Expression of GCAP1 and GCAP2 in the retinal degeneration (*rd*) mutant chicken retina

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Abstract We cloned the guanylate cyclase activating proteins, GCAP1 and GCAP2, from chicken retina and examined their expression in normal and predegenerate *rd/rd* chicken retina. Northern analyses show that the amounts of the single transcripts encoding GCAP1 and GCAP2 are reduced to about 70% of normal levels in *rd/rd* retina. Western analyses reveal that GCAP2 levels appear normal in this retina, while GCAP1 levels are reduced by more than 90%. The specific downregulation of GCAP1 in *rd/rd* retina is consistent with a model for this disease in which activation of guanylate cyclase in the photoreceptors is abnormal, resulting in low levels of cGMP and an absence of phototransduction.

Key words: Guanylate cyclase activating protein; *rd* chicken; Inherited retinal disease; Calcium; Photoreceptor; cGMP

1. Introduction

Photoexcitation of vertebrate photoreceptors leads to a decrease in cytoplasmic calcium which stimulates guanylate cyclase (GC) [1,2] via a Ca^{2+}-sensitive guanylate cyclase activating protein (GCAP) [3-5]. The mammalian retina contains two distinct guanylate cyclase activating proteins, termed GCAP1 and GCAP2 [6,7]. GCAP1 is present in both rod and cone photoreceptor outer segments where phototransduction occurs [6], while GCAP2 appears to be present in both the inner and outer segments of rod cells [7]. The occurrence of two, distinct GCAPs in the photoreceptors parallels the recent discovery of two, distinct Ca^{2+}-regulated GCs in these cells [8-11]. Both GCAPs appear to stimulate photoreceptor GC(s) in a Ca^{2+}-dependent manner [6,7].

In the *rd* (retinal degenerate) chicken, a model for recessively inherited retinal degeneration, blindness precedes photoreceptor degeneration in the retina. The first degenerative changes appear in both the rod and cone outer segments approx. 7–10 days after hatching [12,13]. By 8 months of age, only a few degenerating cone cells remain in the outer retina [13]. The retinal pigment epithelium shows signs of pathology only after the photoreceptor cells have begun to degenerate [12,13]. The absence of both scotopic and photopic electroretinograms at hatch [12,14] suggests failure of the phototransduction mechanism in this mutant. Biochemical analyses of microdissected *rd/rd* retinas show that the levels of cGMP in developing and predegenerate *rd/rd* photoreceptors are only 10–20% of those present in age-matched controls [15,16]. A genetic defect in enzymes involved in photoreceptor cGMP metabolism, that either disables synthesis or accelerates hydrolysis of cGMP, would be consistent with the *rd* phenotype. In this paper, we present the results of experiments that suggest involvement of GCAP1 in the *rd* phenotype. We describe the molecular cloning of chicken GCAP1 and GCAP2, and provide evidence that GCAP1, but not GCAP2, is downregulated in the mutant *rd* retina.

2. Materials and methods

2.1. Library screening and characterization of GCAP cDNA clones

Nick-translated fragments encoding bovine GCAP1 and GCAP2 [5,6] were used to screen +/+ and *rd/rd* chicken retina-pigment epithelium-choroid Uni-ZAP XR cDNA libraries [17] under reduced stringency [18]. The +/+ library yielded several GCAP1 and GCAP2 clones. GCAP1 clone CG5 and GCAP2 clone 6B were further characterized and their coding sequences completely sequenced. The *rd/rd* library yielded two GCAP clones, the coding sequence of one (rCG1) being completely sequenced. The inserts were excised, subcloned into pBluescript, and sequenced as described previously [19,20], or by automatic sequencing using a Perkin Elmer 310 Genetic Analyzer and/or a LiCor sequencer.

2.2. Northern blot analyses

Total RNA [21] and poly(A) RNA prepared using Dynabeads Oligo dT (Dynal, Inc.) were isolated from 1–3-day-old +/+, *rd/+* and *rd/rd* chick retina-pigment epithelium-choroid tissues. Northern blot analyses were performed as described previously [17]. The GCAP1 probes were either a 450 bp DNA fragment amplified with primers W236 and W241, or the full length 2.4 kb CG5 insert amplified with T3/T7 universal primers. The GCAP2 probe was a 1.4 kb *Eco*RI fragment from clone 6B. The actin probe was a 2.0 kb *Pst*I fragment of a chick β-actin cDNA clone [22]. cDNA probes were labeled [23] using a Prime-it II labeling kit (Stratagene). Five independent blots were generated and analyzed. Northern blots were exposed to Kodak XAR-5 film at −70°C in the presence of intensifying screens. In addition, the blots were quantitatively evaluated using an AMBIS radioanalytic imaging system (Molecular Dynamics).

