In vivo studies of the γ subunit of retinal cGMP-phophodiesterase with a substitution of tyrosine-84

Stephen H. TSANG*[1], Clyde K. YAMASHITA*[1], Kentaro DOI†, Daniel J. SALCHOW†, Nicole BOUVIER†, Monica MENDELSOHN‡, Peter GOURAS†, Debora B. FARBER* and Stephen P. GOFF‡[2]

*Jules Stein Eye Institute and Molecular Biology Institute, UCLA School of Medicine, 100 Stein Plaza, Los Angeles, CA 90095-7000, U.S.A., †Edward S. Harkness Eye Institute, Department of Ophthalmology, Columbia University College of Physicians and Surgeons, New York, NY 10032, U.S.A., and ‡Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biophysics, Columbia University College of Physicians and Surgeons, New York, NY 10032, U.S.A.

The inhibitory rod cGMP phosphodiesterase γ subunit (PDEγ) is a major component of the photoresponse and is required to support rod integrity. $Pdeg^{tm1}/Pdeg^{tm1}$ mice (which lack PDEγ owing to a targeted disruption of the *Pdeg* gene) suffer from a very rapid and severe photoreceptor degeneration. The Y84G ($Tyr^{84} \rightarrow Gly$) allele of PDEγ has previously been shown in experiments carried out *in vitro* to reduce the regulatory control of the PDE catalytic core (PDE$\alpha\beta$) exerted by the wild-type γ subunit. To determine the effects of this mutation on *in vivo* function, the murine opsin promoter was used to direct expression to the photoreceptors of $+/Pdeg^{tm1}$ mice of a mutant Y84G and a wild-type PDEγ control transgene. The transgenic mice were crossed with $Pdeg^{tm1}/Pdeg^{tm1}$ mice to generate animals able to synthesize only the transgenic PDEγ. Our results showed that wild-type PDEγ and Y84G transgenes could complement the $Pdeg^{tm1}/Pdeg^{tm1}$ mutant for photoreceptor survival. The mutation caused a significant biochemical defect in PDE activation by transducin. However, the Y84G mutation did not fully eliminate the control of PDEγ on the PDE catalytic core *in vivo*; the expression of the mutant subunit was associated with only a 10-fold reduction in the amplitude of the a-wave and a 1.5-fold decrease in the b-wave of the corneal electroretinogram. Unexpectedly, the mutation caused a much 'milder' phenotype *in vivo* than was predicted from the biochemical assays *in vitro*.

Key words: knockout animals, phosphodiesterase, photoreceptor transduction, retinal degeneration, transgenic animals.

INTRODUCTION

Light, odorants and many neurotransmitters and hormones signal their target cells through G-protein-coupled receptors. The visual response to a single photon [1,2] starts with the activation of rhodopsin, which causes transducin [a heterotrimeric G-protein (T$\alpha\beta\gamma$)] [3] to exchange its bound GDP for GTP. Activated transducin molecules (Tα-GTP) then dissociate from T$\beta\gamma$ molecules and bind to the inhibitory γ-subunits of cGMP phosphodiesterase (PDE; a heterotetrameric enzyme, PDE$\alpha\beta\gamma_2$) [4,5], thereby relieving the inhibition that the two PDEγ subunits exert on the catalytic PDE α and β subunits [3–7]. The resulting activation of PDE$\alpha\beta$ lowers the levels of cGMP [8], which leads to closure of cGMP-gated Na^+/Ca^{2+} channels on the plasma membrane. This reduces the entry of Na^+ and Ca^{2+} into the cytoplasm, causing the entire rod to hyperpolarize.

To examine the *in vivo* function of the inhibitory γ subunits, we generated a mouse line, $Pdeg^{tm1}/Pdeg^{tm1}$, carrying a disruption of the PDEγ gene [9]. The loss of the PDEγ subunit, surprisingly, led to decreased rather than increased PDE activity in homozygous mutant mice. Examination of mutant retinal extracts showed that the PDE$\alpha\beta$ catalytic dimer was formed, but lacked hydrolytic activity. These studies of the PDEγ-deficient mice demonstrated that the inhibitory γ subunit had an unanticipated role in promoting the expression of PDE catalytic activity *in vivo*.

The regions of PDEγ that are required for its various functions were recently studied in reconstituted systems. PDEγ contains a central lysine-rich region, spanning residues Arg^{24} to Gly^{45}, in which 10 of 13 amino acids are basic. These residues contain one site for interaction with Tα [10] and are essential for binding to the PDE catalytic core [11]. The region involved in inhibiting PDE catalytic activity is thought to lie at the C-terminus and is different from the region required for binding [10]. Deletions and point mutations in the C-terminus were shown not to affect the binding of PDEγ to PDE$\alpha\beta$, but to decrease inhibition of PDE activity [12–14]. Further, peptides corresponding to the C-terminus were found to inhibit trypsin-activated PDE [15].

To test the importance of the C-terminus of PDEγ *in vivo*, various mutant and wild-type PDEγ cDNAs under the control of the opsin promoter were constructed and used to generate transgenic mice by conventional means [16]. The transgenes were then transferred to mice homozygous for a targeted disruption of the endogenous PDEγ gene, $Pdeg^{tm1}/Pdeg^{tm1}$, [9] by breeding, and animals of the appropriate genotype were identified by PCR and Southern-blot analysis of genomic DNA. In the present study we examined the substitution of a glycine residue for the tyrosine residue at position 84 (Y84G) previously shown to decrease the inhibitory potential of the PDEγ subunit [12,17].

Abbreviations used: PDE, phosphodiesterase; *Pdeg*, gene encoding the γ subunit of PDE; Tα, transducin α; $Pdeg^{tm1}/Pdeg^{tm1}$, a mouse line carrying a targeted disruption of the *Pdeg* gene; TgY84G, transgenic mice that only express a mutant γ subunit of PDE; ERG, electroretinogram; ROS, rod outer segment(s); GTP[S], guanosine 5′-[γ-thio]triphosphate.

[1] These authors contributed equally to the work.

[2] To whom correspondence should be addressed (e-mail goff@cuccfa.ccc.columbia.edu).

This mutant transgene would be expected to result in the formation of a constitutively active PDE and thus to produce low levels of cGMP in the animals carrying it. These mice would also be expected to show little or no rod response and normal cone responses in electroretinogram (ERG) measurements. Contrary to these expectations, we found that the PDE was inhibited normally and that the newly engineered animals had a significant photoresponse. Thus the Y84G mutation produced a much 'milder' phenotype *in vivo* than predicted from the biochemical assays *in vitro*.

