

Retinoid-Processing in the RPE: Protein Interactions

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ABSTRACT

While the enzymology of the rod visual cycle and its many associated retinal diseases is becoming well characterized, the molecular details of retinoid processing protein interactions in the retinal pigment epithelium (RPE) remain less clear. This is due in part to the difficulties associated with characterizing interactions between RPE cytosolic proteins and membrane-associated enzymes. Toward a better understanding of RPE visual cycle protein interactions, we have refined our mass spectrometric methods to quantify immunoprecipitation (IP) products exhibiting a near-native protein mass and migrating in a single SDS-PAGE band. IPs were performed for RPE microsomes or RPE cytosol with antibodies to CRALBP, RPE65, and RDH5 in the presence or absence of detergents. IP products were separated by SDS-PAGE adjacent to controls without IP, followed by gel excision, in-gel tryptic digestion, ITRAQ labeling and LC-MS/MS. RPE microsomes and cytosol extracts were also fractionated by Blue Native (BN)-PAGE and proteins identified by LC-MS/MS. Protein identification utilized the Mascot search engine and the Swiss-Prot database. Protein quantification and statistical analysis utilized programs written in visual basic or in Excel. Reciprocal IP products from RPE microsomes in either Genapol or sodium cholate exhibited greater relative amounts of CRALBP, RPE65, LRAT, RDH5, and RGR than controls. From RPE microsomes, these proteins also migrated together in BN-PAGE. These results support a retinoid-processing RPE protein complex composed of CRALBP, RPE65, LRAT, RDH5, and RGR. From RPE cytosol without detergent, NHERF1 was recovered above background in anti-CRALBP IP products, consistent with other published results.

INTRODUCTION

A simplified version of the rod visual cycle is shown in Figure 1 along with a few retinal pathologies that have been associated with a malfunctioning visual cycle. Direct protein interactions have been reported between CRALBP and RDH5 (11-cis-retinoid dehydrogenase), between CRALBP and EBP50/NHERF1 [ERM (ezrin, radixin, moesin)-binding phosphoprotein 50, aka sodiumhydrogen exchanger regulatory factor 1] and between RDH5 (11-cis-retinoid dehydrogenase) and RGR (retinal G protein coupled receptor). Functional interactions appear to exist between CRALBP and RPE65 (the isomerohydrolase), and between RPE65 and LRAT (lecithin retinoyl acyltransferase). Other RPE proteins may also be involved in the visual cycle. Here we apply quantitative proteomic methods in combination with reciprocal immunoprecipitation and Blue Native gel electrophoresis to probe for visual cycle protein interactions in bovine RPE.

METHODS

Preparation of RPE Cytosol and Microsome. Bovine eyes were obtained from a local slaughter house and processed within 6 h of death. RPE cells were brushed from the eyecup in Ca²⁺ and Mg²⁺ free PBS containing protease inhibitors and 1 mM PMSF, washed 2-3x in PBS and the cytoplasmic and microsomal fractions prepared according to Saari and Brederberg (1988 J Biol Chem 263, 8084).

Immunoprecipitation. Visual cycle specific antibodies including rabbit polyclonal anti-CRALBP LW55, mouse monoclonal antibodies to RPE65, LRAT, and 11-cis-retinoid dehydrogenase (RDH5). Anti-LRAT and anti-RDH5 mAb were gifts from Dr K Palczewski. Control antibodies included rabbit anti-IgG and mouse anti-IgG. Antibody (200 µg) was covalently coupled to Sepharose Protein A beads using dimethylpimelidate. Prior to use, antibody coupled beads were incubated with ovalbumin (5% solution, 1h) to reduce nonspecific binding. Control RPE antibody and cytosolic fractions were also incubated with ovalbumin treated beads without antibody to evaluate nonspecific background proteins. For immunoprecipitation, 500 µg of RPE cytoplasmic or microsomal protein was incubated with 200 µg antibody coupled to beads in 200 µl of solubilization buffer for 2 hours at 40°C in the dark with gentle rocking. Two detergent-containing solubilization buffers that maintain visual cycle activity were used: (1) 0.1% Genapol, 10 mM phosphate buffered saline (PBS) and 7.5% 30% glycerol; and (2) 6 mM sodium cholate, 25 mM Hepes pH 7.5, 100 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 20 µM leupeptin. Prior to the addition of antibody beads, purified CRALBP (5% w/w) was added to the IP as bait. Following incubation, bound proteins were extracted 3x with solubilization buffer containing 2% SDS.

