

Quantitative Mass Spectrometric Analysis of CRALBP Protein Interactions

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ABSTRACT

Purpose: Previous studies suggest that cellular retinaldehyde-binding protein (CRALBP) functions in the rod visual cycle as a component of a RPE retinoid-processing protein complex. Specific methods are under development for the identification and relative quantification of CRALBP protein interactions.

Methods: CRALBP-protein interactions were sought in bovine RPE microsomes by immunoprecipitation with CRALBP antibody covalently bound to Protein A agarose beads. Immunoprecipitates were digested with trypsin, peptides labeled with ITRAQ amine-specific tags and identified and quantified by LC-MS/MS. IP products were fractionated either by SDS-PAGE prior to tryptic digestion or by SCX ion exchange chromatography after proteolysis. Quantitation was obtained relative to control samples of RPE microsomes.

Results: CRALBP, RPE65, LRAT, RDH5, and RGR often co-precipitate from RPE microsomes with anti-CRALBP antibodies along with a variable number of other proteins. Other proteins include photoreceptor and blood components as well as other potential RPE visual cycle components. Quantified control samples in 11 protein groups are used to provide discrimination between background and authentic CRALBP interaction partners.

Conclusions: Visual cycle proteins CRALBP, RPE65, LRAT, RDH5, and RGR appear to interact in a rod visual cycle protein complex. The ITRAQ labeling and quantification of CRALBP and one disk membrane are shown with the adjacent retinal pigment epithelium (RPE) and the outer nuclear layer. The RPE is shown associated with a continuous, internal membrane in the absence of evidence of its localization. CRALBP is an acceptor of 11-*cis*-retinol or 11-*cis*-retinal generated by RPE65 (the isomerase of RPE65). The RDH, respectively, LRAT, is/are retinoid acyltransferase, RDH, photoreceptor retinoid dehydrogenase, 11-RDH, 11-*cis*-retinyl dehydrogenase (RDH5), RGR, retinal G protein-coupled receptor, CRBP1, cellular retinoid binding protein, 1-RBP, interphotoreceptor matrix retinoid-binding protein.

BACKGROUND

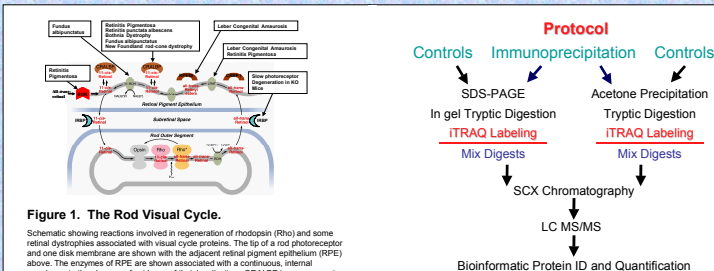


Figure 1. The Rod Visual Cycle.

Schematic showing reactions involved in regeneration of rhodopsin (Rho) and some retinal dystrophies associated with visual cycle proteins. The tip of a rod photoreceptor and one disk membrane are shown with the adjacent retinal pigment epithelium (RPE) and the outer nuclear layer. The RPE is shown associated with a continuous, internal membrane in the absence of evidence of its localization. CRALBP is an acceptor of 11-*cis*-retinol or 11-*cis*-retinal generated by RPE65 (the isomerase of RPE65). The RDH, respectively, LRAT, is/are retinoid acyltransferase, RDH, photoreceptor retinoid dehydrogenase, 11-RDH, 11-*cis*-retinyl dehydrogenase (RDH5), RGR, retinal G protein-coupled receptor, CRBP1, cellular retinoid binding protein, 1-RBP, interphotoreceptor matrix retinoid-binding protein.

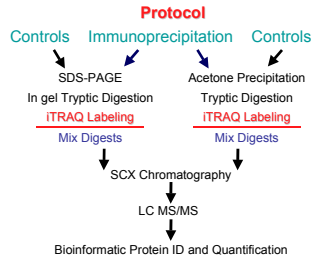


Figure 3. Methods Overview.