EXPERIMENTAL

Generation of mutant mouse lines

The mice studied in these experiments were used in accordance to the ARVO (Association for Research in Vision and Ophthalmology) statement for the Use of Animals in Ophthalmic and Vision Research. DNA constructs for the expression of PDEγ contained 4.4 kb of the mouse opsin promoter, the complete open reading frame of the PDEγ cDNAs [18] and the polyadenylation signal of the mouse protamine gene [19]. The Y84G point mutation was introduced by a standard PCR-based site-specific mutagenesis strategy [20]. The entire PDEγ cDNA coding region in the transgenic construct was sequenced to confirm the introduction of the point mutation and that no other changes had been created inadvertently. Restriction endonucleases *Kpn*I and *Xba*I were used to excise vector sequences from the constructs. Oocytes were obtained from superovulated F1(DBA X C57BL6) females mated with homozygous $Pdeg^{tm1}/Pdeg^{tm1}$ males. The construct was injected into the male pronuclei of oocytes under a depression slide chamber. These microinjected oocytes were cultured overnight in M16 and transferred into the oviducts of 0.5-day-*post coitus* pseudopregnant F1 females. The resulting transgenic mice were then back-crossed to $Pdeg^{tm1}/Pdeg^{tm1}$ mice to place the transgene into the knockout background. The mice were also tested for the absence of the *rd1* mutation [21].

Identification of transgenic mice

DNA was isolated from tail tips or liver samples by homogenizing the tissue, digesting extensively with proteinase K and extracting with phenol. DNAs were analysed by PCR. The DNAs were also digested with *Sac*I and analysed by Southern-blot hybridization with a PDEγ cDNA probe. Additional restriction digests were performed to analyse the structure of the integrated sequences, and to insure that the DNA flanking the transgene was intact.

Rod-outer-segment (ROS) isolation

ROS from dark-adapted mice were isolated under dim red light in Hepes/phosphate-buffered balanced salt solution (HBSS; 4.09 mM sodium phosphate, monobasic, 14.7 mM Hepes, pH 7.2, 148.4 mM NaCl, 4.91 mM KCl, 2.45 mM $CaCl_2$ and 1.23 mM $MgSO_4$). Rhodopsin content was determined by the difference in A_{500} before and after bleaching under a non-saturating halogen light source [22].

Immunoblot analyses

Proteins (185 pmol of rhodopsin/lane) from murine rod outer segments (ROS) were separated by electrophoresis on either a 12 % total acrylamide/1.5 % crosslinker (for the PDEγ subunit) or a 6.5–9.5 % total acrylamide/1.5 % cross-linker (for PDE α and β subunits) inverted gradient polyacrylamide gel previously described by Tsang et al. [9]. Proteins were then transferred to 0.2 μm Immuno-blot PVDF membranes (Bio-Rad Laboratories, Hercules, CA) by the method of Towbin et al. [23], overnight, at 4 V/cm. Membranes were blocked in 3 % BSA in 500 mM NaCl/20 mM Tris (pH 7.6)/0.1 % Tween 20 (BSA-TTBS). For the detection of PDEγ, blots were incubated with a 1:1000 dilution of a mixture of polyclonal peptide antisera directed against the N-terminus (amino acid residues 2–16) of the PDEγ subunit. The PDE α and β subunits were detected by incubation with a polyclonal antiserum raised against a 17-mer peptide [24], 100 % homologous between the rod PDEα and PDEβ subunits as well as the cone PDEα' subunit. Western blots were visualized with the DuoLux Chemiluminescence substrate kit (Vector Laboratories, Burlingame, CA, U.S.A.) utilizing a goat anti-rabbit IgG–alkaline phosphatase conjugate. Blots were exposed to Hyperfilm-MP (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) preflashed to increase sensitivity and linearity according to the Sensitize™ protocol (Amersham Pharmacia Biotech). Signals were quantified by densitometric scanning.

Histology

Mice were killed with an intraperitoneal injection of pentobarbital (100 mg/kg). Each eye was rapidly removed, punctured at the 12 o'clock position along the limbus, and placed in a separate solution of 3 % glutaraldehyde in phosphate-buffered saline. After fixation for 1–2 days, the eyes were washed with saline and the 12 o'clock limbal puncture was used to orient the right and left eyes, which were kept in separate buffer, so that the posterior segment containing the retina could be sectioned along the vertical meridian. A rectangular piece containing the entire retina from superior to inferior ora serrata, including the optic nerve, was prepared for post-fixing in osmic acid, dehydration and epon embedding. A corner was cut out at the superior ora to allow identification of the upper retinal half of the segment. Sectioning proceeded along the long axis of the segment so that each section contained both upper and lower retina as well as the posterior pole. These segments were sectioned semi-serially, stained with Toluidine Blue, mounted, and examined by optical microscopy. Selected areas were trimmed for ultrathin sectioning, stained with uranyl acetate and examined by electron microscopy.

Electroretinography

ERGs were obtained from anaesthetized animals using a saline-moistened cotton-wick electrode that contacted the cornea. Mice were anaesthetized with a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg) administered either intraperitoneally or intramuscularly. The pupils were dilated with 1 % phenylephrine hydrochloride and 1 % cyclopentolate. Body temperature was maintained at 37 °C by a heated stage on which the mouse was laid. A 30-gauge needle was placed subcutaneously on the forehead as a reference electrode and a ground electrode subcutaneously on the trunk. The stimuli were light flashes obtained from a Grass Instruments Inc. (West Warwick, RI, U.S.A.) stroboscope removed from its housing and mounted in a metal box with a circular aperture (3 cm in diameter) placed 9 cm from the centre of the pupil of the mouse. Neutral-density filters were placed between this aperture and the cornea to vary the energy and wavelength of the flash. Responses were detected with a Nicolet Instruments (Lanham, MD, U.S.A.) CA-1000 oscilloscope, averaging from 3–20 responses to the same flash intensity. Mice were dark-adapted for at least 6 h before testing. Stimulation was begun with 4.8 log units of neutral-density filtering before the strobe flash and responses were averaged to one flash every 3 s. At high flash intensities, each flash was

presented every 15 s. (The duration of a flash is nominally 10 ms; the frequency response of the recording system extends from 1 to 500 Hz.)

cGMP RIA

Determination of retinal cGMP content was performed as described by Farber and Lolley [25]. Retinas were isolated under IR illumination or normal room light and homogenized in 0.1 M HCl. The assay was conducted in duplicate and the results expressed in pmol of cGMP/mg of total protein.

PDE activity assay

To measure both dark- (basal) and light-stimulated PDE activities, animals were dark-adapted overnight and dissected under far-red light (Kodak Type 11 filter). Retinas were incubated in hypotonic buffer supplemented with 1 mM ATP [26,27] and 5 μM guanosine 5′-[γ-thio]triphosphate (GTP[S]) [6] for 10 min and were maintained for 2 min extra in the dark or were exposed to room light for 2 min prior to homogenization. Homogenates were centrifuged for 10 min at 8000 ***g*** and the supernatants were collected. Aliquots were removed for protein determination by the method of Peterson [28], using BSA as standard, and cGMP-PDE activity was measured by the method of Farber and Lolley [29] using 250 μM cGMP as substrate. Each reaction was carried out in triplicate. A 3–10 μg portion of retinal protein (depending on the sample) was incubated for 15 min at 37 °C with the substrate in 40 mM Tris buffer, neutral pH, containing 200000 c.p.m. of [^{3}H]cGMP, 5 mM $MgCl_2$ and 1 mM dithiothreitol. The reaction was terminated by heating to 80 °C for 3 min. A 0.4 unit portion of calf intestinal alkaline phosphatase was then added to the sample and incubation allowed to proceed at 37 °C for 10 min. The resulting [^{3}H]guanosine was separated from other nucleotides by a resin slurry (AG1-X2, 50–100 mesh, Bio-Rad Laboratories) and radioactivity was quantified in a liquid-scintillation counter. Results were expressed as nmol of cGMP hydrolysed $\cdot$ min^{-1} $\cdot$ mg^{-1} of total protein.

