GCAP1 rescues rod photoreceptor response in GCAP1/GCAP2 knockout mice

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Visual transduction in retinal photoreceptors operates through a dynamic interplay of two second messengers, Ca^{2+} and cGMP. Ca^{2+} regulates the activity of guanylate cyclase (GC) and the synthesis of cGMP by acting on a GC-activating protein (GCAP). While this action is critical for rapid termination of the light response, the GCAP responsible has not been identified. To test if GCAP1, one of two GCAPs present in mouse rods, supports the generation of normal flash responses, transgenic mice were generated that express only GCAP1 under the control of the endogenous promoter. Paired flash responses revealed a correlation between the degree of recovery of the rod a-wave and expression levels of GCAP1. In single cell recordings, the majority of the rods generated flash responses that were indistinguishable from wild type. These results demonstrate that GCAP1 at near normal levels supports the generation of wild-type flash responses in the absence of GCAP2.

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Introduction

Vision begins with the conversion of light into an electrical response by the retina's photoreceptors (for a recent review see McBee *et al.*, 2001). The receptor light response involves changes in two intracellular second messengers, cGMP and Ca^{2+} . In darkness, high intracellular [cGMP] keeps cyclic nucleotide gated ion channels (CNG channels) open and a steady current (carried by Na^+ and Ca^{2+}) flows into the outer segment. Light activates a G-protein coupled enzyme cascade that stimulates cGMP-specific

phosphodiesterase (PDE). The resulting fall in [cGMP] causes the CNG channels to close and decreases Ca^{2+} influx. Efflux of Ca^{2+} by $\text{Na}^+/\text{Ca}^{2+}, \text{K}^+$ exchange persists, causing a fall in intracellular Ca^{2+} . The fall in Ca^{2+} stimulates synthesis of cGMP by guanylate cyclase (GC) and leads to timely recovery of the light response. Here we examine the molecular mechanism responsible for the Ca^{2+} -regulation of photoreceptor GC.

The mammalian retina contains two particulate GCs (GC1 and GC2), and two different GC-activating proteins (GCAP1 and GCAP2). A third GCAP has been identified only in humans (Haeseleer *et al.*, 1999) and zebrafish (Imanishi *et al.*, 2002). *In vitro*, at low Ca^{2+} , GCAP1 stimulates GC1 while GCAP2 stimulates both GC1 and GC2; both GCAPs are inactive when free Ca^{2+} is high (Haeseleer *et al.*, 1999). The distribution of GC1 and GC2 in rods and cones appears to be species dependent (Yang *et al.*, 1999; Imanishi *et al.*, 2002). Immunocytochemistry in bovine and mouse retina has shown that GCAP1 is present in rods and cones, while GCAP2 is seen nearly exclusively in rods (Cuenca *et al.*, 1998; Kachi *et al.*, 1999).

The genes encoding GCAP1 and GCAP2 are arranged in an autosomal tail-to-tail array (Howes *et al.*, 1998). This arrangement allows for generation of a double knockout mouse (*GCAP^{-/-}*) in which expression of both GCAPs is disabled. Rod outer segment (ROS) membranes from *GCAP^{-/-}* mice show no Ca^{2+} regulation of GC activity. The Ca^{2+} insensitivity of cGMP synthesis causes the flash response in these rods to be larger and slower than in wild type (Mendez *et al.*, 2001). The Ca^{2+} dependence of GC activity is rescued by overexpression of bovine GCAP2 in *GCAP^{-/-}* rods and the time for rod recovery from saturating flashes is returned to normal (Mendez *et al.*, 2001). Overexpression of GCAP2 does not, however, restore the normal kinetics of responses evoked by subsaturating flashes. This suggests that GCAP1, either by itself or in conjunction with GCAP2, participates in the recovery of the rod flash response.

To investigate the role of GCAP1 in rod phototransduction, transgenic mice were generated and bred with *GCAP^{-/-}* mice to produce a mouse that expressed GCAP1 under the control of its endogenous promoter on a GCAP-null background. We present evidence that GCAP1 expression in *GCAP^{-/-}* rods can restore the wild-type properties of the rod light response in the absence of GCAP2.

Results

Generation of transgenic mice expressing GCAP1

To evaluate the role of GCAP1 in phototransduction, we generated mice expressing GCAP1 under the control of the endogenous promoter. A transgene containing the entire

Fig. 1. Gene constructs and promoter analysis of the *GIT⁺* transgenic lines. (A) Wild-type *GCAP* gene array, disrupted gene array and *GCAP1* transgene construct. The arrows above the *GCAP* array indicate the direction of transcription (the *mGCAP1* and *mGCAP2* genes are located on opposite strands). The specific fragments used for genotyping (wild-type fragment 1, neo fragment 3 and transgene fragment 2, see also Figure 2A) are shown as bars below the constructs (for details of sense and antisense primer pairs, see Materials and methods). Black boxes depict *GCAP1* exons, gray boxes *GCAP2* exons. Relevant restriction sites used for the constructs are: H, *HindIII*; X, *XhoI*; E, *EcoRI*. Hatched boxes indicate multiple cloning site sequences derived from the original cloning vector. (B) Amplified fragments of the *GCAP1* promoter region indicating the presence and absence of a 1.8 kb deletion. Primers corresponding to sequences 0.5–3 kb upstream of ATG (in 500 bp increments) together with an anchoring antisense primer located at -170 were used to amplify upstream regulatory fragments. *GCAP^{+/+}* and the *GIT4⁺GCAP^{+/+}* transgenic line show the expected 3.0 kb product when amplified with the anchoring primer and primer -3379. The *GIT3⁺GCAP^{+/+}* transgenic line exhibits an additional band of 1.4 kb. This band was derived from the *GIT3* promoter region from which 1.8 kb were deleted. (C) Schematic depiction of the *GIT* promoter region and identification of the deletion in the upstream region of the *GIT3⁺* line. The wild-type promoter region contains two repeats (a and b), which are developmentally regulated by retinoic acid, a retinoic acid enhancer element (c) on the antisense strand, and an overlapping AP1 enhancer element (d) on the sense strand in a region between -1337 and -3201 (-1 is the first nucleotide upstream of the translation initiator ATG). This region, identified by a gray bar and marked as Δ , is deleted in the *GIT3⁺* line.

