retinas (Figure 4C and 4D). In situ hybridization demonstrated that the RPE expressed a high level of *rbp1* mRNA, while a lower level of mRNA expression was found in the neural retina in a linear pattern indicative of Müller cell expression (Figure 4E and 4F).

Functional analysis of CRBP1

Morpholinos complementary to the initiation start site of *rpb1* mRNA were used to modulate the expression of CRBP1 to determine whether this protein played a role in photoreceptor outer segment folding. Use of a 3'-carboxyfluorescein tagged Morpholino demonstrated a greater than 90% transfection efficiency of retinal cells (data not shown). Quantitative Western blots showed that the Morpholino antisense oligos successfully downregulated CRBP1 protein levels in RPE-supported retinas. By one-way ANOVA, the overall F-test for differences among the five groups was highly significant ($F=82.76$; $p<0.0001$). The decrease in the CRBP1 protein level was proportional to the dose of Morpholino used, with 10 μ M, 20 μ M and 30 μ M downregulating CRBP1 expression by 31%, 55% and 73%, respectively (Figure 5A). Quantification of outer segment organization in eyes exposed to the Morpholinos demonstrated significant differences in the ability of the photoreceptors to properly fold their outer segment membranes. By one-way ANOVA, the overall F-test for differences among the seven groups was highly significant ($F=23.02$; $p<0.0001$). Exposure to control Morpholino had no effect on outer segment assembly (Figure 5A and 5B). In contrast, retinas exposed to the antisense Morpholino had significantly compromised outer segment organization that was statistically different from those of RPE-supported retinas (Figure 5A and 5C). The inability of photoreceptors to properly fold their outer segments was not proportional to the expression level of the protein, but rather exhibited what appeared to be a threshold response (Figure 5A).

11-cis retinal supplementation does not promote outer segment assembly

Because separation of the RPE eliminated the cell type in which all trans-retinol is isomerized to 11-cis retinal, we exposed RPE-deprived tadpole eyes to 11-cis retinal and graded the level of outer segment organization to evaluate if a lack of rhodopsin was responsible for the inability of photoreceptors properly fold outer segments. As a first step to ensure chromophore stability, we measured the absorbance of the 11-cis retinal in Niutwitty medium after three days. The retinoid was stable when maintained in the dark at

23°C. At time 0, the absorbance peak was 400nm and after three days it shifted slightly to 390nm, consistent with that reported by others (Li et al., 1999) (data not shown). We then tested the effect of 11-cis retinal supplementation on outer segment organization. By one-way ANOVA, the overall F-test for differences among the six groups was highly significant ($F=21.38$; $p<0.0001$). The vehicle required to deliver the 11-cis retinal had no bearing on the outer segment grade of RPE-deprived eyes; it neither promoted outer segment assembly nor inhibited it (Figure 6A). Supplementation with 11-cis retinal also had no influence upon the promotion of proper outer segment membrane folding in the RPE-deprived state. The degree of outer segment organization did not differ from that measured in RPE-deprived control conditions (Figures 6A and 6B). To further evaluate the effect of 11-cis retinal on outer segment folding, we next used Morpholinos to selectively downregulate CRBP1 expression while simultaneously supplementing the medium with 11-cis retinal. This experimental manipulation did not promote outer segment assembly (Figure 6A and 6C). Exposure of the RPE-deprived eye to all-trans retinal did not support outer segment membrane folding (Figures 6A and 6D) and it was also toxic to retinal cells, as has been reported in the literature (Lamb and Simon, 2004; Maeda et al., 2009). Maeda and colleagues (Maeda et al., 2009) demonstrated that the mechanism of cell death throughout the retina was likely through an increase in plasma membrane permeability and mitochondria-associated cell death due to caspase activation.

Localization of retinoic acid

Immunohistochemistry was used to localize retinoic acid in RPE-supported and RPE-deprived conditions. In the presence of the RPE, retinoic acid was found in abundance in photoreceptor inner segments and the outer plexiform layer (Figure 7A and 7B). A lower level of retinoic acid was found in a pattern that resembles Müller cells. In contrast, in the absence of the RPE, retinoic acid levels were markedly reduced in all layers of the retina (Figure 7C and 7D).

Retinoic acid supplementation promotes proper outer segment assembly

Because CRBP1 also participates in retinoic acid synthesis, we tested the relationship between CRBP1, retinoic acid and outer segment membrane folding. To do so, we manipulated the levels of retinoic acid and CRBP1 protein and evaluated outer segment organization. By one-way ANOVA, the overall F-test for differences among the seven groups was highly significant ($F=21.52$; p value<0.001). In RPE-deprived retinas, retinoic acid (0.5 μ M) supported outer segment assembly to a level not significantly different from RPE-supported control (Figure 8A and 8B). Simultaneous exposure of RPE-supported eyes to CRBP1 Morpholinos and retinoic acid also supported proper folding of outer segment membranes (Figure 8A and 8C). Citral, an inhibitor of retinoic acid synthesis, was utilized to specifically reduce retinoic acid levels. At 25 μ M, citral disrupted outer segment assembly to a level that was significantly reduced compared to control RPE-supported eyes (Figure 8A and 8D). The addition of retinoic acid to citral-exposed eyes supported outer segment assembly to a level not different from control (Figure 8A and 8E).