Tα-GTP[S] activation

The activation of PDE by Tα was examined by measuring the cGMP-PDE activity in the presence of liposomes (L-α-phosphatidylcholine; type IVS; Sigma Chemical Co., St Louis, MO, U.S.A.) [30] containing increasing amounts of Tα-GTP[S]. The latter was purified by the method of Kroll et al. [31]. Homogenates of dark-adapted retinas from +/+ control and transgenic (Tg) Y84G mutant animals were obtained as described above with the hypotonic extraction buffer also containing 1 mM ATP [8,27,32]. Aliquots were incubated with liposomes (6 mg/ml final concn.) and purified Tα-GTP[S] (0–2.5 μM final concn.). cGMP-PDE assays were conducted at 37 °C using 250 μM cGMP as substrate.

Single-turnover GTPase activity

To minimize individual differences between experimental mice, ROS isolated from several retinas of mice of the same age were pooled. Single-turnover GTPase experiments were performed using modifications of standard methods [20]. Briefly, ROS were bleached and then disrupted by passage through a 28-gauge needle (rather than a hypotonic-shock treatment) as described by Tsang et al. [20]; 10 μl aliquots of the ROS suspension (approx. 10 μM final rhodopsin concn.) were placed in 1.5 ml Microfuge tubes. The reaction was started by addition of 5μl of γ-[^{32}P]GTP (50 fmol, 10000 c.p.m./fmol) and terminated by the addition of 100 μl of 6% (v/v) perchloric acid. A 700 μl portion of an activated charcoal suspension (0.1 g/ml in 50 mM potassium phosphate, pH 7.5) was added to the quenched sample. The mixture was vortex-mixed three times during a 20 min incubation on ice and centrifuged at 12000 ***g*** for 4 min. The radioactivity in 300 μl of supernatant was measured.

RESULTS

Y84G mutant transgene restores the photoreceptor of the PDEγ knockout mice

The Y84G mutation in the C-terminus of PDEγ had been shown to block the inhibition of the catalytic-core PDEαβ that is mediated by the wild-type PDEγ *in vitro*. To test the effects of this C-terminal mutation on PDE function *in vivo*, Tg lines expressing the Y84G mutant allele were generated and crossed with $Pdeg^{tm1}/Pdeg^{tm1}$ to obtain mice that only expressed the mutant PDEγ. Two independent TgY84G lines were used for this study. As controls, eight lines of transgenic mice expressing the wild-type allele in the same background were also developed.

Immunoblots using ROS proteins of each transgenic line, normalized by the amount of rhodopsin present, revealed that the levels of Y84G mutant PDEγ were similar in both lines to the PDEγ levels of control C57BL/6 (+/+) mice (Figure 1). In addition, the levels of PDEαβ were similar to those in C57BL/6 control (+/+) ROS (Figure 1). Therefore we considered both TgY84G lines equivalent for further experiments. Interestingly, normal levels of expression of the transgene were achieved in six out of eight wild-type PDEγ rescue lines. Thus PDEγ levels may be tightly regulated in the photoreceptors.

Retinal histology of sections prepared on 4-month-old mice showed 10–12 rows of photoreceptor nuclei in both heterozygous $Pdeg^{tm1}/+$ mice with the Y84G transgene, and homozygous $Pdeg^{tm1}/Pdeg^{tm1}$ mice with the Y84G transgene

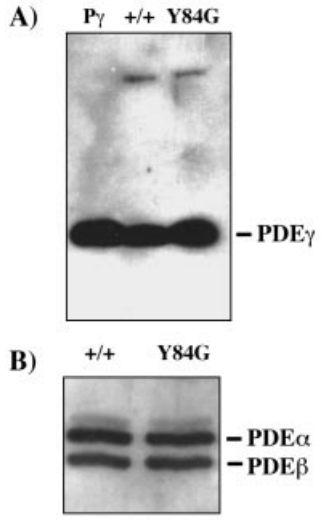

Figure 1 Immunoblot analysis of the expression of PDE subunits in control and TgY84G ROS normalized for rhodopsin content

(**A**) Immunoblot incubated with a polyclonal antibody recognizing the N-terminal of the PDEγ subunit. Protein equivalent to 150 pmol of rhodopsin was loaded. Lane 1, dark-adapted bovine ROS (Pγ); lane 2, control (+/+); lane 3, TgY84G retinas (Y84G). (**B**) Immunoblot incubated with a polyclonal peptide antibody recognizing both the PDE α and β subunits. Protein in both lanes was normalized to 150 pmol of rhodopsin. Lane 1, control (+/+); lane 2, TgY84G (Y84G). Autoradiographs were scanned on a Microtek ScanMaker X6EL with Adobe Photoshop 3.0 software on a PowerMacintosh computer, and the Figure was composited with Adobe PageMaker 6.5 software.

