Proteomic Approaches to Identification of a Mammalian Visual Cycle Protein Complex

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Abstract

Proteomic methods are being used to identify components of the mammalian visual cycle protein complex. The visual cycle is the enzymatic process by which all-*trans*-retinal from photoreceptor bleaching is isomerized to 11-*cis*-retinal in the retinal pigment epithelium (RPE) then shuttled back to the photoreceptor cells for visual pigment regeneration. Protein-protein interactions are sought in bovine RPE microsomes by reciprocal immunoprecipitations and affinity chromatography. Proteins are identified by Western analyses, MALDI-TOF MS and LC MS/MS. Functional interactions are probed using enzyme kinetics. 11-*cis*-retinol dehydrogenase (RDH5), RPE65 and RGR opsin co-precipitate from RPE microsomes with anti-cellular retinaldehyde binding protein (CRALBP) antibodies. CRALBP and RPE65 co-precipitate with anti-RDH5 antibodies. Kinetic data demonstrate a functional interaction between purified recombinant CRALBP and RDH5. We are optimistic that affinity isolation procedures in progress will lead to the identification of other proteins in the visual cycle protein complex.

Introduction

The visual cycle is the enzymatic pathway by which all-*trans*-retinal from photoreceptor bleaching is isomerized to 11-*cis*-retinal in the retinal pigment epithelium (RPE) for visual pigment regeneration (Figure 1). Cellular retinaldehyde binding protein (CRALBP) serves as an acceptor of 11-*cis*-retinol in the isomerization step of visual cycle. Mutations in the CRALBP gene cause retinal degenerations associated with autosomal recessive retinitis pigmentosa (Maw et al., 1997), Bothnia dystrophy (Burstedt et al., 1999), retinitis punctata albescens (Morimura et al., 1999) and Newfoundland rod-cone dystrophy (Eichers et al., 2002). 11-*cis*-retinal dehydrogenase (RDH5) catalyzes the oxidation of 11-*cis*-retinol to 11-*cis*-retinal in the retinal pigment epithelium (RPE) where CRALBP has long been suggested to play a substrate carrier role for the enzyme (Saari and Bredberg, 1982). RDH5 was first detected by co-immunoprecipitation with RPE65, another visual cycle protein essential for enzymatic isomerization of all-*trans*- to 11-*cis*-retinol. RDH5 has also been found to co-immunoprecipitate with RGR opsin (Chen et. al., 2001). Mutations in the RDH5 gene cause delayed dark adaptation and a retinal disease termed fundus albipunctatus (Yamamoto et. al., 1999). It has been suggested that RDH5 is localized to the lumenal side of the endoplasmic reticulum and inaccessible for interaction with cytosolic CRALBP (Simon et. al., 1999). The present work was undertaken to evaluate the possible interaction between CRALBP and RDH5 using purified recombinant proteins. The results support structural and functional interactions between CRALBP with RDH5 and also support the existence of a visual cycle protein complex in the RPE.

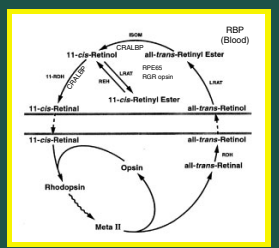

Figure 1. The Visual Cycle

Reactions occurring in RPE and rod photoreceptor cells are shown above and below the horizontal lines, respectively. The interphotoreceptor matrix (IPM) is represented by the space between horizontal lines and contains the interphotoreceptor retinoid-binding protein (IRBP), a protein possibly involved in retinoid transport between photoreceptors and RPE. Absorption of light by rhodopsin in the disk membrane converts 11-*cis*-retinal to all-*trans*-retinal and generates the active photoproduct, metarhodopsin II (Meta II). The Schiff base linking all-*trans*-retinal and opsin is hydrolyzed to release free all-*trans*-retinal. The all-*trans*-retinol dehydrogenase (at-RDH) catalyzes the reduction of all-*trans*-retinal to all-*trans*-retinol by NADPH. The all-*trans*-retinol leaves the photoreceptor cell, traverses the interphotoreceptor matrix and enters the RPE where it is esterified by lecithin: retinol acyltransferase (LRAT). The all-*trans*-retinol ester is converted to 11-*cis*-retinol and free fatty acid by an isomerohydrolase or isomerase (ISOM) with the assistance of RPE 65 and cellular retinaldehyde-binding protein (CRALBP). 11-*cis*-retinol can be esterified by LRAT and stored or oxidized to 11-*cis*-retinal by 11-*cis*-retinol dehydrogenase [11-RDH (or RDH5)]. 11-*cis*-retinyl esters can by hydrolyzed by retinyl ester hydrolase (REH) and utilized for visual pigment regeneration (modified from Saari et. al., 2001). 11-*cis*-retinal is shuttled back to the photoreceptor cell where it associates with opsin to regenerate visual pigment. Retinal G protein-coupled receptor (RGR) opsin is involved in light dependent formation of 11-*cis*-retinal (Chen et. al., 2001). All-*trans*-retinol is synthesized in the liver and transported to the RPE by retinol binding-protein (RBP).