DISCUSSION

Studies from our lab (Stiemke and Hollyfield, 1994; Jablonski and Iannaccone, 2000) and other investigators (Hollyfield and Witkovsky, 1974; Gaur et al., 1992; Reichenbach et al., 1993; Newman and Reichenbach, 1996; Cao et al., 1997; Sheedlo et al., 1998; Harada et al., 2000; Wahlin et al., 2000; Harada et al., 2002) have demonstrated that both Müller cells and the RPE play active roles in supporting photoreceptors and their ability to properly assemble outer segment membranes. In a recent paper, using a differential proteomics approach we identified 23 unique proteins from 27 spots that had significantly altered steady-state levels

when photoreceptor outer segment membrane assembly was disrupted by separation of the RPE from the neural retina (Wang et al., 2009). Of those proteins, 15 of them (65%) were expressed by the RPE, Müller cells or both cell types. One of the plausible protein candidates that was revealed by our differential proteomics study was CRBP1 (Wang et al., 2009). Our localization studies corroborated the findings of others using other species (Bok et al., 1984; Eisenfeld et al., 1985) and showed that within the retina of *Xenopus laevis*, both RPE and Müller cells express CRBP1. The purpose of this investigation was to evaluate the functional role of CRBP1 and to begin to elucidate the mechanism by which a reduction in its steady-state protein level modulates outer segment membrane assembly.

Our data demonstrate that under experimental conditions that would otherwise support proper folding of outer segment membranes, suppression of CRBP1 expression using antisense Morpholinos negatively affect the ability of photoreceptors to properly assemble outer segment membranes. Moreover, our data suggest that a threshold protein level for CRBP1 exists, above which proper outer segment formation is promoted. However, the exact threshold level was not determined in our studies. These data demonstrate that in the isolated intact eye CRBP1 is important for outer segment assembly, but it does not identify the specific mechanism by which this is achieved. Our investigations next sought to determine if CRBP1 functions in its role in the visual cycle or in retinoic acid synthesis.

To determine if CRBP1 supports outer segment folding through its role in 11-cis retinal regeneration and recycling, we exposed retinas in which CRBP1 was selectively downregulated or the RPE was physically separated from 11-cis retinal. The hypothesis of these studies was that lack of CRBP1 reduced the bioavailability of 11-cis retinal, thus leaving opsin in its apoprotein confirmation and rendering outer segments unstable and unable to fold properly. Our data demonstrate that 11-cis retinal supplementation of both RPE-supported retinas in which CRBP1 was downregulated using Morpholinos and RPE-deprived retinas did not result in organized outer segment folding. This suggests that CRBP1 supports outer segment assembly through a function unrelated to rhodopsin regeneration.

To determine if CRBP1 supports outer segment assembly through a mechanism related to retinoic acid, we exposed retinas in which CRBP1 was either selectively downregulated or the RPE was physically separated to all-trans retinoic acid. Because all-trans retinol, the endogenous ligand of CRBP1 (Saari, 1982), is also a precursor of retinoic acid our goal was to determine if retinoic acid was involved in regulating outer segment folding. Although not quantitative, our immunohistochemical data demonstrate that retinoic acid levels are markedly reduced in RPE-deprived eyes. To directly test whether modulation of retinoic acid levels influenced outer segment assembly, we supplemented eyes with retinoic acid under three different experimental conditions where outer segment assembly is disrupted (i.e., RPE-deprived eyes; RPE-supported eyes in which CRBP1 expression was reduced with Morpholinos; and RPE-supported eyes exposed to citral, an inhibitor of retinoic acid synthesis (Hyatt et al., 1996)). In all three paradigms, supplementation of with retinoic acid supported proper folding of nascent outer segment membranes to a level not significantly different from RPE-supported eyes, thus providing evidence that CRBP1 influences outer segment folding in the isolated eye via its role in retinoic acid synthesis.

Approximately a decade ago, a CRBP1 knockout (*Rbp1^{-/-}*) mouse was generated and several groups have carefully evaluated whole animal vitamin A homeostasis and the structure and function of the retina in this mouse (Ghyselinck et al., 1999; Saari et al., 2002; Matt et al., 2005). The data collectively demonstrated that absence of CRBP1 increased the rate of retinol turnover in addition to shortening the half-life of retinyl palmitate, the storage form of retinol (Ghyselinck et al., 1999). When fed a vitamin A-enriched diet, the knockout mice had normal retinal ultrastructure, abundant liver stores of retinyl esters and no signs of

vitamin A deficiency. The authors concluded that with vitamin A abundance, CRBP1 was dispensable for development. In contrast, after being fed a vitamin A deficient diet for 23 weeks until liver stores of retinoids were nearly depleted, outer retinal organization was aberrant in the knockout mice and outer segments contained swollen disoriented membranous whorls (Ghyselinck et al., 1999). In follow up investigations, the same group of investigators measured steady-state levels of retinoic acid in *Rbp1*^{-/-} knockout mice during embryonic development and they concluded that with maternal vitamin A sufficiency, retinoic acid levels are decreased up to 20% (Matt et al., 2005). Based upon in vitro studies using primary embryonic fibroblasts, the authors further concluded that CRBP1 was dispensable for retinoic synthesis (Matt et al., 2005).

Although our data are not in agreement with the conclusions that were derived using *Rbp1*^{-/-} mice, the studies can be reconciled when all factors are taken into account. In the knockout mouse, outer segment organization appeared normal in vitamin A sufficiency (Ghyselinck et al., 1999) despite slightly reduced steady state levels of retinoic acid (Matt et al., 2005). After liver stores of vitamin A have been depleted in *Rbp1*^{-/-} mice, outer segment organization became aberrant and disorganized (Ghyselinck et al., 1999). Unfortunately, retinoic acid levels in the vitamin A deficient state were not evaluated so the true influence of retinoic acid upon outer segment folding was not determined in the *Rbp1*^{-/-} mouse. In contrast, the intact tadpole eyes that we used in our investigations, liver stores of retinyl esters were not present to serve as a depot for retinol which could be converted to retinoic acid. In addition, the medium in which the eyes were cultured is a simple defined medium and contains no vitamin A, its derivatives, or any compounds that could be converted into retinol. Therefore separation of the RPE or downregulation of CRBP1, the intracellular chaperone for retinol and the precursor of retinoic acid, would have significant and potentially dramatic effects on retinoic acid levels. Through a systematic analysis, we have demonstrated that in the isolated but intact eye, modulation of CRBP1 influences outer segment folding through retinoic acid.