2.3. SDS-PAGE and Western blot analyses

Chicken retinas were removed, frozen in liquid nitrogen, and stored

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at −70°C until use. The retinas were processed as described earlier [24]. The concentration of protein was determined by the method of Bradford [25], and SDS-PAGE was performed according to Laemmli [26]. The electrotransfer of proteins onto nitrocellulose membrane and immunodetection was carried as described previously [6].

2.4. Antibodies

Polyclonal antibody (PAb) UW14, immunoreactive with both chicken GCAP1 and GCAP2, was raised against bacterially expressed, truncated bovine GCAP180 [6]. PAbs GS35 and GS31 were raised against bovine N-terminal and C-terminal GCAP1 peptides, respectively [6]. PAb GS35 crossreacts with chicken GCAP1, whereas PAb GS31 is not immunoreactive with either of the chicken GCAPs (used as a control). PAb UW29, specific for GCAP2, was raised against a bovine GCAP2 C-terminal peptide corresponding to residues 170–198 (Fig. 2B).

3. Results and discussion

3.1. Cloning of chicken GCAP1 and GCAP2 cDNA

Clone CG5 encoding GCAP1 was shown to contain a 270 bp 5′-untranslated region (UTR) consisting, in part, of incomplete tandem repeats of 20–23 nt (Fig. 1A). Chicken GCAP2 clone 6B was shown to contain a 175 bp 5′-UTR lacking tandem repeats (Fig. 1B). The translation start codons of both the GCAP1 and GCAP2 cDNA sequences are preceded by in-frame stop codons (Fig. 1A,B) and their coding sequences are 597 and 594 nt in length, respectively. The 1.6 kb 3′-UTR of CG5 includes a polyadenylation signal and a poly(A) tail. The length of CG5, as determined by PCR and sequence analysis, is in agreement with the size of the single GCAP1 transcript (2.4 kb) seen on northern blots (Fig. 3A), and is consistent with the transcription start point being located approx. 280 bp upstream of the translation start codon in the GCAP1 gene. The length of the GCAP2 clone 6B, as determined by PCR is 2.5 kb, slightly smaller than the estimated size of the GCAP2 transcript (Fig. 3B). Clone 6B also contained a poly(A) tail indicating that the 5′-UTR is incomplete.

3.2. GCAP amino acid sequences

The chicken GCAP1 and GCAP2 amino acid sequences are predicted to consist of 199 and 198 residues with calculated molecular masses of 22 822 and 23 128 Da, respectively. Based on similarities to mammalian GCAPs [3,4,6,7], Met^1 is presumably posttranslationally cleaved and Gly^2 is myristoylated in the mature chicken GCAPs. The deduced amino acid sequences of chicken GCAP1 and GCAP2, which are only 43% similar to each other, predict the presence of three EF hand Ca^{2+}-binding sites [27,28], while visinin, another chicken retina specific Ca^{2+}-binding protein of almost identical molecular mass [29], is predicted to have only 2 such sites. On denaturing polyacrylamide gels, depletion of Ca^{2+} in the GCAPs produces characteristic mobility shifts (Fig. 4B,C) not seen in visinin [30]. The sequence similarity between GCAPs and visinin is 30–32% (Fig. 2A), and the similarity between bovine and chicken GCAP1 and GCAP2, is 79 and 77%, respectively (Fig. 2B).

3.3. Sequence alignment of retinal Ca^{2+}-binding proteins

GCAPs and visinin are members of the large and diverse superfamily of calmodulin-like Ca^{2+}-binding proteins [31]. The function of visinin, a recoverin-like Ca^{2+}-binding protein found in retina, is not known, while it is well established that both GCAPs activate photoreceptor GC in low concentrations of Ca^{2+} [6,7]. In addition to differences in the number of functional Ca^{2+}-binding sites, the sequences of the GCAPs and visinin diverge in the C- and N-terminal regions (Fig. 2A). The most conserved domain in these proteins surrounds the first and second Ca^{2+}-binding domains (Fig. 2A). In the GCAPs, the sequences encompassing all of the Ca^{2+}-binding domains are well conserved (Fig. 2B), an observation that suggests a functional role for the third domain, a domain that is not present in visinin. The sequence alignment of bovine and chicken GCAPs (Fig. 2A) demonstrates that the N-terminal domains of GCAP1s are well conserved while the C-termini are divergent. The GCAP2 sequence alignment, in contrast, shows that the N-termini of GCAP2 are divergent while the C termini are conserved (Fig. 2B).