mouse *GCAP1* gene, including the polyadenylation site and a 5.4 kb upstream regulatory region (Figure 1A), was microinjected into single-cell embryos. Four *GCAP1* transgenic (*GIT*) founder mice were generated, two of which (*GIT3⁺* and *GIT4⁺*) passed the transgene to *F*₁ offspring with 50% penetrance, as expected from genetically non-mosaic mice. Lines could not be established from the other two *GIT* founders. Southern blots showed that the *GIT3⁺* founder contained approximately two to three copies, and the *GIT4⁺* founder three to five copies of the transgene (data not shown).

Irregularities in expression patterns (see below) prompted us to examine more closely both transgenes in offspring of the two founders. Analysis of the *GIT3* transgene by primers generated in 500 bp increments revealed a deletion in the upstream region (Figure 1B and C). The deletion removes an ~1.8 kb fragment 1337 bp upstream of the translation start codon, such that the sequence at position -1337 bp was contiguous with the sequence at -3201 bp. The deletion was seen consistently in PCRs of tail DNA from all *GIT3⁺* mice assayed. No alterations were seen in the transgene present in *GIT4⁺* mice, suggesting that the *GIT3* upstream deletion occurred

during integration of the transgene into the mouse genome by an unknown mechanism.

The transcription start point of the mouse *GCAP1* gene is predicted to be 294 bp upstream of ATG [by eukaryotic neural network promoter prediction (Ohler *et al.*, 1999) and 5' RACE; results not shown], and a putative TATA box is present 28 bp upstream of the transcription start point. Proximal regulatory elements, located within a 121 bp region, are highly conserved between the human and mouse *GCAP1* genes. Within this region, retinoic acid and/or thyroid hormone, AP1, possibly RET1 and CRX responsive consensus elements are found, consistent with other photoreceptor-expressed genes. More distally, the mouse promoter region contains a large unsequenceable (CA)*n* repeat (position >-3.7 kb), which spans >500 bp. A similar but much smaller microsatellite sequence consisting of a (GA)*n* repeat is present in the first intron.

The two transgenic lines, one with an intact promoter (*GIT4⁺*) and one with a partial deletion (*GIT3⁺*), provide the desired tools to determine whether *GCAP1* can restore normal rod function and to analyze the consequences of the deletion for effects on *GCAP1* expression levels and cellular distribution.

Fig. 2. Genotyping of transgenic lines and analysis of GCAP1 expression. (A) Mobilities of diagnostic amplicons 1–3 for the various genotypes as outlined schematically in Figure 1A. The fragments were generated in single PCRs containing all primers using genomic DNA isolated from tail biopsies. The amplicons were separated on 1% agarose gels. The control lane contains the three primer pairs, but no genomic template. Fragment 1 is diagnostic of the wild-type *GCAP* array, fragment 2 of the *GIT* transgene (both *GIT3*⁺ and *GIT4*⁺ lines), and fragment 3 for the knockout construct. Genomic DNA amplifying only fragment 1 is *GCAP*^{+/+}, genomic DNA amplifying fragment 3 is *GCAP*^{-/-}, while DNA amplifying both fragments 1 and 3 is *GCAP*^{+/-}. Correspondingly, all lines amplifying fragment 2 carry the *GIT* transgene. (B) Western blot of retinal homogenates. Polyclonal antibody UW14 (Gorczyca *et al.*, 1995) was used to identify GCAP1 transgene expression in *GIT3*⁺/*GCAP*^{-/-} and *GIT4*⁺/*GCAP*^{-/-} retinas relative to wild-type levels. Retinal protein (10 µg) was run for each sample. The slightly faster mobility of GCAP1 in the *GIT3*⁺/*GCAP*^{-/-} retina sample was seen consistently in many western blots. Faster mobilities may be caused by the different amounts of calcium bound (Li *et al.*, 2001) or by differential acylation of the GCAP1 protein (Sokal *et al.*, 2001) in cones, since the *GIT3* line shows predominantly cone expression. WT, wild type.

Generation of transgenic mice expressing GCAP1 on a *GCAP*^{-/-} background

GCAP^{-/-} mice were generated recently by replacing C-terminal exons of the *GCAP1/2* gene array with a neo cassette (Figure 1A) (Mendez *et al.*, 2001). When raised under normal light conditions, the null mice were shown to have normal retinal morphology and reduced a-wave electroretinogram (ERG) responses under dark-adapted conditions (Hurley and Chen, 2001). However, light responses of single *GCAP*^{-/-} rods showed a prolonged rising phase of the light response and a pronounced slowing of the recovery phase (Mendez *et al.*, 2001), consistent with an absence of the Ca²⁺-dependent stimulation of cGMP synthesis that normally accelerates the recovery process.

Crossbreeding of *GIT4*⁺/*GCAP*^{+/-} mice with *GCAP*^{+/-} mice generates offspring expressing GCAP1 on a null background (*GIT4*⁺/*GCAP*^{-/-}), as well as five other genotypes (Figure 2A). A corresponding set of six genotypes was also generated using the *GIT3*⁺ transgenic line. Genotyping of all three alleles (wild-type *GCAP* array, disrupted gene array and *GCAP1* transgene) was

performed simultaneously by PCR amplification, yielding diagnostic fragments of approximately 800, 200 and 500 bp, respectively (Figure 2A). Expression levels for the *GCAP1* transgenes were determined with several western blots using *GIT3*⁺ and *GIT4*⁺ on the null background and comparison with GCAP1 signals in *GCAP*^{+/+} mice. *GIT3*⁺/*GCAP*^{-/-} transgenic retinas always showed ~10–30% of wild-type GCAP1 expression, and GCAP1 expression in *GIT4*⁺/*GCAP*^{-/-} lines was ~30–50% that of wild-type levels (Figure 2B).

