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Tokay Gecko Photoreceptors Achieve Rod-Like Physiology with Cone-Like Proteins[†]

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ABSTRACT

The retinal photoreceptors of the nocturnal Tokay gecko (*Gekko gekko*) consist exclusively of rods by the criteria of morphology and key features of their light responses. Unlike cones, they display robust photoresponses and have relatively slow recovery times. Nonetheless, the major and minor visual pigments identified in gecko rods are of the cone type by sequence and spectroscopic behavior. In the ongoing search for the molecular bases for the physiological differences between cones and rods, we have characterized the molecular biology and biochemistry of the gecko rod phototransduction cascade. We have cloned cDNAs encoding all or part of major protein components of the phototransduction cascade by RT-PCR with degenerate oligonucleotides designed to amplify cone- or rod-like sequences. For all proteins examined we obtained only cone-like and never rod-like sequences. The proteins identified include transducin α ($G\alpha_t$), phosphodiesterase (PDE6) catalytic and inhibitory subunits, cyclic nucleotide-gated channel (CNG α) and arrestin. We also cloned cDNA encoding gecko RGS9-1 (Regulator of G Protein Signaling 9, splice variant 1), which is expressed in both rods and cones of all species studied but is typically found at 10-fold higher concentrations in cones, and found that gecko rods contain slightly lower RGS9-1 levels than mammalian rods. Furthermore, we found that the levels of GTPase accelerating protein (GAP) activity and cyclic GMP (cGMP) phosphodiesterase activity were similar in gecko and mammalian rods. These results place substantial constraints on the critical changes needed to convert a cone into a rod in the course of evolution: The many features of phototransduction molecules conserved between those expressed in gecko rods and those expressed in cones cannot explain the physiological differences, whereas the higher levels of RGS9-1 and GAP activity in cones are likely among the essential requirements for the rapid photoresponses of cones.

INTRODUCTION

An outstanding question in the field of vertebrate phototransduction is the molecular origin of the physiological differences between rods and cones. Rods are exquisitely sensitive but relatively slow, whereas cones are two orders of magnitude less sensitive but have much faster responses (1–4). The cell types also differ significantly in their abilities to survive in the face of progressive neurodegenerative disorders of the retina (5–9).

Qualitatively, the responses of these cell types to light and the mechanisms of phototransduction are similar. Both use G protein-coupled receptors of the rhodopsin family with 11-*cis* retinal covalently attached as photopigments. Both respond to light by activation of a G protein of the transducin type, which in turn activates a cGMP-specific phosphodiesterase of the PDE6 type to lower cytoplasmic cGMP concentrations. Cyclic nucleotide-gated (CNG) channels close as a result, leading to hyperpolarization of the plasma membrane and decreases in intracellular $[Ca^{2+}]$ that initiate a series of inactivation reactions (10). Recovery of the dark current results from phosphorylation of the photoexcited visual pigment, which is then “capped” by rod or cone arrestin as well as Ca^{2+} -regulated cGMP synthesis (11–13), and G-protein inactivation by the action of the GTPase accelerating protein complex consisting of RGS9-1, G β 5, and membrane anchor R9AP (14–16).

In general the sequences of proteins expressed in either rods or cones are more similar across species than are the sequences of rod proteins as compared to cone proteins in the same species; for example, transducin alpha in teleost cones is closer in sequence to transducin alpha in mammalian cones than to teleost rod transducin. This pattern of conservation suggests that the conserved functional differences may derive from some or all of the conserved differences in protein structure. Another factor that may contribute to the different rod and cone physiology is the concentrations (or stoichiometric ratios) of phototransduction components, which are determined by the morphology of the photoreceptors and the expression levels of relevant proteins. One approach to determining which of these differences is important is to direct ectopic expression of cone proteins to rods and vice versa (17). *In the evolution of the Tokay gecko, nature appears to have carried out such an experiment.* Tokay gecko has a pure rod retina and its photoreceptors are rod-like in terms of morphology and electrophysiology (18–21). According to the spectrophotometry

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of the extracted pigments and microspectrophotometry on the photoreceptor cells, gecko has a major visual pigment, P521, and a minor pigment, P467 (22). P521 is a cone-type visual pigment similar to chicken red-sensitive cone visual pigment (iodopsin), belonging to M/LWS (middle/long wavelength sensitive visual pigment) subfamily (23). P467 has the highest sequence similarity to chicken green-sensitive cone visual pigment and is also highly related to a subfamily of rhodopsin, RH2, which are found in both rods and cones (4,23). By molecular cloning, another short-wavelength-sensitive cone-like pigment, P364, was found in gecko rods recently (24). The observation that gecko rods contain primarily cone visual pigments was considered as evidence supporting the transmutation theory (25). The phylogeny of geckos (family of Gekkonidae) suggests that the nocturnal rod-only gecko and modern diurnal cone-only geckos both evolved from a common ancestor whose retina had only cones (26,27). Therefore the essential evolutionary changes to convert cones to rods in gecko have occurred with less evolutionary drift than that associated with the much more ancient divergence of rods and cones in ancestral species.

Although most efforts have been devoted to the analyses of visual pigments in geckos, properties and compositions of the downstream phototransduction components in Tokay gecko's cone-descendent rod-like photoreceptors remain largely unexplored. Herein we report that the major phototransduction proteins of Tokay gecko rods are much more closely related to those associated with cones than to rod proteins. These include the G protein alpha subunit (transducin), the cGMP phosphodiesterase catalytic and inhibitory subunits, the GPCR-capping protein arrestin, and the cGMP-gated cation channels. Concentrations of key proteins, in gecko rods, as assayed by mass or activity, are similar to those in mammalian or amphibian rods, including $G\alpha$, $G\beta$, cGMP phosphodiesterase, RGS9-1 and $G\beta 5L$. Thus the rod-like physiology of gecko photoreceptors must rely on a combination of rod-like expression levels (for RGS9-1 and $G\beta 5L$), a small number of key differences in protein sequences between gecko rod and cone phototransduction proteins, or on proteins other than the major transduction components.