INTRODUCTION

The visual cycle is the complex enzymatic retinoid-processing involved in regenerating bleached rod and cone visual pigments. A simplified version of the rod visual cycle is shown in Figure 1 along with a few of the retinal pathologies that have been associated with abnormal visual cycle proteins. Preliminary proteomic results suggest the existence of a retinoid processing protein complex in the RPE. Direct protein interactions have been reported between CRALBP and RDH5 (11-*cis*-retinyl dehydrogenase) between CRALBP and RPE65 (retinoid G-protein phosphatase) and between CRALBP and RPE65 (the isomerase of RPE65). Other RPE proteins may also be involved in this complex. We used reciprocal immunoprecipitation and ITRAQ quantitative mass spectrometric methods to probe for visual cycle protein interactions in bovine RPE microsomes. The ITRAQ technology utilizes amine-specific tags and yields diagnostic protein ratios (1:14, 1:15, 1:16) during MS/MS analysis of the labeled peptides. The intensity of these ions provides relative peptide quantitation. This is an emerging quantification technology that has now been utilized for comparing protein expression in yeast and *E. coli*, quantifying lysine phosphorylation, measuring circadian changes in peptide levels, assessing protein-protein interactions in vitro, and cancer biomarker discovery. This poster presents initial efforts in applying ITRAQ technology to characterize protein interactions involving both cytosolic and membrane associated retinoid processing proteins.

METHODS

Preparation of RPE Microsomes. Bovine eyes were obtained from a local slaughter house and processed within 6 h of death. Following dissection of the globe behind the limbus, the anterior segment and vitreous were discarded and the retina removed. RPE cells were rapidly but gently brushed from the eye using an artist's 7 mm angular paraffin brush and Ca²⁺ and Mg²⁺ free 1x PBS containing protease inhibitors and 1 mM PMSF (2x 500 μl). The RPE cells were washed 2-3x by centrifugation in PBS and the microsomal fractions prepared according to Saari and Brederberg (1988 J Biol Chem 263, 8084).

Immunoprecipitation. Visual cycle specific antibodies routinely utilized were rabbit polyclonal anti-CRALBP (UW55), mouse monoclonal anti-RPE65, and mouse monoclonal anti-RDH5 (anti-RDH5 mAb was a gift from Dr K Palczewski). Non-specific control antibodies include anti-MOPC. Antibody (200 μg) was covalently coupled to Sepharose Protein A beads using dimethylglycylimide. Prior to use, beads were incubated with ovalbumin (5% solution, 1h) to block non-specific binding. In a typical immunoprecipitation experiment, 500 μg of RPE microsomal protein was incubated with 200 μg antibody coupled to beads in 200 μl of solubilization buffer for 1-2 hour at 4°C in the dark with gentle rotation. Another 500 μg of the microsomes with and without antibody treated beads without antibody. Two detergent solubilization solutions that maintain visual cycle activity were used: (1) 0.1% Genapol, 10 mM phosphate buffered saline (PBS) pH 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 (1.5 μg/ml) was added to the RPE microsomes. Following incubations, the beads were collected by centrifugation, washed with solubilization buffer and bound proteins extracted with solubilization buffer (20 μl x 3) containing 2% SDS.

ITRAQ Labeling. ITRAQ labeling was performed according to the ITRAQ kit from Applied Biosystems. IP and control reaction products and microsomal reference samples were digested with trypsin and peptides labeled with one of four diagnostic ITRAQ tags (ie, m/z 114, 115, 116 or 117). Usually the labeled experimental digest was mixed 1:1 with an unlabeled reference digest and the mixture separated by strong cation exchange (SCX) chromatography and fractions collected. This approach employed acetone precipitation prior to tryptic digestion to lower the SDS concentration. Alternatively, IP and control samples were digested with 5 μg of normal reference digest and the mixture separated by strong cation exchange (SCX) chromatography and fractions collected. This approach employed acetone precipitation prior to tryptic digestion to lower the SDS concentration. Alternatively, IP and control samples were digested with 5 μg of normal reference digest and the mixture separated by strong cation exchange (SCX) chromatography and fractions collected. This approach employed acetone precipitation prior to tryptic digestion to lower the SDS concentration. Alternatively, IP and control samples were digested with 5 μg of normal reference digest and the mixture separated by strong cation exchange (SCX) chromatography and fractions collected. This approach employed acetone precipitation prior to tryptic digestion to lower the SDS concentration.