3.4. Reduction of GCAP1 and GCAP2 mRNA levels in the rd mutant retina

A genetic mutation affecting activation of GC, which has a very low basal activity in bovine ROS in the absence of GCAP1 (<0.1 nmol cGMP/min per mg protein) [9], would be consistent with the *rd* phenotype. We therefore tested the expression levels of both GCAPs in the predegenerate mutant retina at the RNA and protein level. Northern analyses of retinal RNA show single GCAP1 and GCAP2 transcripts of the size expected by cDNA cloning. The transcript levels, however, are approx. 30% reduced in the 1–3-day-old *rd/+* and *rd/rd* retina as compared to normal (Fig. 3). We have previously shown that the transcripts encoding other phototransduction proteins, such as iodopsin [20], visinin [12], the catalytic subunit of cone PDE (α′) [32], and the rod PDEγ subunit (Semple-Rowland and Baehr, unpublished) are normal in 1–3-day-old, predegenerate *rd/rd* retina. These results indicate that both GCAP transcripts are specifically downregulated in the predegenerate mutant retina.

3.5. GCAP1 is nearly absent in the predegenerate rd/rd retina

A panel of polyclonal anti-bovine GCAP antibodies [6] was used for immunodetection of chicken GCAPs (for specificities of antibodies see section 2. Western blots of 1–3-day-old retina homogenates show that GCAP1 is nearly undetectable in the predegenerate *rd/rd* retina while GCAP2 levels are normal (Fig. 4A,D–F). In age-matched *rd/+* retina, GCAP1 levels are slightly reduced (Fig. 4A,D,F). Sequence analysis of *rd/rd* GCAP1 cDNA (Fig. 1A) revealed seven point mutations, none of which altered the amino acid sequence, or could ac-