MATERIALS AND METHODS

Animals and tissues. All experimental procedures were carried out in compliance with National Institutes of Health (NIH) guidelines, as approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine, and conformed to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Retina tissues used in the experiments were from medium to large (14–28 cm) geckos (West Coast Reptile, Fullerton, CA).

Buffers. Standard buffers were: Buffer A (10 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, ~20 mg/L phenylmethylsulfonyl fluoride); Buffer B (67 mM sodium phosphate, pH 7.0, 2.7 mM KCl, 0.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride); Buffer C (10 mM Tris-HCl, pH 8.0, 2.0 mM $MgCl_2$, 2.0 mM cGMP); Buffer D (10 mM MOPS pH 7.0, 30 mM NaCl, 60 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, ~20 mg/L phenylmethylsulfonyl fluoride); Buffer E (5 mM Tris-HCl, pH 7.2, 0.5 mM $MgCl_2$, 1 mM DTT, ~20 mg/L phenylmethylsulfonyl fluoride); Buffer F (25 mM Tris, 192 mM glycine, pH 8.3). Other buffer components and conditions were varied as indicated throughout.

RNA isolation, RT-PCR and cDNA cloning. Total RNA was extracted from gecko whole retina with retina pigment epithelium using Trizol™ reagent (GibcoBRL) by following manufacturer's instructions. Gecko RGS9-1, PDE α , PDE γ , $G\alpha$, arrestin and CNG α cDNAs were cloned by

RT-PCR and rapid amplification of cDNA ends (RACE) strategies as described (28).

A cDNA fragment encoding amino acids 327–394 within the conserved RGS domain of RGS9-1 was amplified by RT-PCR with the use of degenerate primers, gRGS9a, 5'-GGNTT(C/T)TGGGA(A/G)GCNTG(C/T)GAGA-3', and gRGS9b, 5'-CAT(A/G)TA(A/G/T)AT(A/G)TGNGT(C/T)TGNGCNGC(A/G)TC-3'.

To clone gecko $G\alpha$, degenerate primers gGta, 5'-AA(A/G)CA(A/G)ATGAA(A/G)AT(A/T/C)AT(A/T/C)CA(C/T)C-3', and gGtb, 5'-TCNGTNGC(A/G)CANGTCAT(A/G)TG-3', were used to amplify an 833 bp cDNA fragment encoding amino acids 47–108 within conserved domain of transducin alpha subunits. To obtain the coding sequence of $G\alpha$, on the 5' end of this fragment, RT reaction was performed with the use of primer gGt5P1, 5'-ACTGCAGTATCTTGCCATAG-3'. After 5' RACE experiment, primers Adaptor, 5'-GACTCGAGTCGACATCG-3', and gGt5P2, 5'-GACAGATATGAACTCCATAC-3', were used to amplify a cDNA fragment (~500 bp) including untranslated region (UTR) and rest of coding sequence at 5' of gecko $G\alpha$. A 1.2 kb fragment including rest of gecko $G\alpha$ cDNA was cloned by 3' RACE using primers Adaptor and gGt3P, 5'-GACCTGAACCTGAGAAAAG-3'. 1.1 kb full-length gecko $G\alpha$ cDNA fragment including 10 bp UTR at each end was amplified by gGtF1, 5'-AAGCGAGATGGGGAGTG-3', and gGtF2, 5'-AGCTGAAGTCTTA-GAAGAGACC-3'.

A ~700 bp cDNA fragment encoding gecko arrestin was amplified by degenerate primers, gARRa, 5'-TT(C/T)(A/C)GNTA(C/T)GGN(A/C)GN-GA(A/G)G-3', and gARRb, 5'-(C/T)TC(C/T)TT(A/G)TCCATNCNGG-NC-3'. Gecko arrestin UTR and coding sequence at 3' (~500 bp) was cloned by 3' RACE strategy with the use of primers, Adaptor, gARR3P1, 5'-GCACTGGATGGCAAAC-3', and gARR3P2, 5'-CTCAAGCACGAT-GACC-3'.

To clone gecko CNG alpha subunit, degenerate primers A1, 5'-AT(A/T/C)AT(A/T/C)CA(C/T)TGAA(C/T)GCNTG-3', and B2, 5'-TC(A/G)TC-NACNGT(C/T)TT(A/G)TTNGTCCA-3', were used to produce a cDNA fragment about 420 bp, corresponding to conserved putative transmembrane domain S4 to S6 and pore (29). Another pair of degenerate primers, GMP1, 5'-CCIGGIGA(T/C)TA(T/C)AT(T/C)A(T/C)(A/C)(A/G)IAA-3', and GMP2, 5'-(A/G)CA(A/G)AAIA(A/G)(A/G)TCI(G/C)(A/T)(A/G)-TAICC-3' (I = inosine), were used to amplify a cDNA fragment of 222 bp, corresponding to CNBS (cGMP binding region). The sequence between the two cloned regions was PCR amplified by using primers A1 and CNra3, 5'-GCTCCAGATGTTGGCAGTTC-3'.

A 189 bp cDNA fragment encoding the last 62 amino acids of gecko PDE γ was produced with the use of primers PDEgUni, 5'-GGNC-CNCCNAA(A/G)TT(T/C)AA(A/G)C-3'; and cnPDEg3: 5'-TTA(A/T/G)AT(A/T/G)ATNCC(A/G)AA(T/C)TGNGC-3'. The rest of coding sequence and 5' UTR, about 23 bp, was cloned by 5' RACE strategy using cnPDEg3 for RT reaction, and primers Adaptor and gPDE5RA, 5'-GAACTGTCTTGTGTCCTTG-3', for PCR.