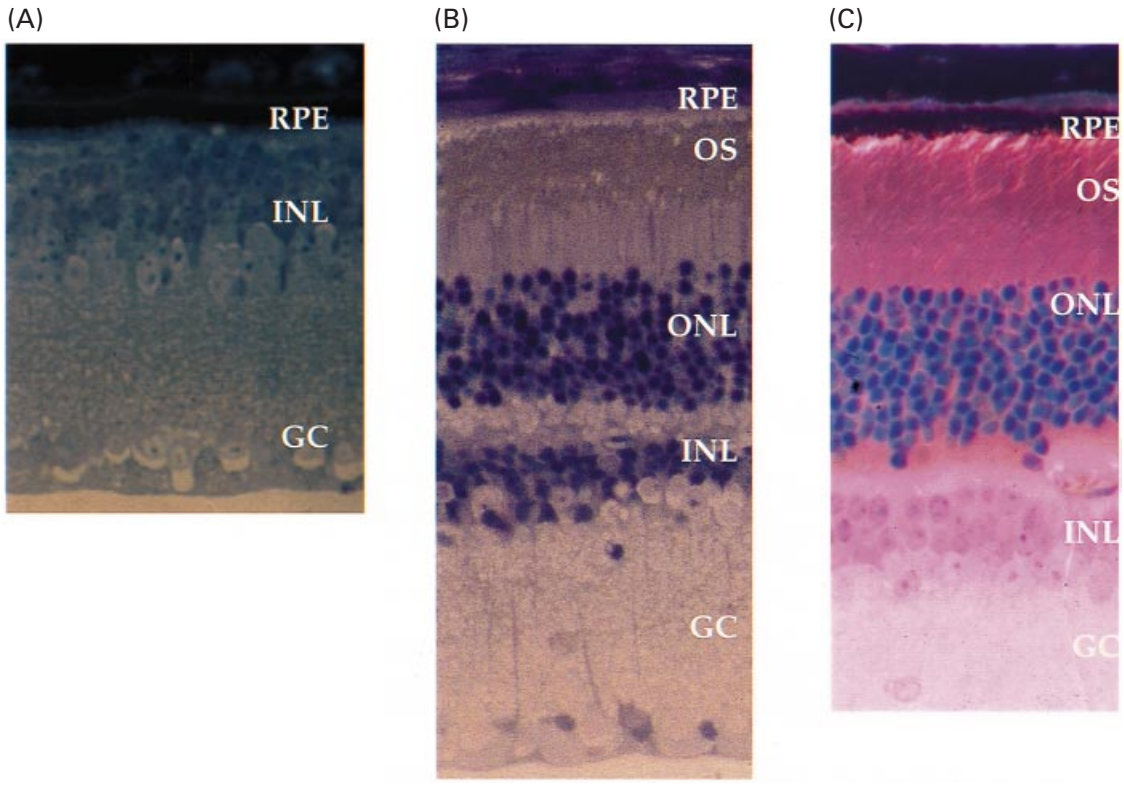

Figure 2 Retinal optical micrographs of (A) an adult homozygote *Pdeg*^{tm1}/*Pdeg*^{tm1}, (B) a homozygote *Pdeg*^{tm1}/*Pdeg*^{tm1} with the Y84G transgene and (C) a wild-type retina at 4 months after birth

Further abbreviations: RPE, retinal pigment epithelium; OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; GC, ganglion-cell layer. The retina of the *Pdeg*^{tm1}/*Pdeg*^{tm1} mutant mouse has lost the photoreceptor layer (OS, IS, ONL) completely by this age.

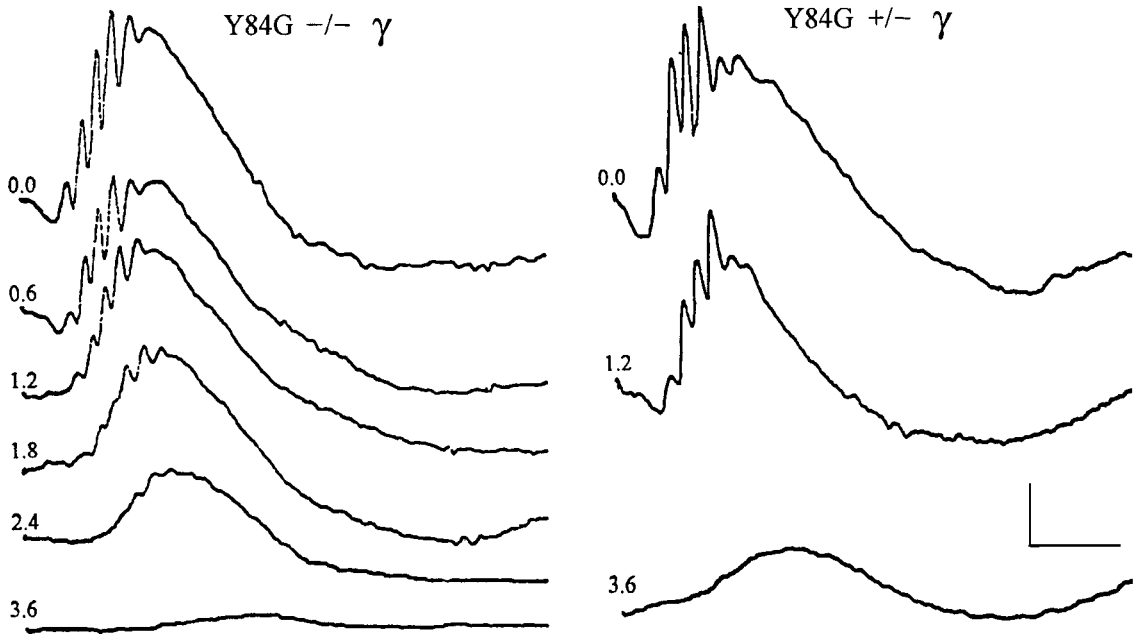

Figure 3 Corneal ERGs to white flashes of different intensities shown by the amount of neutral-density filtering placed before the flash at the left of each trace

The left column of responses are from the homozygous *Pdeg*^{tm1}/*Pdeg*^{tm1} mice (−/−) with the Y84G transgene and the right from heterozygous *Pdeg*^{tm1}/+ mice (+/−) with the Y84G transgene. The calibration at the lower right indicates 100 μV vertically and 50 ms horizontally.

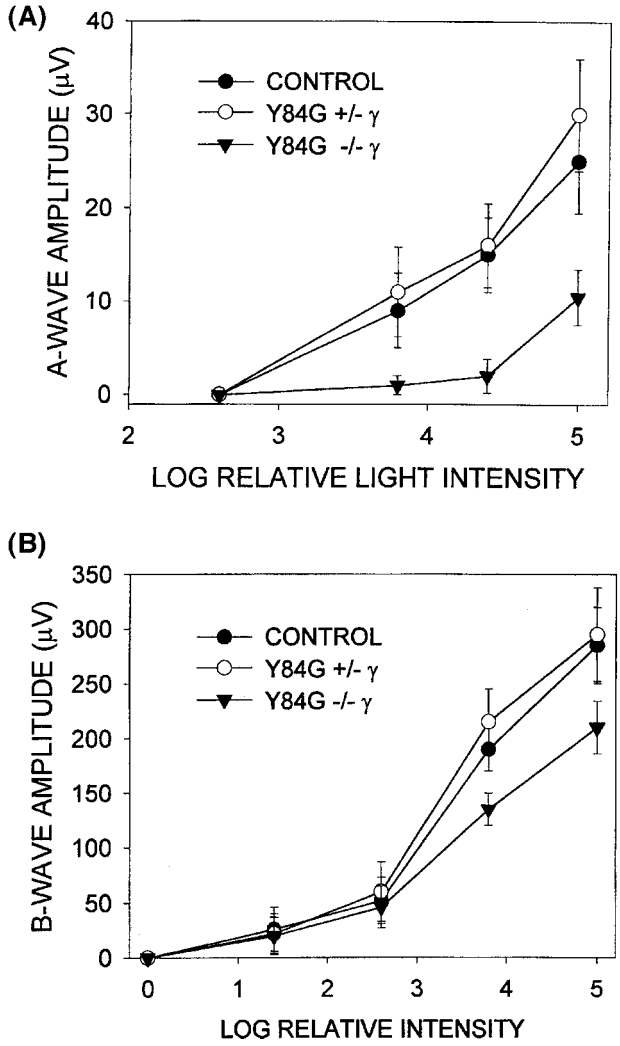

Figure 4 Relationship between the amplitude of the a-wave (A) or the b-wave (B) of the ERG and the log (relative intensity) of a white flash for normal control (●), heterozygous *Pdeg*^{tm1}/+ mice (+/−) with the Y84G transgene (○) and homozygous *Pdeg*^{tm1}/*Pdeg*^{tm1} mice (−/−) with the Y84G transgene (▼)

Each point represents the average responses of 17, 9 and 29 separate ERG measurements respectively. Each vertical line represents the S.E.M.