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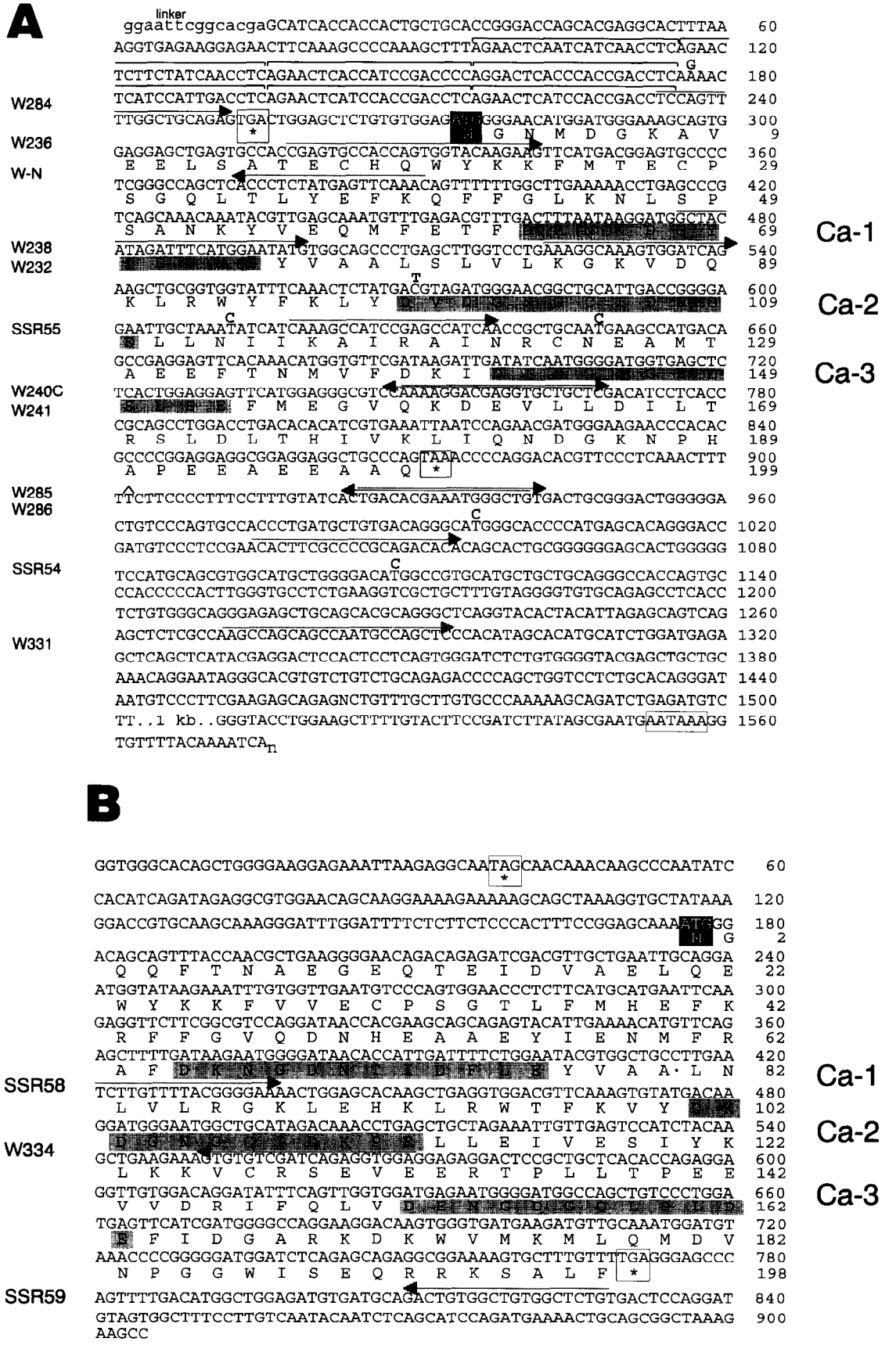
Fig. 1. Chicken retina GCAP cDNA sequences. (A) Partial sequence of GCAP1. The incomplete tandem repeats in the 5′-UTR are bracketed. Nucleotides 1–15 (lower case) are part of the linker used in library construction. In-frame stop codons that define the open reading frames and the polyadenylation signal AATAAA are boxed. Primers used for amplification, named at the left, are indicated by arrows pointing to the right (sense) or left (antisense). Amino acids are depicted in single-letter symbols. The amino acid numbering starts with the first translation initiators (M, black box) of the open reading frames. The three predicted EF-hand motifs for Ca^{2+} binding (Ca-1, Ca-2, and Ca-3) are shaded. The nucleotides deviating in the *rd/rd* GCAP1 cDNA sequence are indicated above the normal sequence. (B) Partial sequence of GCAP2. Details as in panel A.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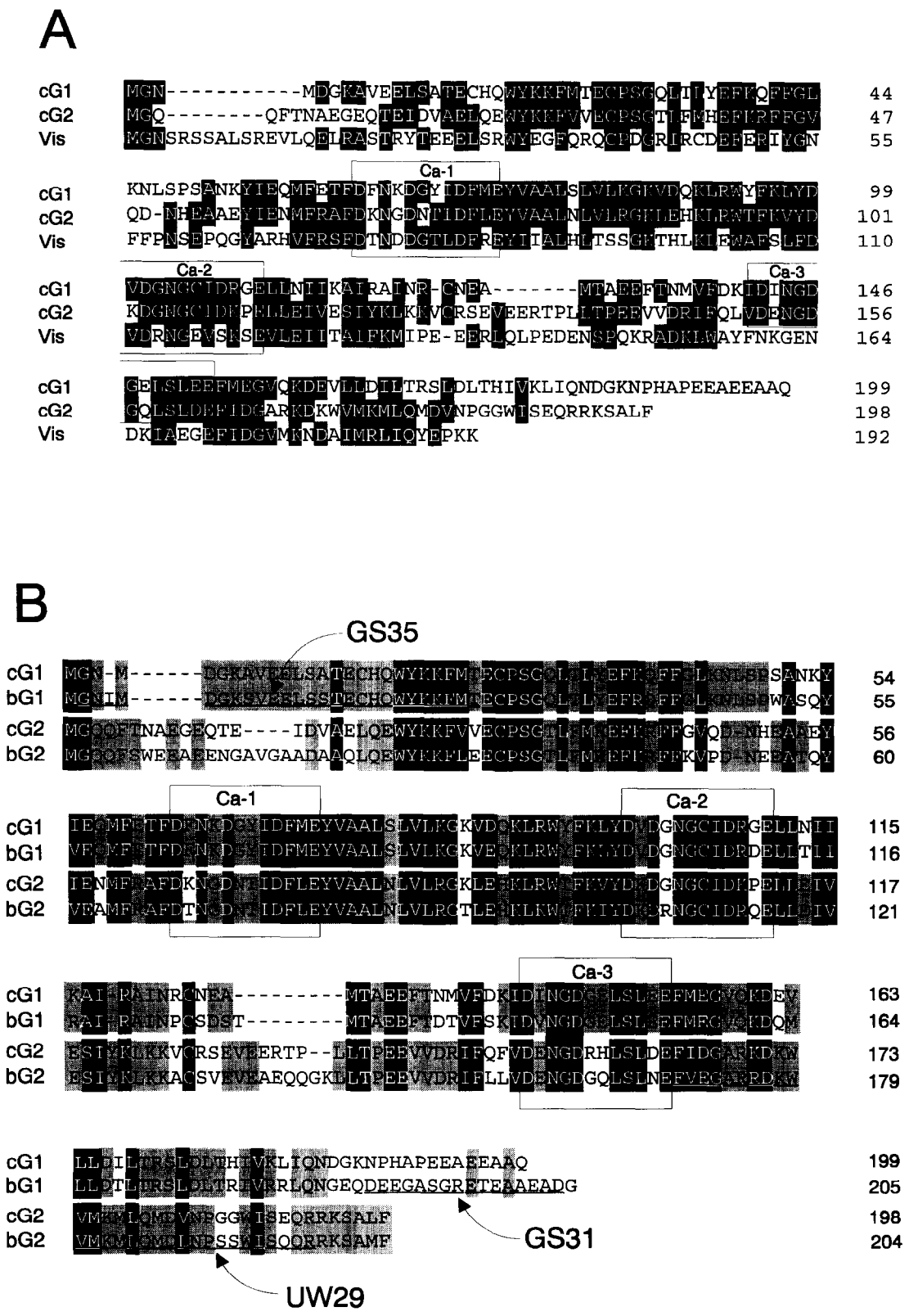

