Retinal Guanylyl Cyclase Isozyme 1 Is the Preferential *In Vivo* Target for Constitutively Active GCAP1 Mutants Causing Congenital Degeneration of Photoreceptors

Elena V. Olshevskaya,* Igor V. Peshenko,* Andrey B. Savchenko,* and Alexander M. Dizhoor

Department of Basic Sciences and Pennsylvania College of Optometry, Salus University, Elkins Park, Pennsylvania 19027

Two calcium-sensitive guanylyl cyclase activating proteins (GCAP1 and GCAP2) activate cGMP synthesis in photoreceptor by retinal membrane guanylyl cyclase isozymes (RetGC1 and RetGC2) to expedite recovery, but calcium-insensitive constitutively active GCAP1 mutants cause photoreceptor degeneration in human patients and transgenic mice. Although GCAP1 and GCAP2 can both activate RetGC1 and RetGC2 *in vitro*, we find that GCAP1 selectively regulates RetGC1 *in vivo*. Furthermore, elimination of RetGC1 but not RetGC2 isozyme reverses abnormal calcium sensitivity of cGMP synthesis and rescues mouse rods in transgenic mice expressing GCAP1 mutants causing photoreceptor disease. Rods expressing mutant GCAP1 not only survive in the absence of RetGC1 but also remain functional, albeit with reduced electroretinography (ERG) amplitudes typical of *RetGC1*^{−/−} genotype. The rod ERG recovery from a strong flash, only slightly affected in both *RetGC1*^{−/−} and *RetGC2*^{−/−} mice, becomes very slow in *RetGC1*^{−/−} but not *RetGC2*^{−/−} mice when GCAP2 is not available to provide Ca²⁺ feedback to the remaining RetGC isozyme. The intrinsic biochemical properties of RetGC and GCAP determined *in vitro* do not explain the observed phenomena. Instead, our results argue that there must be a cellular mechanism that limits GCAP1 access to RetGC2 and makes RetGC1 isozyme a preferential target for the disease-causing GCAP1 mutants. A more general conclusion from our findings is that nondiscriminatory interactions between homologous effector enzymes and their regulatory proteins permitted by their intrinsic biochemical properties can be effectively restricted in a living photoreceptor.

Introduction

Guanylyl cyclase activating proteins (GCAPs) (Dizhoor et al., 1994, 1995; Gorczyca et al., 1994; Palczewski et al., 1994) are Ca²⁺ sensors regulating retinal guanylyl cyclase (RetGC) in photoreceptor membranes (Fig. 1). Mutations in GCAP1 cause congenital cone and cone-rod degenerations in humans (Wilkie et al., 2001; Michaelides et al., 2005; Sokal et al., 2005; Kitaritschky et al., 2009), and constitutively active mutations, Y99C (Payne et al., 1998) and E155G (Wilkie et al., 2001), destroy rods in transgenic mice by altering Ca²⁺ sensitivity of the cGMP synthesis (Olshevskaya et al., 2004; Woodruff et al., 2007).

The rates of cGMP synthesis by RetGC versus decay by phosphodiesterase (PDE6) control the Na⁺ and Ca²⁺ influx in the rod outer segment (ROS), and GCAPs accelerate cGMP synthesis via negative calcium feedback (Pugh et al., 1997, 1999; Fu and Yau, 2007; Dizhoor et al., 2010). In the light, when cGMP hydrolysis blocks Ca²⁺ influx through the cGMP-gated channels, Ca²⁺

dissociates from GCAPs, which enables them to stimulate RetGC and speed up the recovery. Once the influx of Ca²⁺ through the channels reopens and its submicromolar concentrations (Gray-Keller and Detwiler, 1994; Woodruff et al., 2002) in ROS are restored, GCAPs rebinding Ca²⁺ and decelerate RetGC activity. In contrast, Y99C (or E155G) GCAP1 is insensitive to the normal dark intracellular Ca²⁺ levels (Dizhoor et al., 1998; Sokal et al., 1998) and continues to accelerate cGMP synthesis, resulting in elevation of free cGMP and Ca²⁺ in transgenic mouse rods (Olshevskaya et al., 2004; Woodruff et al., 2007). The abnormal sensitivity of cGMP synthesis is the primary cause of the photoreceptor death, which can be averted by increased cGMP hydrolysis (Woodruff et al., 2007; Liu et al., 2009).

Using shRNA suppression of the Y99C GCAP1 activity was successful at moderate levels but not at higher levels of Y99C GCAP1 expression (Jiang et al., 2011). Perhaps the cyclase activity itself also needs to be partially suppressed to treat more severe forms of degeneration, but the actual isozyme—RetGC1 or RetGC2 (Lowe et al., 1995; Yang et al., 1995)—targeted by the mutant GCAP1 has to be first identified.

In vitro, GCAP1 and GCAP2 activate both isozymes with only slightly different affinities and sensitivity to Ca²⁺ (Hwang et al., 2003; Peshenko and Dizhoor, 2004; Peshenko et al., 2011). Biochemical isolation of RetGC/GCAP complexes directly from the normal and diseased photoreceptors remains technically impossible, because they are unstable in detergents (Koch, 1991). Therefore, we tested possible selectivity in GCAP/RetGC interactions *in vivo* by studying retinal degeneration caused by constitu-

Received Feb. 28, 2012; revised April 2, 2012; accepted April 4, 2012.

Author contributions: A.M.D. designed research; E.V.O., I.V.P., A.B.S., and A.M.D. performed research; E.V.O., I.V.P., A.B.S., and A.M.D. analyzed data; A.M.D. wrote the paper.

This work was supported by National Institutes of Health Grant EY11522 and a Pennsylvania Department of Health Formula Grant. We thank Ronald Barrett for help with histological sectioning. A.M.D. is the Martin and Florence Hafler Professor of Pharmacology.

*E.V.O., I.V.P., and A.B.S. contributed equally to this work.

Correspondence should be addressed to Alexander M. Dizhoor, Pennsylvania College of Optometry, Salus University, 8360 Old York Road, Elkins Park, PA 19027. E-mail: adizhoor@salus.edu.

DOI:10.1523/JNEUROSCI.0976-12.2012

Copyright © 2012 the authors 0270-6474/12/327208-10\$15.00/0

Figure 1. The diagram of cGMP synthesis regulation by a negative Ca^{2+} feedback and the role of constitutively active GCAP1 mutants linked to human blindness. Left, Photoreceptors activate cGMP hydrolysis by the light-dependent PDE6 and close the cGMP-gated channels (CNG). In the absence of the influx through the channels, free Ca^{2+} in the cytoplasm of the ROS is rapidly depleted by $\text{Na}^+/\text{Ca}^{2+}$, K^+ -exchanger, and this converts GCAPs to their activator state (GCAP1* and GCAP2*). GCAP1* and GCAP2* activate two isoforms of retinal guanylyl cyclase (RetGC1* and RetGC2*) and allow rods to reopen the CNG channels and recover from excitation. Middle, In the dark, when PDE6 activity is suppressed, a fraction of the CNG channels opens, and the influx of Ca^{2+} converts GCAPs into their inhibitory Ca^{2+} -liganded state. Consequently, the rate of cGMP synthesis remains low in the dark so that only enough CNG channels would remain open to maintain the intracellular Ca^{2+} near the levels at which GCAP1 and GCAP2 stay in their Ca^{2+} -bound form ($\sim 250 \mu\text{M}$ Ca^{2+} ; Woodruff et al., 2002). Right, The Y99C (or E155G) GCAP1 mutant requires much higher Ca^{2+} concentrations to stop RetGC activation. As a result, cGMP synthesis is activated by the Y99C GCAP1 in the dark continues until a larger fraction of CNG channels opens and Ca^{2+} rises to the higher than normal levels (Olshevskaya et al., 2004; Woodruff et al., 2007).

tively active GCAP1 and Ca^{2+} sensitivity of the endogenous GCAP/RetGC complexes in mouse retinas genetically lacking RetGC1 or RetGC2. We also compared the speed of rod recovery in transgenic models expressing only GCAP1 isoform and one of the cyclase isoforms. We find a strong GCAP1 selectivity toward RetGC1 *in vivo* that cannot be explained by their general biochemical properties and must be imposed at the cellular level, thus making RetGC1 the preferred target for the GCAP1 mutants causing retinal disease in humans.

