

Absence of the RGS9-G β 5 GTPase-activating Complex in Photoreceptors of the R9AP Knockout Mouse*

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Timely termination of the light response in retinal photoreceptors requires rapid inactivation of the G protein transducin. This is achieved through the stimulation of transducin GTPase activity by the complex of the ninth member of the regulator of G protein signaling protein family (RGS9) with type 5 G protein β subunit (G β 5). RGS9-G β 5 is anchored to photoreceptor disc membranes by the transmembrane protein, R9AP. In this study, we analyzed visual signaling in the rods of R9AP knockout mice. We found that light responses from R9AP knockout rods were very slow to recover and were indistinguishable from those of RGS9 or G β 5 knockout rods. This effect was a consequence of the complete absence of any detectable RGS9 from the retinas of R9AP knockout mice. On the other hand, the level of RGS9 mRNA was not affected by the knockout. These data indicate that in photoreceptors R9AP determines the stability of the RGS9-G β 5 complex, and therefore all three proteins, RGS9, G β 5, and R9AP, are obligate members of the regulatory complex that speeds the rate at which transducin hydrolyzes GTP.

Timely termination of the light response in retinal photoreceptors is essential for normal vision (reviewed in Refs. 1 and 2). On the molecular level, the normal time course of the light

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response requires rapid deactivation of the G protein transducin, which relays the visual signal to the effector, cyclic GMP phosphodiesterase. Deactivation of transducin occurs when the transducin α subunit hydrolyzes its bound GTP. In normal rods, GTP hydrolysis is catalyzed by the complex of the regulator of G protein signaling protein (RGS9)¹ with type 5 G protein β subunit (G β 5) (reviewed in Refs. 2 and 3). Recent studies have demonstrated that photoreceptors lacking RGS9 or G β 5 produce light responses that recover at an abnormally slow rate (4, 5).

In photoreceptors, the RGS9-G β 5 complex is tightly associated with the transmembrane protein R9AP (RGS9 anchor protein), which anchors RGS9-G β 5 on the surface of the disc membranes of the outer segment, which is the subcellular compartment where visual transduction occurs (6–8). R9AP is a 25-kDa protein structurally related to members of the SNARE (*N*-ethylmaleimide-sensitive factor attachment protein receptor) protein family, which are involved in vesicular trafficking and exocytosis (8–10). In mammals, R9AP is expressed predominantly in the retina (6, 9), whereas in chicken it is also present in cochlear hair cells and dorsal root ganglion neurons (9). R9AP dramatically enhances the ability of RGS9-G β 5 to stimulate transducin GTPase (7, 8, 10) and participates in the delivery of RGS9-G β 5 to photoreceptor outer segment (10).

In this study, we analyzed visual signaling in rods of R9AP knockout mice. The knockout did not affect the overall retinal morphology or photoreceptor development. However, light responses from R9AP knockout rods were very slow to recover and were indistinguishable from those of RGS9 or G β 5 knockout rods. The effect of the R9AP knockout on the photoresponse recovery was explained by a complete absence of any detectable RGS9 in the retinas of knockout mice. On the other hand, the level of RGS9 mRNA was not affected by the knockout. These data indicate that in photoreceptors R9AP determines the stability of RGS9-G β 5, and therefore R9AP should be considered an essential component of the GTPase-activating complex for transducin.

EXPERIMENTAL PROCEDURES

Generation of the R9AP Knockout Mouse—Primers specific for the coding region of the mouse R9AP gene, *Rgs9-1bp*, (forward, 5'-GCGC-GGCTCGTCTTGGAGAC-3'; reverse, 5'-CAGAGGTTTCAGAGCCTGG-TTCC-3') were used for PCR screen of a 129/SvJ mouse bacterial artificial chromosome genomic library (Genome Systems, St. Louis, MO) for a clone containing the complete *Rgs9-1bp* gene with the flanking sequences. The targeting vector contained 2 kb of PCR-amplified genomic sequence directly upstream of the *Rgs9-1bp* start codon followed by a 6.1-kb cassette containing the tau-*lacZ* reporter gene and the neomycin resistance gene, which are flanked by *lox* sites, and a 2.8-kb genomic sequence directly downstream of the stop codon of the gene. The vector was used to transfect E14 embryonic stem cells (11). The targeting of the *Rgs9-1bp* locus was confirmed by screening genomic DNA digested with DraI-resistant embryonic stem cell clones for homologous recombination using a Southern probe specific to sequence outside of the targeted region (Fig. 1A). After germ line transmission was obtained, animals heterozygous for the targeted allele were crossed to create R9AP knockout animals. Analysis was performed in mice that showed 100% penetrance of the retinal phenotypes described in a mixed (129/SvJ x C57BL/6) background.