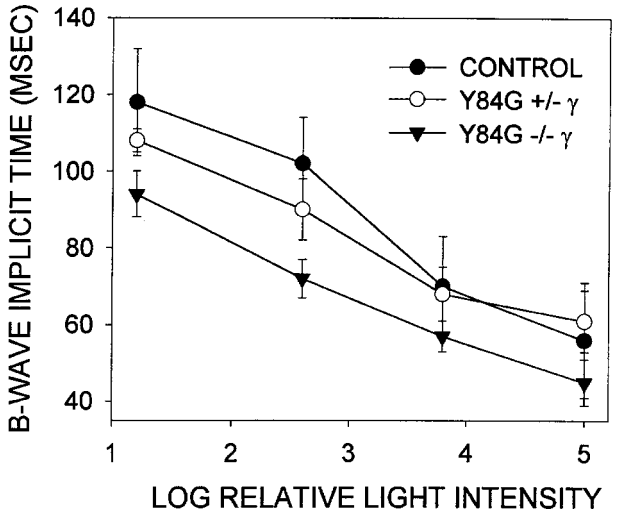

Figure 5 Relationship between the implicit time of the b-wave of the ERG and the log (relative intensity) of a white flash for normal control (●), heterozygous *Pdeg*^{tm1}/+ mice (+/−) with the Y84G transgene (○) and homozygous *Pdeg*^{tm1}/*Pdeg*^{tm1} mice (−/−) with the Y84G transgene (▼)

Each point represents the average responses of 17, 9 and 29 separate ERG measurements respectively. Each vertical line represents the S.E.M.

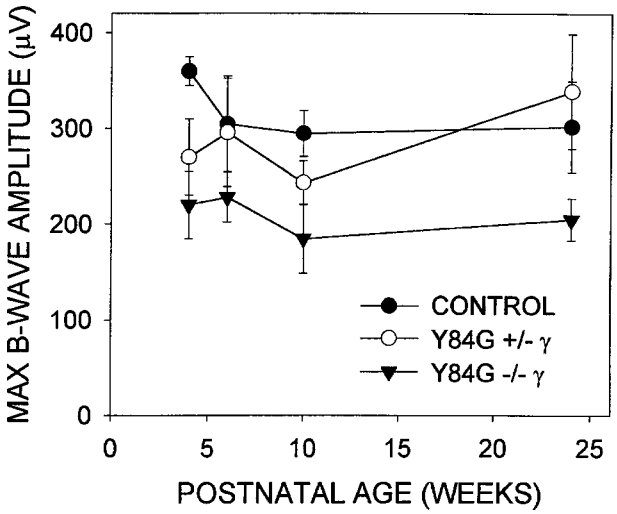

Figure 6 Relationship between the maximum ERG b-wave amplitude and the age of the mouse in weeks

Each point represents the average response of 12 normal controls (●), eight heterozygous *Pdeg*^{tm1}/+ mice (+/−) with the Y84G transgene (○) and six homozygous *Pdeg*^{tm1}/*Pdeg*^{tm1} mice (−/−) with the Y84G transgene (▼). Each vertical line represents the S.E.M.

(Figure 2), whereas the parental homozygous $Pdeg^{tm1}/Pdeg^{tm1}$ mice without the Y84G transgene showed complete degeneration. Thus the degeneration of the parental mutant mice was rescued by the mutant transgene. As expected, the wild-type transgene also rescued the $Pdeg^{tm1}/Pdeg^{tm1}$ mice photoreceptors.

Physiological features of the mutant mice

To assess the photoresponses, ERG measurements were made. The amplitude of both the a- and b-waves in the ERG of the homozygous $Pdeg^{tm1}/Pdeg^{tm1}$ mice (−/−) with the Y84G transgene was decreased compared with that of the heterozygous $Pdeg^{tm1}/+$ mice (+/−) carrying the Y84G transgene (Figure 3) at high light intensities. Plots of the amplitude against the logarithm of relative light intensities showed that this deficit was 10-fold for the a-wave and 1.5-fold for the b-wave (Figures 4A and 4B). The sensitivity for eliciting a threshold b-wave was within normal limits in the homozygote, but was reduced for the a-wave by 10-fold. The relationship between b-wave implicit time and light intensity showed a slightly faster response from the $Pdeg^{tm1}/Pdeg^{tm1}$ mice (−/−) with the Y84G transgene than from controls at similar flash intensities (Figure 5). The reduction in ERG amplitude did not increase with age of the mice, indicating that the abnormality was stationary (Figure 6). The degree of decrease in a- and b-wave sensitivity is relatively mild compared with other PDEγ mutants such as W70A [20] in which Tα activation of PDE is severely impaired. In the W70A mice

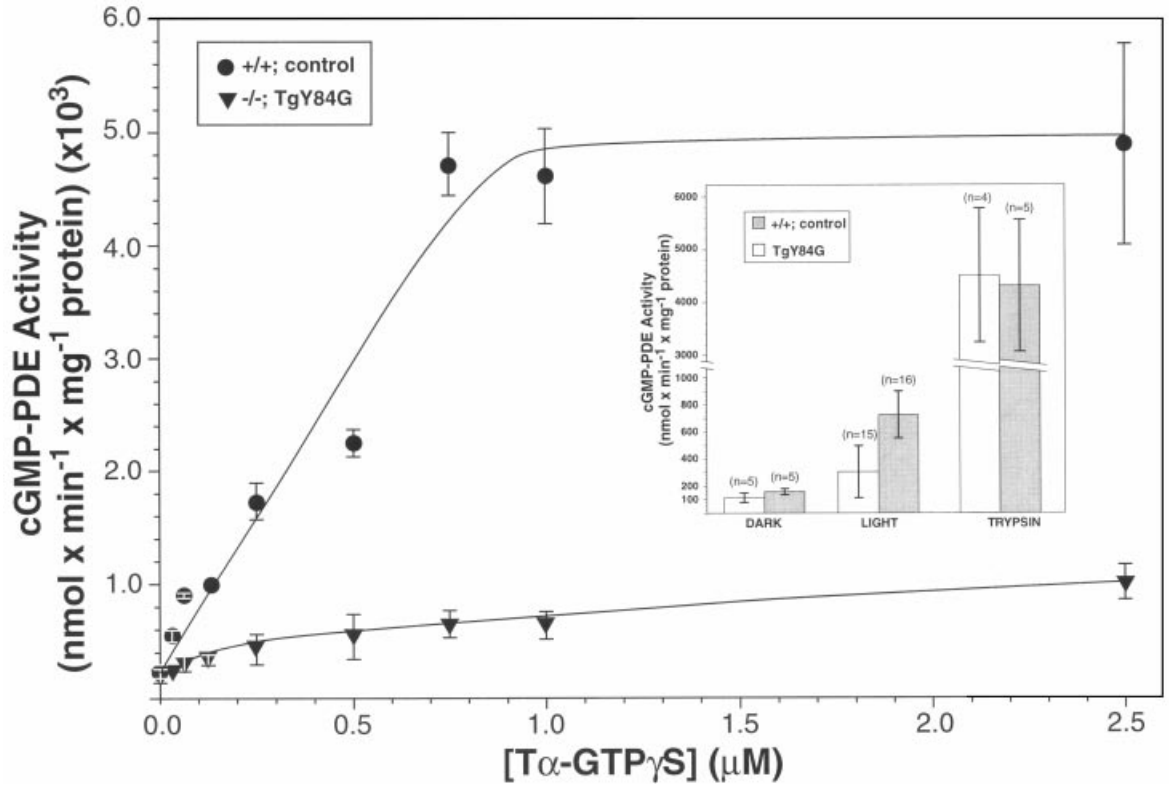

Figure 7 Tα-GTP[S] activation of cGMP-PDE in +/+ control (●) and TgY84G (▼) retinal homogenates