A gecko PDE α cDNA fragment of 988 bp, encoding GAF and PDEase domains, was cloned by RT-PCR using primers PDEAa, 5'-AT(T/C/A)GTNAA(T/C)AA(A/G)AA(A/G)GA(A/G)G-3', and PDEAb, 5'-CNGT-CATCATATNGCC-3'.

Sequence data analyses. The cloned gecko $G\alpha$, PDE γ , arrestin, CNG α , RGS9-1 and PDE α (GenBank accession numbers are AY328525, AY328526, AY328527, AY328528, AY328529, and AY328530, respectively) were compared to the corresponding full-length or partial sequences, which were taken from GenBank or EMBL database with the following accession numbers: bovine (*Bos taurus*) RGS9 (O46469), RGS7 (O46470), cone CNG α (Q29441), rod CNG α (Q00194), olfactory CNG (Q03041), S-antigen (ARRS, P08168), X-arrestin (BAA94344), cone PDE α ' (P16586), rod PDE α (P11541), rod PDE β (P23439), cone $G\alpha$, (P04696), rod $G\alpha$, (P04695), cone PDE γ (AAA30689.1) and rod PDE γ (CAA28507.1); mouse (*Mus musculus*) RGS9 (AAC99481), RGS7 (O54829), RGS11 (Q9Z2H1), RGS6 (Q9Z2H2), cone CNG α (Q9JZ8), rod CNG α (P29974), olfactory CNG (Q62398), S-antigen (P20443), arrestin-C (ARRC, Q9EQP6), cone PDE α ' (NP_291092), rod PDE α (AAH31925), rod PDE β (P23440), cone $G\alpha$, (P50149), rod $G\alpha$, (P20612), cone PDE γ (BAB32255.1) and rod PDE γ (CAA68714.1); human (*Homo sapiens*) RGS9 (AAG09311), RGS7 (P94802), RGS11 (P94810), RGS6 (P49758), cone CNG α (Q16281), rod CNG α (P29973), olfactory CNG (Q16280), S-antigen (NP_000532), arrestin-C (P36575), cone PDE α ' (CAA64079), rod PDE α (P16499), rod PDE β (P35913), cone $G\alpha$, (NP_005263), rod $G\alpha$, (P11488), cone PDE γ (BAA08241.1) and rod PDE γ (AAA03653.1); 13-lined ground squirrel (*Spermophilus tridecemlineatus*)

cone PDE γ (CAA04720.1); northern leopard frog (*Rana pipiens*) S-antigen (P51479), arrestin-C (P51482), cone PDE α' (AAK95401), rod PDE α (AAK95399.1), rod PDE β (AAK95400.1), cone PDE γ (AAK95403.1) and rod PDE γ (AAK95404.1); guinea pig (*Cavia porcellus*) rod PDE γ (AAG43274.1); dog (*Canis familiaris*) rod CNG α (Q28279), S-antigen (Q28281), rod PDE α (Q28263), rod PDE β (P33726), rod G α_t (Q28300) and rod PDE γ (CAA93815.1); rat (*Rattus norvegicus*) RGS9 (P49805), RGS7 (P94803), RGS6 (P49801), RGS11 (P49807), rod CNG α (Q62927), olfactory CNG (Q00195), S-antigen (P15887) and PDE γ (AAG434000); chicken (*Gallus gallus*) RGS7 (AAF00028.1), RGS6 (AAF00030.1), RGS11 (AAF00027), cone CNG α (Q90805), rod CNG α (Q90980), cone PDE α' (P52731), cone G α_t (AAG28573) and rod G α_t (AAG28572); tiger salamander (*Ambystoma tigrinum*) cone arrestin (AAF14637.1), rod arrestin (AAF14636), cone G α_t (AAC67569) and rod G α_t (AAC67568.1); zebrafish (*Danio rerio*) cone (AAL05601) and rod (AAL05600) G α_t ; *Xenopus laevis* RGS9 (BAA95151.1), S-antigen (P51477), arrestin-C (P51483), cone G α_t (P27044) and rod G α_t (P38407); bullfrog (*Rana catesbeiana*) S-antigen (P51478) and arrestin-C (P51481); pig (*Sus scrofa*) S-antigen (P79260); medaka fish (*Oryzias latipes*) rod (BAA82259) and cone (B21719.1) arrestin; rabbit (*Oryctolagus cuniculus*) olfactory CNG (Q28718); catfish (*Ictalurus punctatus*) olfactory CNG (P55934); gold fish (*Carassius auratus*) CNG1 (AAO16601); trout (*Oncorhynchus mykiss*) CNG1 (AAL59140). The sequences were aligned and phylogenetic trees were constructed by CLUSTALW (30) based on neighbor-joining (NJ) method (31).

Preparations of bovine and gecko rod outer segment (ROS) membranes. Bovine ROS membranes were prepared from frozen bovine retinas by sucrose gradient centrifugation (32). Gecko ROS membranes were prepared as described previously (33). Specifically, Tokay geckos were dark-adapted for at least 12 h at 4°C. The cooled animals were then decapitated and double-pithed under dim red light. The retinas were removed from the hemisected eyeballs and placed into Buffer B on ice immediately. After homogenization of the retina with an 18 gauge needle, the ROS membranes were subjected to sucrose floatation twice: first in Buffer B supplement with 45% sucrose, then in Buffer B with 37% sucrose. After bovine or gecko ROS membranes were collected from the sucrose gradient, they were homogenized again, diluted with Buffer A at a 1:5 ratio, spun down by centrifugation at 24,000 g, and then resuspended in Buffer A. The purified gecko and bovine ROS membranes were stored in -80°C until use.