Materials and Methods

Mouse genetic models. All animal procedures were approved by Salus University Institutional Animal Care and Use Committee protocol in compliance with the National Institutes of Health guidelines. Mice of either sex were used for each genotype presented in the study (Table 1). Retinal degeneration models transgenically overexpressing Y99C (line 53) or E155G (line 541) GCAP1 under control of rod opsin promoter were described in previous publications (Olshevskaya et al., 2004; Woodruff et al., 2007). Wild-type (WT) C57BL/6 mice originated from Taconic Farms. The *GCAP1,2*^{−/−} line, produced by simultaneous deletion of the neighboring *GUCA1A* and *GUCA1B* genes (Mendez et al., 2001), was a gift from Dr. Jeannie Chen (University of South California, Los Angeles, CA). The *GCAP2*^{−/−} mouse model developed by selective disruption of *GUCA1B* gene was described in detail previously (Makino et al., 2008). The *RetGC1*^{−/−} (line GC-E null) produced by the disruption of a mouse *GUCY2E* gene (Yang et al., 1999) was a gift from Dr. David Garbers (University of Texas, Dallas, TX), and *RetGC2*^{−/−} mice produced by disruption of *GUCY2F* gene (Baehr et al., 2007) were provided by Dr. Wolfgang Baehr (University of Utah, Salt Lake City, UT). The RetGC1 or RetGC2 knock-outs were bred with the other genotypes to produce *Y99C*⁺*RetGC1*^{−/−}; *Y99C*⁺*RetGC2*^{−/−}; *E155G*⁺*RetGC1*^{−/−}; *RetGC1*^{−/−}*GCAP1,2*^{−/−}; *RetGC2*^{−/−}*GCAP1,2*^{−/−}; *RetGC1*^{−/−}*GCAP2*^{−/−}; and *RetGC1*^{−/−}*GCAP2*^{−/−} genotypes. The WT, *RetGC1*^{−/−}, *RetGC2*^{−/−}, *Y99C*⁺, *Y99C*⁺*RetGC1*^{−/−}, *Y99C*⁺*RetGC2*^{−/−}, and *E155G*⁺*RetGC1*^{−/−} were used in degeneration studies; unlike *Y99C*⁺*RetGC2*^{−/−}, selected as the control line for the degeneration studies, we were unable to breed enough *E155G*⁺*RetGC2*^{−/−}.

The nondegenerating WT; *RetGC1*^{−/−}, *RetGC2*^{−/−}; *RetGC1*^{−/−}*GCAP1,2*^{−/−}; *RetGC2*^{−/−}*GCAP1,2*^{−/−}; *RetGC1*^{−/−}*GCAP2*^{−/−}; and *RetGC1*^{−/−}*GCAP2*^{−/−} lines were used in electroretinography (ERG) paired-flashes experiments.

Electroretinography. Mice were dark-adapted under vented hood overnight and anesthetized by intraperitoneal injection of 20 $\mu\text{g/g}$ ketamine, 8 $\mu\text{g/g}$ xylazine, and 800 $\mu\text{g/g}$ urethane. Scotopic ERG was recorded according to the protocol of Lyubarsky and Pugh (1996) with minor modifications described previously (Woodruff et al., 2007). The saturating ERG responses were evoked by a 1 ms white flash delivering $\sim 10^6$ photon/ μm^2 at the cornea. Rod recovery speed in different genotypes was compared using paired-flashes ERG according to the protocol of Lyubarsky and Pugh (1996) with minor modifications.

Retinal histology and fluorescence microscopy. Mice killed by lethal injection of anesthetic were perfused through the heart with PBS and then with either freshly prepared 4% paraformaldehyde in PBS or 2.5% glutaraldehyde in 0.1 M cacodylate buffer, and the enucleated eyes were then additionally fixed for 4 h on ice in the respective fixative solution and processed for histological sectioning. The paraformaldehyde-fixed eyes were washed with PBS, impregnated with 30% sucrose in PBS, and frozen embedded in OCT medium (Electron Microscopy Sciences). Cryosections were taken using a Hacker-Bright OTF5000 microtome and probed with the polyclonal anti-GCAP1 antibody as described previously (Makino et al., 2008), and the immunofluorescence (IF) images were recorded using an Olympus FV1000 Spectral confocal system. For the *in situ* apoptosis analysis, mice were killed and perfused with PBS and 4% paraformaldehyde on postnatal day 28, and the apoptotic DNA fragmentation in retinal cryosections was detected by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) method using a TMR Red *In Situ* Cell Death Detection kit (catalog #12156792910; Roche Applied Science) according to the protocol of the manufacturer; the nuclei were counterstained with DAPI. The tetramethylrhodamine (TMR) red-labeled nuclei were photographed using an Olympus Magnafire imaging system integrated with BX51 microscope with a 20 $\times$ objective. For histological analysis, glutaraldehyde-fixed retinas were washed in PBS, dehydrated in ethanol solutions, embedded, sectioned, stained, and photographed as described previously (Olshevskaya et al., 2004; Woodruff et al., 2007).

Guanylyl cyclase assays. Mice were dark-adapted overnight and killed under infrared illumination, and the retinas were extracted and assayed for RetGC activity in the dark using [α - ^{32}P]GTP as a substrate using a previously described protocol (Olshevskaya et al., 2004; Peshenko et al., 2011). The assay mixture (25 μl) contained 30 mM 3-(*N*-morpholino)-propanesulfonic acid-KOH, pH 7.2, 60 mM KCl, 4 mM NaCl, 1 mM DTT, 2 mM Ca^{2+} /EGTA buffer, 1 mM free Mg^{2+} , 0.3 mM ATP, 4 mM cGMP, 1 mM GTP, 1 μCi of [α - ^{32}P]GTP, 0.1 μCi of [8 - ^3H]cGMP (PerkinElmer Life and Analytical Sciences), and PDE6 inhibitors zaprinast and dipyrindamole. In some series, 10 mM creatine phosphate and 0.5 U of creatine phosphokinase were added to better maintain the linear time course of the reaction. The resultant [^{32}P]cGMP product was analyzed using fluorescently backed polyethyleneimine cellulose TLC, as described previously (Olshevskaya et al., 1997).

Ca^{2+} /EGTA buffers containing calibrated free Ca^{2+} and Mg^{2+} concentrations were prepared using the method by Tsien and Pozzan (1989) and verified by fluorescent Ca^{2+} indicator dyes as described previously (Peshenko et al., 2006).

Recombinant Y99C GCAP1. The Y99C GCAP1 was expressed from pET11d vector in a BLR(DE3) *Escherichia coli* strain harboring yeast

Table 1. Mouse genotypes used in the study^a

Genotype	Phenotype	Total	Results presented
WT	Nondegenerating	27	Morphology, ERG, paired-flashes ERG, RetGC activity, IF, TUNEL
<i>Y99C⁺</i> (line 53; Olshevskaya et al., 2004)	Fast-degenerating	23	Morphology, ERG, RetGC activity, TUNEL
<i>E155G⁺</i> (line 541; Woodruff et al., 2007)	Fast-degenerating	13	Morphology, ERG, RetGC activity
<i>RetGC1^{-/-}</i> (line GCE null; Yang et al., 1999)	No cone function, rods are preserved	44	Morphology, ERG, RetGC activity, IF
<i>RetGC2^{-/-}</i> (GC2 knock-out; Baehr et al., 2007)	Nondegenerating	16	Morphology, ERG, RetGC activity paired-flashes ERG
<i>Y99C⁺ RetGC1^{-/-b}</i>	No cone function, most of the rods are preserved	46	Morphology, ERG, RetGC activity, IF, TUNEL
<i>Y99C⁺ RetGC2^{-/-b}</i>	Fast-degenerating	12	Morphology, ERG, RetGC activity, TUNEL
<i>E155G⁺ RetGC1^{-/-b}</i>	No cone function, most of the rods are preserved	29	Morphology, ERG, RetGC activity, IF
<i>RetGC1^{-/-} GCAP2^{-/-c}</i>	Slow recovery	16	Paired-flashes ERG
<i>RetGC1^{-/-} GCAP1,2^{-/-d}</i>	Slow recovery	12	Paired-flashes ERG
<i>RetGC2^{-/-} GCAP2^{-/-e}</i>	Fast recovery	11	Paired-flashes ERG
<i>RetGC2^{-/-} GCAP1,2^{-/-f}</i>	Slow recovery	16	Paired-flashes ERG

^aMice of either sex were used.

The new genotypes were bred for this study as follows:

^bfrom fast degenerating lines 53 (Olshevskaya et al., 2004) and 541 (Woodruff et al., 2007) and rod nondegenerating GC-E null (Yang et al., 1999) or GC2 knock-out (Baehr et al., 2007) lines.^cfrom nondegenerating *GCAP2^{-/-}* (Makino et al., 2008) and rod nondegenerating GC-E null (Yang et al., 1999) lines.^dfrom nondegenerating *GCAP1,2^{-/-}* double knock-out (Mendez et al., 2001) and rod non-degenerating GC-E null (Yang et al., 1999) lines.^efrom nondegenerating GC2 knock-out (Baehr et al., 2007) and nondegenerating *GCAP2^{-/-}* (Makino et al., 2008).^ffrom nondegenerating GC2 knock-out (Baehr et al., 2007) and nondegenerating *GCAP1,2^{-/-}* double knock-out (Mendez et al., 2001) lines.

N-mristoyltransferase as described previously (Dizhoor et al., 1998) with modifications described by Peshenko et al. (2006).

Statistical test. When indicated, one-way ANOVA with Bonferroni's *post hoc* test for all-pairs comparison was performed using Kaleidagraph 4 (Synergy Software).