In the retina, retinoic acid influences the fate and differentiation of photoreceptors (Kelley et al., 1999; Prabhudesai et al., 2005), possibly influenced by the transcription factor Nrl. Because Müller cells and the RPE are the sites of retinoic acid synthesis (Edwards et al., 1992; McCaffery et al., 1996), we predict that decreased retinoic acid levels induced by lower CRBP1 expression would incite a photoreceptor response. In our previous study in which we identified CRBP1 as being differentially expressed (Wang et al., 2009), we generated a Müller cell specific proteome network to illustrate the interrelationship between Müller cell differentially expressed proteins and other gene products and metabolites. Central to that network was retinoic acid. Importantly, in the present study, we demonstrate that retinoic acid is necessary and sufficient to support and promote outer segment folding.

The interplay between neurons and their neighboring cells has been an active area of investigation. Within the retina, it has been proposed that Müller cells provide trophic support to promote photoreceptor survival (Reichenbach et al., 1993; Newman and Reichenbach, 1996; Cao et al., 1997; Harada et al., 2000; Harada et al., 2002). Within the brain, glia may regulate synaptogenesis (Ullian et al., 2001; Ullian et al., 2004) and neuronal processing (Newman, 2004) through bidirectional communication (Araque and Perea, 2004). It has been demonstrated that retinoids play a direct role in the relationship between neurons and surrounding cells. For example, in the lateral geniculate eminence, radial glia have been shown to produce retinoids which have a positive effect on the differentiation of neighboring striatal neurons (Toresson et al., 1999). The retina has a similar dependent relationship. When incubated with retinol, the Müller glia synthesize retinoic acid and retinaldehyde (Edwards et al., 1992). Although retinaldehyde was retained intracellularly, large amounts retinoic acid was quickly released into the medium while a minor amount

remained in the Müller cells. The RPE also synthesizes and releases retinoic acid at high levels (McCaffery et al., 1996). Extending these findings, it is likely through secreted retinoic acid that Müller cells and the RPE at least partially modulate the differentiation of neighboring neurons, including the regulation of outer segment membrane folding, and through retained retinoic acid that they modulate their own development.

In summary, our studies reveal that CRBP1 plays an indirect role in the modulation of photoreceptor outer segment folding. Our data also show that it functions through a mechanism that is mediated by retinoic acid. Both the RPE and Müller cells are thus intimately involved in the modulation and support of photoreceptor outer segment assembly. These data provide additional evidence to demonstrate that extrinsic factors provided by the RPE and Müller cells support outer segment assembly and elucidate that retinoic acid is a mediator between the unique cell types.