Southern Blot Analysis—Genomic DNA was extracted from mouse

¹ The abbreviations used are: RGS9, regulator of G protein signaling protein; G β 5, type 5 G protein β subunit; R9AP, RGS9 anchor protein; DEP, *disheveled/Egl-10/pleckstrin*.

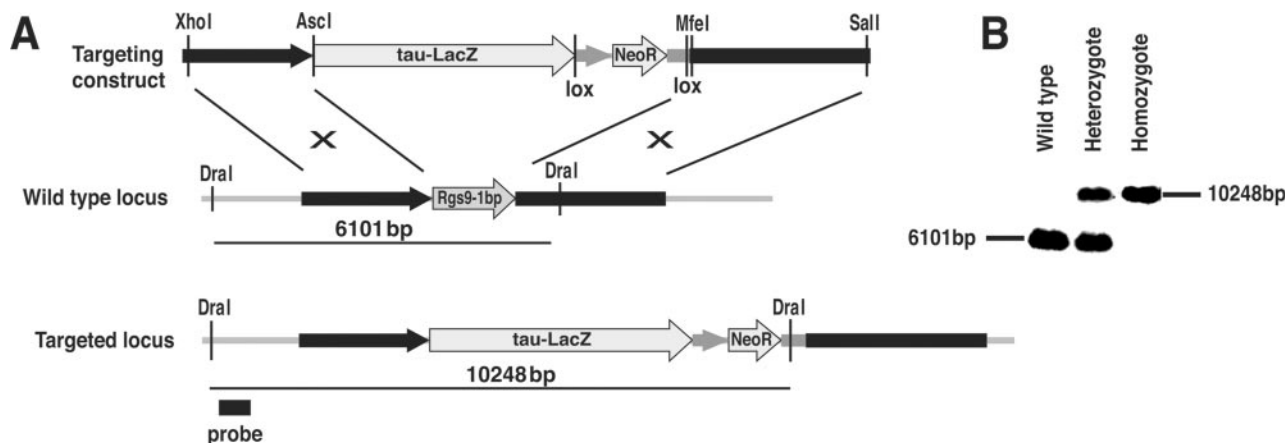


FIG. 1. Targeting strategy of *R9ap* locus. *A*, the complete coding sequence of the *R9ap* gene is intronless and was replaced by homologous recombination with a *tau-lacZ* neomycin resistance reporter/selection cassette. The gene structures of the wild type and targeted loci are depicted below the targeting vector. *B*, Southern blot of mouse tail genomic DNA digested with *Dra*I. Detection with the Southern probe (indicated as "probe" in *A*) reveals the wild type allele at 6101 bp and the targeted allele at 10248 bp. Heterozygote animals carry both alleles.

