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Proteomic Analysis of the Retina: Removal of RPE Alters Outer Segment Assembly and Retinal Protein Expression

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Abstract

The mechanisms that regulate the complex physiologic task of photoreceptor outer segment assembly remain an enigma. One limiting factor in revealing the mechanism(s) by which this process is modulated is that not all of the role players that participate in this process are known. The purpose of this study was to determine some of the retinal proteins that likely play a critical role in regulating photoreceptor outer segment assembly. To do so, we analyzed and compared the proteome map of tadpole *Xenopus laevis* retinal pigment epithelium (RPE)-supported retinas containing organized outer segments with that of RPE-deprived retinas containing disorganized outer segments. Solubilized proteins were labeled with CyDye fluors followed by multiplexed two-dimensional separation. The intensity of protein spots and comparison of proteome maps was performed using DeCyder software. Identification of differentially regulated proteins was determined using nanoLC-ESI-MS/MS analysis. We found a total of 27 protein spots, 21 of which were unique proteins, which were differentially expressed in retinas with disorganized outer segments. We predict that in the absence of the RPE, oxidative stress initiates an unfolded protein response. Subsequently, downregulation of several candidate Müller glial cell proteins may explain the inability of photoreceptors to properly fold their outer segment membranes. In this study we have used identification and bioinformatics assessment of proteins that are differentially expressed in retinas with disorganized outer segments as a first step in determining probable key molecules involved in regulating photoreceptor outer segment assembly.

Keywords

photoreceptor; Müller cell; photoreceptor; proteins; mass spectrometry; cell interaction

INTRODUCTION

Vision begins at the level of the outer segment, the most highly specialized portion of retinal photoreceptors. Both intrinsic and extrinsic factors contribute to the health and survival of this unique neuron, and in particular to the integrity of the outer segments. In addition to photoreceptor-expressed proteins, it is highly likely that other retinal proteins participate in or regulate the complex process of outer segment assembly. To this end, the importance of an intact and fully functional RPE cell layer on photoreceptor integrity and survival has been known for many years. Outer segment synthesis is impaired in the absence of the RPE (Anderson et al. 1983). Moreover, RPE-secreted proteins promote photoreceptor differentiation and survival (Gaur et al. 1992; Jablonski et al. 2000; Sheedlo et al. 1998). Both lines of evidence indicate that interactions between photoreceptors and RPE are of fundamental importance for the support of outer segment assembly (Hollyfield and Witkovsky 1974).

Müller glial cells are also important role players in photoreceptor development and survival. Müller cells are coupled embryologically, physically, and trophically to photoreceptors (Cao et al. 1997; Newman and Reichenbach 1996; Reichenbach et al. 1993). Moreover, glial cells have been shown to regulate synaptogenesis in the brain (Ullian et al. 2001). During development, Müller cells, photoreceptors and a subset of inner retinal neurons descend from a single retinal progenitor cell and arrange themselves in a columnar fashion (Reichenbach et al. 1993; Turner and Cepko 1987) in which Müller cells surround photoreceptors from synaptic terminals to inner segments (Robinson and Dreher 1990). The coupling between Müller cells and photoreceptors is further demonstrated by our studies which document that targeted disruption of Müller cell metabolism with α -amino adipic acid results in disorganization of photoreceptor outer segments despite normal levels of opsin expression (Jablonski and Iannaccone 2000).

In our previous studies on the *Xenopus laevis* tadpole retina, we have demonstrated that removal of the RPE not only disrupts outer segment assembly, but also alters the protein expression profiles of both photoreceptors and Müller cells (Jablonski and Iannaccone 2001; Jablonski et al. 1999; Stiemke and Hollyfield 1994). It is presently unknown if the ability of the RPE to support outer segment assembly is direct upon photoreceptors themselves, or are indirect via Müller cells and/or other retinal neurons. The traditional candidate protein approach that we have used thus far to identify those proteins that are essential for outer segment assembly is inherently limited, labor-intensive, and time consuming. Therefore, to facilitate and expedite the delineation of the mechanisms underlying the complex process of photoreceptor outer segment assembly, we have incorporated 2D DIGE (difference in gel electrophoresis) methodology followed by mass spectrometry to identify those retinal proteins whose steady-state levels were altered in RPE-deprived *Xenopus laevis* tadpole retinas in which outer segments were elaborated, but not properly assembled. The results of this study strongly suggest that Müller cells are intimately involved in the regulation of photoreceptor outer segment assembly.