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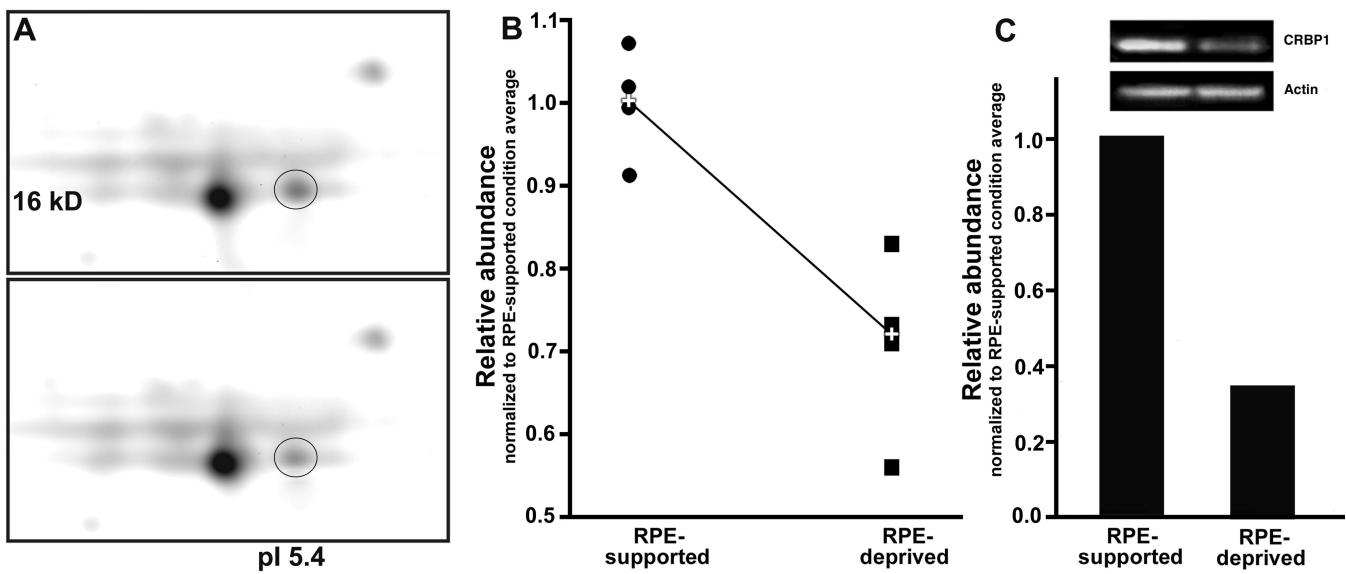
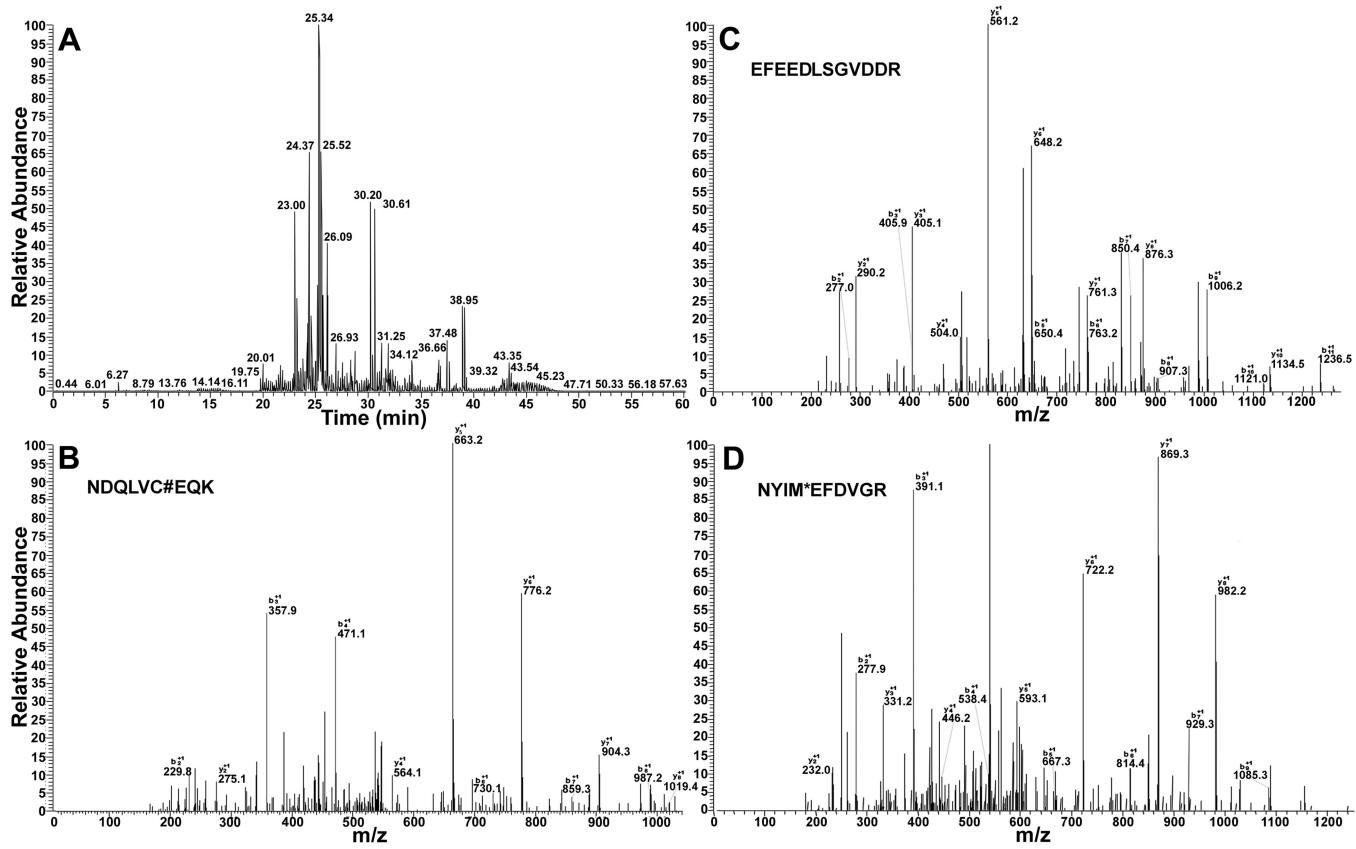


Figure 1.

Representative analysis of spot 22 from our previous investigation (Wang et al., 2009). (A) The Cy3 (RPE-supported; top) and Cy5 (RPE-deprived; bottom) images of the gel area surrounding the spot are indicated by black circles. The spot is located on the gel at a relative molecular weight of 16kD and pI of 5.4 (B) The relative abundance levels for each biological replicate are shown graphically. For spot 22, the levels of protein are consistently higher in samples derived from RPE-supported retinas. This difference is significant at $p=0.039$. (C) Semi-quantitative Western blot analysis performed on another biological replicate confirms the findings of the 2D DIGE analysis. Steady-state CRBP1 levels were normalized to actin levels to account for possible variations in the amount of protein loaded in each lane.

**Figure 2.**

(A) Base peak chromatogram for the LC-MS/MS analysis of spot 22. (B) through (D) MS/MS spectra for the three peptides ions from the LC-MS/MS analysis that matched to *Xenopus laevis* hypothetical protein MGC81232. The peptide ions (doubly-charged, $[M + 2H]^{2+}$, with precursor ion masses 567.25, 705.81, and 630.29) were matched to sequences NDQLVC#EQK (Xcorr 2.98; C# denotes carbamidomethyl-cysteine), EFEEDLSGVDDR (Xcorr 3.92), and NYIM*EFDVGR (Xcorr 3.02; M* denotes oxidized methionine), respectively.

