

Identification of Visual Cycle Protein-Protein Interactions

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Abstract

The rod visual cycle is the process by which all-*trans*-retinal released from rhodopsin during bleaching is enzymatically isomerized to 11-*cis*-retinal in the retinal pigment epithelium (RPE), then shuttled back to the rod photoreceptor cells for rhodopsin regeneration. The mechanism for regeneration of bleached visual pigments in cone photoreceptors appears to be different. Protein-protein interactions were sought in bovine retinal pigment epithelial (RPE) microsomes by reciprocal immunoprecipitations using antibodies to several known visual cycle proteins. Proteins were identified by Western analyses, MALDI-TOF MS and LC MS/MS (QtoF2). Kinetic parameters for 11-*cis*-retinol dehydrogenase (RDH5) activity with and without CRALBP were measured. CRALBP, RDH5, RPE65, RGR opsin, IRBP, CRBP and RBP co-precipitate from RPE microsomes with anti-CRALBP antibodies. Similar results were obtained with anti-RDH5, anti-CRBP and anti-RPE65 antibodies. CRALBP was found to enhance the affinity of purified RDH5 for 9-*cis*- and 11-*cis*-retinoids, supporting a functional interaction. The results support the existence of an RPE retinoid metabolizing protein complex. Further proteomic analyses are directed toward identifying other possible components of visual cycle protein complexes.

Supported in part by NIH grants EY06603, EY014239, EY01730, EY02317, a Research Center Grant from The Foundation Fighting Blindness, and funds from the Cleveland Clinic Foundation and Research to Prevent Blindness, Inc.

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*-retinal 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*-retinol 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 caused delayed dark adaptation and a retinal disease termed fundus albipunctatus (Yamamoto et al., 1999). It has been suggested that RDH5 is localized to the luminal 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.

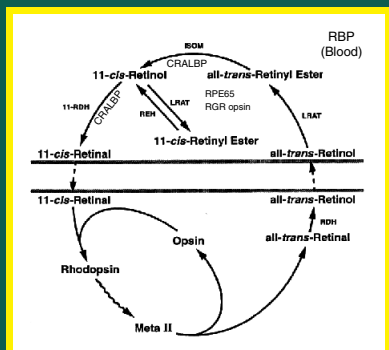


Figure 1. The Visual Cycle

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*-retinal 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). The 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 be 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*-retinyl. (Chen et al., 2001). All-*trans*-retinyl is synthesized in the liver and transported to the RPE by retinoid 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 phenylthiocarbonyl 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), clusternin (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 40C. 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 ~1mm³ 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 37°C.

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-hydroxybenzoic 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.profound.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, 0.5 x 1 mm, LC Packing) with 0.1% formic acid in 2% acetonitrile as loading solvent then eluted onto a capillary C18 column (eg, PicoFit 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 eluent 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.

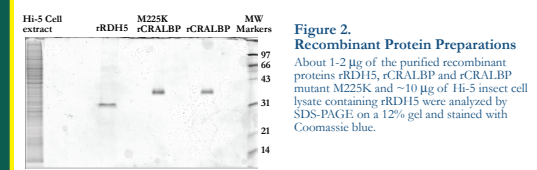


Figure 2. Recombinant Protein Preparations. About 1-2 µg of the purified recombinant proteins rRDH5, cCRALBP and rCRALBP mutant M225K and ~10 µg of Hi-5 insect cell lysate containing rRDH5 were analyzed by SDS-PAGE on a 12% gel and stained with Coomassie blue.