Protein Fractionation and Tryptic Digestion. Proteins in IP products and control samples were separated by SDS-PAGE in adjacent lanes and stained with Coomassie blue. Gel bands were excised from top to bottom, proteins reduced and alkylated with iodoacetamide and digested in gel with trypsin. Tryptic peptides were extracted and quantified with ITRAQ technology.

ITRAQ Labeling. Tryptic peptides were labeled with ITRAQ tags according to the vendor (Applied Biosystems). Following ITRAQ labeling, tryptic digests from adjacent gel bands were separated by SDS-PAGE in adjacent lanes and stained with Coomassie blue. Gel bands were excised from top to bottom, proteins reduced and alkylated with iodoacetamide and digested in gel with trypsin. Tryptic peptides were extracted and quantified with ITRAQ technology.

Protein Identification and Quantification. LC-MS/MS was performed with a CapLC system and a QTOF mass spectrometer (Waters). Peptides were separated on a 75 µm x 5 cm Biobasic C18 column using aqueous formic acid/acetonitrile solutions, a flow rate of ~250 n/min and an acetonitrile gradient. Protein identification utilized the Mascot (Matrix Science) search engines and the Swiss-Prot database. Analysis of ITRAQ labeling utilized a macro written in visual basic. Thresholds for protein quantification included a minimum of two peptides per identified protein with ITRAQ tag ion intensities ≥ 10 and Mascot ion scores ≥ 25. Quantification of proteins exhibiting intact mass by SDS-PAGE were reported.

Blue Native Gel Electrophoresis. Blue-native gel electrophoresis (BN-PAGE) separates protein complexes in their native state. Bovine RPE cytoplasmic and microsomal fractions (~50 µg) were resolved by non-denaturing BN-PAGE according to Invitrogen using precast Native PAGE[®] 3-12% Bis-Tris Gels and NativeMark[™] Protein Standards (Invitrogen). Gel bands were excised from the top to the bottom of the lane, proteins digested in gel with trypsin, and proteins identified by LC-MS/MS.

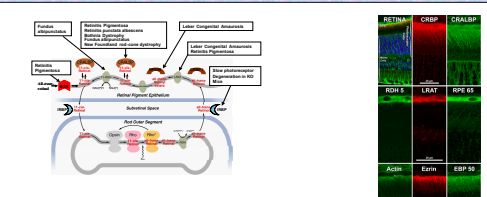


Figure 1. The Rod Visual Cycle. Schematic showing reactions involved in regeneration of rhodopsin (Rh) and some retinal dystrophies associated with the visual cycle. The tip of a rod photoreceptor and one disk membrane are shown with the adjacent retinal pigment epithelium (RPE) above. The enzymes of RPE are shown associated with a continuous, internal membrane in the absence of evidence for their localization. CRALBP is as an acceptor of 11-cis-retinal or 11-cis-retinoyl generated by RPE65 (the isomerohydrolase) or 11-RDh, respectively. LRAT, lecithin retinoyl acyltransferase, RDh, photoreceptor retinoid dehydrogenase; 11-RDh, 11-cis-retinoid dehydrogenase (RDH5); RGR, retinal G Protein coupled Receptor; CRBP1, cellular retinol binding protein 1; IRBP, interphotoreceptor matrix retinoid-binding protein.