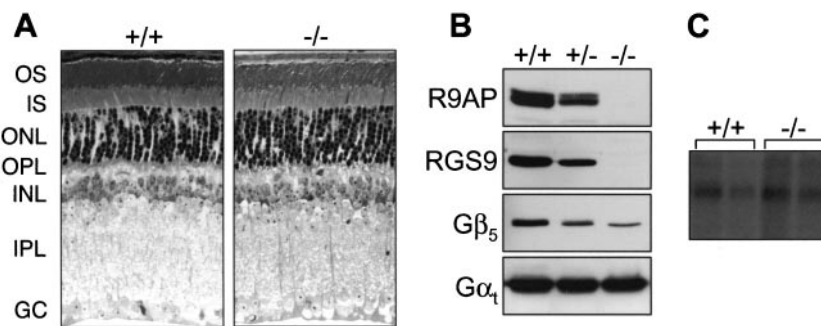


FIG. 2. Characterization of the retinas from *R9AP* knockout mice. *A*, 1- μ m cross-sections of the retinas from 2-month-old wild type and *R9AP* knockout mice stained by toluidine blue. *OS*, outer segment layer; *IS*, inner segment layer; *ONL*, outer nuclear layer; *OPL*, outer plexiform layer; *INL*, inner nuclear layer; *IPL*, inner plexiform layer; and *GC*, ganglion cell layer. *B*, Western blot analysis of *R9AP*, *RGS9*, *G β 5*, and the transducin α subunit (*G α t*) in the retinas of wild type (+/+), heterozygous (+/-), and *R9AP* knockout (-/-) mice. Each lane contained 20 pmol of rhodopsin. The upper band of *R9AP* immunoreactivity represents phosphorylated *R9AP* normally present in photoreceptors (10). *C*, Northern blot analysis of the *RGS9* mRNA in wild type and *R9AP* knockout mice. Decreasing amounts of total retina RNA were hybridized to a specific 32 P-labeled cDNA fragment for *RGS9*. RNA concentrations were 5 and 2.5 μ g.

tails, and 10 μ g of DNA was digested overnight with *Dra*I, electrophoretically fractionated in 0.6% agarose gel, denatured, and transferred to a nylon membrane (Hybond-N, AP Biotech). Hybridization was done overnight at 62 $^{\circ}$ C in hybridization solution (ExpressHyb, Clontech) including 10 μ g/ml denatured herring sperm DNA. The membrane was washed twice with 2 \times SSC, 0.1% SDS at 62 $^{\circ}$ C and twice with 0.2 \times SSC, 0.1% SDS at 62 $^{\circ}$ C for 10–15 min each. Primers for generating the Southern probe were: forward, 5'-CAAATCATTGAGCGGCACC-3'; and reverse, 5'-AGTATTGGAGAGGTCCTTG-3'.

Northern Blot Analysis—Denatured total RNA was isolated from retinas with Trizol reagent (Invitrogen). After separation on 0.8% formaldehyde-agarose gels, RNA was transferred to nylon membrane (Hybond-N, AP Biotech) and incubated at 60 $^{\circ}$ C with *RGS9*-specific probe in hybridization solution (ExpressHyb, Clontech). The probe was generated by PCR amplification of the 1–1300 region of the *RGS9* gene and labeled with 32 P by random priming. After the membranes were washed with 0.1% SDS in 0.2 \times SSC at 68 $^{\circ}$ C, the blots were exposed to XAR-5 film (Eastman Kodak).

Western Blot Analysis—Two mouse retinas were removed from the eyes, placed in 100 μ l of deionized water, and homogenized by sonication. Rhodopsin concentration in retinal homogenates was determined spectrophotometrically from the difference in the absorption at 500 nm before and after bleaching the sample using the extinction coefficient of $\epsilon_{500} = 40500$. Samples containing 20 pmol of rhodopsin were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. For protein detection, membranes were incubated with one of the following antibodies: rabbit antibody against the *R9AP*-(102–144) fragment (10), sheep anti-*RGS9c* antibody (12), sheep anti-*G β 5* NT_L antibody (12), and rabbit anti-*G α t* antibody (Santa Cruz Biotechnology). After incubation with horseradish peroxidase-conjugated secondary antibodies, the signals were detected using the West Pico ECL Western blot detection system (Pierce).

Preparation of Plastic-embedded Cross-sections of the Retina—Eyes were enucleated, cleaned of outside tissue, and fixed for 1 h in freshly prepared 2% paraformaldehyde with 2.5% glutaraldehyde in 0.1 M cacodylate buffer containing 2.5 mM CaCl_2 (pH 7.4). The eye globe was then hemisected along the vertical meridian and allowed to fix overnight at the same buffer. The eye cup was rinsed with excess 0.1 M cacodylate buffer (pH 7.4) and placed into 2% osmium tetroxide. The eye cup was gradually dehydrated in an increasing ethanol series (25–100%) and embedded in Epon. 1- μ m cross-sections were obtained and stained with alkaline toluidine blue for light microscopy.