GC stimulation levels at low Ca²⁺ were found consistently to be in between those for knockout and wild-type retinas (data not shown). Expression levels of 30–50% are consistent with expression of a heterozygous transgene (homozygous transgenics were not generated) and represent an average expression level in all photoreceptors. However, as shown below, expression in individual rods varies significantly.

Cellular distribution of GCAP1 in *GCAP1* transgenic lines

GCAP1 transgene expression in photoreceptors of *GIT3*⁺ and *GIT4*⁺ lines on the wild-type background were determined by immunocytochemistry (Figure 3) and by functional analyses (Figures 4–6). To identify cellular and subcellular locations of transgenic *GCAP1*, we performed immunocytochemistry with a GCAP1-specific polyclonal antibodies (UW101 and UW14) on retinas collected from the nine genetic variations produced in this study (Figure 3A–I). As expected, *GCAP*^{-/-} retinas were immunonegative for GCAP1 (Figure 3G). *GIT* lines on a *GCAP*^{-/-} background were immunopositive for GCAP1 and immunonegative with the anti-GCAP2 antibody (data not shown) as opposed to wild-type GCAP2 staining. *GCAP1* transgene expression was specifically targeted to photoreceptor cells in both *GIT3*⁺ and *GIT4*⁺ lines. The results are consistent with the ability of the intact endogenous *GCAP1* promoter (5.4 kb) to direct expression of GCAP1 to both rods and cones. The *GIT3*⁺ *GCAP*^{+/-} and *GIT4*⁺/*GCAP*^{+/-} transgenic mice (Figure 3B and C) showed additional GCAP1 expression, seen as intense immunoreactive packets in the outer segments, particularly in cones, relative to wild-type levels. Interestingly, GCAP1 immunolocalization was quite variable across photoreceptor outer segments in all lines except those expressing near normal GCAP1 levels, including the wild-type and *GIT4*⁺/*GCAP*^{+/-} mice.

GIT4⁺/*GCAP*^{+/-} mice, which express ~30–50% more GCAP1 than wild type (Figure 2B), exhibit a loss of two to three photoreceptor cell layers by 4 weeks of age (Figure 3C), but do not exhibit any further degeneration even as late as 6 months of age (Baehr *et al.*, 2000; and data not shown). The number of cone photoreceptors at 6 months of age appears to be normal. The degenerative effect in some rods is presumably due to overexpression of GCAP1, since *GIT4*⁺ mice on heterozygous and *GCAP*^{-/-} backgrounds (with reduced wild-type GCAP1 levels) showed normal outer nuclear layer (ONL) thickness at advanced ages. Photoreceptor degeneration due to overexpression has commonly been seen in transgenic rhodopsin lines (Olsson *et al.*, 1992). The lower *GIT3* transgene expression level does not appear to exhibit an additive deleterious affect on the ONL in *GIT3*⁺ mice on a

Fig. 3. Immunocytochemical localization of GCAPs in retinas from all genotypes generated. GCAP1 was identified with polyclonal antibody UW101 by indirect immunofluorescence (FITC), and mouse cones were labeled with rhodamine-conjugated peanut agglutinin (PNA). The first row (A, D, G) contains *GCAP*^{+/+}, *GCAP*^{+/-} and *GCAP*^{-/-} retinas without transgene, the second row (B, E, H) retinas from *GIT3*⁺*GCAP*^{+/+}, *GIT3*⁺*GCAP*^{+/-} and *GIT3*⁺*GCAP*^{-/-}, and the third row (C, F, I) from the *GIT4*⁺*GCAP*^{+/+}, *GIT4*⁺*GCAP*^{+/-} and *GIT4*⁺*GCAP*^{-/-} retinas. Bar = 20 μ m for all figure parts. Retinal layers are identified as OS (outer segments), IS (inner segments), ONL (outer nuclear layer containing photoreceptor nuclei) and OPL (outer plexiform layer).

Fig. 4. Rod response families in different mouse lines. Panels show superimposed mean responses ($n \geq 10$), recorded from either *GCAP*^{+/+} (A), *GCAP*^{-/-} (B) or *GIT4*⁺*GCAP*^{-/-} (C–F) rods, to 10 ms flashes that increase in intensity in ~2-fold steps from starting values (equivalent 500 nm photons/ μ m²/flash) of: 0.6, 2.0, 8.4, 2.0, 8.4 and 8.4, respectively. In all panels, responses were normalized to the rod's resting dark current ($R/R_{\max}$), which was: (A) –21.8 pA, (B) –13.6 pA, (C) –13.1 pA, (D) –12.8 pA, (E) –21.4 pA and (F) –11.3 pA. All flashes were delivered at 0.1 s.

GCAP^{+/+} background. Interestingly, the *GIT3*⁺ transgenic line exhibits enhanced cone GCAP1 expression, while rod expression is greatly reduced (Figure 3H). Unlike the immunostaining seen in wild-type retina cone cells, GCAP1 in the *GIT3*⁺*GCAP*^{-/-} retina is localized intensely throughout cone cells.