Results

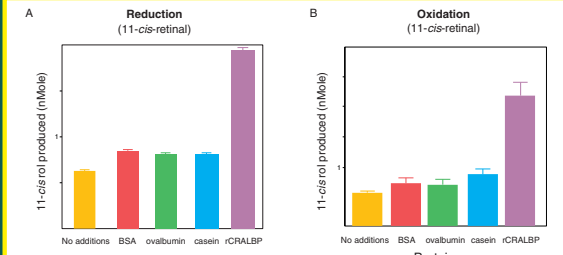


Figure 3. Effect of various proteins on RDH5 activity. (A) Reduction of 11-*cis*-retinal to 11-*cis*-retinol. The reactions were performed at pH 5.5 in 100 µl using 1 µg of purified rRDH5, 1.97 nmole 11-*cis*-retinal and β -NADH plus 5 µg of the indicated proteins. (B) Oxidation of 11-*cis*-retinol to 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- <i>cis</i> -retinal		9- <i>cis</i> -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 cCRALBP	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 cCRALBP. 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- <i>cis</i> -retinal		9- <i>cis</i> -retinal	
	Km	Vmax	Km	Vmax
No addition	7.5±0.3	117±5 (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 cCRALBP	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*-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 cCRALBP. CRALBP M225K mutant lacks retinoid binding capacity and has no effect on RDH5 kinetic parameters.

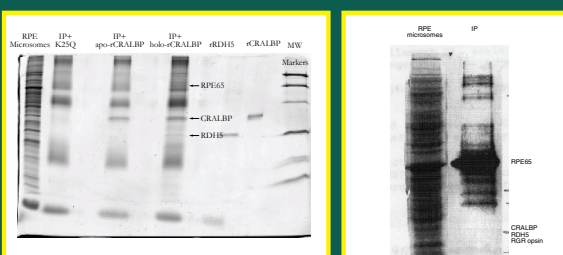


Figure 4. Anti-CRALBP Immunoprecipitation. Immunoprecipitation (IP) was performed with bovine RPE microsomes and anti-CRALBP pAb and with holo-CRALBP or apo-CRALBP added as bait and after antibody pre-incubation with an excess of antigenic peptide K25Q. The IP products were separated by SDS-PAGE (10% 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.

Figure 5: Anti-RPE65 Immunoprecipitation showing Western blot analysis of RPE microsomes immunoprecipitated with anti-RPE65 antibodies.

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.

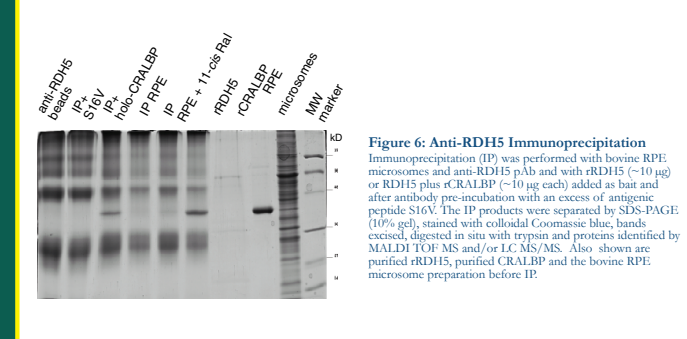


Figure 6. Anti-RDH5 Immunoprecipitation. Immunoprecipitation (IP) was performed with bovine RPE microsomes and anti-RDH5 pAb and with rRDH5 (~10 µg) or RDH5 plus cCRALBP (~10 µg each) added as bait and after antibody pre-incubation with an excess of antigenic peptide S16V. The IP products were separated 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 cCRALBP and the bovine RPE microsome preparation before IP.

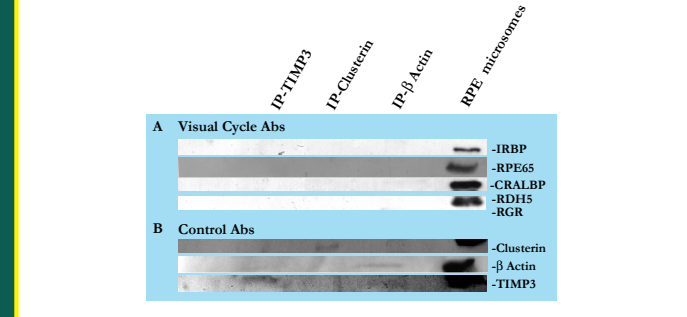


Figure 7. Control Immunoprecipitations and Western Analyses. Western analyses are shown of immunoprecipitation (IP) products from bovine RPE microsomes reacted with anti-TIMP3 pAb, anti-clusternin mAb or anti-actin pAb. The membrane blot was probed sequentially with (A) anti-visual cycle antibodies to IRBP, RPE65, CRALBP, RDH5 and RGR opsin then (B) with antibodies to clusternin, 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.