Methods

Preparation of recombinant proteins. Recombinant human wildtype CRALBP and M225K mutant CRALBP were produced in *E. coli* and purified by nickel affinity chromatography (Crabb et al., 1998a). Protein was quantified by the Bradford method and/or phenylthiocarbamyl amino acid analysis using Applied Biosystems models 420H, 120, 900 instrumentation (Crabb et al., 1998b). Human RDH5 was produced in Hi-5 insect cells using baculovirus vectors and purified to apparent homogeneity by nickel affinity chromatography (Jang et. al., 2000).

Determination of Kinetic parameters. Kinetic parameters for RDH5 catalyzed reduction of 11-*cis* (or 9-*cis*) retinal were determined using the thiobarbiturate assay method (Futterman and Saslaw, 1961). Either free 11-*cis* (or 9-*cis*) retinal or holo-CRALBP were used as substrate and the reaction monitored spectrophotometrically at 530 nm. Kinetic parameters for RDH5 catalyzed oxidation of 11-*cis*- or 9-*cis*-retinol were determined using a phase partition radioactive assay (Saari et. al., 1993).

Immunoprecipitation. Visual cycle protein interactions were probed by immunoprecipitation using bovine retinal pigment epithelial (RPE) microsomes, polyclonal anti-peptide antibodies to CRALBP and RDH5 and a monoclonal antibody to RPE 65 (all produced by our laboratories). Commercially available antibodies to actin (Zymed Laboratories), clusterin (Chemicon International) and TIMP-3 were used with RPE microsomes for control immunoprecipitations. Antibodies were covalently coupled to protein A sepharose beads, incubated with RPE microsomes, extensively washed, boiled and the immunoprecipitated protein subjected to SDS-PAGE. The identity of immunoprecipitated proteins was determined by Western analysis and mass spectrometry.

Western Analysis. Western Analysis was performed as described in detail elsewhere (West et al., 2001; Miyagi et al., 2002). Briefly, proteins were electroblotted to PVDF membrane at 70 volts for 30 minutes. Nonspecific interactions were blocked with 5% milk. Primary antibody incubations were typically overnight at 4oC. Membranes were washed extensively with TBS buffer containing 0.5% Tween-80 before and after incubation with secondary antibody (1 h at room temperature). Immunoreactivity was detected by chemiluminescence system (Amersham Pharmacia BioTech, Inc).

In Gel Tryptic digestion. SDS-PAGE gel bands were excised, cut into ~1mm^3 cubes and Coomassie blue washed away with 250 µl aliquots of 60% acetonitrile (1x 30 min), 50% acetonitrile in 50 mM ammonium bicarbonate (1x 30 min), and 50% acetonitrile in 15 mM N-ethylmorpholine acetate pH 8 (3x 15 min). After destaining, gel pieces were dried in a Speed Vac then rehydrated in 15 µl 15 mM N-ethylmorpholine acetate pH8 containing 0.1 µg modified trypsin (Promega) and incubated overnight at 37ºC.

Protein identification by MALDI-TOF MS. An aliquot of the digested peptide mixture (1 µl) was spotted onto the MALDI target plate with 1 µl of matrix α-cyano-4-hydroxycinnamic acid. A Voyager-DE Pro MALDI-TOF mass spectrometer (Applied Biosystems) equipped with delayed extraction was employed for peptide mass mapping in positive reflector mode as described in detail elsewhere (West et. al., 2001). Peptide masses were searched against the NCBI protein sequence data base using ProFound (www.proteometrics.com)and MS-Fit (www.prospector.ucsf.edu) software.