The results seen with the *GIT3*⁺ line indicate that regulatory elements directing expression to rod photo-

receptors or rod-specific enhancer elements may be located in a region –1337 to –3201 bp upstream of the translation start site. This region revealed numerous repetitive elements, including direct repeats, palindromes and developmentally regulated retinoic acid repeats (DR) (Sam *et al.*, 1996). This region therefore is highly susceptible to structural folding, which may have contributed to rearrangement and subsequent deletion of this area during transgene insertion in the *GIT3*⁺ founder. Two DR repeats lie at positions –2244 to –2036 bp and –1883 to –1803 bp upstream of the ATG start site (Figure 1C, a and b). DR expression has been shown to be induced by retinoic acid in cultured ES cells and is expressed with tissue specificity in embryos as well as the adult mouse brain. Interestingly, between these two repetitive regions, on opposite strands, lie overlapping consensus binding sites for retinoic acid and AP1 enhancers (Figure 1C, c and d, respectively). Although little is understood concerning rod versus cone specificity in gene expression, there is evidence that retinoic acid can induce progenitor photoreceptor cells to adopt a rod cell fate (Kelley *et al.*, 1999). Loss of this region and subsequent lack of expression of GCAP1 in rods in the *GIT3*⁺ transgenic line may have revealed a developmentally regulated program for rod expression of GCAP1 by retinoic acid. In addition, although less intense than in the *GIT3*⁺ line, the *GIT4*⁺ line also appears to show slightly higher GCAP1 staining in cone cell bodies and synaptic pedicles. This line, however, does not show a promoter deletion. It is conceivable that the transgene containing the 5.4 kb upstream regulatory sequence does not contain the necessary

Table I. Flash response properties for all recorded cells

Rod type	Integ. time (ms) $\pm$ SEM	Range (low–high)	Time to peak (ms) $\pm$ SEM	Range (low–high)	Intensity $I_{0.5}$ $\pm$ SEM	Range (low–high)	I_{max} (pA) $\pm$ SEM	Range (low–high)	No. of cells
Wild type	313 $\pm$ 20	192–472	206 $\pm$ 11	157–320	29.5 $\pm$ 1.5	21.5–41.9	–14 $\pm$ 0.9	–6.4 to –21.8	17
GCAP ^{–/–}	759 $\pm$ 32	624–1009	609 $\pm$ 20	481–731	6.9 $\pm$ 0.4	4.8–10.2	–12.7 $\pm$ 0.4	–9 to –14.7	13
GIT4*GCAP ^{–/–} (all cells)	410 $\pm$ 33	121–654	284 $\pm$ 26	164–542	24.5 $\pm$ 2.2	9.4–40.4	–15 $\pm$ 0.7	–10.1 to –21.4	22
GIT3*GCAP ^{–/–}	694 $\pm$ 65	567–970	513 $\pm$ 25	483–618	6.7 $\pm$ 0.7	4.5–8.3	–12.1 $\pm$ 0.4	9.1 to –14.7	6
GIT4*GCAP ^{+/+}	408 $\pm$ 24	339–607	245 $\pm$ 23	188–411	26.9 $\pm$ 1.8	16.1–35.2	–14.4 $\pm$ 1.1	–10.7 to –22.7	10
GIT4*GCAP ^{–/–} with integ. time <472 ms	314 $\pm$ 27	122–453	217 $\pm$ 21	163–480	30.9 $\pm$ 1.7	18–40.4	–15.1 $\pm$ 0.8	–10.8 to –21.4	14
GIT4*GCAP ^{–/–} with integ. time >472 ms	578 $\pm$ 17	518–653	402 $\pm$ 31	246–542	13.1 $\pm$ 1.3	9.4–19.3	–14.9 $\pm$ 1.3	–10.1 to –21.3	8

The mean $\pm$ SEM and range of individual measures are reported for each rod type. Integ. time, integration time (response area/peak amplitude); I_{max} , maximal current [pA].

‘fine-tuning’ regulatory elements, which may be located further upstream and contribute to the lower levels of expression in cones.

Rescue of rod function by GCAP1

Light responses of rods from GCAP^{–/–} mice are markedly slower than those of GCAP^{+/+} rods. Overexpression of bovine GCAP2 on a GCAP^{–/–} background restored normal rod responses to saturating flashes but failed to restore the normal recovery kinetics to responses evoked by sub-saturating flashes (Mendez *et al.*, 2001). We used two recording techniques to determine whether GCAP1 in the absence of GCAP2 could rescue normal rod function. Responses of single rods to dim and moderate strength flashes were recorded using suction electrodes. Responses to bright flashes were evaluated by recording the ERG responses to paired flashes. Together these functional assays indicated that GCAP1 alone could rescue normal rod behavior over a broad range of light levels.

Rod single-cell recordings

Suction electrodes (Yau *et al.*, 1977) were used to record light-evoked changes in circulating dark current from the outer segments of rods from wild-type and genetically modified mice. Representative response families are shown in Figure 4. As reported previously (Mendez *et al.*, 2001), flash responses in GCAP^{–/–} rods were more sensitive to light and slower to reach peak and recover than responses in wild-type rods (Figure 4A and B).

To compare rods from mice of different genotypes, three features of the flash response were measured: the light intensity that evokes a half-saturating response ($I_{0.5}$), and the time-to-peak (t_{peak}) and integration time (t_{integ}) of the linear range response (see Table I). The three response parameters were determined for recorded rods and are plotted against each other in Figure 5. In the three panels, data from wild-type (open circles) and GCAP^{–/–} rods (filled circles) fall into separate clusters. Compared with wild-type cells, GCAP^{–/–} rods generate flash responses that take on average about three times longer to reach peak, are nearly four times more sensitive to light and have 2- to 3-fold longer integration times (Table I). In contrast, the three properties of the flash responses recorded from the

GIT4*GCAP^{–/–} rods show large rod-to-rod variations. In some rods the responses are indistinguishable from wild-type while in others they are slower, with properties between those of wild-type and GCAP^{–/–} rods. This is apparent in Figure 4C–F, where response families have been selected from GIT4*GCAP^{–/–} rods to illustrate the range of rod-to-rod variation in the kinetics of the responses.

Measurements of the three standard response parameters for all the recordings from GIT4*GCAP^{–/–} rods are plotted in Figure 5 (filled triangles). The results from the different rods do not form a single cluster of data points. The data points intersperse with wild-type, but also extend into part of the range of the results from GCAP^{–/–} rods. This observation is consistent with variable expression of GCAP1 in the GIT4*GCAP^{–/–} rods (see below). In the bottom rows of Table I, GIT4*GCAP^{–/–} rods are divided into two groups based on whether the integration time of the linear range response is less than or greater than the longest wild-type integration time (472 ms). Fourteen of 22 GIT4*GCAP^{–/–} rods that fall into the group with shorter integration times and have mean values of $I_{0.5}$, t_{peak} and t_{integ} that do not differ significantly from wild type (Table I). This indicates that despite rod-to-rod variation, the majority of GIT4*GCAP^{–/–} rods generate flash responses with wild-type properties.