A

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1 MSTNFNGYWK MLSNEHFEDY MKALDVNIAI RKIANLLKPD KEIIQNGDHM IIKTLSTFKN
61 YIMEFDVGRE FEEDLSGVDD RKCMTTVNWR NDQLVCEQKG EKIGRGWTQW MEGDELHLEM
121 RVGDRVVKQV FKKVQQ

```

B 77.6% identity in 134 residues overlap; Score: 594.0; Gap frequency: 0.0%

Xenopus mouse	1 MSTNFNGYWKMLSNEHFEDYMKALDVNIAIRKIANLLKPDKEIIQNGDHMIIKTLSTFKN
	1 MPVDFNGYWKMLSNEFEEYLRALDVNVALRKIANLLKPDKEIVQDGDHMIIRTLSTFRN
	* ***** *
Xenopus mouse	61 YIMEFDVGREFEEDLSGVDDRKCMTTVNWRNDQLVCEQKGEKIGRGWTQWMEGDELHLEM
	61 YIMDFQVGKEFEEDLTGIDDRKCMTTVSWDGDKLQCVQKGEKEGRGWTQWIEGDELHLEM
	* *
Xenopus mouse	121 RVGDRVVKQVFKKVQQ
	121 RAEGVICKQVFKKVH
	* * * * * * * * *

Figure 3.

(A) Protein sequence of *Xenopus laevis* hypothetical protein MGC81232. The peptides from our MS/MS analysis are shown in grey. A total of 3 peptides were matched for 24% sequence coverage throughout the length of the protein. (B) BLAST analysis of the protein sequence of *Xenopus laevis* hypothetical protein MGC81232 revealed that the *Xenopus* protein was 77.6% identical to mouse CRBP1. Identical matches between the two sequences are marked with an asterisk.

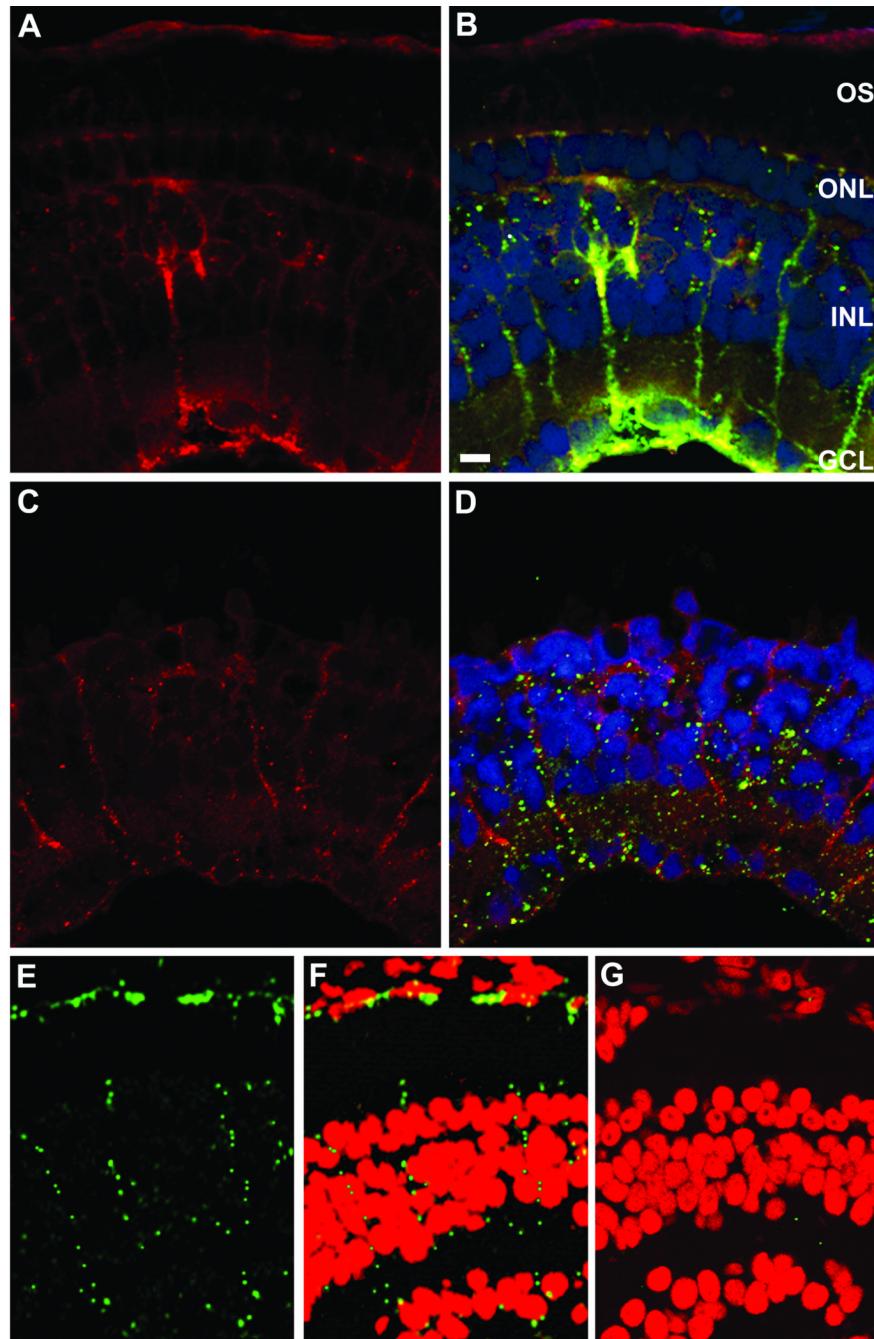
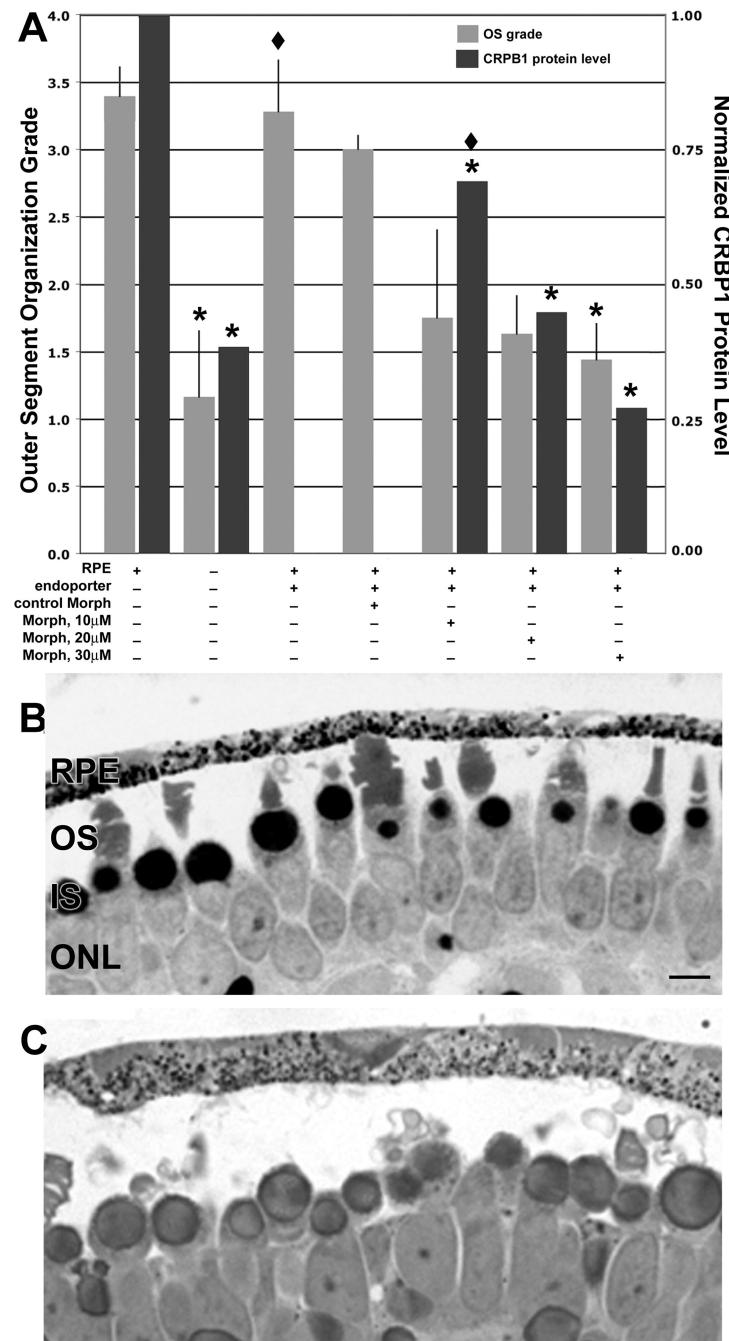


Figure 4.