Fig. 2. Amino acid sequence alignments. (A) Alignment of chicken GCAP1, GCAP2, and visinin. L=I=V=M; Y=F; E=D; R=K; A=T=S are considered conservative substitutions. For best fit, several gaps were introduced (shown by hyphens). Conserved residues are printed white on black background. (B) Chicken and bovine GCAPs. Identical or conservatively substituted residues in GCAP1s only, or in GCAP2s only, are shaded. Residues conserved in all shown sequences are white on black background. The epitopes for polyclonal anti-peptide antibodies (GS35, GS31, UW29) are underlined in the bovine GCAP1 and chicken GCAP2 sequence. In A and B, EF-hand Ca^{2+}-binding domains (Ca-1, Ca-2, Ca-3) predicted by sequence analysis (program PROSITE of PC Gene, Intelligenetics, Inc) are boxed.

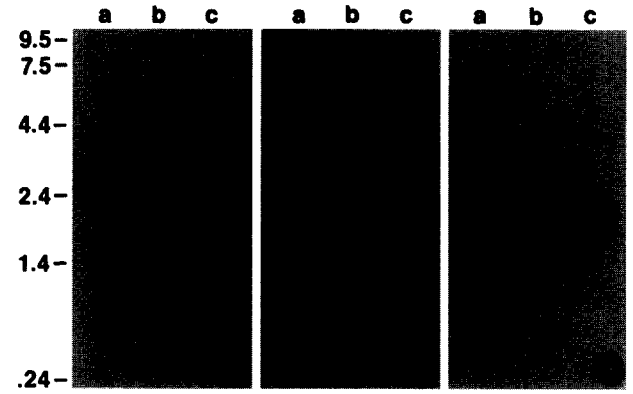

Fig. 3. Northern blot probed with GCAP1 and GCAP2. Northern blot of total RNA extracted from (lane 1) +/+, (lane 2) *rd*/+ and (lane 3) *rd*/*rd* chicken retina-pigment epithelium-choroid. Each lane was loaded with 20 μg of RNA. Panel A was probed with random-primer labeled GCAP1, panel B with GCAP2 cDNA, and panel C with a chicken β-actin fragment. A 0.24–9.5 kb RNA ladder (BRL/Gibco) was used to determine the size of the transcripts (indicated on the left).