Flash responses of rods from the GIT3⁺ line (GIT3*GCAP^{–/–}) do not show a similar rescue of wild-type properties. Indeed these cells generate flash responses that were more similar to the responses recorded from GCAP^{–/–} rods than wild type (Figure 5, open triangles). Responses from rods of mice overexpressing GCAP1 (GIT4*GCAP^{+/+}) were also measured. The light responses of these rods closely resemble wild type (Figure 5, filled squares), suggesting that the amount of endogenous GCAP1 in wild-type mice is sufficient to cause maximal activation of GC.

Paired flash ERG responses in transgenic rods

The single-cell recordings described above indicate that GCAP1 restores normal rod responses to subsaturating flashes. ERGs allowed us to determine whether the ability

Fig. 5. Flash response properties. Collected results were plotted for each recorded rod half-saturating flash intensity ($I_{0.5}$ in equivalent 500 nm photons/ $\mu\text{m}^2/\text{flash}$), dim flash response integration time (response area/peak amplitude), and time-to-peak for $GCAP^{+/+}$ (open circles), $GCAP^{-/-}$ (filled circles), $G1T4^+GCAP^{-/-}$ (filled triangles), $G1T3^+GCAP^{-/-}$ (open triangles) and $G1T4^+GCAP^{+/+}$ (filled squares).

of GCAP1 to rescue normal function extended to bright flashes.

We used the paired flash ERG method (Lyubarsky and Pugh, 1996) to monitor the time course of recovery of the photoresponse following a strongly saturating conditioning flash (5500 isomerizations). Response recovery was estimated by measuring the amplitude of the a-wave evoked by a probe flash (350 000 isomerizations) delivered at different intervals following the conditioning flash (Pepperberg *et al.*, 1997). Although both rods and cones are excited by the conditioning and probe flashes, the recording primarily reflects rod activity, since cones represent only ~3% of the receptor population and have been reported to contribute <5% to the saturated a-wave (Lyubarsky *et al.*, 1999). Hence, the amplitude of the a-wave evoked by the second flash depends on the extent to which the rod response to the first flash has recovered.

Figure 6A shows an example of this procedure for a 1 s interval between the conditioning and probe flashes. The response to the probe flash alone is shown on the right. The

Fig. 6. Paired flash ERGs. (A) Left, paired flash response of different lines of transgenic mice at 1000 ms. A conditioning flash produced 5500 isomerizations/rod and a probe flash produced 350 000 isomerizations/rod. Each trace represents the average of three mice. Right, response to the probe flash alone. (B) Graphical representation of normalized a-wave recovery of the probe flash for varying times after the test flash. Each point represents the average for 4–10 mice. Data were fit to a single exponential with the form: $e^{-(t-T)/\tau}$. $GCAP^{+/+}$ mice: $T = 481$ ms, $\tau = 481$ ms; $G1T4^+GCAP^{-/-}$ mice: $T = 511$ ms, $\tau = 567$ ms; $G1T3^+GCAP^{-/-}$ mice: $T = 722$ ms, $\tau = 1102$ ms; $GCAP^{-/-}$: $T = 511$ ms, $\tau = 2476$ ms. The response of each probe flash alone has been normalized to 1, and other traces are scaled accordingly.

extent of a-wave recovery in the 1 s interval between conditioning and probe flashes depends on the genotype of the mouse. Wild-type mice showed substantial a-wave recovery in 1 s, while $GCAP^{-/-}$ mice showed little recovery. $G1T3^+$ and $G1T4^+$ mice showed levels of recovery between wild type and null.

The differences in recovery time course among different genotypes are summarized in Figure 6B. To correct for differences in the response to the probe flash in different mouse lines, a-wave amplitudes were normalized to the amplitude of the response evoked by the probe flash alone. Figure 6B plots these normalized amplitudes against the time interval between the conditioning and probe flashes. All lines of mice show suppression of the rod a-wave for an interval of 600 ms. Wild-type mice demonstrate a nearly complete recovery of a wave amplitude when the inter-stimulus interval is 3 s. Mice lacking both GCAP1 and GCAP2 ($GCAP^{-/-}$) show incomplete recovery, even after 5 s. Mice from the $G1T3$ line ($G1T3^+GCAP^{-/-}$) show a somewhat faster a-wave recovery than those lacking GCAP, while $G1T4^+GCAP^{-/-}$ mice showed a-wave recovery similar to wild type (Figure 6). Mice expressing the $G1T4^+$ transgene on the wild-type background showed a-wave recoveries very similar to wild type.

Levels of GCAP1 expression in single rods

Due to the variability in single-cell recordings in the $G1T4^+$ lines, immunocytochemistry was performed and

Fig. 7. Relative GCAP1 signal profiles. Retinas were immunostained for GCAP1 (UW101 FITC) and recoverin (RCV10 rhodamine). Double-labeled confocal images were split for analyzing relative GCAP1 expression levels among the $G1T4^{+}GCAP^{-/-}$ (A), $GCAP^{+/+}$ (B) and $G1T3^{+}GCAP^{-/-}$ (C) retinas. Recoverin immunolocalization (red signal) within the outer segments in each retina is invariant, while *GCAP1* transgene expression shows variable staining among the outer segments. The relative GCAP1 (green) signal was normalized for each retina such that signals ranged from 0 to 250% signal intensity for each image. Normalized signals were then compared across ~20 outer segments for each retina. The $GCAP^{+/+}$ retina clearly shows a fairly equivalent intensity of staining for GCAP1 across all outer segments. Outer segments from $G1T4^{+}GCAP^{-/-}$ retinas exhibit a range of GCAP1 intensities, while $G1T3^{+}GCAP^{-/-}$ retinas show few intensely stained rod outer segments. Co-localization is shown in yellow.