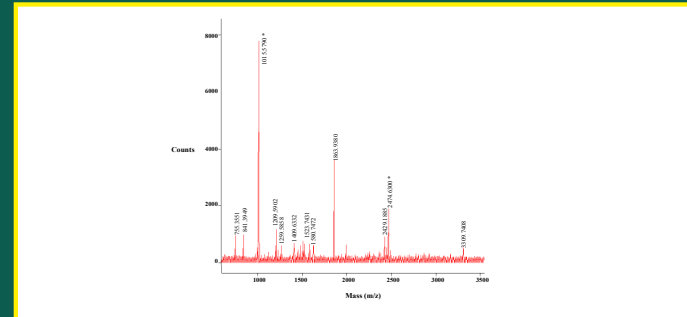


Figure 8. MALDI-TOF MS Identification of CRALBP. As an example, the MALDI-TOF mass spectra of tryptic peptides from the CRALBP gel band in Figure 8 is shown above and peptides identified by mass mapping are listed below. Internal calibration standards are identified with asterisks.

MH+ obs.	MH+ calc.	Error (ppm)	Start	End	Peptide Sequence
755.3551	755.3840	38.3	116	121	(R) GYVNFV (L)
841.3949	841.4208	30.8	92	98	(K) DSGFQLR(F)
1209.5902	1209.6050	12.3	206	216	(K) GFTMQEQAASLR (I)
1259.5858	1259.5942	6.7	10	19	(R) MIVPEEQELR (A)
1409.6332	1409.6557	15.9	223	234	(R) MVDMLQDSFAPR (I)
1523.7431	1523.7528	7.0	138	151	(R) CTHEAGYPGVLSR (D)
1580.7472	1580.7644	5.9	129	142	(K) DHGPIVFGPQSLPR (H)
1863.9380	1863.9493	6.0	122	137	(R) LKQVPELFDLSPEAVR (C)
2429.1885	2429.1982	4.0	65	87	(R) ELQVEIVQAQASGEELAAVAER (V)
3309.7408	3309.5956	43.9	266	295	(R) VYVHGDDLSGIFVQEIDENILPSDGGTLPK (Y)

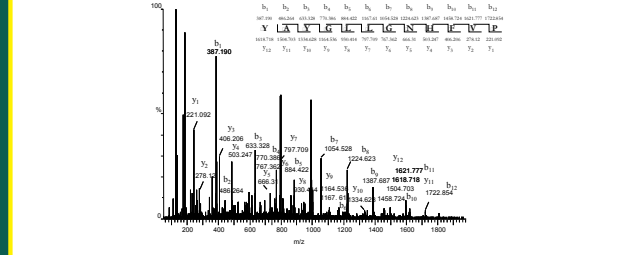


Figure 9. LC MS/MS Identification of RPE65. As an example, a QTOF LC MS/MS spectra is shown for the tryptic peptide YANGLLGNHIVP obtained from an excised gel band in Figure 9 following immunoprecipitation of RPE microsomes with anti-CRALBP pAb. This MS/MS spectra identifies RPE65 as a component of the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

Table 3. Proteins Identified in RPE Visual Cycle Immunoprecipitates. Identification Method.