count for instability of *rd*/*rd* GCAP1. In addition, genomic Southern blotting of +/+, *rd*/+, and *rd*/*rd* DNA did not reveal detectable insertions, deletions, or rearrangements in the mutant GCAP1 gene (results not shown). Since GCAP1 mRNA is produced to near normal levels in the predegenerate mutant retina, a defect in a site that regulates transcription of the GCAP1 gene, or splicing and polyadenylation of the gene transcript, seems unlikely. Furthermore, the absence of disease causing mutations in the GCAP1 coding sequence suggests that the genetic defect responsible for downregulation of GCAP1 may be inherent to a distinct candidate gene that specifically affects GCAP1, but not GCAP2. Additional transduction proteins whose synthesis is unaffected in predegenerate *rd*/*rd* retina include visinin [12], iodopsin [20], and rhodopsin [33]. Based on data presented here and the observation that cGMP levels in predegenerate *rd*/*rd* retina are only 10–20% of normal [15,16], potential candidates for the *rd* gene include genes encoding guanylate cyclase and those encoding proteins involved in the posttranslational processing, intracellular transport, or stabilization of GCAP1.

cGMP levels in photoreceptors are tightly regulated by a cell specific cGMP phosphodiesterase, guanylate cyclase(s), and their regulatory proteins. In situations where regulation of cGMP has been impaired, the development and function of the affected photoreceptor cells has been severely compromised. Reduced levels of cGMP in dark-adapted rods have been suggested to cause human congenital stationary night-blindness (CSNB) [34-36]. In the PDEγ gene knockout mouse model [37], the inhibitory subunit of rod PDE, PDEγ, is not