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. 2005). In brief, *Xenopus laevis* embryos were obtained through induced breeding and embryos were staged by external morphologic criteria (Nieuwkoop and Faber 1956). Eyes were removed from stage

33/34 tadpoles because at this developmental stage, outer segments are just beginning to be synthesized and the sclera has not yet formed allowing for ease of removing the RPE (Stiemke et al. 1994). While our experimental paradigm allows us to remove the RPE from its normal position next to photoreceptors, it does not eliminate the RPE. Rather, the RPE retracts away from the neuroepithelium and collects at the limbus (Supplement 1). Thus the influence of the RPE upon photoreceptor development is greatly minimized, yet the cell population remains present and it is therefore not necessary to subtract the RPE proteome from that of the RPE-supported condition to equalize the proteomes of the experimental conditions. In addition, because phagocytosis of outer segments by the RPE is not initiated until several stages after the harvesting of eyes (Stiemke and Hollyfield 1994), any potential proteome differences due to the absence of outer segment engulfment by the RPE are non-existent.

Groups of individual eyes were cultured in Niu-Twitty media at 23°C for three days. Approximately 300 tadpole eyes were collected and pooled for each experimental condition. Four biological replicates from four separate frog matings were analyzed, each replicate consisting of one pool of RPE-supported retinas and one pool of RPE-deprived retinas that were obtained from the same clutch of eggs.

2D DIGE and electrophoretic protein separation

Our 2D electrophoresis protocol has been previously published (Wohabrebbi et al. 2002) and was followed in these studies with minor modifications. Retinas were solubilized in lysis buffer (pH 8.5) containing Tris-HCl, thiourea, urea, and CHAPS. The PlusOne 2D Clean-Up Kit (GE Healthcare) was used prior to labeling protein extracts with CyDye DIGE fluors (GE Healthcare). Samples were multiplexed as outlined in Figure 2 and a total of 500 µg of protein was loaded onto each IPG strip (pH 4–7; GE Healthcare); approximately 167 µg of Cy3-labeled sample, 167 µg of Cy5-labeled sample and 167 µg of Cy2-labeled pooled internal standard. As shown in Supplement 2, dye-swapping was performed to eliminate potential dye bias. To further facilitate the reproducibility of the 2D gels, we used the Ettan DALTweleve system (GE Healthcare).

Gel Imaging and DeCyder™ analysis

Each 2D gel was imaged using a Typhoon 9410 Imager (GE Healthcare) and the appropriate filter sets. Using DeCyder™ software (GE Healthcare), each protein spot from all four gels was matched and the relative abundance of each CyDye-labeled protein was quantitatively compared. Because the identical Cy2-labeled pooled standard was included in all gels, any abundance differences that were due to inherent gel-gel variability were normalized, thus providing enhanced confidence in the variability of protein levels. After imaging, gels were silver stained using the PlusOne silver Staining kit (GE Healthcare) with modifications to ensure compatibility with mass spectrometry. Differentially expressed proteins were determined using Student's t-test that is part of the DeCyder™ software. Spots with p values of ≤0.1 were selected for mass spectrometric analysis. The average ratio of standardized log abundance change between RPE-deprived and RPE-supported eyes was expressed as a fold-change.

Mass Spectrometry and Database Searches

Differentially expressed protein spots were 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) as described previously (Giorgianni et al. 2004). TurboSEQUENT, part of Bioworks v.3.2 (ThermoFinnigan), was the peaklist-generating and search engine software used to analyze the nanoLC-MS/MS data. The following search parameters were used: **1.** enzyme specificity – trypsin; **2.** number

of missed cleavages permitted – 2; **3.** fixed modifications – none; **4.** variable modifications – methionine oxidation (+ 16 AMU) and cysteine carbamidomethylation (+ 57 AMU); **5.** mass tolerance of precursor ions - 2 AMU; **6.** mass tolerance of fragment ions - 1 AMU; **7.** SwissProt (May 2007 release, 245,534 protein entries) was the database searched with no species restrictions; **8.** the MS/MS data were also searched against the *Xenopus laevis* subset of the *nr* database (May 2007 release, 17,048 protein entries). All peptide sequences with statistically significant scores were verified by “blasting” them against the *All non-redundant GenBank CDS* database; and **9.** the Xcorr versus charge state cut-off scores for individual peptides were set at 5.0, 2.0 and 3.5, for single, double and triple charged peptide, respectively; these values have proven in our experience to be an excellent compromise between false and negative assignments. The five single-peptide matched proteins reported in this work were validated by manually analyzing the individual MS/MS spectra. All spectra contained expected fragment ion peaks whose height was at least 4 fold above the average noise level. All major peaks were assigned to the matched peptide sequences. For spots in which more than one protein was detected by nanoLC-ESI-MS/MS analysis, we selected as the differentially protein the one that was most represented by the ion peaks and had the best Xcorr values. Human or mouse orthologs for all *Xenopus* proteins that were identified in this study were determined by searching the Swiss-Prot database.