Results

Elimination of RetGC1 effectively preserves rods in Y99C and E155G mice

Rods in mouse retinas expressing Y99C GCAP1 under control of rod opsin promoter (line 53) (Olshevskaya et al., 2004) progressively degenerate, and scotopic ERG a-wave in these mice disappears by the age of 4–6 months, concomitant with the loss of the outer nuclear layer. Even faster progressing degeneration occurs in rods overexpressing E155G GCAP1 mutant, line 541 (Woodruff et al., 2007). In sharp contrast to the Y99C⁺ and the E155G⁺ mice that express the mutant GCAP1 in *RetGC1^{+/+} RetGC2^{+/+}* background and lose the entire rod population between 4 and 6 months of age (Fig. 2), most of the rods survive and retain the outer segment layer in the *Y99C⁺ RetGC1^{-/-}* and *E155G⁺ RetGC1^{-/-}* retinas (Fig. 2*Ad,Af*). Compared with the parental Y99C⁺ line 53 lacking photoreceptor outer nuclear layer, the rescue of rods in the *Y99C⁺ RetGC1^{-/-}* mice was robust and fairly uniform in different parts of the retina, typically within 60–80% normal linear nuclei density (Fig. 2*Ba,Bb*). On average, rod preservation in *Y99C⁺ RetGC1^{-/-}* mice at the age of 5–6 months was near 72%. Likewise, there was a dramatic (~56%) preservation of rods in *E155G⁺ RetGC1^{-/-}* retinas when the parental E155G⁺ line had virtually no photoreceptor nuclei left at that age (Fig. 2*C*).

Along with the robust preservation of the retinal morphology, the rescued Y99C and E155G rods remained functional when tested by scotopic ERG (Fig. 3). Although the scotopic ERG is a sum of both rod and cone activity, only 3% of total photoreceptors in a mouse retina are cones (Jeon et al., 1998), so the mass cone hyperpolarization signal is rather minuscule compared with that of rods and is hard to detect in WT mouse retinas (Sakurai et al., 2011). Therefore, we here refer to the scotopic a-wave as primarily rod ERG. In *RetGC1^{-/-}* mice, there are no functional cones and their ERG originates exclusively from rods (Yang et al., 1999; Baehr et al., 2007). The lack of RetGC1 is known to strongly reduce the amplitude of the rod ERG a-wave without causing rods to degenerate (Yang et al., 1999; Baehr et al., 2007). Conse-

quently, the amplitude of the rod ERG a-wave in *RetGC1^{-/-}* mice expressing Y99C or E155G GCAP1 in our experiments was also reduced (Fig. 3*A*). However, both the a-wave (Fig. 3*A–C*) and the b-wave (Fig. 3*A,D*) remained clearly defined in mice who expressed the mutated GCAP1 in *RetGC1^{-/-}* background, at the age when the a-wave was no longer detectable and only low-amplitude residual b-wave was occasionally present in the degenerated parental Y99C⁺ or E155G⁺ mice. The average a-wave amplitudes in WT, *RetGC1^{-/-}*, *Y99C⁺ RetGC1^{-/-}*, and *E155G⁺ RetGC1^{-/-}* mice were ~430, 127, 220, and 160 μV, respectively. The a-wave exceeded 100 μV in two *Y99C⁺ RetGC1^{-/-}* mice tested as late as 13 months of age (data not shown). The ANOVA/Bonferroni's all-pairs comparison test performed on genotypes summarized in Figure 3, *C* and *D*, indicated high statistical significance ($p < 0.0001$) at $\alpha = 0.01$ of ERG rescue in Y99C⁺ or E155G⁺ mice after elimination of RetGC1. We emphasize that the rescue effect in that case needs to be compared against the parental *RetGC1^{-/-}* genotype rather than WT mice, because RetGC1 knock-out itself strongly diminishes the ERG (Yang et al., 1999). Compared with *RetGC1^{-/-}*, there was even a small increase of the average a-wave amplitude in *Y99C⁺ RetGC1^{-/-}* mice (by ~93 μV), which was statistically significant (at $\alpha = 0.01$), and in *E155G⁺ RetGC1^{-/-}* mice (by ~33 μV), which was not statistically significant (at $\alpha = 0.05$).

In contrast, *RetGC2^{-/-}* rods harboring constitutively active GCAP1 degenerated in a rapid progressive manner (Fig. 4). Compared with the *Y99C⁺ RetGC1^{-/-}* mice, the *Y99C⁺ RetGC2^{-/-}* photoreceptor nuclei in most parts of the retina after 5 months were reduced to less than one full layer (Fig. 4*Aa–Ac*), although in some regions there were short clusters in which the residual nuclei were more abundant (Fig. 4*Ac*, bottom). The average linear nuclei density in the *Y99C⁺ RetGC2^{-/-}* retinas (Fig. 4*Bd*) was therefore slightly elevated compared with the Y99C⁺ mice (44 vs 24 per 200 μm retina length). However, in ANOVA/Bonferroni's all-pairs comparison test applied to the group (*Y99C⁺ RetGC2^{-/-}*, *Y99C⁺ RetGC1^{-/-}*, Y99C⁺, and WT), those two genotypes were not different at $\alpha = 0.05$. In contrast, the difference from both *Y99C⁺ RetGC1^{-/-}* (249 per 200 μm) and the WT (358 per 200 μm) was highly significant for both Y99C⁺ and the *Y99C⁺ RetGC2^{-/-}* mice ($p < 0.0001$ at $\alpha = 0.01$). Evidently, a small increase in linear nuclei density in Y99C⁺ *RetGC2^{-/-}* retinas did not constitute a substantial rescue effect.

Figure 2. The lack of RetGC1 preserves photoreceptors in mice expressing Y99C or E155G GCAP1. **A**, Cross-sections of glutaraldehyde-fixed retinas from WT (**a**), *RetGC1*^{-/-} (**b**), *Y99C*⁺ (**c**), and *Y99C*⁺*RetGC1*^{-/-} (**d**) mice aged 5.5 months and *E155G*⁺ (**e**) or *E155G*⁺*RetGC1*^{-/-} (**f**) mice aged 4.5 months; methylene blue/azur stain; 40× objective. Scale bar, 50 μm. For additional details, see Materials and Methods. Notice that, in contrast to the WT (**a**), the *Y99C*⁺ (**c**) and *E155G*⁺ (**e**) retinas demonstrate typical obliteration of the outer nuclear layer caused by constitutively active GCAP1 (Woodruff et al., 2007), whereas in *RetGC1*^{-/-} retinas (**b**), the outer nuclear layer remains primarily intact, consistent with the original report by Yang et al. (1999). Most of the rods survive in *Y99C*⁺*RetGC1*^{-/-} (**d**) and *E155G*⁺*RetGC1*^{-/-} (**f**) retinas and retain clearly identifiable layer of outer segments. onl, Outer nuclear layer; inl, inner nuclear layer. **B**, Preservation of photoreceptors in different areas of the retina. Full-size retinas section of the *Y99C*⁺ and *Y99C*⁺*RetGC1*^{-/-} retinas at 5.5 months of age (**a**), with area between the arrows marking the thickness of the photoreceptor layer showing that the outer nuclear layer of photoreceptors is mostly eliminated in the *Y99C*⁺ retinas but remains uniformly well detectable in all parts of the retina outside the optic nerve; each photograph is a composite of a series of micrographs taken with 10× objective. Scale bar, 500 μm. The distribution of the photoreceptor nuclei along the retina (**b**) was quantified in WT (□), *Y99C*⁺ (●), *E155G*⁺ (◆), *Y99C*⁺*RetGC1*^{-/-} (○), and *E155G*⁺*RetGC1*^{-/-} (◇) by counting identifiable photoreceptor nuclei per 200 μm increment distances, on both sides from the optic nerve (0 mm on the diagram), averaged from three sections in each retina. **C**, Nuclei density per 200 μm length in the mid-portion of the retina (0.5–1.5 mm from the optic nerve) averaged from random sampling of 40× objective frames from five different WT, *RetGC1*^{-/-}, *Y99C*⁺, *Y99C*⁺*RetGC1*^{-/-} (aged 5–6 months), and *E155G*⁺ and *E155G*⁺*RetGC1*^{-/-} (aged 4.5–5 months) retinas: 358 ± 56 (mean ± SD), $n = 11$; 346 ± 64, $n = 16$; 24 ± 8, $n = 17$; 249 ± 58, $n = 20$; 2 ± 2, $n = 10$; and 194 ± 23, $n = 10$, respectively.