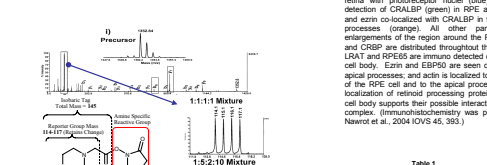


Table 1
ITRAQ Validation: RPE Microsomes Mixed 1:1

Accession	Protein	Ratio	Std Dev	Peptides
Q21975	RPE65	1.02	0.20	11
Q27979	RDH5	0.86	0.34	9
P12661	RBP	0.83	0.58	5
P10123	CRALBP	1.02	0.52	4
P42763	RGR	1.18	0.21	3
Q21982	LRAT	1.01		

Figure 2. Protein Identification and ITRAQ Based Quantification. The isotopic ITRAQ labeling chemistry is shown below a peptide precursor MS spectra and MS/MS spectra. The masses of ITRAQ reagent groups (114-117) and balance group are varied by the number of ¹³C, ¹⁵N and ¹⁶O within the structures. Quantitation is derived from the intensity of the reporter groups which are detected in an underlabeled region of the MS/MS spectra. Examples are shown of ITRAQ tag interferences from a six-peptide digest mixed in two different stoichiometries from 2004 Mol Cell Proteomics 3:12, 1154.

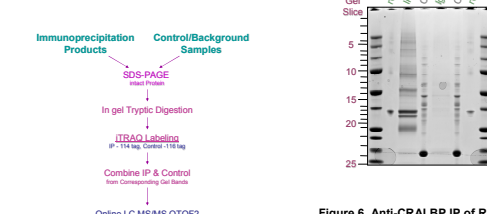


Figure 3. Subcellular Localization of Retinoid Processing Proteins. For orientation, the upper left panel shows immunohistochemical analysis of the albino mouse retina with photoreceptor nuclei (blue) and immunodetection of CRALBP (green), RPE and Müller cells and ezrin co-localized with CRALBP in the RPE apical processes (orange). All other panels represent enlargements of the region around the RPE. CRALBP and CRBP are distributed throughout the RPE. RDH5, LRAT and RPE65 are immunodetected only in the RPE cell body. Ezrin and EBP50 are seen only in the RPE apical processes, and ezrin is localized to the basal side of the RPE cell and to the apical processes. The co-localization of retinoid processing proteins in the RPE cell body supports their possible interaction in a protein complex. (Immunohistochemistry was performed as in Nawrot et al., 2004 IOVS 45, 393.)

Figure 6. Anti-CRALBP IP of RPE Cytosol. IP products from anti-CRALBP and from controls (ovalbumin or mouse anti-IgG treated RPE cytosol) were fractionated side-by-side with 0.1% Genapol digested RPE microsomal protein (~5 µg). Gel slices were excised from the top to the bottom of each lane. Following in-gel tryptic digestion, peptides were labeled with ITRAQ tags and IP and control digests from adjacent gel bands, corresponding in apparent mass, were combined and subjected to LC-MS/MS. Quantitative proteomic results are shown in Table 4.

Table 4. Anti-CRALBP Immunoprecipitation in RPE Cytosol

SwissProt Accession	Protein	CRALBP			Background			IP/Background		
		ITRAQ ratio	Unique Peptides	RSD	ITRAQ ratio	Unique Peptides	RSD	CRALBP		
P10123	CRALBP	12.33	7	57.1%	2.59	6	74.5%	4.76		
Q21975	RPE65	0.76	1	100%	0.58	3	7.7%	2.02		
Q27979	RDH5	1.62	3	19.7%						
Q32K28	EBP50/NHERF1	7.93	3	66.3%						
P31576	Ezrin	6.27	1	72.5%						
A52968	Heat shock 70kDa protein 8	1.39	0	53.6%	0.25	11	33.1%	5.47		
A52961	Actin	0.68	11	44.4%	0.15	16	31.5%	4.63		
A6H7E0	Lactate dehydrogenase A	0.42	1	100%	0.18	10	69.7%	2.40		
Q56G13	Perovirion-2	0.78	0	7.2%	0.34	5	64.2%	2.27		
Q5E4E1	Creatine kinase B-type	0.28	9	37.3%	0.12	11	38.3%	2.21		
ASP_38	Lactate dehydrogenase B	0.34	5	40.8%	0.16	16	31.5%	2.01		
Q310P3	Phosphoglycerate kinase 1	0.20	0	30.4%	0.10	6	39.3%	1.95		
Q9R9J5	Chaperlin D	0.31	1	100%	0.16	3	29.4%	1.92		
P09211	Carbonic anhydrase 2	0.17	6	31.5%	0.10	14	34.0%	1.67		
A52984	Pyruvate kinase	0.21	25	43.3%	0.13	25	34.2%	1.66		
Q7LVZ2	Heat shock protein HSP 90-alpha	0.26	11	33.9%	0.16	14	34.5%	1.59		
Q9KJL4	Enolase alpha	0.18	19	86.0%	0.13	20	42.7%	1.35		
Q21H86	Tubulin alpha	4.89	3	49.8%						