GTPase single-turnover assays. GTPase single-turnover assays were performed to test bovine or gecko RGS9-1/G β 5 GAP activity, essentially as described before (34). Specifically, 14 μ L of bovine or gecko rod outer segments in Buffer A containing a varied amount of visual pigments (2, 5, 10, 20 μ M) were exposed to light. Then GTP hydrolysis was initiated by adding 7 μ L of [γ -³²P] GTP (Amersham Pharmacia Biotech) to the ROS by vortexing. The final concentration of GTP is 25 nM in each reaction. The reaction was quenched by 100 μ L of 5% trichloroacetic acid at various times, and P_i released from hydrolyzed GTP was determined by activated charcoal assay. The first order rate constants for GTP hydrolysis (k_{inact}) were obtained by fitting data to single exponential curves.

Immunoprecipitation of RGS9-1/G β 5 GAP complex. Purified anti-RGS9 antibody anti-RGS9-1c (14) was covalently attached to CNBr-activated Sepharose 4B-CL as described previously (34). One milliliter of gecko ROS with 16 μ M visual pigments or 530 μ L bovine ROS with 30 μ M rhodopsin was diluted by Buffer A to 3 mL, and ROS membranes were collected by centrifugation at 100,000 g. After resuspension in 1 mL Buffer A supplemented with 1% Nonidet P-40, ROS membranes were incubated with 20 μ L of anti-RGS9-1c IgG coupled beads for 4 h at 4°C upon mixing on a shaker. The beads were separated from the supernatant by a brief centrifugation and washed three times with Buffer A plus 1% Nonidet P-40. Bound proteins were redissolved in 50 μ L SDS-PAGE sample buffer and separated from the beads by a brief centrifugation. Ten microliters of bovine and gecko sample were subjected to SDS-PAGE, together with bovine serum albumin (BSA, Sigma) standards. After Coomassie staining, the gel was dried and scanned, and the density of protein bands was quantified by software UN-SCAN-IT (Silk Scientific Corporation, UT). The amounts of immunoprecipitated gecko and bovine proteins were quantified with the use of the standard curve generated with BSA standards.

PDE assays. PDE catalytic activity was measured with the pH recording method (35) as modified previously (36). Specifically, assays were carried out in 96-well-microtiter plates, and pH changes were monitored with MI-410 microelectrodes (Microelectrodes, Inc.). To test gecko cone PDE and bovine rod PDE activity, the hydrolysis of 2 mM cGMP was recorded as decrease of pH in a final volume of 200 μ L. Full PDE cGMP hydrolysis activity was acquired by removal of inhibitory subunits, PDE γ , using

trypsin (Sigma) in a final concentration of 0.2 mg/mL. Assays were performed in Buffer C containing different amounts of bovine ROS (with 0.5, 1.0, 1.5, 2.0 μ M rhodopsin) or gecko ROS (with 0.2, 0.4, 0.7, 0.9 μ M visual pigment).

Purification of transducin. To quantify the expression levels of transducin in gecko rods and compare that in bovine rods, transducin was isolated from gecko and bovine ROS with modification as described before (37). Specifically, 1 mL gecko or bovine ROS membranes, each with 16 μ M visual pigment, in Buffer D, were homogenized with the use of an 18 gauge syringe at 4°C and collected by centrifugation at 100,000 g for 20 min. The ROS were resuspended in Buffer D, and the washing steps were repeated. Then the washed ROS membranes were resuspended in Buffer E. After homogenization, the ROS membranes were spun down by centrifugation at 100,000 g for 30 min. The ROS were washed in Buffer E another two times. After collection of the ROS by centrifugation at 100,000 g for 20 min, pellets were resuspended in 1 mL buffer E containing 100 μ M GTP, and homogenized thoroughly. The GTP wash supernatants that contain transducin were collected by centrifugation at 100,000 g for 30 min. The washing steps were repeated with the use of Buffer E with GTP and combine the GTP wash supernatants to 2 mL. The purified transducin samples were concentrated in Centricon (Millipore) to 30 μ L. Twelve microliters of the concentrated gecko and bovine transducin samples, together with same fractions of Buffer E wash supernatants, were subjected to SDS-PAGE and stained by Coomassie brilliant blue.

Immunoblotting. After spectrophotometry to quantify visual pigments, gecko and bovine ROS membranes were analyzed by SDS-PAGE, followed by immunoblotting according to a standard protocol (38). Buffer F was used for electrophoretic transfer of PDE γ , and buffer F supplemented with 0.1% SDS for transfer of RGS9-1 and G β 5. The membranes for immunoblotting were supported nitrocellulose (NitroPure, Osmonics, Inc.). Transfer was at 350 mA at 4°C and lasted 60 min for RGS9-1 and G β 5 or 45 min for PDE γ , membranes were blocked by 5% nonfat dry milk-TBS/T solution (20 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.1% [vol/vol] Tween 20) for 1 h, followed by incubation with primary antibody for 4 h. Polyclonal antibodies anti-RGS9-1c and anti-G β 5 (34) were used at a 1:1000 dilution, and anti-PDE γ was used at a dilution of 1:500. The secondary antibody used was horseradish peroxidase-conjugated (Promega) anti-rabbit IgG, with detection by chemiluminescence, with the use of the ECL system (Amersham Pharmacia Biotech).

Molecular graphics of gecko transducin. The molecular model of gecko transducin was visualized with Protein Explorer (<http://www.umass.edu/microbio/chime/pe/protexpl/frntdoor.htm>) based on the crystal structure of Gt/i chimera (PDB file: 1GOT.pdb). Sequences of transducin alpha subunit from gecko, cone and rod transducins from salamander, human, chicken and *Xenopus laevis* were aligned to identify cone- and/or rod-specific amino acids. Residues conserved within cones are highlighted in cyan color; conserved residues but different between cone and rod sequences are colored in blue, and residues in gecko that are the same as in rod G α_t are highlighted in red color.

RESULTS

Cloning and sequence analyses of the cDNA encoding gecko G α_t , arrestin, PDE α , PDE γ , RGS9-1 and CNG α

We cloned full-length cDNA sequences of gecko G α_t and PDE γ , and partial cDNA sequences of PDE α , arrestin, CNG α and RGS9-1 by RT-PCR and RACE from gecko retina RNA (Table 1). Although both rod and cone-specific degenerate primers and primers encoding conserved regions of rod and cone homologues were used for RT-PCR, only a single PCR product was obtained for each phototransduction protein.