The *RetGC2*^{-/-} mice retain near-normal ERG responses (Baehr et al., 2007) (Fig. 4Ca). In contrast to that, *Y99C*⁺*RetGC2*^{-/-} mice continue to demonstrate typical for *Y99C*⁺ rapid decline of scotopic ERG within the first 4 months of age (Fig. 4Cb,Cc). Concomitant with the progressive obliteration of the outer nuclear layer, the a-wave became undetectable in eight mice tested between 5 and 6 months of age (Fig. 4Cd), and only one of them demonstrated a small identifiable b-wave (Fig. 4Cd). That low-amplitude b-wave sustained a 30 lux background and therefore was likely a residual cone activity, occasionally detectable at that age in the parental *Y99C*⁺ line (Olshevskaya et al., 2004; Woodruff et al., 2007). Overall, retinal degeneration pattern in *Y99C*⁺ in *RetGC2*^{-/-} genetic background was similar to that originally reported for *Y99C*⁺ mice (Olshevskaya et al., 2004; Woodruff et al., 2007).

Deregulation of cGMP synthesis by Y99C GCAP1 leading to a progressive apoptotic cell death is accompanied by DNA fragmentation in matured photoreceptors (Dizhoor et al., 2006). We could not confidently identify apoptotic signals in differentiated WT and *Y99C*⁺*RetGC1*^{-/-} retinas at 1 month of age (Fig. 4D), and what we could regard as possible occurrence of TUNEL-positive nuclei was no higher than 1 per 2000 μm of the retina section length in both cases. We could, however, quite clearly observe TUNEL-positive nuclei scattered in the layer of differentiated photoreceptors in both *Y99C*⁺*RetGC2*^{-/-} and *Y99C*⁺ age-matched retinas (Fig. 4Dc,Dd). When randomly sampled in three sets of retinas (two of each genotype per experiment), the difference was significant (ANOVA/Bonferroni's test, $\alpha = 0.01$, $p < 0.0001$) between *Y99C*⁺*RetGC2*^{-/-} (linear density of 4.8 ± 2 SD per 400 μm, $n = 9$) and *Y99C*⁺*RetGC1*^{-/-} (0.2 ± 0.4 SD per 400 μm, $n = 5$) retinas but not between the *Y99C*⁺*RetGC2*^{-/-} and *Y99C*⁺ (5.1 ± 2.3 per 400 μm, $n = 7$) retinas. A relatively modest frequency of the apoptotic nuclei in the *Y99C*⁺ outer nuclear layer at that stage does not appear particularly surprising considering that it takes their photoreceptors several months to completely degenerate (Olshevskaya et al., 2004; Dizhoor et al., 2006).

Ca²⁺ sensitivity of cGMP synthesis does not change in Y99C or E155G GCAP1 rods lacking RetGC1

A strong decrease in Ca²⁺ sensitivity of the retinal guanylyl cyclase shown in Fig-

ure 5A, shifted outside the normal intracellular range of Ca^{2+} concentrations ($[\text{Ca}]_{1/2} > 0.2 \mu\text{M}$ vs $0.06 \mu\text{M}$ in WT), is the hallmark of the rods expressing Y99C or E155G GCAP1 transgene (Olshevskaya et al., 2004; Makino et al., 2006; Woodruff et al., 2007). It is technically impossible to accurately quantify the absolute activity of RetGC in normal and degenerating rods, because the severe early degeneration in $Y99C^{+}$ and $E155G^{+}$ retinas eliminates most of the activity simply because of the photoreceptor loss (Olshevskaya et al., 2004; Woodruff et al., 2007). However, the experiments presented in Figure 5 argue that the protective effect caused by the RetGC1 isozyme deletion coincides with a dramatic reversal of the abnormal Ca^{2+} sensitivity of the cGMP synthesis in transgenic mice (Fig. 5B). When Y99C or E155G GCAP1 is expressed in $\text{RetGC1}^{-/-}$ background, the cyclase sensitivity to Ca^{2+} becomes virtually the same as in WT or $\text{RetGC1}^{-/-}$ retinas ($[\text{Ca}]_{1/2}$ of $\sim 0.06 \mu\text{M}$) (Fig. 5B). In other words, RetGC2 isozyme remaining in the $\text{RetGC1}^{-/-}$ retinas is not affected by the constitutively active GCAP1 expressed in their rods. If there is any interaction between RetGC2 and the mutated GCAP1, it is too weak to be detected by our method. In contrast, elimination of RetGC2 isozyme does not reverse the abnormal sensitivity of the cGMP synthesis caused by the transgenic expression of the Y99C GCAP1 ($[\text{Ca}]_{1/2}$ of $\sim 0.3 \mu\text{M}$) (Fig. 5C).

The recombinant Y99C GCAP1 constitutively activates RetGC2 *in vitro* but not in a living retina

Both GCAP1 and GCAP2 efficiently activate mouse RetGC1 and RetGC2 isozymes *in vitro* (Peshenko et al., 2011). Therefore, it was surprising to find that the Ca^{2+} sensitivity of RetGC2 in the retinas expressing Y99C or E155G GCAP1 remained essentially normal. This could indicate that GCAP1 altered by the constitutive mutation has lost its ability to interact with RetGC2 as a result of the mutation per se. Alternatively, RetGC2 could be modified *in vivo* in such a manner that it no longer recognizes the mutated GCAP1. However, the results presented in Figure 6 reject both possibilities and argue that RetGC2 isozyme in the $Y99C^{+} \text{RetGC1}^{-/-}$ retinas can bind Y99C GCAP1 if such opportunity is presented. When the assay mixture containing $Y99C^{+} \text{RetGC1}^{-/-}$ retinal sample ($[\text{Ca}]_{1/2}$ of $\sim 0.077 \mu\text{M}$) was supplemented by purified Y99C GCAP1, the sensitivity of the endogenous RetGC2 was shifted far outside the normal range ($[\text{Ca}]_{1/2} > 0.6 \mu\text{M}$) (Fig. 6A). Essentially the same effect occurred in $E155G^{+} \text{RetGC1}^{-/-}$ retinas: the $[\text{Ca}]_{1/2}$ value measured directly in the retinal homogenate ($0.05 \mu\text{M}$) increased

Figure 3. The $Y99C^{+}$ and $E155G^{+}$ rods rescued by RetGC1 gene knock-out remain functional. **A**, Representative scotopic ERG responses to a saturating flash: **a**, WT (5.5 months of age); **b**, $Y99C^{+}$ mice (5.5 months); **c**, $E155G^{+}$ (3.5 months); **d**, $\text{RetGC1}^{-/-}$ (5 months); **e**, $Y99C^{+} \text{RetGC1}^{-/-}$ (5.5 months); **f**, $E155G^{+} \text{RetGC1}^{-/-}$ (5 months). **B**, Scotopic ERG a-wave amplitudes (saturating flash) in mice of different genotypes: **a**, WT ($\circ$) and $\text{RetGC1}^{-/-}$ ($\bullet$); **b**, $Y99C^{+}$ ($\triangle$) and $Y99C^{+} \text{RetGC1}^{-/-}$ ($\blacktriangledown$); **c**, $E155G^{+}$ ($\diamond$) and $E155G^{+} \text{RetGC1}^{-/-}$ ($\blacklozenge$). Each data point represents a separate mouse from the 4–6 month age group. The asterisks correspond to those mice whose ERG represent close to the average amplitude for the respective genotype and are shown in **A**. **C**, Scotopic a-wave amplitude (mean $\pm$ SEM) in WT, $\text{RetGC1}^{-/-}$, $Y99C^{+} \text{RetGC1}^{-/-}$, and $E155G^{+} \text{RetGC1}^{-/-}$ mice was $430 \pm 17 \mu\text{V}$, $n = 27$; $127 \pm 10 \mu\text{V}$, $n = 44$; $220 \pm 15 \mu\text{V}$, $n = 46$; and $160 \pm 13 \mu\text{V}$, $n = 29$, respectively. No measurable a-wave was recorded from 23 $Y99C^{+}$ and 13 $E155G^{+}$ mice. **D**, Scotopic b-wave amplitudes (mean $\pm$ SEM) in WT, $\text{RetGC1}^{-/-}$, $Y99C^{+}$, $Y99C^{+} \text{RetGC1}^{-/-}$, $E155G^{+}$, and $E155G^{+} \text{RetGC1}^{-/-}$ mice was $880 \pm 40 \mu\text{V}$, $n = 27$; $423 \pm 36 \mu\text{V}$, $n = 38$; $10 \pm 5 \mu\text{V}$, $n = 23$; $658 \pm 61 \mu\text{V}$, $n = 40$; $14 \pm 8 \mu\text{V}$, $n = 13$; and $465 \pm 50 \mu\text{V}$, $n = 27$, respectively. For additional details, see Materials and Methods.

almost 10-fold ($0.49 \mu\text{M}$) when purified recombinant $E155G^{+} \text{GCAP1}$ was added in the assay (Fig. 6B).