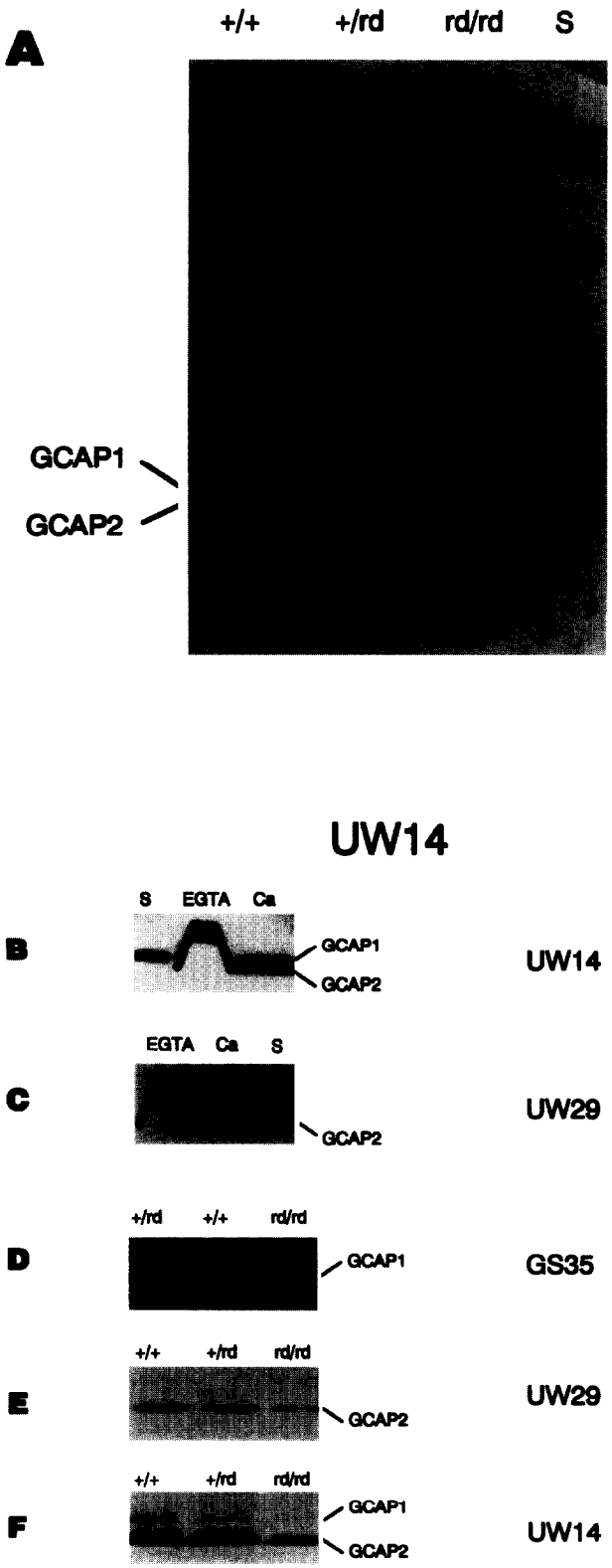

Fig. 4. Western blot of +/+, *rd*/+, and *rd*/*rd* retinal homogenates with GCAP1 and GCAP2 specific antibodies. (A) Blot probed with PAb UW14 (polyclonal against expressed truncated bovine GCAP1) which recognizes both chicken GCAP1 and GCAP2. The immunoreactive polypeptide at 70 kDa is unidentified. Prestained mobility standards (S) (BioRad) are shown on the right (107, 76, 52, 37, 27 and 19 kDa). (B,C) Panels depicting the change in SDS-PAGE mobility of GCAPs in the presence and absence of EGTA. GCAP proteins were purified from 100 normal chick retinas using G4 affinity chromatography as previously described [6]. The panels were stained with UW14 (B) or UW29 (C), a polyclonal against C-terminal GCAP2 peptide. (D) Blot stained with GS35 (polyclonal against N-terminal peptide from GCAP1) specifically showing the decrease in GCAP1 levels in *rd*/+ and *rd*/*rd* retina. (E) Blot stained with UW29 showing no change in GCAP2 levels in *rd*/+ and *rd*/*rd* retina. (F) Blot stained with UW14 (repeat of A with different retina samples). As a control, a blot stained with PAb GS31 (polyclonal against C-terminal peptide of bovine GCAP1 not present in chicken GCAP1) showed no immunostaining (not shown).

expressed, leading to permanent activation of PDE and very low levels of cGMP. As a result, the photoreceptors of these mice undergo rapid degeneration. Abnormally high levels of cGMP have also been found to be detrimental, leading to the rapid degeneration of photoreceptor cells in the *rd* (retinal degeneration) mouse [38]. In the *rd* chicken, the near absence of GCAP1 is consistent with a model for this disease in which the synthesis of cGMP by a photoreceptor GC is disabled, resulting in low levels of cGMP and an absence of phototransduction, conditions which lead to degeneration of the photoreceptor cells. The results of this study exemplify the uniqueness of the *rd* chicken, both as a model system for studies of the fundamental mechanisms underlying photoreceptor transduction and of autosomal recessive photoreceptor disease.