Ingenuity Pathways Analysis

To investigate whether these differentially expressed gene products belonged to specific pathways, we conducted Ingenuity Pathways Analysis on two separate lists of gene products, one of which consisted of photoreceptor proteins and the other of Müller cell proteins. This was done to enable us to discover, visualize and explore networks within each cell type that were relevant to our proteome analyses. The file containing Swiss-Prot identifiers was uploaded to Ingenuity.com. The genes on the uploaded lists, called Focus Genes, were then used as the starting point for generating biological networks. The application queried the Ingenuity Pathways Knowledge Base for interactions between Focus Genes and all other gene objects stored in the knowledge base, and generated two separate networks, one for photoreceptors and one for Müller cells, that are displayed graphically as nodes (genes/gene products/metabolites) and lines (the biological relationships between the nodes). Human, mouse, and rat orthologs of a gene, while stored as separate objects in the knowledge base, are represented as a single node in the network. Although phylogenetically distinct, we have made the assumption that similar pathways were acting in the *Xenopus*. Based upon the p-value calculated from the Fischer's exact test, a score was computed to indicate the probability that the network was generated by chance alone. A score of 2 or higher indicated at least a 99% confidence that the Focus Genes are not together in a network due to random chance.

Semi-Quantitative Western blot confirmation of differential protein expression

To confirm differential steady state protein levels of our focus proteins, we performed semi-quantitative Western blots. 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). SeaBlue (Invitrogen) prestained protein marker was used to estimate relative molecular weights. Proteins were transferred to PVDF membrane and analyses were performed using the Dylight 680/800 Western blotting kit (Pierce). The following primary antibodies were used in this analysis: anti-HSPD1 (Millipore); anti-PCNA (Santa Cruz); anti-RLBP1 (generously provided by Dr. John Saari, University of Washington); anti-YWHAZ (Millipore); anti-NDUFS3 (Mitosciences); anti-FABP7 (Millipore); anti-SOD1 (Novus Biologicals); and anti-actin (Millipore). Antibodies for other proteins were not available or they did not recognize the *Xenopus* antigen. The fluorescently-labeled secondary antibodies were applied and the blots were protected from

light. Membranes were scanned using the 700nm and 800nm channels of the LI-COR Odyssey Infrared imager. Anti-actin was included in all the blots to allow for normalization.

Western blot analysis of Müller cell line extract

To determine and/or confirm that our list of differentially expressed proteins was found in Müller cells, we performed Western blots on protein extracts from a rat Müller cell line (generously provided by Dr. Vijay Sarthy, Northwestern University). Identical amounts of protein were separated under reduced conditions using pre-cast 12% Tris glycine gels (Lonza) and proteins were transferred to nitrocellulose membranes. The following primary antibodies were used in this analysis: anti-HSPD1; anti-PCNA; anti-YWHAZ; anti-PSMA; anti-SOD1; and anti-actin. Antibodies for other proteins were not available or they did not recognize the *Xenopus* antigen. Western blots were performed using HRP-conjugated goat anti-mouse or anti-rabbit antibodies (Pierce) and developed using enhanced chemiluminescence substrate (SuperSignal WestFemto; Pierce). Blots were scanned on a Kodak 4000MM Image Station.

RESULTS

In RPE-supported eyes, newly elaborated photoreceptor outer segments were highly structured and formed typical cylindrical and cone-shaped profiles (Supplement 3A). In contrast, outer segments elaborated by RPE-deprived eyes were disorganized and lacked proper folding of nascent membranes (Supplement 3B).

The reproducibility of the 2D gels was excellent, thus facilitating spot matching. The use of 2D DIGE allowed us to determine that 27 unique spots contained protein amounts that differed statistically between RPE-supported and RPE-deprived eyes (Figure 1). Collectively, the spots varied both in isoelectric point and relative molecular mass and were present throughout the gel. There was one area that was consistently congested (approximate relative molecular mass range of 45–60 kD and isoelectric point range of 6.2–6.8), thus impeding our ability to resolve individual spots and determine which spots varied in steady-state protein levels. In other areas of the gel, a trail of spots at varying isoelectric points but the same relative molecular mass was noted (e.g., spots 1 and 2, and spots 3–6). Analysis of the mass spectrometry data obtained from the spots revealed the identities of all 27 proteins (Table 1). Although 27 unique spots were determined, 21 different proteins were identified. Four proteins were contained in two or more spots each. Two of these proteins differed only by isoelectric point (spots 1 and 2, and spots 3–6, Figure 1 and Table 1), while the other two differed by both isoelectric point and relative molecular mass (spots 13 and 14, and 24 and 25, Figure 1 and Table 1). Confirmation of the differential steady state levels was obtained for seven proteins (Supplement 4). We were unable to perform a similar analysis of several other proteins due to the lack of available antibodies that recognized the *Xenopus* antigen. Nonetheless, we predict that those would have also confirmed our 2D DIGE results.