(A–D) Localization of CRBP1 protein in the RPE and Müller cells using immunohistochemistry. Immunopositive labeling is shown in RPE-supported retinas in A & B and in RPE-deprived retinas in C & D. Red =CRBP1; green=glutamine synthetase; blue=nuclei; and yellow=co-localization of CRBP1 and glutamine synthetase in Müller cells. Labeling of CRBP1 protein is shown individually in A & C and overlapped with nuclei in B & D. (E–G) Localization of *rbp1* mRNA in the RPE and Müller cells using *in situ* hybridization. Green=*rbp1* mRNA; and red=nuclei. Labeling of *rbp1* mRNA is shown individually in E and overlapped with nuclei in F. G is a sense control. RPE=retinal pigment

epithelium; OS=outer segments; ONL=outer nuclear layer; INL=inner nuclear layer; GCL=ganglion cell layer. Mag bar=10 μ m.

**Figure 5.**

(A) Graphic representation of outer segment organization (grey bars) and normalized CRBP1 protein levels (black bars) in retinas exposed to Morpholinos. Exposure of retinas to endporter, which facilitates entry of the Morpholino into the cytoplasmic compartment, is included as a negative control. By one-way ANOVA, the overall F-test for differences among the groups was highly significant ($F=23.02$; p value <0.0001 for outer segment organization; $F=82.76$; p value <0.001 for CRBP1 protein levels). * p value <0.05 vs. RPE-supported retinas; ◆ $=$ p value <0.05 vs. RPE-deprived retinas. (B) Representative image of RPE-supported eye exposed to control Morpholino. (C) Representative image of RPE-

supported eye exposed to CRBP1 Morpholino. RPE=retinal pigment epithelium; OS=outer segments; IS=inner segments; ONL=outer nuclear layer. Mag bar=10 μ m.

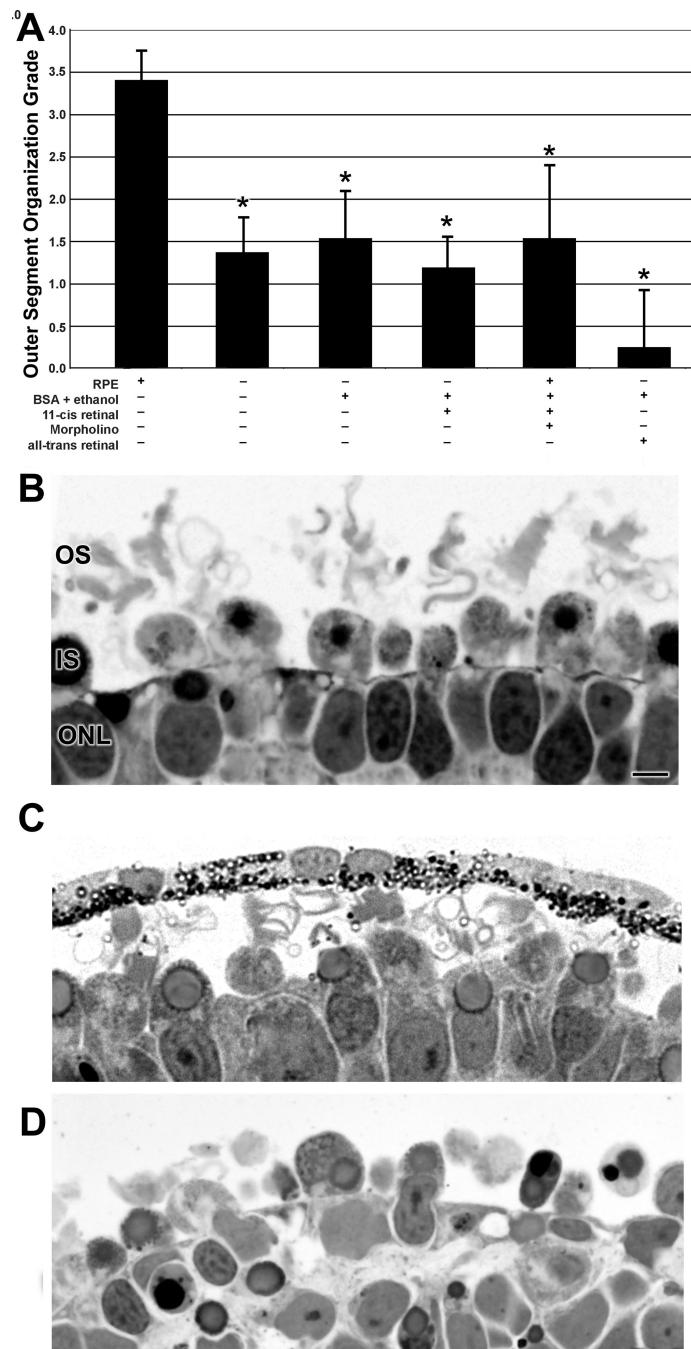


Figure 6.

(A) Graphic representation of the degree of organization of the outer segments of retinas exposed to 11-cis retinal. By one-way ANOVA, the overall F-test for differences among the groups was highly significant ($F=21.38$; p value<0.0001 for outer segment organization). * = p value<0.05 vs. RPE-supported retinas; ♦= p value<0.05 vs. RPE-deprived retinas. (B) Representative image from a retina exposed to 11-cis retinal. (C) Representative image from a retina exposed to Morpholino and 11-cis retinal. (D) Representative image from a retina exposed to all-trans retinal. OS=outer segments; IS=inner segments; ONL=outer nuclear layer. Mag bar=10μm.

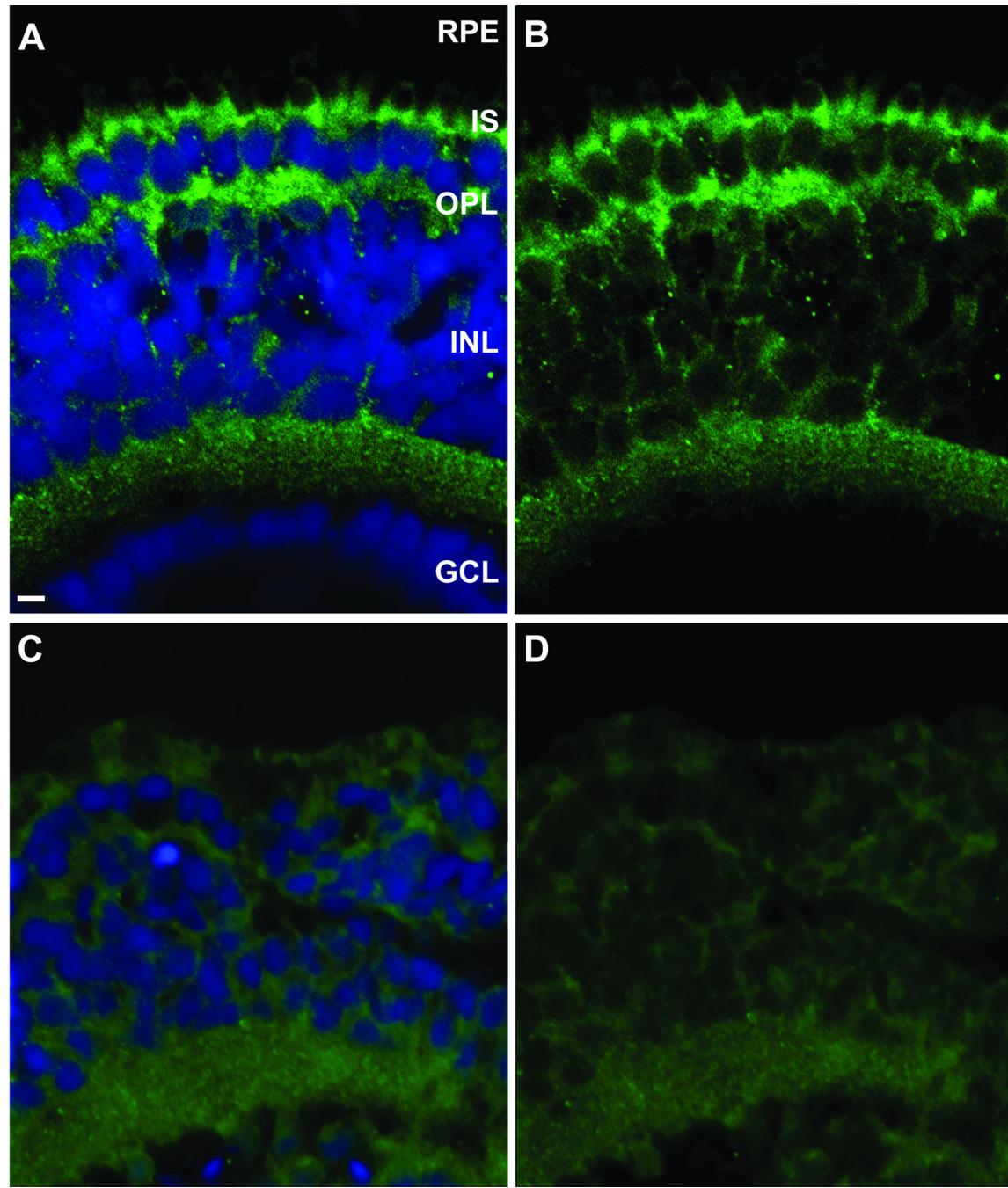
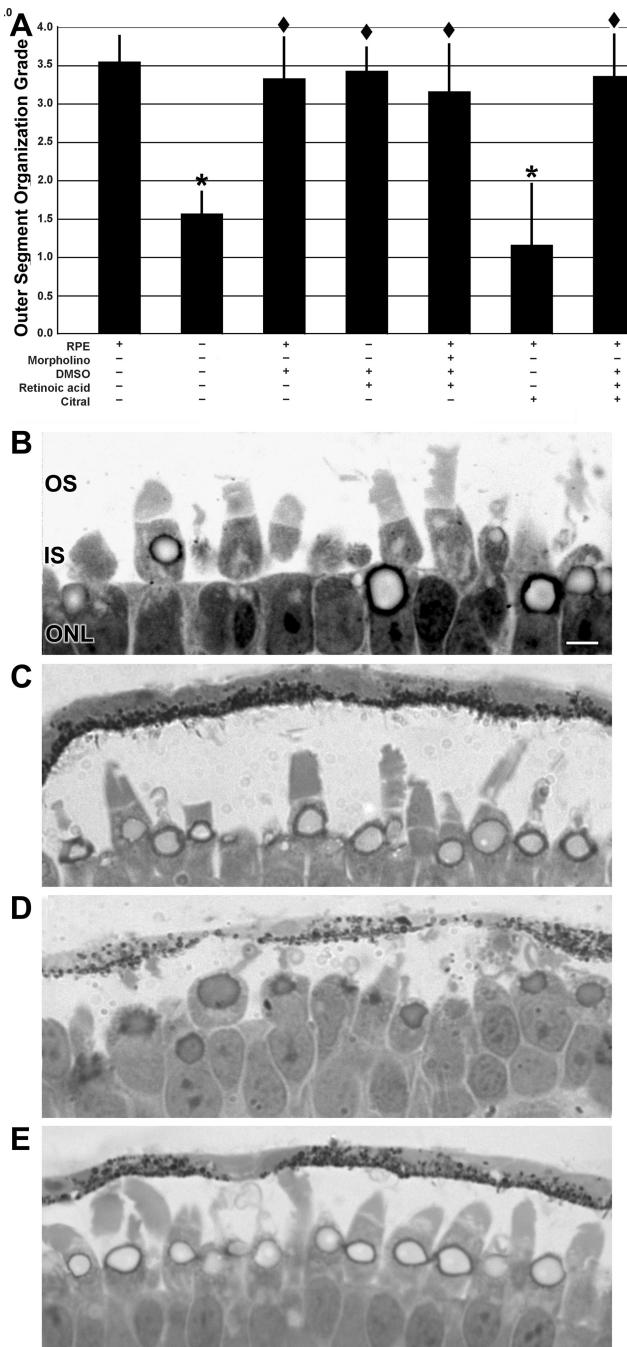


Figure 7.