Suction Electrode Recordings—Mice were housed in 12-h cyclic light conditions and dark-adapted overnight before an experiment. Under infrared light, animals were anesthetized and euthanized, and the retinas were removed and stored in Leibovitz's L-15 medium (Invitrogen) with 10 mM glucose and 0.1 mg/ml bovine serum albumin on ice. Small pieces of retina were placed in the recording chamber and perfused with bicarbonate-buffered Locke's solution, bubbled with 95% O_2 , 5% CO_2 , and warmed to 35–37 $^{\circ}$ C (pH 7.4). Responses to flashes (500 nm, 10 ms) were recorded from individual rods using suction electrodes as described (5). Briefly, individual outer segments were drawn into a glass pipette containing 140 mM NaCl, 3.6 mM KCl, 2.4 mM MgCl_2 , 1.2 mM CaCl_2 , 3 mM HEPES, 0.2 mM EDTA, and 10 mM glucose (pH 7.4). The bath and suction electrodes were connected to calomel half-cells by agar bridges, and the bath voltage was maintained at ground potential by an active feedback circuit. The rod membrane current was amplified (Axopatch 1B, Axon Instruments, Union City, CA) and filtered at 20 Hz with an 8-pole Bessel filter. Data were digitized continuously at 200 Hz using NiDAQ (National Instruments, Austin, TX) for IgorPro (Wavemetrics, Lake Oswego, OR). Light intensities were controlled with neutral density filters and calibrated daily (United Detector Technology, Baltimore, MD).

The average response to a high number (>30) of flashes was consid-

ered to be a dim flash response (linear response) if its mean amplitude was less than 20% of the maximal response amplitude. These dim flash responses were used to estimate the form of the single photon response using the “variance to mean” method as described previously (13). Integration time, used as a measure of the duration of the dim flash response, is defined as the time integral of the average linear response divided by its peak amplitude (14). The time that a bright flash response remained in saturation was calculated as the time interval between the midpoint of the flash and the time at which the current recovered by 10%.

RESULTS

Generation and Characterization of the R9AP Knockout Mouse—To test the function of R9AP in mice, we induced a null mutation into the R9AP gene (*Rgs9-1bp*) by gene targeting (Fig. 1A). We replaced the complete gene with a reporter gene and a neomycin resistance gene by homologous recombination in embryonic stem cells. The targeting of the *Rgs9-1bp* locus was confirmed by Southern blot analysis of *Dra*I-digested genomic DNA (Fig. 1B) and by PCR (not shown). After germ

line transmission was obtained, the animals heterozygous for the targeted allele were intercrossed to generate R9AP knockout mice. Mice that were homozygously lacking *Rgs9-1bp* were viable and fertile and displayed no obvious behavioral abnormalities. They also had normal retinal morphology up to at least 2 months of age (Fig. 2A). The total amount of rhodopsin in their retinas was also normal (384 ± 89 pmol/retina *versus* 406 ± 103 pmol/retina in wild type mice; S.E., $n = 2$).

The Absence of RGS9 from the Retinas of R9AP Knockout Mice—We analyzed the effects of the R9AP knockout on the expression of the proteins constituting the GTPase-activating complex for transducin. No detectable amount of R9AP was present in the retinas of R9AP knockout animals, consistent with targeted disruption of the R9AP locus in the genome (Fig. 2B). Strikingly, the retinas lacking R9AP also lacked any detectable amount of RGS9, and had significantly reduced levels of G β 5. The amounts of R9AP, RGS9, and G β 5 in the retinas of R9AP^{+/-} heterozygous animals bearing only one functional R9AP allele were about one-half of that present in their wild type littermates. In contrast, the amount of transducin in the retinas of knockout and heterozygous animals was normal.

Our data indicate that R9AP is required for the expression of RGS9 and G β 5. This is similar to the lack of G β 5 expression in RGS9 knockout mice (4) and the reciprocal lack of RGS9 expression in G β 5 knockouts (15). In both RGS9 and G β 5 knockout mice, this regulation was not caused by a reduction of G β 5 or RGS9 mRNA and was argued to occur on the posttranslational level. To test whether the absence of R9AP affects RGS9 expression in a similar fashion, we compared RGS9 mRNA levels in retinas of R9AP knockout mice and their wild type littermates. As shown in Fig. 2C, these mRNA levels were indeed similar.