Figure 2 and Figure 3 illustrate our methodological approach to the analysis of all 27 differentially expressed protein spots using spot 6 as an example. The portion of a representative 2D gel containing spot 6 was enlarged and the location of the spot was marked with an arrow. In this example, the protein sample from the RPE-supported and RPE-deprived eyes were labeled with Cy3 and Cy5, respectively and the intensity of the signal from spot 6 of the RPE-deprived sample was greater than that of the RPE-supported lysate (compare Figures 2A and 2B). Shown graphically in Figure 2C are the relative abundance levels of spot 6 from all four biological replicates that have been normalized to the average of the signal from the RPE-supported eyes. On average, the relative abundance of the protein in spot 6 was 39% greater in RPE-deprived samples ($p=0.044$, Table 1). The chromatogram from the nanoLC-MS/MS analysis for spot 6 is shown in Figure 2D. The MS/

MS spectrum of the peptide ion (doubly-charged; precursor ion mass 672.9 Da) that matched to the sequence TVIIEQSWGSPK is shown in Figure 2E. Illustrated in Figure 3 is the sequence of the full *Xenopus* hspd1 protein (black text) and those peptide matches that were obtained by our MS/MS analysis (grey text). This same methodology was used to identify the protein contained in the remaining 26 spots. A summary of this data is shown in Table 1.

Based upon the number of identified peptides, the peptide probability scores, the magnitude of the XCorr values and the quality of the spectrum, identities were obtained for all 27 spots. Because some spots contained the same protein as found in neighboring spots, only 21 unique proteins were identified. Of those 21 spots, 90% of them (19 spots) were identifiable in the subset of Swiss-Prot proteins containing only sequences from *Xenopus* (Table 1). The remaining two spots were identified through human or mouse orthologs. The number of peptides matched and percent amino acid coverage for each spot ranged from 1 to 13 and 4% to 41%, respectively (see Table 1). Single-peptide-based identifications are presented in Supplement 5.

The major functional groups to which the differentially expressed proteins belong, as determined from their Entrez Gene Summaries, are cell morphology/development, cell communication/signaling, cell cycle/DNA replication, nervous/visual system function, general cell function, or cell growth/proliferation/death (Table 2). Only one protein (i.e., MYL3) was a structural protein and therefore it did not fit within the broad functional categories represented in Table 2. One of the 21 unique proteins was an integral membrane protein (i.e., RHO), thus indicating that our experimental procedure was able to facilitate 2D separation of some hydrophobic proteins. We included GFAP and GLUL to our list of Müller cell proteins, because we have previously determined them to be altered in RPE-deprived *Xenopus laevis* tadpole eyes (Jablonski and Iannaccone 2001). Based upon their respective relative molecular weights and isoelectric points, it is highly probable that these two proteins were in the congested area of the gel that was previously described. A literature review revealed that 17 of the proteins that were differentially expressed under our experimental conditions are known to be expressed by and play a functional role in the eye (see Table 1 for expression patterns in various cell types). Those proteins are: HSPA8 (Al-Ubaidi et al. 2008;Dean et al. 1999), HSPD1 (Al-Ubaidi et al. 2008;West et al. 2001), ATP5B (Al-Ubaidi et al. 2008;Lupien et al. 2007;West et al. 2001), PCNA (Fimbel et al. 2007), RLBP1 (Saari 2000), GNB1 (Al-Ubaidi et al. 2008;Lupien et al. 2007;Whelan and McGinnis 1988), YWHAZ (Ivanov et al. 2006) (West et al. 2001), PSMA1 (Ferrington et al. 2008), NDUFS3 (Huang et al. 2004;Lupien et al. 2007), WNT2 (Fokina and Frolova 2006), LYZ (Bonilha et al. 2004;Tsai et al. 2006), RBP1 (Saari 2000), FABP7 (Hauck et al. 2003;Helle et al. 2003), SOD1 (Diehn et al. 2005;Huang et al. 2004;Imamura et al. 2006) (Lupien et al. 2007), RHO (Hargrave 2001), CRKRS (<http://www.dsi.univ-paris5.fr/genatlas/fiche.php?n=30221>), GLUL (Moscona et al. 1970), and GFAP (Lewis et al. 1989). It is striking that Müller cells rather than photoreceptors express the vast majority of differentially expressed proteins, 14 of 17 and 6 of 17, respectively. In Supplement 6 we provide confirmation of the expression of PCNA, PSMA and SOD1 in Müller cells along with the first direct evidence that Müller cells also express HSPD1 and YWHAZ. The remaining five of the proteins (i.e., LPLUNC1, RPSA, MYL3, FGF4 and WNT11) have not previously been shown to be expressed in the eye. We were unable to confirm the abundance of these five proteins and/or localize them due to the lack of available antibodies that cross-react with *Xenopus* tissues.

To begin to unravel the subcellular mechanism(s) responsible for the complex process of photoreceptor outer segment assembly, we created lists of photoreceptor- and Müller cell-expressed proteins and submitted them for Ingenuity Pathways Analysis (Gerling et al.