Constitutively active GCAP1 remains expressed in $\text{RetGC1}^{-/-}$ rods

Another possibility that needs to be addressed is the reduction of GCAP1 expression typically observed in $\text{RetGC1}^{-/-}$ but not $\text{RetGC2}^{-/-}$ background (Baehr et al. 2007). Indeed, if the mutated GCAP1 were merely not expressed in the $\text{RetGC1}^{-/-}$ background, there would be no reason to expect the remaining RetGC2 to alter its Ca^{2+} sensitivity. However, in contrast to the

Figure 4. RetGC2 knock-out does not prevent rod degeneration in $Y99C^+$ mice. **A**, Retinal histology: **a**, full cross-section of $Y99C^+RetGC2^{-/-}$ retina aged 5 months (a composite from a series of photographs taken with $10\times$ objective; scale bar, 500 μm ; the space between the arrows marks the thickness of the photoreceptor layer); **b**, a typical distribution of the photoreceptor nuclei in the $Y99C^+RetGC2^{-/-}$ retina (Δ) compared with that of $Y99C^+$ ($\bullet$) and $Y99C^+RetGC1^{-/-}$ ($\circ$), analyzed as described in Figure 2; **c**, representative cross-sections of the $Y99C^+RetGC2^{-/-}$ retinas ($40\times$ objective). The most typical pattern was elimination of the outer nuclear layer to less than one complete row of photoreceptor nuclei (top), with occasional clusters containing up to two rows of nuclei but without identifiable ROSs (bottom). **B**, The density of randomly sampled photoreceptor nuclei per 200 μm retina length within 0.5–1.5 mm eccentricity in five $Y99C^+RetGC2^{-/-}$ mice aged 5 to 6 months was strongly reduced (44 ± 24 SD per 200 μm , $n = 19$) compared with the $Y99C^+RetGC1^{-/-}$ retinas (249 ± 58 , $n = 20$) and close to that of the $Y99C^+$ genotype (24 ± 8 , $n = 17$). **C**, Scotopic ERG in 5.5-month-old $RetGC2^{-/-}$ (**a**) and in $Y99C^+RetGC2^{-/-}$ (**b–d**) mice at 1 month (**b**), 3.5 months (**c**), and 5.5 months (**d**) of age. A low-amplitude b-wave ($\sim 30 \mu V$) (**d**) was detected in one of eight tested $Y99C^+RetGC2^{-/-}$ mice; it remained in the presence of a 30 lux background incandescent light (**e**). There was no measurable a-wave in eight $Y99C^+RetGC2^{-/-}$ mice aged 5–6 months, similarly to the parental $Y99C^+$ line (Fig. 3). **D**, Photoreceptor apoptotic nuclei are detectable by TMR red TUNEL in $Y99C^+RetGC2^{-/-}$ mice but not in $Y99C^+RetGC1^{-/-}$ mice. Paraformaldehyde-fixed cryosections of WT (**a**), $Y99C^+RetGC1^{-/-}$ (**b**), $Y99C^+RetGC2^{-/-}$ (**c**), and $Y99C^+$ (**d**) retinas (postnatal day 28) were probed for DNA fracture using TUNEL reaction (red fluorescence) in the same experiment; the nuclei were counterstained with DAPI (blue). Scale bar, 50 μm . onl, Outer nuclear layer. For additional details, see Materials and Methods.

endogenous GCAP1, the Y99C mutant, whose transgenic expression is driven by rod opsin promoter, robustly accumulates in $RetGC1^{-/-}$ rods (Fig. 7A). There was also the anticipated loss of RetGC1 but not RetGC2 or GCAP2 expression in the $Y99C^+RetGC1^{-/-}$ retinas (Fig. 7Aa,Ab). The total expression level of GCAP1 was not only well detectable but was also markedly higher than normal. Similar to the original Y99C line 53 (Olshevskaya et al., 2004), the transgene expression exceeded more than fivefold the level of the endogenous WT GCAP1 (note that the transgenic Y99C migrates in SDS gel as the upper band) (for details, see Olshevskaya et al., 2004). Furthermore, anti-GCAP1 IF in the outer segments of $RetGC1^{-/-}$ mice was noticeably dimmer compared with the WT rods, as described previously by Baehr et al. (2007), yet it was well detectable in $RetGC1^{-/-}$ rods expressing the transgenic mutants of GCAP1 (Fig. 7B). The overall brightness of the anti-GCAP1 fluorescence in $Y99C^+RetGC1^{-/-}$ rods (Fig. 7Bc) was visibly increased compared with the WT. Likewise, $E155G^+RetGC1^{-/-}$ mice under the same conditions displayed a robust GCAP1 IF in rods, brighter than in the WT (Fig. 7Bd). Evidently, the normal Ca^{2+} sensitivity of RetGC2 in $Y99C^+RetGC1^{-/-}$ or $E155G^+RetGC1^{-/-}$ retinas (Fig. 5) cannot be explained by a lack of mutant GCAP1 expression in their rods.

GCAP2 gene knock-out has different effect on rod recovery in $RetGC1^{-/-}$ and $RetGC2^{-/-}$ mice

One could argue that the *in vivo* selectivity between RetGC1 and RetGC2 exists only for the two tested constitutively active GCAP1 mutants, not necessarily for the normal GCAP1. To evaluate the general possibility that GCAP1 selectively targets RetGC1 *in vivo*, we compared the rates of rod recovery using gene knock-out hybrids that expressed only one of the RetGC isozymes and only GCAP1 to mediate the Ca^{2+} feedback. To produce those hybrids, $RetGC1^{-/-}$ or $RetGC2^{-/-}$ mice were crossed with $GCAP2^{-/-}$ mice, which have a disrupted *GUCA1B* gene but continue to express GCAP1, RetGC1, and RetGC2 (Makino et al., 2008). For the comparison with the mice completely lacking Ca^{2+} feedback to the particular cyclase isozyme, we also combined either RetGC1 or RetGC2 gene knock-out with the $GCAP1,2^{-/-}$ double knock-out, in which a portion of the chromosome containing the adjacent *GUCA1A* and *GUCA1B* genes was deleted (Mendez et al., 2001). The $GCAP1,2^{-/-}$ ret-

Figure 5. Calcium sensitivity of cGMP synthesis in mouse retinas expressing Y99C or E155G GCAP1 varies depending on the RetGC isozymes present in rods. **A**, In WT retinas (●), cGMP synthesis is sensitive to Ca^{2+} within the normal range (shadowed) of free Ca^{2+} between the light and the dark (Woodruff et al., 2002), but in Y99C^{+} (○) and E155G^{+} (◇) retinas, the sensitivity shifts outside the upper limit of the normal Ca^{2+} range. **B**, Ca^{2+} sensitivity of guanylyl cyclase in $\text{Y99C}^{+}\text{RetGC1}^{-/-}$ (◇) or $\text{E155G}^{+}\text{RetGC1}^{-/-}$ (◇) retinas becomes indistinguishable from that of the WT (●) or $\text{RetGC1}^{-/-}$ (△). **C**, Ca^{2+} sensitivity of retinal guanylyl cyclase in $\text{Y99C}^{+}\text{RetGC2}^{-/-}$ retinas (▲) remains right-shifted compared with the WT (●), just like in Y99C^{+} (○) mice. The cyclase activity was assayed as described in Materials and Methods. For comparison on the same scale, the activities in each series were normalized by the corresponding maximal RetGC activity measured in each genotype. The data were fitted by the equation $A = (A_{\max} - A_{\min}) / (1 + ([\text{Ca}]/[\text{Ca}]_{1/2})^h) + A_{\min}$, where $A_{\max}$ and $A_{\min}$ are the maximal and the minimal activity of guanylyl cyclase, respectively, $[\text{Ca}]_{1/2}$ is the concentration of Ca^{2+} producing 50% inhibition, and h is a Hill coefficient.

inas express normal complement of RetGC isozymes but recover very slowly, because their basal activity is not accelerated after photoexcitation (Mendez et al., 2001; Burns et al., 2002).

The $\text{RetGC1}^{-/-}$ or $\text{RetGC2}^{-/-}$ mice bred into $\text{GCAP1,2}^{-/-}$ or $\text{GCAP2}^{-/-}$ background were tested for their mass rod response recovery after a bright flash using a paired-flashes ERG paradigm (Lyubarsky and Pugh, 1996) (Fig. 8). It needs to be emphasized that $\text{RetGC1}^{-/-}$ a-wave recovers slightly slower than in the WT in paired-flashes ERG experiments, despite its amplitude being greatly diminished (Yang et al., 1999; Baehr et al., 2007). The amplitudes of the test flash response varied between different genotypes (380, 98, 137, 47, 224, 320, and 171 μV in WT, $\text{RetGC1}^{-/-}$, $\text{RetGC1}^{-/-}\text{GCAP2}^{-/-}$, $\text{RetGC1}^{-/-}\text{GCAP1,2}^{-/-}$, $\text{RetGC2}^{-/-}$, $\text{RetGC2}^{-/-}\text{GCAP2}^{-/-}$, and $\text{RetGC2}^{-/-}\text{GCAP1,2}^{-/-}$ mice, respectively). Note that, although WT rods would recover