The cGMP-PDE assay was conducted using 250 μM cGMP in the presence of liposomes (6 mg/ml). Results represent the means of four separate experiments carried out in triplicate ± S.E.M. Curves were hand-fitted. Inset: determination of cGMP-PDE activity in dark-adapted, light- and trypsin-activated +/+ control and TgY84G retinas. Dark-adapted and light-activated retinas were incubated in the presence of 1 mM ATP and 5 μM GTP[S] under conditions stated in the Experimental section. Results represent the means ± S.E.M. for *n* independent experiments performed in quadruplicate using 250 μM cGMP as substrate.

more than a 100-fold loss in b-wave sensitivity was observed in previous measurements.

Biochemical analyses of mutant mice

The levels of cGMP were measured for both dark- and light-adapted retinas by RIA. The cGMP levels of dark-adapted +/+ control and TgY84G retinas were 68.0 ± 24.6 ($n = 10$) and 52.6 ± 12.4 ($n = 7$) pmol·mg of total protein^{-1} respectively, and those of light-adapted control and TgY84G retinas were 26.4 ± 9.9 ($n = 8$) and 26.8 ± 7.8 ($n = 7$) pmol of cGMP·mg of total protein^{-1} respectively. The results are the means of several determinations performed in duplicate ± S.E.M. Although there is an apparent decrease (22.6%) in cGMP levels between dark +/+ control and TgY84G retinas, this change is not statistically significant by the two-tailed Student's test. The similar cGMP concentrations are consistent with the observed normal morphology of the TgY84G animals, in contrast with the increased cGMP levels that cause retinal degeneration in the *rd1/rd1* and the $Pdeg^{tm1}/Pdeg^{tm1}$ mice.

To determine whether the Y84G transgene affected the activation and function of PDE, the basal (dark), light-stimulated (in the presence of GTP[S]) and trypsin-activated PDE activities were measured in control and Y84G mutant ROS (inset to Figure 7). Trypsin-activated PDE reflects total PDE activity and it is much higher than light-, transducin-, protamine- or histone-stimulated PDE activities [6,8]. Basal cGMP-PDE activity was comparable in +/+ control and mutant Y84G animals, (162 ± 17 and 168 ± 33 nmol·min^{-1}·mg of total protein^{-1} respectively). In the presence of GTP[S] [6], light was found to be more efficient in activating the +/+ control PDE (746 ± 284 nmol·min^{-1}·mg^{-1}; 4.5-fold increase) than the mutant enzyme (340 ± 266 nmol·min^{-1}·mg^{-1}; 1.8-fold increase), with a calculated two-tailed Student's t-test value of 4.04. The control PDE activity was in the range of that determined by Wensel and Stryer [6], and the light-activated PDE was 32% that of the trypsin-activated activity in the presence of 10 μM GTP[S] and 3.8 μM rhodopsin. However, trypsin-induced PDE activity, (PDEγ-independent) [33], was comparable between TgY84G mutant and control mice, showing that the Y84G mutation does not interfere with the assembly of a functional catalytic core. The PDE activity in the dark, which is inhibited by PDEγ [33], was also similar in TgY84G mutants and control retina, demonstrating that the Y84G mutation did not grossly alter PDEγ's ability to bind and inhibit the PDE$\alpha\beta$ catalytic core *in vivo*.

In vitro, Tα-GTP[S] can bind to PDEγ and stimulate PDE activity [34]. We determined the ability of Tα-GTP[S] to activate PDE in the Y84G transgenic animals by reconstituting increasing amounts of Tα-GTP[S] with light-adapted retinal extracts in liposomes. As expected, the maximal level of Tα-GTP[S] activation for the wild-type PDEγ was similar to that caused by trypsin. Both mutant and wild-type PDE levels responded to Tα-GTP[S] stimulation. However, the titration curves showed that the TgY84G mutant enzyme was about 5-fold less efficiently

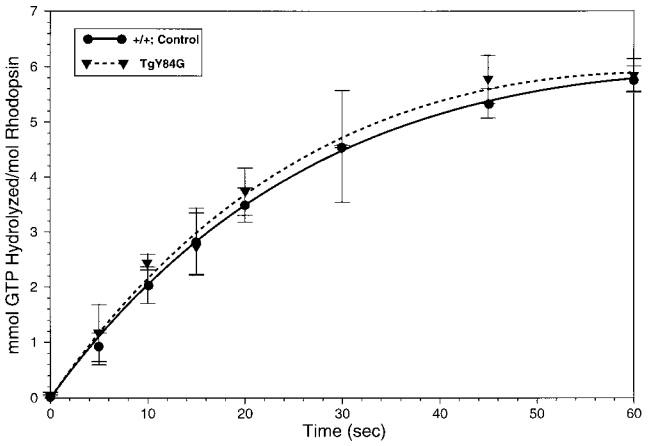

Figure 8 GTPase activity of +/+ control (○) and TgY84G (▼) ROS normalized to 10 mM final rhodopsin concentration

Data represents the means ± S.E.M. for three separate ROS preparations; experiments were conducted in duplicate. Results were curve-fitted to an exponential rise-to-maximum equation.

activated by Tα-GTP[S] than the normal +/+ retina (Figure 7). GTP[S] in conjunction with light was also found to be less effective in activating the mutant PDE complex. These results, therefore, substantiated the depression observed in the ERG for the TgY84G a- and b-wave amplitudes (Figure 4).

Since PDEγ accelerates the GTPase activity of the Tα subunit in the presence of ROS membranes, a defect in interaction of PDEγ with Tα should adversely affect Tα GTPase activity [20], and could explain the reduced efficiency of Tα-GTP[S] activation of the enzyme. To test if the Y84G mutation altered the interaction of PDEγ with Tα *in vivo*, the rate of GTPase activity turnover was measured in control and TgY84G retinas. The measured activities were plotted as mmol of GTP hydrolysed/mol of rhodopsin versus time and the rate constants were calculated from a curve fitted to an exponential rise-to-max equation (Figure 8). The rate constants obtained were similar for both the +/+ control and TgY84G ROS under the conditions of the assay (0.031 ± 0.007 s^{-1} and 0.024 ± 0.005 s^{-1} respectively), suggesting that the mutation does not significantly alter the interaction of PDEγ and Tα-GTP.