Protein Identification by LC MS/MS. Tryptic peptides were analyzed by LCMS/MS using a quadrupole-time of flight (QTOF2) mass spectrometer equipped with a CapLC system (Micromass), Protein Lynx Global Server acquisition and processing software as described in detail elsewhere (Miyagi et al., 2002). Peptide digests were trapped on a C18 precolumn (eg, 0.3 x 1 mm, LC Packing) with 0.1% formic acid in 2% acetonitrile as loading solvent then eluted onto a capillary C18 column (eg, PicoFrit 0.050 x 50 mm, 15µ tip ID, New Objective, Inc.). Chromatography was performed at 250 nl/min with aqueous acetonitrile/formic acid solvents and 100% of the eluant directed into the mass spectrometer. Data-dependent survey scans selected the three most intense precursor ions on each MS scan for MS/MS data acquisition. MS/MS spectra was collected over the range m/z 50-2000.

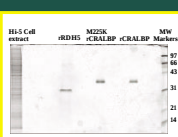

Figure 2. Recombinant Protein Preparations

About 1-2 µg of the purified recombinant proteins rRDH5, rCRALBP and rCRALBP mutant M225K and ~10 µg of Hi-5 insect cell lysate containing rRDH5 were analyzed by SDS-PAGE on a 12% gel and stained with Coomassie blue.

Results

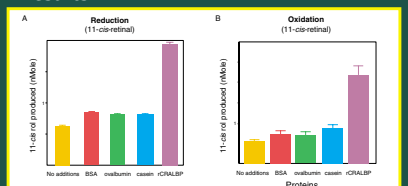

Figure. 3: Effect of various proteins on RDH5 activity

(A) Reduction of 11-cis-retinal to 11-cis-retinol. The reactions were performed at pH 5.5 in 100 µl using 1 µg of purified rRDH5, 1.97 nmole 11-cis-retinal and β-NADH plus 5 µg of the indicated proteins. (B) Oxidation of 11-cis-retinol to 11-cis-retinal. The reactions were performed at pH 7.5 in 100 µl using 0.2 µg of purified rRDH5, 3.0 nmole 11-cis-retinol and β-NAD, plus 5 µg of the indicated proteins.

Table 1
CRALBP Substrate Carrier Function

RDH5 Reduction Activity

Substrate	11-cis-retinal		9-cis retinal	
	Km	Vmax	Km	Vmax
No addition	6.0±0.2	440±40 (n=4)	2.7±0.2	154±23 (n=4)
BSA	5.2±0.3	348±51 (n=4)	2.7±0.14	151±21 (n=2)
WT rCRALBP	1.7±0.3	411±44 (n=4)	1.2±0.1	157±30 (n=4)
M225K	5.7±0.2	333±50 (n=4)		

Enzymatic reduction of 11-cis or 9-cis-retinal was measured with purified recombinant RDH5 at different substrate concentrations in the presence of the indicated purified proteins. The number of independent kinetic analyses (n) is indicated.

These results show that the affinity of RDH5 for 11-cis and 9-cis-retinal is increased (lower Km) when the retinoid is complexed with wildtype rCRALBP. CRALBP M225K mutant lacks retinoid binding capacity and has no effect on RDH5 kinetic parameters.

Table 2
CRALBP Substrate Carrier Function

RDH5 Oxidation Activity

Substrate	11-cis-retinol		9-cis-retinol	
	Km	Vmax	Km	Vmax
No addition	7.5±0.3	117±3 (n=4)	8±0.3	222±48 (n=4)
BSA	6.3±0.3	113±4 (n=4)	3.5±0.2	197±35 (n=2)
WT rCRALBP	2.5±0.3	143±10 (n=4)	3.5±0.4	188±42 (n=4)
M225K	6.9±0.5	122±7 (n=4)		

Enzymatic oxidation of 11-cis or 9-cis-retinol was measured with purified recombinant RDH5 at different substrate concentrations in the presence of the indicated purified proteins. The number of independent kinetic analyses (n) is indicated.