Protein ¹	MALDI TOF MS		LC MS/MS		Western blot	Molecular Weight (kDa)	Accession Number ⁴
	Peptide Matches	Coverage %	Peptide Matches	Coverage %			
Immunoprecipitation with anti-CRALBP							
IRBP	7	25	3	3	125	49.8	P12611
RPE65	7	25	7	30	578	60.0	J66277
CRALBP	7	25	2	8	103	35.0	P10123
RDH5	7	29	2	7	572	31.9	Q27979
RGR			2	12	706	25.6	A5275044
LRAT			1	6	425	21.0	P18522
RBP							
Immunoprecipitation with anti-RPE65							
IRBP	1	3	3	3	520	49.8	P12611
RPE65	13	32	3	3	800	60.0	J66277
CRALBP	2	8	2	8	346	35.0	P10123
RDH5	9	12	1	1	1411	35.0	Q27979
RGR	1	5	1	5	404	31.9	A5275044
LRAT	2	10	1	10	416	25.6	A5275044
Immunoprecipitation with anti-RDH5							
IRBP							
RPE65			4	22	447	60.0	J66277
CRALBP	7	25	2	7	193	35.0	P10123
RDH5			5	20	495	35.0	Q27979
RGR						51.8	A5275044

1. Immunoprecipitates were performed with bovine RPE microsomes and anti-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; RGR, Retinal G Protein-coupled Receptor; LRAT, lecithin:retinol acyl transferase; RBP, serum retinoid-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.

References

Burstedt MS, Sandgren O, Holmgren G, Forsman-S. K. (1999) Invest Ophthalmol Vis Sci. 40, 995-1000
 Chen P, Lee TD, Fong HK (2001) J Biol Chem. 276: 21098-104.
 Crabb, JW, A. Carlson, Y. Chen, S. Goldflam, R. Intes, K.A. West, J.D. Holmes, J.T. Kapron, L.A. Luck, J. Horwitz and D. Bok (1998a) Protein Science 7: 746-757.
 Crabb JW et al. (1998b) J. Biol. Chem. 273. 33: 20712-20720.
 Eichers, E. R., J. S. Green, D. W. Stockton, C. Jackson, J. Whelan, J. A. McNamara, G. J. Johnson, J. R. Lupski, and N. Katsanis. (2002) Amer J Human Genetics. 70: 955-964.
 Futterman, S. and Saslaw, S.D. (1961) J. Biol. Chem. 236, 1652-57.
 Jang GF, McBeet JK, Alekseev AM, Haelecker F, Palczowski K. (2000) J Biol Chem. 275, 28128-38.
 Maw MA, Kennedy B, Knight A, Bridges R, Roth KE, Mann EJ, Mukkadan JK, Nancarrow D, Crabb JW, Denton MJ. (1997) Nat Genet. 17: 198-200.
 Miyagi M, H. Sakaguchi, RM Darrou, L. Yan, KA West, KS Adlak, DJ Stuehr, JG Holyfield, DT Orgniasnik and JW Crabb (2002) Molecular and Cellular Proteomics (In Press)
 Morimura H, Beson EL, Deyra TP. (1999) Invest Ophthalmol Vis Sci. 40:1004-1012
 Saari, J. C. and Bredberg, D.L. (1982) J Biol Chem. 257: 266-272
 Saari JC, Bredberg DL, Garwin GG, Buczynski J, Wheeler T, Palczowski K. (1993) Anal Biochem. 213: 128-32
 Saari J. C., Nawrot M., Kennedy B. N., Garwin G. G., Hurley J. B., Huang J., Pessin D.E., Crabb J. W. (2001) Neuron. 29: 739-48.
 Simon, A., Romert, A., Gustafson, A.L., McCaffery, J. M., Eriksson, U. (1999) J Cell Sci. 112: 549-558.
 West KA, Yan L, Miyagi M, Crabb JS, Marmorstein AD, Marmorstein L, Crabb JW. (2001) Exp Eye Res. 73: 479-91.
 Yamamoto H, Simon A, Eriksson U, Harris E, Berson EL, Deyra TP. (1999) Nat Genet. 22: 188-91.

Acknowledgements

We thank Drs Krzysztof Palczowski and Françoise Haelecker for providing anti-RDH5 monoclonal antibody and RDH5 baculovirus expression clones. We also thank Dr Henry Fong for anti-RGR polyclonal and monoclonal antibodies and Drs Joe Holyfield and Bela Anand-Apte for antibodies to clusternin and TIMP3, respectively.