2006). The Ingenuity database contained information on all 23 proteins, and created two networks with highly significant scores of 23 and 41 for photoreceptor and Müller cell proteins, respectively. The final networks are shown in Figure 4 and Figure 5.

Because the Ingenuity network expanded upon our list of proteins to show a more global perspective of direct and indirect relationships between known proteins/metabolites that are differentially expressed in the absence of the RPE, some central nodes in the network are not our focus proteins. We accept that our analytical strategy is not able to determine 100% of those proteins present at different levels in retinas with disorganized outer segments because of technical limitations and rely on the Ingenuity analysis to bridge the gaps in the data generated in this analysis. Many proteins we identified in our analysis are ubiquitously expressed, therefore they are included in the pathways analysis of both cell types. Proteins that were central to both networks and likely play important roles in the regulation of gene product expression of both cell types are beta-estradiol and nuclear factor-kappa B (NFκB). Other shared proteins that are more peripherally located in each network are SOD1, HSPA8, HSPD1, ATP5B, NDUFS3 and PSMA1. Focus proteins that are unique to each pathway are RHO for photoreceptors and PCNA, GFAP, YWHAZ, FABP7, RBP1 and GLUL for Müller cells.

DISCUSSION

The retina is a complex structure composed of several neuronal cell types, the Müller glia, and the adjacent RPE. Within this tissue, photoreceptors are highly polarized and ultrastructurally unique neurons with a specialized structure called the outer segment. Functional and anatomical integrity of outer segments is essential for optimal vision. Outer segment membranous discs are continuously being renewed at the proximal end, while the distal ends are phagocytized by the RPE (Young 1967), therefore the mechanisms that govern outer segment assembly must operate effectively throughout the life of an individual. Despite the importance of its proper structure, the complex physiologic task of photoreceptor outer segment assembly remains an enigma. One limiting factor in revealing the mechanism(s) by which this process is regulated is that not all of the proteins or cell types that participate in this process are known. Because of this, determining the sequence of events that regulate this task and therefore possible steps at which therapeutic intervention might promote this process is quite difficult.

In this study, we sought to determine and identify those proteins that were differentially expressed in an experimental condition in which proper outer segment assembly was disrupted, so that many of the proteins that are involved in and possibly critical for the regulation of outer segment assembly could be determined. For this task, we selected the *Xenopus laevis* tadpole explant system. While it is a non-mammalian system, the intact *Xenopus* tadpole eye has many advantages, the most important being that amphibian photoreceptors are the only ones capable of elaborating significant amounts of outer segment material in the absence of the RPE (Stiemke et al. 1994). In all other vertebrate models, photoreceptors elaborate very few rudimentary outer segment membranous leaflets when separated from the RPE (Adler 1986; Adler 1987; Adler and Politi 1989; Araki et al. 1987; Caffé et al. 1989; Lahav 1987; LaVail and Hild 1971; Politi et al. 1988; Roof et al. 1991; Saga et al. 1996; Sehgal et al. 2006; Watanabe and Raff 1990). An additional feature of the *Xenopus* intact eye model is that in the RPE-deprived state, the membranes are disorganized (Jablonski and Ervin 2000; Jablonski et al. 1999; Stiemke and Hollyfield 1994; Stiemke et al. 1994), but abundant enough to make it possible to determine which proteins may be critical for assembly of outer segments. Moreover, eyes remain intact with no dissociation into single cells, therefore photoreceptors maintain their association with Müller radial glial cells and neighboring neurons. Accordingly, comparisons of photoreceptor

development in RPE-supported and RPE-deprived states can be assessed with minimal disturbance of photoreceptor interactions with other retinal elements and thus the contributions of all retinal cell types can be assessed simultaneously. In the present study, we exploited these unique features to reveal those proteins whose steady-state expression levels differed between RPE-supported retinas with organized and highly structured outer segments and RPE-deprived retinas with disorganized outer segments lacking properly folded membranes.

The results of this study demonstrate that proteomic studies can be readily performed using *Xenopus* as a model system, despite the fact that neither the *Xenopus laevis* nor *Xenopus tropicalis* genomes have been fully sequenced. The vast majority (90%) of the *Xenopus* proteins we identified were already listed in the Swiss-Prot database. Regarding the two proteins for which the *Xenopus* protein sequences were not known, identification of the protein of interest was readily accomplished by searching for mouse and/or human orthologs.

We have also generated a list of likely candidates that may be involved in regulating the ability of photoreceptors to assemble properly their outer segments. Most of the proteins are regulatory rather than structural proteins. MYL3 is the only exception. Importantly, MYL3 has never before described in the eye and its function in this organ is not known. This study has also contributed to the list of proteins expressed in the eye. Of the 21 differentially expressed proteins, five have not previously been reported as ocular proteins in the literature. Of course, it will be necessary to immunolocalize those proteins to determine whether they are expressed in cell types that may play a role in the regulation of outer segment assembly. Unfortunately, antibodies that recognize *Xenopus* orthologs of these proteins are not available, so the ocular cell types that express them remain unknown.