Protein Identification. Proteins were identified by LC MS/MS using a CapLC system and a QTOF2 mass spectrometer (Waters). Peptides were separated on a 75 μm x 5 cm Biobasic C18 column (New Objective, Cambridge, MA) using aqueous formic acid/acetonitrile solutions, a flow rate of ~250 n/min and an acetonitrile gradient. Bioinformatics included ProteinLynx, TM Global Server (Waters Corporation) and Mascot (Matrix Science) search engines and the Swiss-Prot and NCBI protein sequence databases. Analysis of ITRAQ labeling utilized a macro written in visual basic.

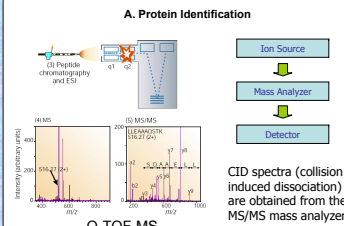


Figure 2. Protein Identification and Quantification.

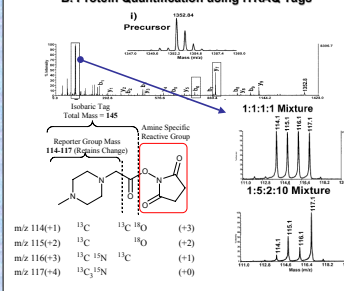


Figure 2. Protein Identification and Quantification.

(A) Protein identification was by MS/MS peptide sequence analysis using a QTOF2 mass spectrometer. (B) ITRAQ Protein Quantification. The isotopic ITRAQ labeling chemistry is shown below a peptide precursor MS spectra and MS/MS spectra. The masses of ITRAQ reporter groups (114-117) and balance groups are varied by the number of ¹³C, ¹⁵N and ²H in and the structures. Quantitation is derived from the intensity of the reporter groups which are detected in an unlabeled region of the MS/MS spectra. Examples are shown of ITRAQ tag intensities from a 500 μg protein digest mixed in two different stoichiometries from 2004 Mass Cell Problems 3.12, 1154).



Figure 4. Subcellular localization of RPE proteins.

For orientation, the upper left panel shows immunohistochemical analysis of the albino mouse retina with photoreceptor nuclei (blue) and immunodetection of CRALBP (green) in RPE and Muller cells and exrn 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 actin 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. However, based on these localization results, we expect the composition of such a protein complex to be different in the RPE apical process. (Immunohistochemistry was performed as in Nawrot et al., 2004 CV95 45, 393.)

Table 1 Protein IP'd from RPE Microsome without Quantification

Protein	Accession number	anti-CRALBP	anti-RPE65	anti-RDH5	anti-RGR	anti-Ezrin	anti-EBP50	anti-Actin	Sum
Cellular retinaldehyde binding protein (CRALBP)	P1020	+	+	+	+	+	+	+	5
Interphotoreceptor retinoid binding protein (IRBP)	P1261	+	+	+	+	+	+	+	5
Retinal pigment epithelium-specific protein (RPE65)	Q2979	+	+	+	+	+	+	+	5
11-cis Retinyl dehydrogenase (RDH5)	Q2979	+	+	+	+	+	+	+	5
Actin	R0276	+	+	+	+	+	+	+	5
Glyoxylate 3-phosphate dehydrogenase	P1066	+	+	+	+	+	+	+	5
Actin	P1066	+	+	+	+	+	+	+	5
Tubulin beta	P0407	+	+	+	+	+	+	+	5
Tubulin beta	P0407	+	+	+	+	+	+	+	5
Interphotoreceptor matrix retinoid-binding protein	P1261	+	+	+	+	+	+	+	5
Hemoglobin beta	P0102	+	+	+	+	+	+	+	4
Hemoglobin beta	P0102	+	+	+	+	+	+	+	4
RPE-retinal G protein coupled receptor (RGR)	P4703	+	+	+	+	+	+	+	4
Leixin retinoid acyltransferase (LRAT)	Q2982	+	+	+	+	+	+	+	4
Leixin retinoid acyltransferase 1 (LRAT1)	Q2982	+	+	+	+	+	+	+	4
Endocase alpha	Q8244	+	+	+	+	+	+	+	3
Frustrin beta/intermediate filin A	P0475	+	+	+	+	+	+	+	3
Haemoglobin 2 beta	P0102	+	+	+	+	+	+	+	3
Rhodopsin	P0058	+	+	+	+	+	+	+	3
Vimentin	P04616	+	+	+	+	+	+	+	3
Total Protein Identified		327	109	235	344	129	827		

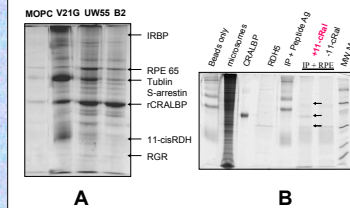


Figure 5. Representative Immunoprecipitations.