DISCUSSION

In vitro experiments carried out previously had shown that deletions in the C-terminal tail blocked PDEγ's ability to inhibit trypsin-activated PDE activity [1,13,35] and that a mutation of the Tyr^{84} residue reduced PDEγ inhibition [12,13,35,36], possibly due to a decrease in its binding affinity for the PDE catalytic core [37]. Therefore, the PDEγ C-terminal tail was thought to be necessary and sufficient to inhibit the PDE$\alpha\beta$ catalytic activity [12,13,38–40]. On the basis of these studies, we hypothesized that the TgY84G mutant retina would display a higher basal level of PDE activity. Surprisingly, we found that the PDE activity in the dark, which is inhibited by PDEγ [1,33], was similar in TgY84G mutants and control retina (inset to Figure 7), demonstrating that the Y84G mutation did not affect PDEγ's ability to bind and inhibit the PDE$\alpha\beta$ catalytic core. In fact, the mutation at the C-terminal Tyr^{84} residue of PDEγ still allows the formation of a PDE$\alpha\beta\gamma$ complex with regulated PDE activity.

However, our studies showed that PDE in the mutant retinas could only be activated by Tα-GTP[S] to a level about 5-fold lower than that in control retinas (Figure 7). Similarly, GTP[S] in conjunction with light was also less effective in activating the mutant PDE complex. Thus these results suggest that it is more difficult for Tα to remove the mutant Y84G PDEγ than the wild-type PDEγ from the PDE$\alpha\beta$ core. Previous measurements showed that, *in vitro*, trypsin-activated PDE was inhibited by nanomolar concentrations of PDEγ [6], but that this activation required micromolar concentrations of Tα [3]. These reports are consistent with our findings in that the affinity of the PDE core for PDEγ was much higher than for Tα-GTP in retinal homogenates. In fact, the formation of an inhibited PDE heterotetramer was 1000-fold more favoured in *in vitro* assays. Nevertheless, there are likely to be other factors that increase the affinity of Tα-GTP for PDEγ *in vivo*.

Takemoto et al. [13] reported that Y84G did not affect PDEγ's interaction with Tα *in vitro*, and Brown [37] found that a PDEγ Y84L mutation did not affect the Tα activation of the reconstituted enzyme. These observations were confirmed by the similar Tα GTPase activities that we measured in both normal and TgY84G retinas (Figure 8). The levels of GTPase hydrolysis were lower than previously reported [20] because more diluted ROS extracts obtained after passage through a 28-gauge needle were used in the reactions (only 10 μM of the rhodopsin equivalent of ROS membrane) for both mutant and control. These extracts were prepared from the disruption of ROS through the passage through a 28-gauge needle. This is consistent with the results of reports indicating that dilution of cellular components, most likely RGS9 (regulator of G-protein signalling 9), diminished the rate of GTP hydrolysis [41,42].

Animals expressing the Y84G mutant allele did exhibit a substantial photoresponse as judged by the ERG, although the a-wave and b-wave responses were reduced. Normal dark levels of PDE activity implied that the PDE core was properly inhibited. The surprisingly intact physiological photoresponse suggests that, contrary to predictions based on *in vitro* experiments, either the Y84G mutant PDEγ or other factors exert proper regulation of PDE activity *in vivo*. One of such factors could be the glutamic acid-rich protein 2 ('GARP2'), since it was shown to interact more strongly with light-activated than inactive PDE [43]. Alternatively, the discrepancies between the *in vitro* and *in vivo* data may reflect differences in the concentrations and nature of the contacts between components present on a membrane.

We thank Marie E. Burns, Akio Yamazaki, Bernard K.-K. Fung, C.-S. Lin, Frank D. Costantini, and members of their laboratories, for sharing ideas, cell lines, antisera and equipment as well as critical reading of the manuscript before its submission. We are also grateful to Hild Kjeldbye for guidance and advice, as well as to members of the Farber, Goff and Gouras laboratories for support, especially Sarah Freeman, Jian Ma and Douglas J. Wiener. This work was supported by National Institutes of Health grants RO1 EY 11510 (to P.G.), EY02651 (to D.B.F.) and by the Foundation Fighting Blindness (D.B.F.). S.H.T. was supported by Foundation Fighting Blindness, Fight-for-Sight, Research to Prevent Blindness (RPB) and T32 EY07105. D.J.S. was supported by a fellowship from ProRetina Deutschland e.B.K.D. is supported by the Human Frontiers program. D.B.F. is the recipient of an RPB Senior Scientific Investigators Award. S.P.G. is an investigator at the Howard Hughes Medical Institute.

REFERENCES

1 Stryer, L. (1991) Visual excitation and recovery. J. Biol. Chem. **266**, 10711–10714
2 Yarfitz, S. and Hurley, J. B. (1994) Transduction Mechanisms of Vertebrate and Invertebrate Photoreceptors. J. Biol. Chem. **269**, 14329–14332
3 Fung, B. K.-K., Hurley, J. B. and Stryer, L. (1981) Flow of information in the light-triggered cyclic nucleotide cascade of vision. Proc. Natl. Acad. Sci. U.S.A. **78**, 152–156
4 Baehr, W., Devlin, M. J. and Applebury, M. L. (1979) Isolation of bovine ROS phosphodiesterase. J. Biol. Chem. **254**, 11669–11677

5 Fung, B. K. K., Young, J. H., Yamane, H. K. and Griswold-Prenner, I. (1990) Subunit stoichiometry of retinal rod cGMP phosphodiesterase. Biochemistry **29**, 2657–2664

6 Wensel, T. G. and Stryer, L (1986) Reciprocal control of retinal rod cyclic GMP phosphodiesterase by its γ subunit and transducin. Proteins: Struct. Funct. Genet. **1**, 90–99

7 Deterre, P., Bigay, J., Forquet, F., Robert, M. and Charbre, M. (1988) cGMP phosphodiesterase of retinal rods is regulated by two inhibitory subunits. Proc. Natl. Acad. Sci. **85**, 2424–2428

8 Miki, N., Baraban, J. M., Keins, J. J., Boyce, J. J. and Bitensky, M. W. (1975) Purification and properties of the light-activated cyclic nucleotide phosphodiesterase of rod outer segments. J. Biol. Chem. **260**, 6320–6327

9 Tsang, S. H., Gouras, P., Yamashita, C. K., Kjeldbye, H., Fisher, J., Farber, D. B. and Goff, S. P. (1996) Retinal degeneration in mice lacking the γ subunit of rod cGMP phosphodiesterase. Science **272**, 1026–1029

10 Lipkin, V. M., Dumler, I. L., Muradov, K. G., Artemyev, N. O. and Etingof, R. N. (1988) Active sites of the cyclic GMP phosphodiesterase γ subunit of retinal rod outer segments. FEBS Lett. **234**, 287–290

11 Artemyev, N. O. and Hamm, H. E. (1992) Two-site high-affinity interaction between inhibitory and catalytic subunits of rod cyclic GMP phosphodiesterase. Biochem. J. **283**, 273–279

12 Gonzalez, K., Cunnick, J. and Takemoto, D. (1991) Retinal cyclic-GMP phosphodiesterase γ-subunit: identification of functional residues in the inhibitory region. Biochem. Biophys. Res. Commun. **181**, 1094–1096