BACKGROUND AND RESULTS

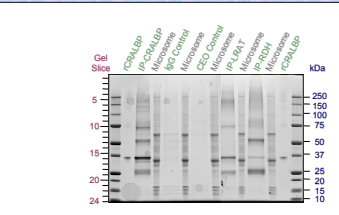


Figure 4. Reciprocal Immunoprecipitation of RPE Microsomes in Genapol. Reciprocal IP products from anti-CRALBP, RDH5, LRAT, and RPE65 and from controls (ovalbumin or mouse anti-IgG treated microsomes) were fractionated side-by-side with 0.1% Genapol digested RPE microsomal protein (~5 µg). Gel slices were excised from the top to the bottom of each lane. Following in-gel tryptic digestion, peptides were labeled with ITRAQ tags and IP and control digests from adjacent gel bands, corresponding in apparent mass, were combined and subjected to LC-MS/MS. Quantitative proteomic results are shown in Table 2.

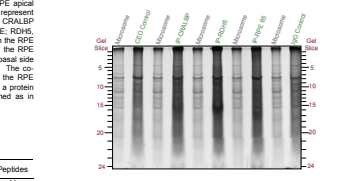


Figure 5. Reciprocal Immunoprecipitation of RPE Microsomes in Cholate. Reciprocal IP products from anti-CRALBP, RDH5, and RPE65 and from controls (ovalbumin or mouse anti-IgG treated microsomes) were fractionated side-by-side with 0.1% Sodium Cholate digested RPE microsomal protein (~5 µg). Gel slices were excised from the top to the bottom of each lane. Following in-gel tryptic digestion, peptides were labeled with ITRAQ tags and IP and control digests from adjacent gel bands, corresponding in apparent mass, were combined and subjected to LC-MS/MS. Quantitative proteomic results are shown in Table 2.

Table 2. Anti-CRALBP, -RDH5, -LRAT and -RPE65 Reciprocal Immunoprecipitation of RPE Microsomes in Genapol¹