Examination of the differentially expressed proteins reveals seven candidates that are shared by photoreceptors and Müller cells, six of which are active participants in the unfolded protein response (UPR). UPR is a conserved mechanism that serves to preserve cellular function and promote survival in response to oxidative stress and the resultant accumulation of misfolded proteins in the endoplasmic reticulum (Malhotra and Kaufman 2007). The purpose of this response is two-fold: (1) to activate the expression of molecular chaperones to assist in protein folding; and (2) to reduce the translation of proteins so as to diminish the burden on the endoplasmic reticulum (Schröder and Kaufman 2005). We propose that our experimental results can best be explained by the retina experiencing an UPR in the RPE-deprived state (Figure 6). For the purposes of our discussion we will limit our theory to the photoreceptors and Müller cells, the cells most likely to influence outer segment assembly. We predict that the removal of the RPE leaves the retina susceptible to oxidative stress. The retina responds by increasing SOD1. The oxidative burden then leads to protein misfolding and upregulation of heat shock proteins as cells attempt to compensate for the presence of an increased load of unfolded proteins, which is an ATP-dependent process. Our data supports this by our demonstrated increase in HSPA8 and HSPD1, both heat shock proteins and ATP5B and NDUFS3, both mitochondrial ATP-generating enzymes. When proteins are not properly folded, they are targeted for proteosomal degradation. We measured an increase in PSMA1, the 20S proteasome alpha 5 subunit.

To compensate for an increased burden on protein folding mechanisms, transcription of some proteins is downregulated. Our study documents a decrease in expression of GNB1 and RHO, both photoreceptor proteins, along with RLBP1, RBP1, FABP7 and GLUL, all Müller cell proteins. Expression of other proteins is upregulated in our analyses. The known Müller cell proteins that show increased expression in the RPE-deprived state are PCNA and YWHAZ, both involved in cell survival (Chaum 2003; Paunesku et al. 2001) as well as

GFAP. None of the proteins we measured as upregulated is known to be expressed by the photoreceptors. Once the balance of normal steady-state expression levels has shifted due to UPR, we hypothesize that the other proteins unrelated to the UPR may play an active role in the regulation of outer segment assembly. These proteins, which will be evaluated in the following paragraphs, are GNB1, RHO, FABP7, GLUL, RLBP1, and RBP1.

It is unlikely that a decrease in the protein amounts of either GNB1 or RHO are responsible for improperly folded membranes. While disease-causing mutations have been documented for RHO (Dryja et al. 1993; Dryja et al. 1991; Dryja et al. 1990a; Dryja et al. 1990b; Farrar et al. 1990; Kijas et al. 2002; McWilliam et al. 1989; Nathans and Hogness 1984; Rosenfeld et al. 1992), a reduction in the amount of wild type RHO, as in RHO +/- mice, has no effect on outer segment structure (Liang et al. 2004). In addition, although a disruption of the *Gnb1* gene in the *Rd4* mouse is predicted to lead to retinal degeneration (Kitamura et al. 2006), at P8 when *Gnb1* levels are approximately half of that of wild type mice, outer segment structure appears normal (Roderick et al. 1997). It is only after *Gnb1* levels are immeasurable that outer segment structure is compromised (Kitamura et al. 2006; Roderick et al. 1997). Based upon these data, it is highly improbable that a reduction in either photoreceptor protein, in the absence of a mutation, would have dire consequences on outer segment membrane folding.

In contrast, several of the Müller cell protein we found to be dysregulated by RPE deprivation could be important for this critical physiological process. One likely candidate is FABP7. It is known that FABPs function to transfer long chain fatty acids. Elegant studies have documented that FABP7 has a high affinity for DHA (Politi et al. 2001) and that the membranes of the photoreceptor outer segments are highly enriched in DHA (Fliesler and Anderson 1983). Moreover Müller cells have been shown to take up DHA, incorporate it into their phospholipids and transfer them to photoreceptors (Politi et al. 2001). A decrease in this protein, as we document in the RPE-deprived state, could have negative consequences for outer segment folding and stability.

Another plausible Müller cell candidate is GLUL, which functions to convert glutamate to glutamine (Shaked et al. 2002). A reduced level of GLUL may impact neighboring neurons due to a resultant increase in glutamate. Studies indicate that while glutamate is neurotoxic at elevated levels, ganglion cells rather than photoreceptors are the cell type most affected (Sucher et al. 1997). GLUL expression has been widely hailed as an indicator of Müller cell differentiation (Piddington and Moscona 1965) that is dependent upon intercellular interactions (Linser and Moscona 1979; Linser et al. 1982). A more recent study has teased apart the intricacies of this relationship and has indicated that GLUL expression within the retina is tied not specifically to Müller cell differentiation, but rather to the development of synapses with neighboring photoreceptor cells in the outer plexiform layer and contact between photoreceptors and Müller cells at the outer limiting membrane, the site of the adherens junctions (Prada et al. 1998). Previously we demonstrated a very high correlation between the presence of adherens junctions at the outer limiting membrane and the ability of photoreceptors to properly assemble their outer segments (Jablonski and Ervin 2000). It is possible that the presence of the adherens junctions, rather than GLUL itself, promotes the formation of proper subcellular cytoarchitecture that may have downstream effects on outer segment membrane assembly and stability.