GCAP1 immunointensities were compared among rod outer segments (Figure 7). Using a recoverin monoclonal antibody to counterstain rod outer segments, the relative intensity of GCAP1 signal was determined on a scale of 0–250%. Whereas wild-type signal intensities are consistently high across the rod outer segments, $G1T4^{+}GCAP^{-/-}$ rods show variable intensities among the outer segments, and $G1T3^{+}GCAP^{-/-}$ signals are consistently diminished in all rod outer segments (ROS). These results are in agreement with the electrophysiological data. GCAP1 transgene expression in the $G1T4^{+}GCAP^{-/-}$ line shows a range of immunointensities, suggesting that the transgene is variably expressed among the rod photoreceptors. The lowered $G1T3^{+}$ transgene expression in ROS may be explained by the deletion in the regulatory sequence of the transgene.

Variations in expression patterns have been observed previously and addressed in other transgenic studies. Most recently, variegated expression of a rhodopsin–GFP (green fluorescent protein) fusion protein was described in the retinas of transgenic *Xenopus laevis* (Moritz *et al.*, 2001). Variable transgene expression was first reported in non-mosaic transgenic mice ~10 years ago (Lem *et al.*, 1991; Zack *et al.*, 1991). These authors suggested that chromatin structure surrounding the transgene integration site may be causative to variable expression among the targeted cell population. We suggest that a similar mechanism causes variable expression of GCAP1 in $G1T4^{+}GCAP^{-/-}$. This can account for the fact that GCAP1 expression can rescue wild-type response properties in most but not all rods in

$G1T4^{+}GCAP^{-/-}$ mice, and incompletely restores normal recovery from a strongly saturating flash in the ERG measurements.

Discussion

To convert light into an electrical signal, vertebrate photoreceptors use an enzymatic transduction cascade to control the circulating current that flows into the cell through cGMP-gated channels. The intracellular level of cGMP depends on the relative activities of light-stimulated PDE and Ca^{2+} -dependent GC. The present study investigates the molecular mechanisms responsible for the Ca^{2+} regulation of cGMP synthesis. This issue has not been resolved and is complicated by the fact that rods contain two distinct particulate guanylate cyclases (GC1 and GC2) and two different Ca^{2+} -sensitive activators (GCAP1 and GCAP2).

The GCAPs are among the best characterized Ca^{2+} binding proteins of the calmodulin superfamily, many of which are specifically expressed in neurons. The genes encoding GCAP1 and GCAP2 are related evolutionarily. They were generated by ancient gene duplication/inversion events and share an identical gene structure. On the protein level, however, the two GCAPs share only ~45% homology, although they have identical domain distributions (EF hands) and similar three-dimensional structures. There are also several characteristic biochemical and functional differences between them. GCAP1 stimulation is specific for GC1, while GCAP2 stimulates both GC1

and GC2, consistent with the two activator proteins and enzymes having different interaction sites (reviewed in Palczewski *et al.*, 2000). In addition, GCAP1 is active as a monomer (Sokal *et al.*, 2001) and is associated with the membrane and/or GC1 over the full range of physiological $[Ca^{2+}]$ (Gorczyca *et al.*, 1995). GCAP2, on the other hand, is active as a dimer and its solubility has been reported to increase upon binding of Ca^{2+} (Olshevskaya *et al.*, 1999).

Immunocytochemistry using monospecific antibodies has shown that both GCAP1 and GCAP2 are expressed in mouse ROS (Howes *et al.*, 1998), raising questions about their relative roles in the transduction process. In mouse rods lacking both GCAPs, cGMP synthesis occurred at its normal basal dark rate but was not stimulated by exposure to low Ca^{2+} (Mendez *et al.*, 2001). The GCAP double knockout rods had normal morphologies yet generated light responses with greatly slowed recovery phases (Mendez *et al.*, 2001) (Figure 4B). Overexpression of bovine GCAP2 in the *GCAP^{-/-}* rods speeded recovery from strongly saturating flashes but did not restore to normal the recovery kinetics of responses evoked by dim to moderate intensity flashes and, in particular, failed to rescue the fast initial phase of recovery that is a characteristic feature of the wild-type flash response (Mendez *et al.*, 2001).

We used a conceptually similar approach to evaluate the role of GCAP1 in the rod recovery process. First, a *GCAP1* transgenic mouse line was established in which GCAP1 expression, governed by the endogenous mouse *GCAP1* promoter, was accurately targeted to photoreceptor cells. Secondly, mice were generated expressing only GCAP1 on a *GCAP^{-/-}* background. We found that in the majority of cases, expression of GCAP1 in *GCAP^{-/-}* rods restored wild-type recovery kinetics to responses evoked by dim, moderate and strongly saturating flash intensities. This was apparent in the results of both single-cell electrophysiology and ERG recordings from the intact retina (Figures 4–6). A small but significant fraction of the rods that expressed GCAP1 on a null background (*GIT4⁺GCAP^{-/-}*) generated responses that recovered with a time course between that of wild-type and double knockout rods. The variation in recovery kinetics was attributed to cell-to-cell differences in GCAP1 expression levels, as shown by immunocytochemistry (Figure 7). In spite of this variation, in most cells GCAP1 expression in the absence of GCAP2 rescued the phenotype of the wild-type rod flash response, including the initial rapid phase of recovery that follows the peak of the response (Figures 4 and 5). This was not the case in rods from the *GIT3⁺GCAP^{-/-}* line, in agreement with the near absence of GCAP1 expression in these cells. The recovery of cone photoreceptors in these different transgenic mouse lines is currently under investigation using the paired flash ERG method. Preliminary results indicate that GCAP1, as expected, also plays a key role in cone recovery.