SwissProt Accession	Protein	CRALBP			RDH5			LRAT			RPE65			Background ²			IP Products/Background					
		ITRAQ ratio	Unique Peptides	RSD	ITRAQ ratio	Unique Peptides	RSD	ITRAQ ratio	Unique Peptides	RSD	ITRAQ ratio	Unique Peptides	RSD	ITRAQ ratio	Unique Peptides	RSD	CRALBP	RDH5	LRAT	RPE65	Average	
Q21975	RPE65	1.02	11	19.7%	0.21	4	21.2%	0.24	5	52.2%	0.19	3	81.2%	4.55	147	2.25	2.80					
A52961	Actin	0.48	4	52.0%	0.39	2	59.0%	0.16	2	16.2%	0.25	5	21.2%	0.15	4	15.0%	3.18	2.00	1.00	1.60	2.10	
Q27979	RDH5	0.80	2	38.2%	0.54	2	50.7%	0.24	1	10.0%	0.25	4	30.7%	0.20	2	23.0%	1.00	0.50	0.50	0.50	0.50	
Q21982	LRAT	1.00	4	33.3%	0.39	5	28.2%	0.33	5	24.2%	1.08	4	30.1%	0.74	2	8.7%	4.08	0.50	2.00	1.50	1.50	
P10123	CRALBP	0.80	2	24.0%	0.75	2	25.0%	0.22	3	29.2%	0.22	3	29.2%	0.20	2	19.1%	1.00	0.50	0.50	0.50	0.50	
Q27979	RDH5	0.38	15	61.3%	0.38	16	113.0%	0.27	14	31.4%	0.11	9	65.8%	0.07	2	7.8%	1.04	1.05	1.05	1.05	1.05	
Q21982	LRAT	1.28	1	100%																		
P31576	Ezrin	1.02	6	19.7%	0.87	1	5.6%				5.27	7	34.3%									
Q222C6	Smilee to sporadic hyaline 1	0.70	6	27.1%	0.23	4	21.2%				0.34	5	62.2%	0.19	3	81.2%	4.55	1.47	2.25	2.80		
A52961	Actin	0.48	4	52.0%	0.39	2	59.0%	0.16	2	16.2%	0.25	5	21.2%	0.15	4	15.0%	3.18	2.00	1.00	1.60	2.10	
Q27979	RDH5	0.80	2	38.2%	0.54	2	50.7%	0.24	1	10.0%	0.25	4	30.7%	0.20	2	23.0%	1.00	0.50	0.50	0.50	0.50	
Q21982	LRAT	1.15	3	36.2%	0.59	2	9.5%	0.07	2	16.0%	0.24	5	21.2%									
Q27979	RDH5	0.80	2	38.2%	0.54	2	50.7%	0.24	1	10.0%	0.25	4	30.7%	0.20	2	23.0%	1.00	0.50	0.50	0.50	0.50	
A20106	Calnexin-transporter Atras	0.33	7	54.7%				1.13	2	2.1%	0.34	7	49.0%									
Q27979	RDH5	0.80	2	38.2%	0.54	2	50.7%	0.24	1	10.0%	0.25	4	30.7%	0.20	2	23.0%	1.00	0.50	0.50	0.50	0.50	
Q21982	LRAT	1.00	4	33.3%	0.39	5	28.2%	0.33	5	24.2%	1.08	4	30.1%	0.74	2	8.7%	4.08	0.50	2.00	1.50	1.50	

¹ Bovine RPE microsomes were solubilized in 0.1% Genapol, immunoprecipitated with the indicated antibodies, fractionated by SDS-PAGE and analyzed by LC-MS/MS as described in methods. Contaminants are not shown (n = 6) from photomicrographs. Blank, microtubule, nucleus and lysates.

² Background measurements are the bound components of Genapol solubilized RPE microsomes incubated with ovalbumin treated Sepharose protein A beads.

Table 3. Anti-CRALBP, -RPE65, and -RDH5 Reciprocal Immunoprecipitation of RPE Microsomes in Cholate¹

SwissProt Accession	Protein	CRALBP			RPE65			RDH5			Background ²			IP Products/Background							
		ITRAQ ratio	Unique Peptides	RSD	ITRAQ ratio	Unique Peptides	RSD	ITRAQ ratio	Unique Peptides	RSD	ITRAQ ratio	Unique Peptides	RSD	CRALBP	RPE65	RDH5	Average				
P10123	CRALBP	209.14	1	100%	21.14	1	58.4%	19.70	11	61.7%	14.90	11	24.2%	20.20	2.11	5.37	1.18				
Q21975	RPE65	2.51	2	28.0%	0.23	6	32.0%	0.11	4	23.0%	0.21	2	21.0%	0.11	2	11.0%	2.00	1.00	1.00	1.00	1.00
Q27979	RDH5	14.10	11	29.1%	0.53	8	29.6%	0.35	9	29.2%	1.08	3	16.2%	7.30	4.70	7.40	1.40				
Q21982	LRAT	14.27	12	27.5%	0.44	4	24.0%	0.21	11	11.1%											
P42763	RGR	2.46	2																		