Two other Müller cell proteins that are also expressed by the RPE, RLBP1 and RBP1, are both involved in retinoid recycling that is required for vision (Saari 2000). Previous studies in the RLBP1 knockout mouse indicate that even in complete absence of the gene product, photoreceptor structure is not affected, thus making it unlikely that RLBP1 is playing an active role in outer segment assembly (Saari et al. 2001). In contrast RBP1 may be a more

critical protein for this process. It has been demonstrated in the RBP1 knockout mouse that retinal function and outer segment ultrastructure were compromised (Ghyselinck et al. 1999), thus making RBP1 a likely candidate to influence the proper folding of outer segment membranes.

Previously we demonstrated that disruption of Müller cell metabolism hampers the ability of photoreceptors to assemble properly (Jablonski and Iannaccone 2000). Moreover, other groups have shown that Müller cells, rather than photoreceptors, respond directly to survival factors (Wahlin et al. 2000). These data together with the outcomes of our present study provide strong evidence to demonstrate that Müller cells actively play a role in promoting the ability of photoreceptors to properly fold their outer segment membranes. The outcomes of this investigation have revealed specific candidate proteins that may be the intermediaries between the two cell types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Unfolded Protein Response

Figure 6.

Table 1

List of differentially expressed proteins

Spot No.	SwissProt ID of <i>Xenopus</i> protein	SwissProt ID of Human or Mouse Homolog	ID in Ingenuity Network	Protein Name	Expression pattern	Number of peptides matched	% Amino acid coverage	Average ratio ^{*,*}	P value
1	Q91993	P63017	HSPA8	Heat shock cognate 70 protein	PR ^{1,3} , MC ³ , RPE ³ , O ³	13	20	1.43	0.046
2	Q91993	P63017	HSPA8	Heat shock cognate 70 protein	PR ^{1,3} , MC ³ , RPE ³ , O ³	13	21	1.41	0.026
3	Q61P60	P63038	HSPD1	Hspd1 protein	PR ¹ , MC ^{2,1} , RPE ^{1,8}	5	9	1.42	0.041
4	Q61P60	P63038	HSPD1	Hspd1 protein	PR ¹ , MC ^{2,1} , RPE ^{1,8}	3	8	1.59	0.002
5	Q61P60	P63038	HSPD1	Hspd1 protein	PR ¹ , MC ^{2,1} , RPE ^{1,8}	7	17	1.71	0.021
6	Q61P60	P63038	HSPD1	Hspd1 protein	PR ¹ , MC ^{2,1} , RPE ^{1,8}	12	29	1.39	0.044
7	ND	Q8TDL5	LPLUNC1	Long palate, lung & nasal epithelium carcinoma associated	Unknown ⁺	4	18	1.39	0.036
8	Q6NWX0	P56480	ATP5B	Mitochondrial ATP synthase beta subunit	PR ¹ , MC ^{1,5} , RPE ^{1,8}	7	12	1.62	0.047
9	Q7ZX07	P14206	RPSA	Lamr1-prov protein	Unknown ⁺	6	24	1.44	0.043
10	P18248	P17918	PCNA	Proliferating Cell Nuclear Antigen	MC ^{6,2,1} , GC ⁶	1	4	1.53	0.081
11	Q6GLC8	Q9Z275	RUBP1	Cellular retinaldehyde-binding protein	MC ^{1,7} , RPE ^{1,7}	1	4	-1.37	0.047
12	P79959	P62874	GNB1	Guanine nucleotide binding protein	PR ^{1,20} , MC ^{1,5}	2	6	-1.34	0.041
13	Q91896	P63101	YWHAZ	14-3-3 protein zeta	MC ^{2,1} , RPE ^{1,8} , GC ^{1,3}	2	10	-1.48	0.035
14	Q91896	P63101	YWHAZ	14-3-3 protein zeta	MC ^{2,1} , RPE ^{1,8} , GC ^{1,3}	9	41	1.36	0.041
15	Q68A89	Q9Z2U1	PSMA1	20S proteasome alpha 5 subunit	PR ⁵ , MC ^{5,2,1} , RPE ⁵ , GC ⁵	1	5	1.42	0.055
16	Q6P613	Q53FM7	NDUFS3	NADH dehydrogenase Fe-S protein 3	PR ^{1,1} , MC ^{1,1,15} , GC ^{1,1} , O ^{1,1}	1	5	1.14	0.042
17	Q6P7Y5	P09542	MYL3	Myosin light polypeptide 3	Unknown ⁺	3	21	-1.26	0.054
18	P48806	P11403	FGF4	Fibroblast growth factor 4	Unknown ⁺	5	18	-1.55	0.034
19	P31283	P21552	WNT2	Protein Wnt-2 precursor	O ⁷	3	24	-1.67	0.038
20	Q5M8G0	P00702	LYZ	Lysozyme C precursor	RPE ² , O ^{1,8}	2	24	-1.31	0.055