Figure 6. RetGC2 reacts with the purified Y99C or E155G GCAP1 *in vitro* but is insensitive to the Y99C or E155G GCAP1 transgenically expressed in the rods. **A**, The cyclase activity was assayed at different free Ca^{2+} concentrations in the retinas obtained from the WT (●), $\text{RetGC1}^{-/-}$ (■), and $\text{Y99C}^{+}\text{RetGC1}^{-/-}$ (○) mice; the $\text{Y99C}^{+}\text{RetGC1}^{-/-}$ retinas were also assayed in the presence of 10 μM purified Y99C GCAP1 (◇). **B**, The cyclase activity was assayed at different free Ca^{2+} concentrations in the retinas obtained from the WT (●), $\text{RetGC1}^{-/-}$ (■), and $\text{E155G}^{+}\text{RetGC1}^{-/-}$ (○) mice; the $\text{E155G}^{+}\text{RetGC1}^{-/-}$ retinas were also assayed in the presence of 10 μM purified E155G GCAP1 (△).

faster from “test” flashes of lower amplitude (which is, by decreasing the flash strength), the average “test” a-wave amplitude and the speed of the recovery among the genotypes in our experiments (0.6, 0.7, 1.2, 1.4, 0.65, 0.7, and 1.8 s half-recovery time, respectively) did not correlate. The strength of the flashes was always standard and the difference was only in genotype. Consistent with previous reports (Yang et al., 1999; Baehr et al., 2007), rod responses in $\text{RetGC1}^{-/-}$ and $\text{RetGC2}^{-/-}$ mice were markedly different from each other in their a-wave amplitudes, but in both they recovered only slightly behind the WT. Likewise, in both $\text{RetGC1}^{-/-}$ and $\text{RetGC2}^{-/-}$, the recovery became very sluggish after elimination of the two GCAPs together (Fig. 8B,C). However, elimination of GCAP2 alone affected the speed of recovery in $\text{RetGC1}^{-/-}$ versus $\text{RetGC2}^{-/-}$ mice in a very different manner. Whereas $\text{RetGC2}^{-/-}\text{GCAP2}^{-/-}$ recovered at nearly the same rate as $\text{RetGC2}^{-/-}$, the recovery in $\text{RetGC1}^{-/-}\text{GCAP2}^{-/-}$ was strongly delayed compared with $\text{RetGC1}^{-/-}$ (Fig. 8A). These results argue that, in the absence of GCAP2, the RetGC1 isozyme of the cyclase can and the RetGC2 isozyme cannot effectively receive Ca^{2+} feedback through the remaining GCAP1.

Discussion

Two photoreceptor calcium-sensor proteins, GCAP1 and GCAP2, activate membrane guanylyl cyclase in a “relay” manner (Makino et al., 2008), but mutations in GCAP1 cause photoreceptor degeneration in humans. Ca^{2+} -insensitive GCAP1 mutants causing the disease trigger apoptotic photoreceptor death in transgenic mice by deregulating cGMP synthesis (Olshevskaya et al., 2004; Woodruff et al., 2007). Because GCAP1 effectively regulates both RetGC1 and RetGC2 *in vitro* (Peshenko et al., 2011), the goal of our study was to identify the target isozyme for the

Figure 7. Constitutively active GCAP1 transgene is strongly expressed in *RetGC1*^{−/−} rods. **A**, Immunoblotting of retina samples from WT and *Y99C*⁺*RetGC1*^{−/−} mice (**a**), from top to bottom: GCAP1, the arrows indicate the position of the transgenic Y99C GCAP1 (top band) and the endogenous WT GCAP1 (bottom band) (for details, see Olshevskaya et al., 2004); GCAP2; RetGC1; RetGC2; β -actin; and their respective semiquantitative comparison (**b**) by the relative chemiluminescence intensity (*R*) to the WT. **B**, FITC anti-GCAP1 IF (left). The cryosections from WT (**a**), *RetGC1*^{−/−} (**b**), *Y99C*⁺*RetGC1*^{−/−} (**c**), and *E155G*⁺*RetGC1*^{−/−} (**d**) retinas at 1 month of age were probed with anti-GCAP1 antibody as described in Materials and Methods. In the right portion of each image, the nuclei counterstained with TO-PRO-3 (red) are also shown in the same frame to mark the histological layers. Scale bar, 50 μ m. The eyes were fixed and processed using the same protocol, and the GCAP1 fluorescence images were acquired using identical settings for excitation and image acquisition. The staining in **a–c** was processed simultaneously and in **d** under identical conditions using the same reagents and staining procedure.

mutant GCAP1 *in vivo* (Fig. 1). GCAP and RetGC cannot interact in the presence of detergents required for extraction of RetGC from the membrane (Koch, 1991), which makes biochemical isolation of GCAP/RetGC complexes (e.g., by coimmunoprecipitation) impossible. Instead, we used a comparative study of retinal degeneration and cyclase regulation in transgenic mice expressing a constitutively active GCAP1 but lacking one of the RetGC isozymes. We reasoned that, in the absence of the target isozyme, the Ca^{2+} sensitivity of cGMP synthesis would return to the normal Ca^{2+} range even in the presence of Ca^{2+} -insensitive GCAP1.

Transgenic expression of constitutively active GCAP1 in the absence of RetGC1 provides massive rescue of rod photoreceptors—both morphological (Fig. 2) and functional (Fig. 3). In contrast, no evidence for similar rescue was found in mice lacking RetGC2 isozyme (Fig. 4). Last, transgenically expressed constitutively active GCAP1 fails to shift Ca^{2+} sensitivity of cGMP synthesis in *RetGC1*^{−/−} but not *RetGC2*^{−/−} retinas (Fig. 5). These findings implicate RetGC1 as a preferential target for the mutant GCAP1 *in vivo*.

It is not completely clear why most, but not all, *RetGC1*^{−/−} rods expressing mutant GCAP1 were rescued (Fig. 2). Inactivation and rapid loss of *RetGC1*^{−/−} cones (Yang et al., 1999) could only account for ~3% reduction of photoreceptor nuclei count in *Y99C*⁺*RetGC1*^{−/−} retinas, which leaves >25% photoreceptors unaccounted for. As a likely reason, the levels of the transgenic overexpression, which usually varies between different cells, may become high enough in some of them to provoke protein overload response, thus eliminating such cells before their complete differentiation. The rest of the *RetGC1*^{−/−} rods evidently tolerate high levels of the mutant GCAP1 expression—they escape early death and mature by 1 month of age (Fig. 7). It is particularly important to reiterate that the cyclase sensitivity to Ca^{2+} in the matured *Y99C*⁺*RetGC1*^{−/−} or *E155G*⁺*RetGC1*^{−/−} rods overexpressing mutant GCAP1 becomes normal (Fig. 5B), and this can explain why TUNEL does not reveal apoptotic activity in the differentiated *Y99C*⁺*RetGC1*^{−/−} rods (Fig. 4D). In contrast, mutant GCAP1 still causes abnormal regulation of the cyclase (Fig. 5C) and continues to exert apoptotic DNA fracture in mature *RetGC2*^{−/−} photoreceptors (Fig. 4D).

RetGC2 activity in *Y99C*⁺*RetGC1*^{−/−} or *E155G*⁺*RetGC1*^{−/−} rods is sufficient to preserve their photoresponse, albeit at lower than WT levels (Fig. 3). It is important to note that full restoration of this function could not be expected even if all rods were rescued from degeneration, because the parental *RetGC1*^{−/−} genotype per se has diminished rod ERG, a previously established phenomenon, which appears paradoxical considering the near-normal single rod responses in this genotype (Yang et al., 1999; Baehr et al., 2007). Taking this into account, the functional rescue of *Y99C*⁺*RetGC1*^{−/−} and *E155G*⁺*RetGC1*^{−/−} rods appears quite efficient. The average a-wave amplitude in the *Y99C*⁺*RetGC1*^{−/−} ERG was even slightly larger than in the parental *RetGC1*^{−/−} mice (Fig. 3C), perhaps as a result of outcrossing of a highly inbred *RetGC1*^{−/−} line.