In the cloned gecko phototransduction protein cDNA sequences (Table 1), G α_t consists of 1074 nucleotides, including the full-length coding region corresponding to 350 amino acids, 11 nucleotides of the 5'-untranslated region (UTR) and 10 nucleotides of 3'-UTR sequence; PDE γ consists of 423 nucleotides including the full-length coding region of 92 amino acids, and 144 nucleotides of 5'-UTR region; arrestin consists of 1174 nucleotides including the partial coding region of 336 amino acids, and 163

Table 1. Cloned gecko phototransduction proteins

	Full-length (f) or partial (p) sequence	cDNA (bp)	Amino acids	Identity to cone-type proteins*	Identity to rod-type proteins*
G α_t	f	1074	350	88–89%	76–77%
PDE γ	f	423	92	97% (chick)	90% (chick)
arrestin	p	1174	336	70% (salamander)	50% (salamander)
PDE α	p	988	329	89% (chick)	60%
CNG α	p	806	266	87% (chick)	80% (chick)
RGS9-1†	p	204	68	90%	90%

*The species in parentheses indicate the source of proteins gecko proteins were compared to. Gecko proteins were compared to bovine, human, and mouse phototransduction proteins if without indication. †The same isoform of RGS9 is expressed in rods and cones.

nucleotides of 3'-UTR sequence; PDE α subunit cDNA fragment consists of 988 nucleotides encoding 329 amino acids, which range from the end of GAF domain to PDEase domain (39,40); CNG α subunit consists of 806 nucleotides encoding 266 amino acids that cover part of the conserved cyclic nucleotide monophosphate binding domain and gecko RGS9-1 RGS domain consists of 195 nucleotides encoding 65 amino acids.

By comparing to their homologues and orthologs, phylogenetic trees showed the cloned gecko phototransduction proteins were cone-type (Fig. 1; Table 1). The deduced amino-acid sequence of gecko G α_t shows about 90% identity to human, mouse, chicken, bovine and salamander cone G α_t , and 77% identity to their rod counterparts. The gecko PDE γ is longer than a typical rod or cone-type PDE γ , which consists of 84–87 amino acids. The gecko PDE γ shows 97% identity to chicken cone PDE γ and 90% to chicken rod homologue. Although only partial sequences of gecko arrestin, PDE α' and CNG α were cloned, the cloned parts were at least 60% of the full-length sequences, which gave reliable phylogenetic tree results. By comparing to the corresponding sequences, gecko arrestin shows 70% identity to tiger salamander cone arrestin and 50% to its rod counterpart; gecko PDE α is 89% identical to chicken cone PDE α' , and about 60% to mammalian rod PDE α homologues; and the deduced amino-acid sequence of gecko CNG α shows 87% identity to chicken cone CNG α and 80% identity to its rod homologue. In addition, the cloned gecko RGS9-1 RGS domain is 90% identical to known mammalian and *Xenopus* orthologs.

Composition and quantification of RGS9-1/G β 5 GAP complex in gecko ROS

RGS9-1/G β 5 GAP complex regulates the deactivation of G protein transducin in rod and cone photoreponses. There are two splicing variants of G β 5, long-isoform G β 5L and short isoform G β 5S. Both G β 5 splicing variants were shown to be able to bind RGS9-1 forming a functional GAP complex *in vitro*, and little difference was found between the values of the physiologically relevant parameter for catalytic efficiency, k_{cat}/K_m , for full-length RGS9-1 complexed with G β 5L as compared to the complex with the short isoform G β 5S (34,41). G β 5L was suggested to be the only partner binding RGS9-1 to form GAP complex in rods (15,42). Immunoprecipitation of RGS9-1 GAP complex from cone-dominant chipmunk retina homogenates found that both G β 5L and G β 5S were coprecipitated with RGS9-1, indicating G β 5S also bound

RGS9-1 to form GAP complex in cones (43). To find out the composition of the GAP complex in gecko rods, the complex was immunoprecipitated from detergent-solubilized gecko ROS with the use of RGS9-1 antibody (Fig. 2A). The immunoblots of the precipitates showed that as in bovine ROS, only G β 5L was coprecipitated with RGS9-1 (Fig. 2B), indicating the composition of GAP complex in gecko rods was same as that in mammalian rods.

The expression levels of RGS9-1 in gecko and bovine ROS were also quantified and compared. RGS9-1 was immunoprecipitated from bovine or gecko ROS containing the same amounts of visual pigments. The amounts of visual pigments were determined by differential absorption spectra and calculated from the extinction coefficients of bovine rhodopsin and gecko P521 pigment. After resolving the immunoprecipitates on SDS-PAGE and quantifying the amounts of RGS9-1 by using a standard curve generated with Coomassie-stained BSA (Fig. 2A), the molar ratios of RGS9-1 to visual pigments in bovine and gecko ROS were quantified as 0.00109 ± 0.00010 and 0.00083 ± 0.00018 (Fig. 2C), respectively, indicating similar expression levels of RGS9-1/G β 5 GAP complex in gecko and bovine rods. The molar ratio in bovine ROS was consistent with the previous results obtained by quantitative Western blots (43).

Comparison of expression levels of PDE γ and G α_t in bovine and gecko ROS

By immunoblots, we compared expression levels of bovine rod and gecko cone PDE γ . Similar intensities of immunostaining for PDE γ were observed from bovine and gecko ROS containing the same amounts of visual pigments (Fig. 2D). In addition, by purification of transducin from bovine and gecko ROS containing the same amounts of visual pigments, the expression levels of G α_t were compared by Coomassie staining (Fig. 2E). The result showed similar levels of transducin in bovine and gecko ROS, as well.