Our results show that the phenotype of the wild-type flash response can be rescued by GCAP1 expression in the absence of GCAP2. They also demonstrate that GCAP1 is responsible for the initial rapid phase of response recovery. The key to this role of GCAP1 is most likely its close association with GC1 under high- and low- Ca^{2+} conditions. This would enable GC1 to be stimulated as soon as the light-evoked fall in Ca^{2+} is sufficient for Ca^{2+}

dissociation from GCAP1, which has been shown to occur in <100 ms (Sokal *et al.*, 1999). While the results have established a prominent role for GCAP1 in rod phototransduction, they provide little information about the role played by GCAP2. Although clearly present, its contribution to rod function is uncertain and thus is an important subject for future research.

Materials and methods

GCAP1 transgene and GCAP1/GCAP2 knockout constructs

The λ MG3 murine *GCAP1* genomic clone and *GCAP1/2* double knockout constructs have been described previously (Figure 1). Briefly, the genomic clone contains the complete coding region with intervening introns, a 5.4 kb upstream *GCAP1* regulatory sequence, and an ~2.5 kb downstream region, which includes the endogenous polyadenylation site. After subcloning into *pBSKS(+)*, the *NorI*-digested fragment was used as the transgene for injection into fertilized single-cell mouse embryos. The murine *GCAP* genes are arranged in a tail-to-tail array, separated by ~2.5 kb. *XbaI* digest of a 129/SV clone and insertion of a neocassette resulted in the elimination of *GCAP1* exons 2–4 and *GCAP2* exons 3 and 4. Generation and characterization of *GCAP1/GCAP2* knockouts are described elsewhere (Mendez *et al.*, 2001).

PCR genotyping transgenic and endogenous GCAP1/GCAP2 alleles

In order to discriminate between the endogenous *GCAP1* allele and the *GCAP1* transgene, a PCR primer was generated to include the pBluescript multiple cloning site (MCS) downstream of the *NorI* site, which was used to cut out the transgene insert for microinjection. The MCS transgene primer 5'-CAC TAT AGG GCG TCG ACT CGA TCA CGC AGC (bold letters, MCS; italicized letters, genomic sequence) and the *GCAP1* transgene primer 5'-AAG GGC GGA AGA TCA CGG AGG TAG CCC TC, located 500 bp downstream, were used to amplify the transgene. The primers used to identify the double knockout were the *GCAP2* intron B primer 5'-GAA GCA GCC AGG CCA AGC CAC ACC CAT CACA and the Neo primer 5'-CGG TGG ATG TGG AAT GTG TGC GAG GCC AGA. For identification of the endogenous *GCAP1/GCAP2* allele, *GCAP2* exon 3 primer 5'-GGC AGG GTT TCT TCA GCT TGT AAA TCG CCT GCC AGA was used. All five primers were used in single PCRs (Promega) for each tail DNA sample. Results from a tail DNA PCR in which the mouse was transgene positive, on a wild-type and knockout endogenous *GCAP1/GCAP2* background, would yield 500, 800 and 200 bp products, respectively (Figure 2A). For deletion analysis of the *GIT3* promoter, a 3' antisense anchoring primer at position -170 (5'-CAC CCA AAC TAG AGA TGC) was used in separate reactions with the following 5' primers: -1185 (5'-TGA GTA TTG AAG GGA GCC), -1716 (5'-GAC TGC AGA CTG TAA GTC), -2252 (5'-CCA TTA GGA AGG TAG CCA), -2773 (5'-GGA TCT GCC ATC TCA TTC) and -3379 (5'-CCC TCT CCT TTG TGT AAC).

Immunocytochemistry

Eyes were fixed in 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.4) overnight at 4°C and then cryoprotected in 30% buffered sucrose. Cryosections (14 μ m thick) were preincubated with 10% normal goat serum (NGS). Primary and secondary antibodies were diluted with 0.1 M phosphate buffer/0.05% Triton X-100 for all reactions. Sections were incubated overnight at 4°C with UW101 or UW14 antibodies at dilutions of 1:2000 and 1:3000, respectively. Polyclonal antibodies UW14 and UW101 were raised against bacterially expressed, truncated bovine *GCAP1* as described previously (Gorczyca *et al.*, 1995) and characterized for mouse retinal tissue (Howes *et al.*, 1998). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Vector Laboratories, Inc.) was used at a dilution of 1:100 for 1 h at room temperature. Immunofluorescence was photographed with an LSM510 Inverted Laser Scan confocal microscope (Zeiss, Germany) and pictures were composed using Adobe Photoshop. In some sections, rhodamine-conjugated PNA (Vector Laboratories, Inc.), which specifically labels cone photoreceptor outer segment sheaths, was used at a concentration of 25 μ g/ml for 1 h at room temperature. For double labeling experiments, a monoclonal antibody for mouse recoverin (Rcv10) (kindly provided by Dr Jim McGinnis, University of Oklahoma) was used at a concentration of 1:2000 and indirectly labeled with a rhodamine-conjugated goat anti-mouse secondary antibody.