The most unexpected biochemical finding from this study is that only RetGC1 and not RetGC2 becomes deregulated by the constitutively active GCAP1 (Figs. 5, 6). Evidently, this difference cannot be explained by much higher intrinsic binding affinity of GCAP1 to RetGC1, because mouse GCAP1 activates both native isozymes with fairly similar apparent affinities in purified mouse outer segment membranes (Peshenko et al., 2011). As we discussed above, the RetGC/GCAP complex cannot be directly isolated from the retina but can be detected functionally, based on the Ca^{2+} sensitivity of the cyclase, because in the absence of GCAPs both RetGC isozymes are completely insensitive to Ca^{2+} (Peshenko et al., 2011). The normal Ca^{2+} sensitivity of the cy-

Figure 8. Scotopic ERG recovery of *RetGC1*^{−/−} and *RetGC2*^{−/−} retinas in *GCAP1,2*^{−/−} and *GCAP2*^{−/−} backgrounds. **A**, Rod recovery was assessed by paired-flashes scotopic ERG a-wave using a white “probe” flash ($\sim 500 \times 10^3$ photons/ μm^2 at cornea), delivered at different interstimulus time intervals (IST) after the conditioning test flash suppressing the circulating current (510 nm, $\sim 5 \times 10^3$ photons/ μm^2 at cornea). Two sets of representative raw traces for *RetGC1*^{−/−} (**a**) and *RetGC1*^{−/−} *GCAP2*^{−/−} (**b**) mice, respectively, are shown as an example; note the delayed recovery of the probe flash ERG (arrows) in the *RetGC1*^{−/−} *GCAP2*^{−/−} genotype. **B**, **C**, Average fractional recovery (*r*) in 16 WT (○), 10 *RetGC1*^{−/−} (△), 16 *RetGC1*^{−/−} *GCAP2*^{−/−} (▲), 12 *RetGC1*^{−/−} *GCAP1,2*^{−/−} (●), 13 *RetGC2*^{−/−} (□), 11 *RetGC2*^{−/−} *GCAP2*^{−/−} (■), and 16 *RetGC2*^{−/−} *GCAP1,2*^{−/−} (◆) mice aged 2 months. The lack of GCAP2 markedly extends the recovery in *RetGC1*^{−/−} mice (**B**) but not in *RetGC2*^{−/−} mice (**C**). The probe flash a-wave amplitude at indicated interstimulus intervals was normalized by that of a probe flash response amplitude delivered without the test flash. The time for a 50% recovery in WT, *RetGC1*^{−/−}, *RetGC1*^{−/−} *GCAP2*^{−/−}, *RetGC1*^{−/−} *GCAP1,2*^{−/−}, *RetGC2*^{−/−}, *RetGC2*^{−/−} *GCAP2*^{−/−}, and *RetGC2*^{−/−} *GCAP1,2*^{−/−} mice was 0.6, 0.7, 1.2, 1.4, 0.65, 0.7, and 1.8 s, respectively.

clase in the *Y99C*⁺ *RetGC1*^{−/−} and *E155G*⁺ *RetGC1*^{−/−} retinas argues that mutated GCAP1 in living rods can form a stable complex with RetGC1 but not with RetGC2 (Fig. 5). Based on the study by Hwang et al. (2003), we can expect (Peshenko et al., 2011) that GCAP concentrations in mouse rods are as high as 7–9 μM , which would nearly saturate RetGC activity *in vitro*. Consistent with this, 10 μM purified recombinant Y99C or E155G GCAP1 added to the cyclase assay containing Y99C⁺ *RetGC1*^{−/−} or E155G⁺ *RetGC1*^{−/−} retina drastically shifts its Ca^{2+} sensitivity curve (Fig. 6). Hence, the mutated GCAP1 can effectively form a complex with RetGC2 when this isozyme becomes accessible to GCAP1 in retinal homogenate. However, no such shift occurs because of the transgenically expressed GCAP1. This indicates that not the intrinsic binding affinities of RetGC and GCAPs are at play, but rather presently unknown spatial organization or

assembly processes *in vivo* preclude RetGC2 from tight association with the mutant GCAP1.

Is such restriction only characteristic of the Y99C and E155G GCAP1 or does it reflect a more general rule? This is a principal question, because multiple mutations in GCAP1 have already been linked to human retinal diseases, and it would be important to find whether or not mutations making GCAP1 into a constitutive activator would likely act via RetGC1 isozyme. The data in Figure 8 argue that, in contrast to the nearly indiscriminate RetGC/GCAP interactions *in vitro*, there is a strong selectivity imposed on GCAP1 interactions *in vivo*. RetGC1 can account for $\sim 70\%$ and RetGC2 nearly 30% of the total maximal cGMP production in mouse rods (Peshenko et al., 2011), and each isozyme alone is evidently able to support their timely, almost as quickly as the WT, recovery (Yang et al., 1999; Baehr et al., 2007). However, elimination of GCAP2 causes rod ERG in the *RetGC1*^{−/−} mice to recover much slower—nearly as slow as in a complete absence of the Ca^{2+} feedback (Fig. 8B). This indicates that even the normal endogenous GCAP1 has limited access to the remaining RetGC2 isozyme and regulates it with a very low efficiency (if any). At the same time, the absence of GCAP2 has little effect on *RetGC2*^{−/−} rod recovery (Fig. 8C), so GCAP1 in these rods evidently continues to regulate the remaining RetGC1 isozyme in a normal manner. Consequently, from the general ability of GCAP1 to effectively activate only RetGC1 *in vivo*, we should expect that not just Y99C or E155G but other disease-related GCAP1 as well would also selectively target RetGC1.

GCAP1 fails to provide efficient Ca^{2+} feedback to RetGC2 *in vivo* not because its intrinsic binding affinity or the extent of RetGC2 activation are too low—they are both comparably high when tested at physiological concentrations *in vitro* (Peshenko et al., 2011). The selectivity would rather come from structural and/or spatial organization of the RetGC/GCAP complexes in living rods, limiting GCAP1 access to RetGC1. It could be imposed by a presently unidentified sorting mechanism that allows RetGC1/GCAP1 but not RetGC2/GCAP1 complex into the outer segment. GCAP1 localization in the inner segment and cell body of the *RetGC1*^{−/−} rods (Fig. 7B) supports such a possibility. Whereas the endogenous GCAP1 and the Y99C mutant transgenically expressed in mouse rods both tend to accumulate in the outer segment (Olshevskaya et al., 2004), the Y99C GCAP1 is instead accumulated in the cell body and inner segment in nondegenerating *RetGC*^{−/−}, Y99C⁺ *RetGC1*^{−/−}, and E155G⁺ *RetGC1*^{−/−} rods (Fig. 7B). Evidently, accumulation in the cell body per se does not reflect the events leading to rod degeneration but rather suggests that RetGC1 is needed to guide GCAP1 to the outer segment. Alternatively (or additionally), factor(s) destabilizing GCAP1 complex with RetGC2 or hampering their mutual access may be present directly in the outer segment, because some of the mutant GCAP1 likely diffuses into the Y99C⁺ *RetGC1*^{−/−} outer segment (Fig. 7B) but nonetheless does not affect Ca^{2+} sensitivity of the remaining RetGC2 isozyme (Fig. 5). Only future studies could identify cellular factors involved in the GCAP1 selectivity, but we can already rule out an irreversible post-translational modification of RetGC2 as one of them, because native RetGC2 binds purified normal GCAP1 (Peshenko et al., 2011) and constitutively active GCAP1 (Fig. 6) in crude retinal homogenates.

The absence of a major effect of GCAP2 knock-out in *RetGC2*^{−/−} ERG recovery (Fig. 8C) could make it tempting to speculate that GCAP2 does not provide Ca^{2+} feedback to RetGC1 *in vivo*. However, we should emphasize that such speculation cannot be derived from the present study, because it is quite possible that GCAP2 and GCAP1 compete for RetGC1 in

RetGC2^{−/−} rods and that, in the absence of GCAP2, the cyclase regulation can be taken over by the remaining GCAP1. This uncertainty could only be resolved by using *RetGC1*^{−/−} and *RetGC2*^{−/−} mice lacking GCAP1.