GTPase single-turnover assays in gecko ROS

To test gecko transducin recovery kinetics, we carried out GTPase single-turnover assays. Similar to the results in bovine ROS, the hydrolysis of GTP in gecko ROS was concentration dependent, consistent with endogenous RGS9-1 accelerating the hydrolysis of GTP by G α_t (Fig. 3). In addition, similar GTP hydrolysis rates were observed in gecko and bovine ROS containing equal amounts of visual pigments. Given that there are similar levels of transducin and RGS9-1 in bovine and gecko ROS, these results indicate that gecko RGS9-1 has similar GAP activity to the bovine ortholog.

Hydrolysis of cGMP by PDE in gecko ROS

To measure the efficiencies of hydrolysis of cGMP by cone PDE in gecko rods and compare the results with those in mammalian rods, we performed pH-based PDE assays in gecko and bovine ROS. Figure 4 shows pH recordings in bovine or gecko samples with 0.5 μ M visual pigments. Both gecko cone and bovine rod PDE displayed low basal activity and were similarly activated by addition of trypsin to remove the inhibitory subunit, PDE γ . Both basal and maximal PDE activities in gecko ROS were similar to those in bovine samples. The results showed bovine rod PDE in ROS with 1.0 μ M rhodopsin hydrolyzed 21.0 ± 2.3 μ M cGMP per

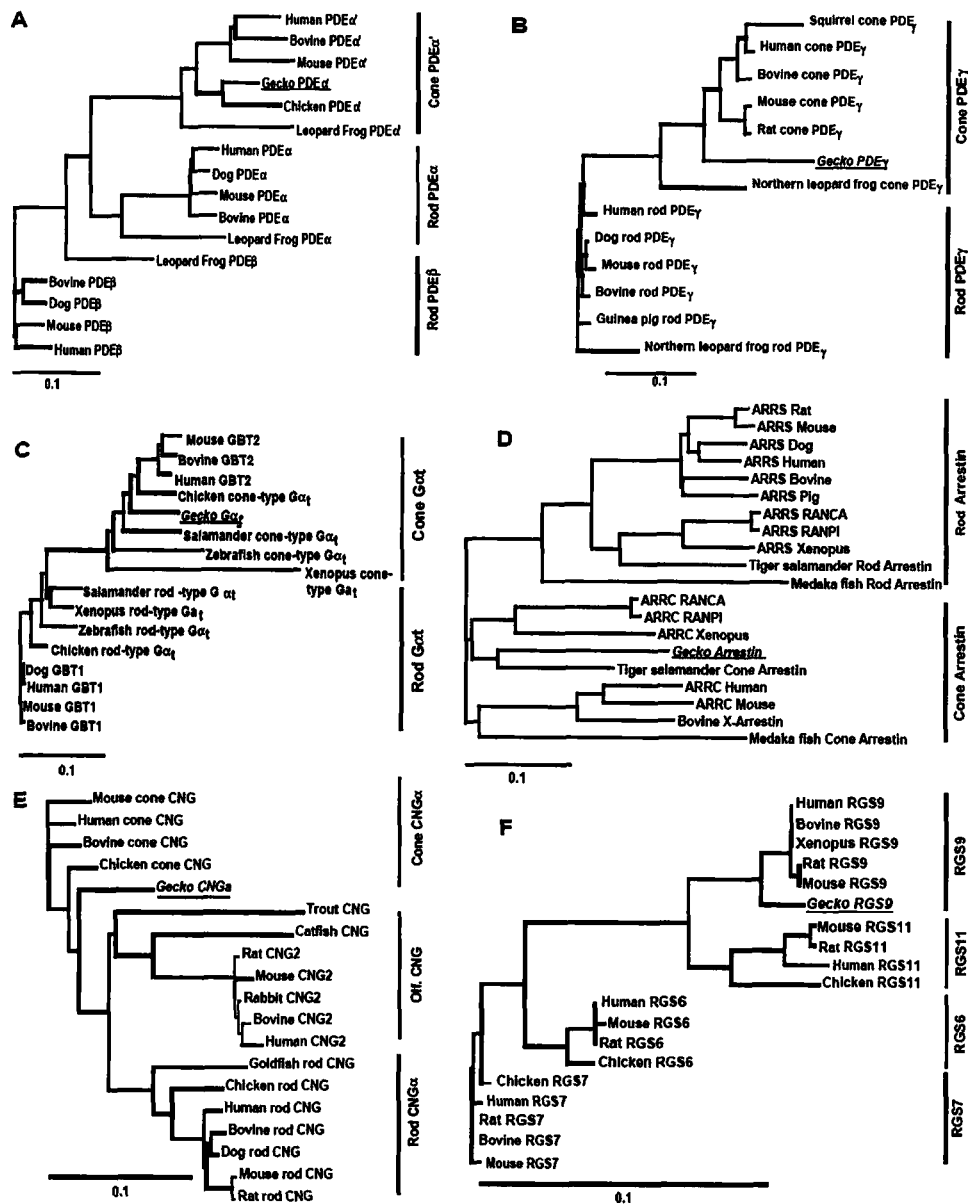


Figure 1. Phylogenetic trees calculated from the amino acid sequences of A: PDE α , B: PDE γ , C: G α_t , D: arrestin, E: CNG α and F: RGS9 with the neighbor-joining method. The horizontal distances are proportional to the percent differences in amino-acid sequences, and the scale bar indicates 10% replacement of an amino acid per site.

second, and gecko PDE hydrolyzed $16.2 \pm 3.6 \mu\text{M cGMP per } \mu\text{M}$ visual pigment per second. Based on the ratio of PDE γ to rhodopsin in bovine ROS (0.0096 ± 0.0016) (43), and assuming similar expression levels of PDE γ in bovine and gecko ROS (Fig. 2B), the calculated maximum turnover numbers of gecko and bovine PDE were 3365 and 4375 cGMP hydrolyzed per PDE per second, respectively.