These results show that the affinity of RDH5 for 11-cis and 9-cis-retinal is increased (lower Km) when the retinoid is complexed with wildtype rCRALBP. CRALBP M225K mutant lacks retinoid binding capacity and has no effect on RDH5 kinetic parameters.

Figure 4: Anti-CRALBP Immunoprecipitation

Immunoprecipitation (IP) was performed with bovine RPE microsomes and anti-CRALBP pAb and with holo-rCRALBP or apo-CRALBP added as bait and after antibody pre-incubation with an excess of antigenic peptide K25Q. The IP products were separated by SDS-PAGE (10% gel), stained with colloidal Coomassie blue, bands excised, digested in situ with trypsin and proteins identified by MALDI TOF MS and/or LC MS/MS. Also shown are purified rRDH5, purified CRALBP and the bovine RPE microsome preparation before IP.

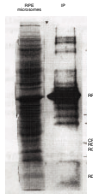

Figure 5: Anti-RPE65 Immunoprecipitation

Immunoprecipitation (IP) was performed with bovine RPE microsomes and anti-RPE65 mAb. The IP products were separated by SDS-PAGE (10% gel), stained with colloidal Coomassie blue, bands excised, digested in situ with trypsin and proteins identified by MALDI TOF MS and/or LC MS/MS.

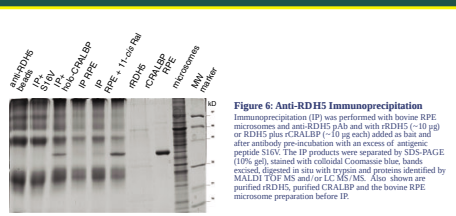

Figure 6: Anti-RDH5 Immunoprecipitation

Immunoprecipitation (IP) was performed with bovine RPE microsomes and anti-RDH5 pAb and with rRDH5 (~10 µg) or RDH5 plus rCRALBP (~10 µg each) added as bait and after antibody pre-incubation with an excess of antigenic peptide S16V. The IP products were separated by SDS-PAGE (10% gel), stained with colloidal Coomassie blue, bands excised, digested in situ with trypsin and proteins identified by MALDI TOF MS and/or LC MS/MS. Also shown are purified rRDH5, purified CRALBP and the bovine RPE microsome preparation before IP.

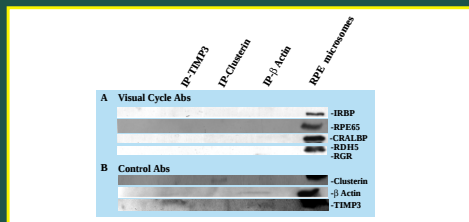

Figure 7: Control Immunoprecipitations and Western Analyses

Western analyses are shown of immunoprecipitation (IP) products from bovine RPE microsomes reacted with anti-TIMP3 pAb, anti-clusterin mAb or , anti-actin pAb. The membrane blot was probed sequentially with (A) anti-visual cycle antibodies to IRBP, RPE65, CRALBP, RDH5 and RGR opsin then (B) with antibodies to clusterin, actin and TIMP3. No stripping was performed between antibody reactions.

This figure shows that no visual cycle proteins co-precipitate with unrelated antibodies. In contrast, specific visual cycle antibodies co-precipitate a number of visual cycle proteins. These results support specific interactions among the visual cycle proteins.

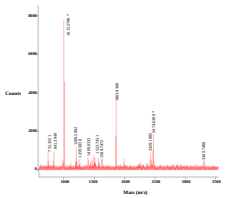

Figure 8: MALDI-TOF MS Identification of CRALBP

As an example, the MALDI-TOF mass spectra of tryptic peptides from the CRALBP gel band in Figure 8 is shown above and peptides identified by mass mapping are listed below. Internal calibration standards are identified with asterisks.