References

- Baehr W, Karan S, Maeda T, Luo DG, Li S, Bronson JD, Watt CB, Yau KW, Frederick JM, Palczewski K (2007) The function of guanylate cyclase 1 and guanylate cyclase 2 in rod and cone photoreceptors. *J Biol Chem* 282:8837–8847.
- Burns ME, Mendez A, Chen J, Baylor DA (2002) Dynamics of cyclic GMP synthesis in retinal rods. *Neuron* 36:81–91.
- Dizhoor AM, Lowe DG, Olshevskaya EV, Laura RP, Hurley JB (1994) The human photoreceptor membrane guanylyl cyclase, RetGC, is present in outer segments and is regulated by calcium and a soluble activator. *Neuron* 12:1345–1352.
- Dizhoor AM, Olshevskaya EV, Henzel WJ, Wong SC, Stults JT, Ankoudinova I, Hurley JB (1995) Cloning, sequencing, and expression of a 24-kDa Ca²⁺-binding protein activating photoreceptor guanylyl cyclase. *J Biol Chem* 270:25200–25206.
- Dizhoor AM, Boikov SG, Olshevskaya EV (1998) Constitutive activation of photoreceptor guanylate cyclase by Y99C mutant of GCAP-1. Possible role in causing human autosomal dominant cone degeneration. *J Biol Chem* 273:17311–17314.
- Dizhoor AM, Olshevskaya EV, Peshenko IV (2006) Calcium sensitivity of photoreceptor guanylyl cyclase (RetGC) and congenital photoreceptor degeneration: modeling in vitro and in vivo. In: *Neuronal calcium sensor proteins* (Philippov PP, Koch KW, eds), pp 203–219. Hauppauge, NY: Nova Science Publishers.
- Dizhoor AM, Olshevskaya EV, Peshenko IV (2010) Mg²⁺/Ca²⁺ cation binding cycle of guanylyl cyclase activating proteins (GCAPs): role in regulation of photoreceptor guanylyl cyclase. *Mol Cell Biochem* 334:117–124.
- Fu Y, Yau KW (2007) Phototransduction in mouse rods and cones. *Pflügers Arch* 454:805–819.
- Gorczyca WA, Gray-Keller MP, Detwiler PB, Palczewski K (1994) Purification and physiological evaluation of a guanylate cyclase activating protein from retinal rods. *Proc Natl Acad Sci U S A* 91:4014–4018.
- Gray-Keller MP, Detwiler PB (1994) The calcium feedback signal in the phototransduction cascade of vertebrate rods. *Neuron* 13:849–861.
- Hwang JY, Lange C, Helten A, Höppner-Heitmann D, Duda T, Sharma RK, Koch KW (2003) Regulatory modes of rod outer segment membrane guanylate cyclase differ in catalytic efficiency and Ca²⁺-sensitivity. *Eur J Biochem* 270:3814–3821.
- Jeon CJ, Strettoi E, Masland RH (1998) The major cell populations of the mouse retina. *J Neurosci* 18:8936–8946.
- Jiang L, Zhang H, Dizhoor AM, Boye SE, Hauswirth WW, Frederick JM, Baehr W (2011) Long-term RNA interference gene therapy in a dominant retinitis pigmentosa mouse model. *Proc Natl Acad Sci U S A* 108:18476–18481.
- Kitiratschky VB, Behnen P, Kellner U, Heckenlively JR, Zrenner E, Jägle H, Kohl S, Wissinger B, Koch KW (2009) Mutations in the GUCA1A gene involved in hereditary cone dystrophies impair calcium-mediated regulation of guanylate cyclase. *Hum Mutat* 30:E782–E796.
- Koch KW (1991) Purification and identification of photoreceptor guanylate cyclase. *J Biol Chem* 266:8634–8637.
- Liu X, Pawlyk BS, Adamian M, Olshevskaya EV, Dizhoor AM, Makino CL, Li T (2009) Increased light exposure alleviates one form of photoreceptor degeneration marked by elevated calcium in the dark. *PLoS One* 4:e8438.
- Lowe DG, Dizhoor AM, Liu K, Gu Q, Spencer M, Laura R, Lu L, Hurley JB (1995) Cloning and expression of a second photoreceptor-specific membrane retina guanylyl cyclase (RetGC), RetGC-2. *Proc Natl Acad Sci U S A* 92:5535–5539.
- Lyubarsky AL, Pugh EN Jr (1996) Recovery phase of the murine rod photoresponse reconstructed from electroretinographic recordings. *J Neurosci* 16:563–571.
- Makino CL, Wen XH, Michaud N, Peshenko IV, Pawlyk B, Brush RS, Soloviev M, Liu X, Woodruff ML, Calvert PD, Savchenko AB, Anderson RE, Fain GL, Li T, Sandberg MA, Dizhoor AM (2006) Effects of low AIPL1 expression on phototransduction in rods. *Invest Ophthalmol Vis Sci* 47:2185–2194.
- Makino CL, Peshenko IV, Wen XH, Olshevskaya EV, Barrett R, Dizhoor AM (2008) A role for GCAP2 in regulating the photoresponse. Guanylyl cyclase activation and rod electrophysiology in GUCA1B knock-out mice. *J Biol Chem* 283:29135–29143.
- Mendez A, Burns ME, Sokal I, Dizhoor AM, Baehr W, Palczewski K, Baylor DA, Chen J (2001) Role of guanylate cyclase-activating proteins (GCAPs) in setting the flash sensitivity of rod photoreceptors. *Proc Natl Acad Sci U S A* 98:9948–9953.
- Michaelides M, Wilkie SE, Jenkins S, Holder GE, Hunt DM, Moore AT, Webster AR (2005) Mutation in the gene GUCA1A, encoding guanylate cyclase-activating protein 1, causes cone, cone-rod, and macular dystrophy. *Ophthalmology* 112:1442–1447.
- Olshevskaya EV, Hughes RE, Hurley JB, Dizhoor AM (1997) Calcium binding, but not a calcium-myristoyl switch, controls the ability of guanylyl cyclase-activating protein GCAP-2 to regulate photoreceptor guanylyl cyclase. *J Biol Chem* 272:14327–14333.
- Olshevskaya EV, Calvert PD, Woodruff ML, Peshenko IV, Savchenko AB, Makino CL, Ho YS, Fain GL, Dizhoor AM (2004) The Y99C mutation in guanylyl cyclase-activating protein 1 increases intracellular Ca²⁺ and causes photoreceptor degeneration in transgenic mice. *J Neurosci* 24:6078–6085.
- Palczewski K, Subbaraya I, Gorczyca WA, Helekar BS, Ruiz CC, Ohguro H, Huang J, Zhao X, Crabb JW, Johnson RS, Walsh KA, Gray-Keller MP, Detwiler PB, Baehr W (1994) Molecular cloning and characterization of retinal photoreceptor guanylyl cyclase-activating protein. *Neuron* 13:395–404.
- Payne AM, Downes SM, Bessant DA, Taylor R, Holder GE, Warren MJ, Bird AC, Bhattacharya SS (1998) A mutation in guanylate cyclase activator 1A (GUCA1A) in an autosomal dominant cone dystrophy pedigree mapping to a new locus on chromosome 6p21.1. *Hum Mol Genet* 7:273–277.
- Peshenko IV, Dizhoor AM (2004) Guanylyl cyclase-activating proteins (GCAPs) are Ca²⁺/Mg²⁺ sensors: implications for photoreceptor guanylyl cyclase (RetGC) regulation in mammalian photoreceptors. *J Biol Chem* 279:16903–16906.
- Peshenko IV, Dizhoor AM (2006) Ca²⁺ and Mg²⁺ binding properties of GCAP-1. Evidence that Mg²⁺-bound form is the physiological activator of photoreceptor guanylyl cyclase. *J Biol Chem* 281:23830–23841.
- Peshenko IV, Olshevskaya EV, Savchenko AB, Karan S, Palczewski K, Baehr W, Dizhoor AM (2011) Enzymatic properties and regulation of the native isozymes of retinal membrane guanylyl cyclase (RetGC) from mouse photoreceptors. *Biochemistry* 50:5590–5600.
- Pugh EN Jr, Duda T, Sitaramayya A, Sharma RK (1997) Photoreceptor guanylate cyclases: a review. *Biosci Rep* 17:429–473.
- Pugh EN Jr, Nikonov S, Lamb TD (1999) Molecular mechanisms of vertebrate photoreceptor light adaptation. *Curr Opin Neurobiol* 9:410–418.
- Sakurai K, Chen J, Kefalov VJ (2011) Role of guanylyl cyclase modulation in mouse cone phototransduction. *J Neurosci* 31:7991–8000.
- Sokal I, Li N, Surgucheva I, Warren MJ, Payne AM, Bhattacharya SS, Baehr W, Palczewski K (1998) GCAP1 (Y99C) mutant is constitutively active in autosomal dominant cone dystrophy. *Mol Cell* 2:129–133.
- Sokal I, Dupps WJ, Grassi MA, Brown J Jr, Affatigato LM, Roychowdhury N, Yang L, Filipek S, Palczewski K, Stone EM, Baehr W (2005) A novel GCAP1 missense mutation (L151F) in a large family with autosomal dominant cone-rod dystrophy (adCORD). *Invest Ophthalmol Vis Sci* 46:1124–1132.
- Tsien R, Pozzan T (1989) Measurement of cytosolic free Ca²⁺ with quin2. *Methods Enzymol* 172:230–262.
- Wilkie SE, Li Y, Deery EC, Newbold RJ, Garibaldi D, Bateman JB, Zhang H, Lin W, Zack DJ, Bhattacharya SS, Warren MJ, Hunt DM, Zhang K (2001) Identification and functional consequences of a new mutation (E155G) in the gene for GCAP1 that causes autosomal dominant cone dystrophy. *Am J Hum Genet* 69:471–480.
- Woodruff ML, Sampath AP, Matthews HR, Krasnoperova NV, Lem J, Fain GL (2002) Measurement of cytoplasmic calcium concentration in the rods of wild-type and transducin knock-out mice. *J Physiol* 542:843–854.
- Woodruff ML, Olshevskaya EV, Savchenko AB, Peshenko IV, Barrett R, Bush RA, Sieving PA, Fain GL, Dizhoor AM (2007) Constitutive excitation by Gly90Asp rhodopsin rescues rods from degeneration caused by elevated production of cGMP in the dark. *J Neurosci* 27:8805–8815.
- Yang RB, Foster DC, Garbers DL, Fülle HJ (1995) Two membrane forms of guanylyl cyclase found in the eye. *Proc Natl Acad Sci U S A* 92:602–606.
- Yang RB, Robinson SW, Xiong WH, Yau KW, Birch DG, Garbers DL (1999) Disruption of a retinal guanylyl cyclase gene leads to cone-specific dystrophy and paradoxical rod behavior. *J Neurosci* 19:5889–5897.