13 Takemoto, D. J., Hurt, D., Oppert, B. and Cunnick, J. (1992) Domain mapping fo the retinal cyclic GMP phosphodiesterase γ-subunit. Biochem. J. **281**, 637–643

14 Berger, A. L., Cerione, R. A. and Erickson, J. W. (1997) Real time conformational changes in the retinal phosphodiesterase γ subunit monitored by resonance energy transfer. J. Biol. Chem. **272**, 2714–2721

15 Granovsky, A. E., Natochin, M. and Artemyev, N. O. (1997) The γ subunit of rod cGMP-phosphodiesterase blocks the enzyme catalytic site. J. Biol. Chem. **272**, 11686–11689

16 Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994) Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

17 Lipkin, V. M., Udovichenko, I. P., Bondarenko, V. A., Yurovskaya, A. A., Telnykh, E. V. and Skiba, N. P. (1990) Site-directed mutagenesis of the inhibitory subunit of retinal rod cyclic GMP phosphodiesterase. Biomed. Sci. **1**, 305–308

18 Tuteja, N. and Farber, D. B. (1988) γ-Subunit of mouse retinal cyclic-GMP phosphodiesterase cDNA and corresponding amino acid sequence. FEBS Lett. **232**, 182–186

19 Lem, J., Applebury, M. L., Falk, J. D., Flannery, J. G. and Simon, M. I. (1991) Tissue-specific and developmental regulation of rod opsin chimeric genes in transgenic mice. Neuron **6**, 201–210

20 Tsang, S. H., Burns, M. E., Calvert, P. D., Gouras, P., Baylor, D. A., Goff, S. P. and Arshavsky, V. Y. (1998) Role of the target enzyme in deactivation of photoreceptor G protein *in vivo*. Science **282**, 117–121

21 Pittler, S. J. and Baehr, W. (1991) Identification of a nonsense mutation in the rod photoreceptor cGMP phosphodiesterase β-subunit gene of the rd mouse. Proc. Natl. Acad. Sci. **88**, 8322–8326

22 Zimmerman, W. F. and Godchaux, W. D. (1982) Preparation and characterization of sealed bovine rod cell outer segments. Methods Enzymol. **81**, 52–57

23 Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354

24 Piriev, N. I., Yamashita, C., Samuel, G. and Farber, D. B. (1993) Rod photoreceptor cGMP-phosphodiesterase: analysis of α and β subunits expressed in human kidney cells. Proc. Natl. Acad. Sci. U.S.A. **90**, 9340–9344

25 Farber, D. B. and Lolley, R. N. (1982) Measurement of cyclic nucleotides in retina. Methods Enzymol. **81**, 551–556

26 Bitensky, M. W., Miki, N., Keirns, J. J., Keirns, M., Baraban, J. M., Freeman, J., Wheeler, M. A., Lacy, J. and Marcus, F. R. (1975) Activation of photoreceptor disk membrane phosphodiesterase by light and ATP. Adv. Cyclic Nucleotide Res. **5**, 213–240

27 Kamps, K. M. and Hofmann, K. P. (1986) ATP can promote activation and deactivation of the rod cGMP-phosphodiesterase: kinetic light scattering on intact rod outer segments. FEBS Lett. **208**, 241–247

28 Peterson, G. L. (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. **83**, 346–356

29 Farber, D. B. and Lolley, R. N. (1976) Enzyme basis for cyclic GMP accumulation in degenerative photoreceptor cells of mouse retina. J. Cyclic Nucleotide Res. **2**, 139–148

30 Kutuzov, M. and Pfister, C. (1994) Activation of the retinal cGMP-specific phosphodiesterase by the GDP-loaded alpha-subunit of transducin. Eur. J. Biochem. **220**, 963–971

31 Kroll, S., Phillips, W. J. and Cerione, R. A. (1989) The regulation of the cyclic GMP phosphodiesterase by the GDP-bound form of the α subunit of transducin. J. Biol. Chem. **264**, 4490–4497

32 Chader, G. J., Herz, L. R. and Fletcher, R. T. (1974) Light activation of phosphodiesterase activity in retinal rod outer segments. Biochim. Biophys. Acta **347**, 491–493

33 Hurley, J. B. and Stryer, L. (1982) Purification and characterization of the γ regulatory subunit of the cyclic GMP phosphodiesterase from retinal rod outer segments. J. Biol. Chem. **257**, 11094–11099

34 Fung, B. K. and Griswold, P. I. (1989) G protein–effector coupling: binding of rod phosphodiesterase inhibitory subunit to transducin. Biochemistry **28**, 3133–3137

35 Artemyev, N. O., Natochin, M., Busman, M., Schey, K. L. and Hamm, H. E. (1996) Mechanism of photoreceptor cGMP phosphodiesterase inhibition by its γ-subunits. Proc. Natl. Acad. Sci. U.S.A. **93**, 5407–5412

36 Berger, A. L., Cerione, R. A. and Erickson, J. W. (1999) Delineation of two functionally distinct gammaPDE binding sites on the bovine retinal cGMP phosphodiesterase by a mutant γ PDE subunit. Biochemistry **38**, 1293–1299

37 Brown, R. L. (1992) Functional regions of the inhibitory subunit of retinal rod cGMP phosphodiesterase identified by site-specific mutagenesis and fluorescence spectroscopy. Biochemistry **31**, 5918–5925

38 Skiba, N. P., Artemyev, N. O. and Hamm, H. E. (1995) The carboxyl terminus of the γ-subunit of rod cGMP phosphodiesterase contains distinct sites of interaction with the enzyme catalytic subunits and the α-subunit of transducin. J. Biol. Chem. **270**, 13210–13215

39 Natochin, M. and Artemyev, N. O. (1996) An interface of interaction between photoreceptor cGMP phosphodiesterase catalytic subunits and inhibitory γ subunits. J. Biol. Chem. **271**, 19964–19969

40 Lipkin, V. M., Bondarenko, V. A., Zagranichny, V. E., Dobrynina, L. N., Muradov, K. G. and Natochin, M. (1993) Site-directed mutagenesis of the cGMP phosphodiesterase γ subunit from bovine rod outer segments: role of separate amino acid residues in the interaction with catalytic subunits and transducin α subunit. Biochim. Biophys. Acta **1176**, 250–256

41 Dratz, E. A., Lewis, J. W., Schaechter, L. E., Parker, K. R. and Kliger, D. S. (1987) Retinal rod GTPase turnover rate increases with concentration: a key to the control of visual excitation? Biochem. Biophys. Res. Commun. **146**, 379–386

42 Arshavsky, V., Antoch, M. P., Lukjanov, K. A. and Philippov, P. P. (1989) Transducin GTPase provides for rapid quenching of the cGMP cascade in rod outer segments. FEBS Lett. **250**, 353–356

43 Korschen, H. G., Beyermann, M., Muller, F., Heck, M., Vantler, M., Koch, K. W., Kellner, R., Wolfrum, U., Bode, C., Hofmann, K. P. and Kaupp, U. B. (1999) Interaction of glutamic-acid-rich proteins with the cGMP signalling pathway in rod photoreceptors. Nature (London) **400**, 761–766

Received 8 August 2000/26 October 2000; accepted 13 November 2000