The gecko cone maximum PDE activity, 16.2 ± 3.6 cGMP hydrolyzed per pigment per second or 3365 cGMP hydrolyzed per PDE per second, is very close to that of carp cone PDE, 17.8 ± 1.5 cGMP hydrolyzed per pigment present per second (44). It is also similar to previously reported bovine rod PDE k_{cat} value of about 4000 cGMP hydrolyzed per PDE per second (45,46) and frog rod PDE k_{cat} value of 4400 cGMP hydrolyzed per PDE per second (47). These results are consistent with previous observa-

tions that bovine rod, bovine cone and chipmunk cone PDE have similar maximum activities (43,48).

Cone-/rod-specific residues

Our data from biochemical assays and immunoblots suggest that the phototransduction processes in gecko photoreceptor closely resemble those in rods, including transducin GTPase kinetics. However, it remains possible that a small subset of amino-acid residues in gecko G α_t dictates the rod-like phenotypes. To identify such candidates, we conducted a sequence alignment with the use of G α_t sequences from gecko, human, chicken, salamander and frog presented in the molecular graphic format based on the published transducin Gt/i crystal structure (Fig. 5). Residues that are conserved within cone transducin alpha subfamily are colored

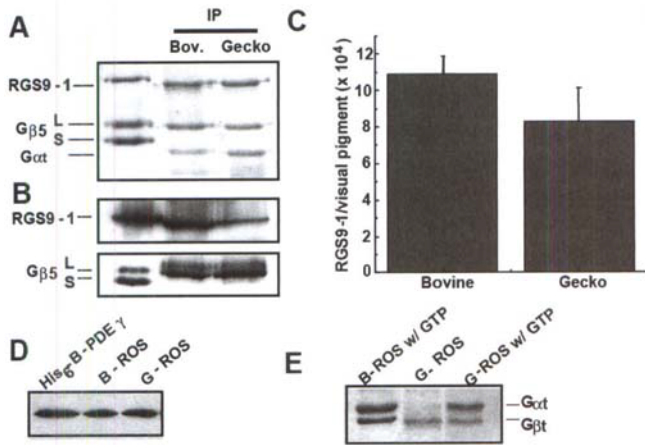


Figure 2. Comparisons of expression levels of gecko A–C: RGS9-1, D: PDE γ and E: transducin to that of bovine counterparts. A: Immunoprecipitates from detergent extracts of bovine (“Bov.”) and gecko (“Gecko”) ROS by RGS9 antibody were analyzed by SDS-PAGE and stained by Coomassie brilliant blue. B: Immunoblots of RGS9-1 (upper) and G β 5 (lower). The far left lanes of A and B are His-tagged RGS9-1/G β 5L, G β 5S purified from insect cells. C: the ratio of RGS9-1 to visual pigment, D: immunoblots of PDE γ in gecko (G-ROS) and bovine (B-ROS) ROS containing same amounts of visual pigments. E: transducin was extracted from gecko and bovine ROS containing same amounts of visual pigments with the use of low salt plus GTP for bovine (B-ROS w/GTP), low salt (G-ROS), followed by low salt plus GTP (G-ROS w/GTP) for gecko. Identical proportions of bovine and gecko samples were analyzed by SDS-PAGE gel and stained by Coomassie brilliant blue.

in cyan. Residues that are conserved but different from cones in rods sequences are colored in blue. The distribution of these two kinds of residues is dispersed throughout the structure of G α_t but was mostly excluded from the switch domains or active site. On the other hand, two residues (Ile66 and Thr166, highlighted in red color) in gecko transducin are identified to be the same as in rods but not in cones and reside within close vicinity (Fig. 5A). Beside the two rod-specific residues, the N-terminus in gecko G α_t also shares sequence similarity to other rod transducins with

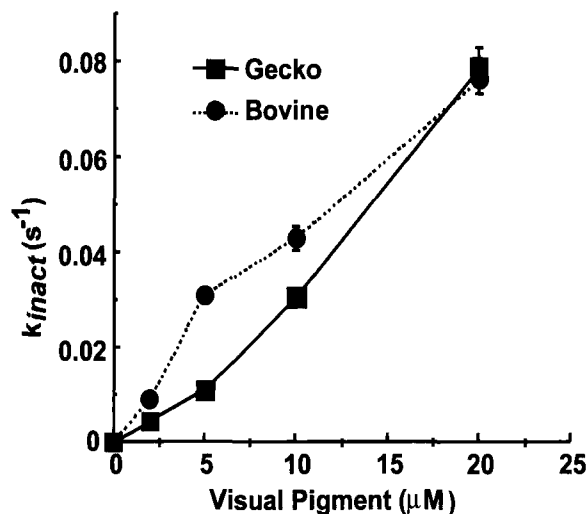


Figure 3. G α_t GTPase single-turnover assays in gecko and bovine ROS. The first-order rate constants for GTP hydrolysis in bovine rod outer segment membranes measured under single-turnover conditions are plotted as a function of concentrations of visual pigments from gecko (squares) or bovine (circles) ROS.

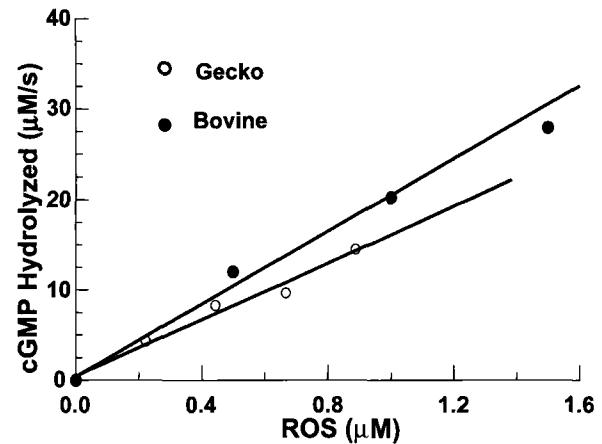


Figure 4. cGMP hydrolysis assays to measure and compare the maximum PDE activities of gecko cone PDE and bovine rod PDE. pH recordings from gecko (open circles) and bovine (closed circles) ROS containing different amounts of visual pigments as indicated were carried out to measure the maximum PDE activities following trypsin activation. The curves are linear least-squares fits. The slopes reflect the maximum activities of gecko cone PDE and bovine rod PDE as $16.2 \pm 3.6 \text{ s}^{-1}$ and $21.0 \pm 2.3 \text{ s}^{-1}$, respectively.

four residues deleted (Fig. 5 and Table 2), compared to cone transducins.