Electrophysiological Properties of the Rods from R9AP Knockout Mice—To study the effects of inactivating the R9AP gene on the electrophysiological properties of intact rods, we used suction electrodes to record rod responses to flashes of light. A representative family of responses from a R9AP knockout rod is shown in Fig. 3A. On average, R9AP knockout rods displayed normal maximal response amplitudes and normal sensitivity to light (Table I). However, the responses of R9AP knockout rods were slow to recover, similar to the responses of rods with known defects in transducin deactivation, such as those of RGS9 and G β 5 knockout mice (4, 5).

To quantify the effect on photoresponse kinetics, we measured the time to peak, the duration, and the recovery time constant of responses of many R9AP knockout rods. The average duration of the dim flash response, as measured by integration time (see “Experimental Procedures”), was on the order of 2 s (Table I). This is 10-fold slower than the integration time that has been observed for dim flash responses of wild type mouse rods but indistinguishable from the integration times measured for both RGS9 and G β 5 knockout rods (4, 5). The recovery time constant was assessed by fitting a single exponential curve to the falling phases of the average dim flash response. The average time constant of this exponential recov-

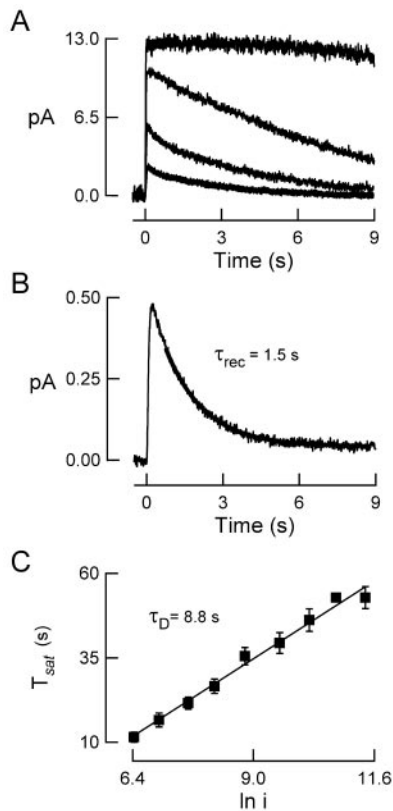


FIG. 3. **Photoresponses of R9AP knockout rods.** A, family of flash responses from a representative R9AP knockout rod. Flashes were delivered at $t = 0$ and ranged in strength from 15 to 606 photons/ μm^2 , roughly in 4-fold increments. B, population mean single photon response of 11 R9AP knockout rods. The *bold curve* is an exponential function fitted to the final falling phase. C, average dominant time constant of recovery from 13 R9AP^{-/-} rods. Each data point is the average of 7–12 points. Error bars represent S.E.

TABLE I
Electrophysiological properties of R9AP knockout rods

I_o , flash strength that elicits a half-maximal response; τ_{rec} , dominant time constant for recovery of saturating flash responses. Error indicates S.E., and number of rods is given in parentheses. Wild type values are reproduced from Ref. 5.

Strain	Maximal amplitude	I_o	Integration time	Dim flash τ_{rec}	Time to peak	Elementary amplitude	Bright flash τ_{rec}
	pA	photons/ μm^2	s	s	ms	pA	s
R9AP ^{-/-}	11.2 ± 1.0 (15)	54 ± 9 (10)	2.2 ± 0.4 (11)	2.0 ± 0.5 (11)	282 ± 62 (11)	0.54 ± 0.09 (11)	9.3 ± 0.5 (13)
Wild type	11.3 ± 0.5 (34)	70 ± 5 (27)	0.26 ± 0.02 (30)	0.17 ± 0.02 (28)	89 ± 3 (30)	0.34 ± 0.04 (28)	0.19 ± 0.01 (21)

ery was 2.0 ± 0.5 s (Fig. 3; Table I), very similar to the time constant of recovery observed in RGS9 (2.6 ± 0.3 s, from Ref. 4) and G β 5 (2.5 ± 0.2 s, from Ref. 5) knockout rods.