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References

[1] Lagnado, L. and Baylor, D. (1992) Neuron, 8, 995–1002.
[2] McNaughton, P.A. (1990) Physiol. Rev. 70, 847–884.
[3] Gorczyca, W.A., Gray-Keller, M.P., Detwiler, P.B. and Palczewski, K. (1994) Proc. Natl. Acad. Sci. USA 91, 4014–4018.
[4] Subbaraya, I., Ruiz, C.C., Helekar, B.S., Zhao, X., Gorczyca, W.A., Pettenati, M.J., Rao, P.N., Palczewski, K. and Baehr, W. (1994) J. Biol. Chem. 269, 31080–31089.
[5] Palczewski, K., Subbaraya, I., Gorczyca, W.A., Helekar, B.S., Ruiz, C.C., Ohguro, H., Huang, J., Zhao, X., Crabb, J.W., Johnson, R.S., Walsh, K.A., Gray-Keller, M.P., Detwiler, P.B. and Baehr, W. (1994) Neuron 13, 395–404.
[6] Gorczyca, W.A., Polans, A.S., Surgucheva, I., Subbaraya, I., Baehr, W. and Palczewski, K. (1995) J. Biol. Chem. 270, 22029–22036.
[7] Dizhoor, A.M., Olshevskaya, E.V., Henzel, W.J., Wong, S.C., Stults, J.T., Ankoudinova, I. and Hurley, J.B. (1995) J. Biol. Chem. 270, 25200–25206.
[8] Shyjan, A.W., de Sauvage, F.J., Gillett, N.A., Goeddel, D.V. and Lowe, D.G. (1992) Neuron 9, 727–737.
[9] Goraczniak, R.M., Duda, T., Sitaramayya, A. and Sharma, R.K. (1994) Biochem. J. 302, 455–461.
[10] Garbers, D.L. and Lowe, D.G. (1994) J. Biol. Chem. 269, 30741–30744.
[11] Yang, R.-B., Foster, D.C., Garbers, D.L. and Fülle, H.-J. (1995) Proc. Natl. Acad. Sci. USA 92, 602–606.
[12] Ulshafer, R.J., Allen, C.B., Dawson, W.W. and Wolf, E.D. (1984) Exp. Eye Res. 39, 125–135.
[13] Ulshafer, R.J. and Allen, C.B. (1985) Exp. Eye Res. 40, 865–877.
[14] Dawson, W.W., Ulshafer, R.J., Parmer, R. and Lee, N.R. (1990) Clin. Vis. Sci. 5, 285–292.
[15] Lee, N.R., Ulshafer, R.J. and Cohen, R.J. (1987) Invest. Ophthalmol. Vis. Sci. 28, 344.
[16] Lee, N.R. (1991) Cyclic nucleotides in the (*rd*) retinal degenerate chicken retina. Ph.D. Dissertation, University of Florida, Gainesville, FL.
[17] Semple-Rowland, S.L. and Van der Wel, H. (1992) Biochem. Biophys. Res. Commun. 183, 456–461.
[18] Pittler, S.J., Baehr, W., Wasmuth, J.J., McConnell, D.G., Champagne, M.S., VanTuinen, P., Ledbetter, D. and Davis, R.L. (1990) Genomics 6, 272–283.
[19] Pittler, S.J. and Baehr, W. (1991) Proc. Natl. Acad. Sci. USA 88, 8322–8326.
[20] Semple-Rowland, S.L. and Green, D.A. (1994) Invest. Ophthalmol. Vis. Sci. 35, 2550–2557.
[21] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[22] Cleveland, D.W., Lopata, M.A., MacDonald, R.J., Cowan, N.J., Rutter, W.J. and Kirschner, M.W. (1980) Cell 20, 95–105.
[23] Feinberg, A.P. and Vogelstein, B. (1984) Anal. Biochem. 137, 266–267.
[24] Polans, A.S., Buczylko, J., Crabb, J. and Palczewski, K. (1991) J. Cell Biol. 112, 981–989.
[25] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[26] Laemmli, U.K. (1970) Nature 227, 680–685.
[27] Kretsinger, R.H. and Nockolds, C.E. (1973) J. Biol. Chem. 248, 3313–3326.
[28] Falke, J.J., Drake, S.K., Hazard, A.L. and Peerson, O.B. (1994) Q. Rev. Biophys. 27, 219–290.
[29] Yamagata, K., Goto, K., Kuo, C.-H., Kondo, H. and Miki, N. (1990) Neuron 2, 469–476.
[30] Polans, A.S., Burton, M.D., Haley, T.L., Crabb, J.W. and Palczewski, K. (1993) Invest. Ophthalmol. Vis. Sci. 34, 81–90.
[31] Nakayama, S., Moncrief, N.D. and Kretsinger, R.H. (1992) J. Mol. Evol. 34, 416–448.
[32] Semple-Rowland, S.L. and Green, D.A. (1994) Exp. Eye Res. 59, 365–372.
[33] Ulshafer, R.J., Adamus, G., Clausnitzer, E. and Hargrave, P.A. (1990) Invest. Ophthalmol. Vis. Sci. 31, 546.
[34] Dryja, T.P., Berson, E.L., Rao, V.R. and Oprian, D.D. (1993) Nature Genet. 4, 280–283.
[35] Gal, A., Orth, U., Baehr, W., Schwinger, E. and Rosenberg, T. (1994) Nature Genet. 7, 64–68.
[36] Sieving, P.A., Richards, J.E., Naarendorp, F., Bingham, E.L., Scott, K. and Alpern, M. (1995) Proc. Natl. Acad. Sci. USA 92, 880–884.
[37] Tsang, S.H. and Goff, S.P. (1995) Invest. Opthalmol. Vis. Sci. 36, S642.
[38] Farber, D.B. and Lolley, R.N. (1974) Science 186, 449–451.