Localization of retinoic acid in photoreceptors, the outer plexiform layer and Müller cells using immunohistochemistry. Immunopositive labeling is shown in RPE-supported retinas in A & B and in RPE-deprived retinas in C & D. Green=retinoic acid; and blue=ToPro III iodide labeling of nuclei. Labeling of retinoic acid is shown individually in right-hand panels and overlapped with nuclear stain in left-hand panels. RPE=retinal pigment epithelium; IS=inner segments; OPL=outer plexiform layer; INL=inner nuclear layer; GCL=ganglion cell layer. Mag bar=10 μ m.

**Figure 8.**

(A) Graphic representation of the degree of organization of the outer segments of retinas exposed to retinoic acid. By one-way ANOVA, the overall F-test for differences among the groups was highly significant ($F=21.52$; p value <0.0001). * $=p$ value <0.05 vs. RPE-supported retinas; ◆ $=p$ value <0.05 vs. RPE-deprived retinas. (B) Representative image from an RPE-deprived retina exposed to 0.5 μ M retinoic acid. (C) Representative image from an RPE-supported retina exposed to Morpholino and retinoic acid. (D) Representative image from an RPE-supported retina exposed to citral. (E) Representative image from an RPE-supported retina exposed to citral and 0.5 μ M retinoic acid. OS=outer segments; IS=inner segments; ONL=outer nuclear layer. Mag bar=10 μ m.