Guanylate cyclase activity assays

Three different preparations of GC were carried out (Tsang *et al.*, 1998). The retinas were carefully dissected from mouse eyes, homogenized with 50 mM HEPES pH 7.8, 20 mM NaCl, 60 mM KCl (50 μ l per eye), and used directly in the GC assay (Otto-Bruc *et al.*, 1997). Intact ROS was prepared according to the procedure described by Arshavsky and colleagues with some modifications (Tsang *et al.*, 1998). The mouse retinas from 12 animals were removed from the eyecups, placed in 120 μ l of 8% OptiPrep (Nycomed, Oslo, Norway) in Ringer's buffer, and vortexed at maximum speed for 1 min. The samples were then centrifuged at 1500 r.p.m. for 1 min (Eppendorf centrifuge), and the supernatant containing the ROS was gently removed. The retina tube was added again to 120 μ l of 8% OptiPrep, vortexed and centrifuged using the same method. The vortexing and sedimentation sequence was repeated five times. The collected ROS supernatant (~1200 μ l) was loaded on the Optiprep discontinuous gradient made with 10, 20 and 30% OptiPrep in Ringer's solution in a 15 ml centrifuge tube. The tube was centrifuged at 13 000 r.p.m. for 50 min using swing bucket rotor JS13.1. The ROS was collected from 20%/30% OptiPrep interface. The ROS harvest was divided between three tubes and diluted three times with Ringer's solution. It was then centrifuged for 30 min at 13 000 r.p.m. on the same rotor. The sedimented material contained ROS. Crude preparations of mouse ROS were obtained under dim red light by vortexing 4–12 retinas in 45% sucrose, 67 mM potassium phosphate pH 7.5 for 5 min at room temperature. Samples were centrifuged for 7 min at 7000g. The supernatants were collected, diluted 1.5 times with 67 mM potassium phosphate pH 7.5 and centrifuged at 10 000g for 10 min. The outer segment pellets were resuspended in 50 mM HEPES pH 7.8, 20 mM NaCl, 60 mM KCl, and used immediately for GC assays or frozen at -80°C . The protein concentration in each sample was determined by Bradford assay. GC activity from mouse ROS was assayed under dim red light at either low (45 nM) or high (2 μ M) free Ca^{2+} as described previously (Haeseleer *et al.*, 1999).

Single cell recordings

All animal procedures conformed with protocols approved by the Administrative Panel on Laboratory Animal Care at the University of Washington. Mice were dark adapted for 12–15 h, killed by cervical dislocation, and the eyes removed. The retina was isolated and stored on ice for up to 12 h in HEPES-buffered Ames solution (10 mM HEPES, pH adjusted to 7.4 with NaOH). Isolated rods were obtained by shredding a small piece of retina (~1 mm²) with fine needles in a 160 μ l drop of solution. The drop was then injected into a recording chamber mounted on the stage of an inverted microscope (Nikon Eclipse) equipped with an infrared video viewing system. The recording chamber was continuously superfused at 2–3 ml/min with bicarbonate-buffered Ames solution warmed to 37°C (pH 7.4 when equilibrated with 5%CO₂/95% O₂). The entire dissection was carried out under infrared illumination using a dissecting microscope equipped with infrared/visible image converters.

An isolated rod was drawn by suction into a heat-polished, silanized borosilicate electrode with an opening 1.2–1.5 μ m in diameter. The electrode was filled with HEPES-buffered Ames media. Electrical connections to the bath and suction electrode were made by NaCl-filled agar bridges that contacted calomel half-cells. Bath voltage was held at ground by an active clamp circuit (Baylor *et al.*, 1984). Membrane current collected by the suction electrode was amplified by an Axopatch 200A patch clamp amplifier (Axon Instruments, Foster City, CA), filtered at 30 Hz (–3 dB point) with an 8-pole Bessel low-pass filter, and digitized at 1 kHz.

Light from light-emitting diodes (LEDs) with peak outputs at 470, 570 and 640 nm were combined using a trifurcated fiber optic and focused on the preparation using a water-immersion lens (20 $\times$ NA 0.4) in place of the microscope condensor. The light stimulus was spatially uniform and illuminated a circular area, 0.57 mm in diameter, centered on the recorded cell. Light intensities measured at the preparation were converted to equivalent photons at the wavelength of the rod's peak sensitivity (500 nm) using the absorption spectrum of rhodopsin and the measured LED spectrum. Equivalent intensities for each experiment are given in the figure legends.

Paired flash analysis

Prior to testing, mice were allowed to adapt to the dark overnight. Under dim red light, mice were anaesthetized with a solution of ketamine (100 mg/ml) and xylazine (10 mg/ml). Pupils were dilated with a single drop of 0.5% mydriacyl and 2.5% phenylephrine. Mice were placed on a heating pad maintained at 39°C, inside a Ganzfeld dome coated with reflective white paint. A small amount of 1% methylcellulose gel was

applied to the eye and a platinum electrode was placed in contact with the center of the cornea. Similar platinum reference and ground electrodes were placed in the forehead and tail, respectively. After placement in the dome, mice were allowed to remain in complete darkness for 5 min. Signals were amplified with a Grass P122 amplifier (bandpass 0.1–1000 Hz). Data was acquired using a National Instruments Lab-PC DAQ board (sampling rate 10 000 Hz). Traces were averaged and analyzed with custom software written in Matlab.

Light stimulation was provided by two Novatron xenon flash lamps positioned at the top of the dome. The intensity of the test flash was ~5500 photoisomerizations/rod. The intensity of the probe flash was calculated as 350 000 photoisomerizations/rod. Flashes were calibrated in a manner similar to that described in Lyubarsky *et al.* (1996). In order to deliver probe flashes intense enough to saturate the rod photocurrent, it was necessary to use white light. To calculate the number of photoisomerizations produced by the white flashes, the 500 nm to white efficacy was calculated using the methods described in Lyubarsky and Pugh (1996) and Pugh *et al.* (1998). A conversion factor of 1.35 log units was used.

The protocol for paired flashes was adapted from methods published previously (Lyubarsky and Pugh, 1996; Pepperberg *et al.*, 1997). First, a test flash was delivered to suppress the circulating current of the rod photoreceptors. The recovery of this current was monitored by delivering a second flash, termed the probe flash. The interstimulus interval between the two flashes was varied from 500 to 5000 ms. For an interstimulus of 500 ms the conditioning flash completely suppressed the rod a-wave. Each trial was separated by at least 120 s of darkness to allow the retina time to dark-adapt. The amplitude of the probe flash alone was monitored throughout the experiment to ensure that this time was sufficient. These 'probe alone' flashes were also used to normalize the response of probe flashes following a test flash. The normalized amplitude of the probe flash a-wave versus the interstimulus interval were plotted and fit to the following equation: $1 - e^{-(t-T)/\tau}$. T is the critical time before which there is no rod recovery, and τ is the recovery time constant.

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