It is well known that the recovery of responses of rods lacking RGS9 and G β 5 slows further as the flash strength increases (4, 5, 10). R9AP knockout rods also showed this phenomenon. For bright flashes that produce saturating responses, the dominant time constant of recovery can be determined by measuring the slope of the dependence of saturation time (T_{sat}) on the natural log of the flash strength (16). We found that in R9AP knockout rods, the dominant time constant of recovery, τ_D , was 9.3 ± 0.5 s (Fig. 3C; Table I), indistinguishable from the τ_D values reported for both RGS9 (8.99 ± 0.22 s, from Ref. 4) and G β 5 knockout rods (8.8 ± 0.3 s, from Ref. 5).

The striking similarity of the R9AP knockout responses to those of the RGS9 and G β 5 knockouts indicates that inactivating the R9AP gene has the same functional consequences as inactivating either the catalyst, RGS9, or its binding partner, G β 5. We conclude that all three proteins, RGS9, G β 5, and R9AP, are obligate members of the regulatory complex that speeds the rate at which transducin hydrolyzes GTP.

DISCUSSION

In this study we report that knocking out the gene for R9AP results in a functional knockout of RGS9, which is evidenced by the lack of RGS9 protein in photoreceptors and abnormally slow recovery of the light response. In addition, the reduction in the R9AP protein level in the retinas of heterozygous mice causes a proportional reduction of RGS9-G β 5. Provided that the levels of RGS9 mRNA were not affected by the R9AP knockout, the most plausible explanation for these effects is that the association of RGS9 with R9AP plays a crucial role in stabilizing the entire GTPase-activating complex and that this interaction can determine the amount of functionally active RGS9-G β 5 in the cell. These data also suggest that all RGS9-G β 5 in rods is present as the complex with R9AP, making it unlikely that photoreceptors contain less R9AP than RGS9 as suggested previously (6). In principle, one could argue that the absence of RGS9 in the knockout may be explained by a drastically reduced translation of RGS9 mRNA without R9AP. However, this is unlikely because RGS9 mRNA is efficiently translated without R9AP in several eukaryotic protein expression systems (17–19).

Interestingly, the effect of the R9AP knockout on the expression level of RGS9 is not entirely reciprocal. RGS9 knockout causes only a modest reduction in the R9AP levels and does not affect the delivery of R9AP to rod outer segments (10). There is also a difference in the levels of G β 5 in R9AP and RGS9 knockouts. Although undetectable in the photoreceptors of RGS9 knockout (4), an appreciable fraction of G β 5 is present in the R9AP knockout photoreceptors (Fig. 2B). This is somewhat curious because the stability of the G β 5 molecule is known to be dependent on its binding to the G protein γ subunit-like (GGL) domain of RGS9 (17, 18). One possible explanation is that in the R9AP knockout, RGS9 and G β 5 interact with one another prior to the degradation of RGS9 and that this early interaction somehow makes G β 5 more resistant to subsequent degradation. To the contrary, no G β 5 is ever formed in photoreceptors of RGS9 knockout mice, perhaps because the RGS9-G β 5 complex is never formed.

Along with the results reported in our other recent study

(10), our data allow us to define the part of the RGS9 molecule that is primarily responsible for targeting unanchored RGS9 for degradation. Previously, we expressed RGS9 lacking the N-terminal DEP (disheveled/Egl-10/pleckstrin) domain in the rods of RGS9 knockout mice. This RGS9 mutant was not able to interact with R9AP (see also Refs. 7 and 8) but was not degraded. The results of the present study suggest that the DEP domain *per se* plays a decisive role in the cellular fate of the RGS9 molecule. The DEP domain binding to R9AP allows RGS9 survival and delivery to the site of its function in the rod outer segment. Conversely, we suggest that the presence of exposed DEP domain lacking its R9AP partner affects RGS9 expression, most likely by leading to degradation of RGS9.

Finally, the strong expression of R9AP in avian hair cells (9) led us to address whether R9AP knockouts display deficiencies of the inner ear function. Our initial assessment of auditory brainstem responses and distortion product otoacoustic emissions (20) did not reveal any significant differences between the knockout and wild type littermates (data not shown). This indicates that cochlear mechanics, transduction, and synaptic transmission at low and moderate sound levels are not significantly altered by the R9AP knockout. These results are consistent with our comparative analysis of R9AP expression, which is detectable in several neural cell types of the chicken but is restricted to photoreceptors in the mouse (9).

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