Identified CRALBP peptides

MH+ obs.	MH+ calc.	Error (ppm)	start	end	Peptide Sequence
755.3551	755.3840	38.3	116	121	(R) GYVNFR (L)
841.3949	841.4208	30.8	92	98	(K) DSGFFLR(F)
1209.5902	1209.6050	12.3	206	216	(K) GFTMQQAASLR (T)
1259.5858	1259.5942	6.7	10	19	(R) MVPEEEQELR (A)
1409.6332	1409.6557	15.9	223	234	(K) MVDMLQDSFPAR (F)
1523.7431	1523.7528	7.0	138	151	(R) CTIEAGYPGVLSSR (D)
1580.7472	1580.7644	5.9	29	42	(K) DHGPVFGPCSQLPR (H)
1863.9380	1863.9493	6.0	122	137	(R) LQYPELFDSLSPEAVR (C)
2429.1885	2429.1982	4.0	65	87	(R) ELQEMVQAQAASGEELAVAVAER (V)
3309.7408	3309.5956	43.9	266	295	(K) VFVHGDDLSGFYQEIDENILPSDFGGTLPK (Y)

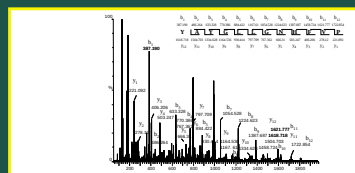

Figure 9: LC MS/MS Identification of RPE65

As an example, a QTOF LC MS/MS spectra is shown for the tryptic peptide YAYGLLGNHFVP obtained from an excised gel band in Figure 9 following immunoprecipitation of RPE microsomes with anti-CRALBP pAb. This MS/MS spectra identifies RPE65 as a component of the gel band.

Table 3 Proteins Identified in RPE Visual Cycle Immunoprecipitates [1]

Protein [2]	Identification Method					Molecular Weight (KDa)		Accession Number [4]
	MALDI TOF MS		LC MS/MS		Western blot			
	Peptide Matches	Coverage %	Peptide Matches	Coverage %	Score [3]	Calculated	On Gel	
Immunoprecipitation with anti-CRALBP								
IRBP			3	3	125	+ 137	>97	P12981
RPE65	7	25				+ 60.9	65	X66277
CRALBP	7	25	7	30	578	+ 36.3	40	P10123
RDH5	7	29	2	8	103	+ 35.0	35	Q27979
RGR			5	7	572	+ 31.9	35	P47803
LRAT			2	12	760	25.6	21	AF275344
RBP			1	6	425	21.0	21	P18902
Immunoprecipitation with anti-RPE65								
IRBP			1	3	520	+ 137	>97	P12981
RPE65			13	32	8909	+ 60.9	65	X66277
CRALBP			2	8	346	+ 36.3	40	P10123
RDH5			9	12	1411	+ 35.0	35	Q27979
RGR			1	5	404	+ 31.9	35	P47803
LRAT			2	10	419	25.6	21	AF275344
Immunoprecipitation with anti-RDH5								
IRBP						+ 137	>97	P12981
RPE65			4	22	447	+ 60.9	65	X66277
CRALBP	7	25	2	7	193	+ 36.3	40	P10123
RDH5			5	20	495	+ 35.0	35	Q27979
RGR						+ 31.9	35	P47803

1. Immunoprecipitations were performed with bovine RPE microsomes and antibodies to visual cycle proteins CRALBP, RPE65 and RDH5 as shown in Figs 4-6. Immunoprecipitated proteins were identified by MALDI TOF MS, LC MS/MS and Western analysis as described in Methods. 2. IRBP, Interphotoreceptor Retinoid-binding Protein; RPE65, Retinal Pigment Epithelium 65; CRALBP, Cellular Retinaldehyde-binding Protein; RDH5, 11-cis -Retinol Dehydrogenase 5; RGR, Retinal G Protein-coupled Receptor; LRAT, lecithin:retinol acyl transferase; RBP, serum retinol-binding protein. 3. Score calculated by ProteinLynx Global Server, Mass Lynx software (Micromass). 4. Swiss Protein database accession numbers are shown in plain font and NCBI accession numbers are in italics.

Conclusions

- CRALBP interacts with RDH5 based on reciprocal co-immunoprecipitations from RPE.
- CRALBP modulates RDH5 activity by increasing the affinity between the enzyme and retinoid substrates evidenced by lower K_ms in enzyme assays using the purified proteins.
- Several known visual cycle and retinoid-binding proteins co-immunoprecipitate from RPE microsomes including CRALBP, RDH5, RPE65, RGR opsin, LRAT, IRBP and RBP. These proteins co-precipitate with anti-visual cycle antibodies but not with non visual cycle, control antibodies.
- Overall, the results support a RPE visual cycle protein complex as a functional component of visual pigment regeneration.

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