Spot No.	SwissProt ID of <i>Xenopus</i> protein	SwissProt ID of Human or Mouse Homolog	ID in Ingenuity Network	Protein Name	Expression pattern	Number of peptides matched	% Amino acid coverage	Average ratio [*]	P value
21	Q66KR3	O96014	WNT11	Wnt-11-related protein	Unknown ⁺	1	5	1.32	0.034
22	Q6NU58	P09455	RBP1	Cellular retinol-binding protein	MC ¹⁷ , RPE ¹⁷	3	24	-1.36	0.039
23	Q8CE2	P51880	FABP7	Fatty acid binding protein, brain	MC ^{8,10} , GC ¹⁰	3	23	-1.58	0.044
24	P13926	P08228	SOD1	Cu-Zn superoxide dismutase	PR ¹² , MC ^{12,15,21} , O ¹¹	3	27	-1.20	0.014
25	P13926	P08228	SOD1	Cu-Zn superoxide dismutase	PR ¹² , MC ^{12,15,21} , O ¹¹	6	41	1.14	0.054
26	P29403	P15409	RHO	Rhodopsin	PR ⁸	2	18	-1.28	0.054
27	ND	Q9NYV4	CRKRS	Cell division cycle 2-like protein kinase 7	Unknown ⁺	3	26	-1.43	0.042
n/a	P51121	P15104	GLUL	Glutamine synthetase	MC ¹⁴	-	-	-	-
n/a	ND	P14136	GFAP	Glial fibrillary acidic protein	MC ¹⁴	-	-	-	-

^{*} = The average ratio shows the relative abundance level from all four biological replicates that have been normalized to the average of the signal from the RPE-supported eyes. A negative ratio indicates a decrease in the abundance of the protein in the RPE-deprived state, while a positive ratio indicates an increase in the abundance in the RPE-deprived state.

⁺ = The retinal expression pattern is not known. The data was not available in the literature and an antibody that recognizes the *Xenopus* protein was not available for us to determine within our lab which retinal cell type expresses it.

Key for references: 1=Al-Ubaidi et al. 2008; 2= Bonilha et al. 2004; 3= Dean et al.1999; 4= Diehn et al. 2005; 5=Ferrington et al. 2008; 6=Fimbel et al. 2007; 7=Fokina and Frolova 2006; 8=Hargrave 2001; 9=Hauck et al. 2003; 10=Helle et al. 2003; 11=Huang et al. 2004; 12=Imamura et al. 2006; 13=Ivanov et al. 2006; 14=Lewis et al. 1989; 15=Lupien et al. 2007; 16=Moscona et al. 1970; 17=Saari 2000; 18=Tsay 2006; 19=West et al. 2001; 20=Whelan and McGinnis 1988; 21= the present study.

Table 2

Functional categories to which differentially expressed proteins belong

Function/Role	Proteins involved [†]	Number involved
Cell Morphology and development	FABP7, FGF4, GFAP [*] , GNB1, HSPA8, HSPD1, MYL3, RBP1, RHO, RPSA, SOD1, WNT2, YWHAZ	13
Cell communication/signaling	FABP7, FGF4, GFAP [*] , GNB1, HSPD1, SOD1, RHO, RPSA, WNT11	9
Cell Cycle, DNA replication	CRKRS, FGF4, GNB1, HSPA8, HSPD1, PCNA, RHO, SOD1, YWHAZ	9
Nervous and Visual System function	FGF4, FABP7, GFAP [*] , GNB1, HSPD1, RBP1, RLBP1, RHO, SOD1	9
Cell Function	ATP5B, CRKRS, FABP7, FGF4, GLUL [*] , GNB1, HSPA8, HSPD1, MYL3, NDUFS3, PCNA, LPLUNC1, PMSA1, RBP1, RPSA, SOD1, LYZ	17
Cell Growth, Proliferation and Death	FGF4, HSPD1, PCNA, RBP1, RHO, SOD1, YWHAZ	7

^{*} GFAP and GLUL were not isolated through the DIGE analysis as described here, but have been previously shown to be dysregulated under RPE-deprived conditions. The protein names corresponding to the abbreviations can be found in Table 1.

[†] the protein names corresponding to the abbreviation used can be found in Table 1