Consistent bias stained SDS-PAGE of immunoprecipitation products, microsomal preparations and reference proteins are shown. Gels were excised for in situ tryptic digestion and protein identification by LC MS/MS. Select protein bands are labeled. A. Immunoprecipitations of RPE microsomal proteins with three different anti-CRALBP antibodies and control anti-MOPC V21G. pAb anti-peptide antibody UW55. pAb to intact CRALBP. B2. mAb to intact CRALBP. The UW55 pAb is our antibody of choice. B. Immunoprecipitation with anti-RDH5 mAb. C. Immunoprecipitation with anti-RPE65 mAb.

Table 2 ITRAQ Validation - RPE Microsomes Mixed 1:1

Accession	Protein	Ratio	St Dev	Peptides (115/116)
Q28175	RPE65	0.20	11	
Q2979	RDH5	0.86	0.34	9
P12611	IRBP	0.83	0.26	5
P10123	CRALBP	1.02	0.52	4
P47803	RGR	1.18	0.21	3
Q08212	LRAT	1.01	-	2

Two aliquots of a tryptic bovine RPE microsomes were labeled with 115 or 116 ITRAQ tags, mixed 1:1, fractionated by SCX and analyzed by LC MS/MS. Quantitation of retinoid processing proteins yielded ratios of ~1.

Table 4 ITRAQ Quantification anti-CRALBP IP Products after SCX Fractionation

Accession number	Protein	anti-CRALBP	anti-RPE65	anti-RDH5	anti-RGR	anti-Ezrin	anti-EBP50	anti-Actin	Background
P1020	CRALBP	13.9	0.28	0.48	ND	ND	ND	ND	14
P1020	CRALBP	ND	0.27	ND	ND	ND	ND	ND	14
P62739	Actin	0.22	0.25	0.36	0.13	ND	ND	ND	14
Q28175	RPE65	0.19	0.29	0.19	ND	ND	ND	ND	14
Q2979	11-cis RDH (RDH5)	0.11	0.18	0.39	ND	ND	ND	ND	14
P0102	Hemoglobin beta	0.61	0.2	ND	ND	ND	ND	ND	14
P38657	Protein disulfide-isomerase A3 precursor	ND	0.22	ND	ND	ND	ND	ND	14
P0407	78 kDa glucose-regulated protein (GRP78)	ND	0.18	0.33	ND	ND	ND	ND	14
Background from photoreceptors:									
P0407	Actin	0.22	0.10	0.11	ND	ND	ND	ND	14
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Q2979	11-cis RDH (RDH5)	0.11	0.18	0.39	ND	ND	ND	ND	14
P0102	Hemoglobin beta	0.61	0.2	ND	ND	ND	ND	ND	14
P38657	Protein disulfide-isomerase A3 precursor	ND	0.22	ND	ND	ND	ND	ND	14
P0407	78 kDa glucose-regulated protein (GRP78)	ND	0.18	0.33	ND	ND	ND	ND	14
Background from blood:									
P0102	Hemoglobin beta	0.61	0.2	ND	ND	ND	ND	ND	14
P0102	Hemoglobin beta	0.61	0.2	ND	ND	ND	ND	ND	14
Q2979	11-cis RDH (RDH5)	0.11	0.18	0.39	ND	ND	ND	ND	14
P0102	Hemoglobin beta	0.61	0.2	ND	ND	ND	ND	ND	14
P38657	Protein disulfide-isomerase A3 precursor	ND	0.22	ND	ND	ND	ND	ND	14
P0407	78 kDa glucose-regulated protein (GRP78)	ND	0.18	0.33	ND	ND	ND	ND	14
Background from nucleus:									
P0407	Actin	0.22	0.10	0.11	ND	ND	ND	ND	14
P0407	Actin	0.22	0.10	0.11	ND	ND	ND	ND	14
Q2979	11-cis RDH (RDH5)	0.11	0.18	0.39	ND	ND	ND	ND	14
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Background from blood:									
P0102	Hemoglobin beta	0.61	0.2	ND	ND	ND	ND	ND	14
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