DISCUSSION

The large size and shape of nocturnal gecko (*Gekko*, *Hemidactylus*, *Tarentola*) rods are typical of rod characteristics (18,19), in contrast to the recent report finding occasional cone-like open disc features in geckos from other genera (49). Whether the rods in gecko retina are genuine rods or “modified” cones (49), necessary changes have to be engineered during evolution to fulfill the requirements for rod-like physiology. We have found that many of the key players in phototransduction in gecko rods closely resemble their counterparts in cones, and do not share most of the sequence differences common to the rod isoforms in other species. Thus the high sensitivity and slow kinetics of gecko rods cannot be attributable to those major differences in the primary structures of rod and cone phototransduction components, but rather, must be due to one or more of the following: (1) the few conserved differences between rod and cone proteins that are found in gecko proteins; (2) significant functional differences in other proteins not studied here; and (3) differences in concentrations or posttranslational modifications of certain proteins between rods and cones.

An interesting candidate sequence for Option 1 is the tetrapeptide EM/LKA near the N terminus of cone transducin alpha subunits, which is missing in all rod transducins, including gecko. The function of this motif is not known, but the amino terminal region of transducin alpha is known to be important for interactions with the disk membrane and with photoexcited rhodopsin (50).

Candidates for Option 2 include rhodopsin kinase and unknown proteins controlling outer segment morphology. The phosphorylation of R* and subsequent binding of arrestin are important steps in inactivation of the photoresponse. It has been reported that R* phosphorylation occurs much more rapidly in carp cones than in rods (44,51), a difference that could lead to both lower sensitivity and faster kinetics. Despite our repeated attempts, unfortunately

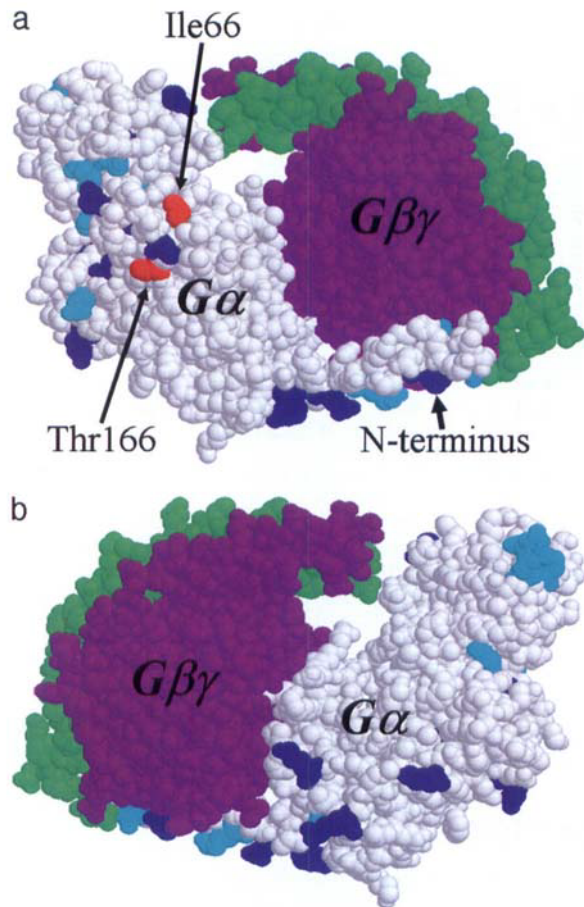


Figure 5. Molecular graphics of transducin structure. The molecular model of transducin $G\alpha$, was visualized with Protein Explorer based on the crystal structure of Gt/i chimera (1GOT.pdb). Residues conserved within cones are highlighted in cyan color; conserved residues different between cone and rod sequences are colored in blue, and residues in gecko that are the same as in rod $G\alpha$, are highlighted in red.

we were unable to obtain even partial sequence of gecko visual pigment kinase by degenerate RT-PCR (data not shown). Therefore we can not rule out that gecko visual pigment kinase may contribute to the rod-like photoreponse. It is not known how the morphological differences between rods and cones are determined at the molecular level but the morphology of gecko photoreceptors is clearly of the rod type, and proteins yet to be identified that control cell structure may differ in sequence or in expression levels between rods and cones.

The importance of enzyme concentrations in the phototransduction cascade has been demonstrated by a computational model in which the response characteristics can be modulated by changing the abundance of different enzymes (52). An example of the important concentration difference (Option 3) is the difference in concentration of the GAP complex composed of RGS9-1, G β 5 and R9AP. A previous study (43) revealed a 10-fold higher concentration of this complex in cones than in rods, but we have found that gecko rods have levels and activities of these proteins similar to those in mammalian rods. Furthermore, overexpression of RGS9 complex in transgenic mice was able to speed up the light response of rods (53). This complex probably makes an important contribution to faster recovery in cones, but seems unlikely to account for all or even most of the sensitivity and kinetic differences.

Table 2.

Gecko	1
Cone Human	1
Cone Xenopus	1
Cone Chicken	1
Cone Salamander	1
Rod Human	1
Rod Xenopus	1
Rod Chicken	1
Rod Salamander	1

Concentrations and activities of the proteins mediating activation, $G\alpha_t$ and PDE in gecko rods are similar to those in rods of other species, but previous studies have suggested that these levels do not differ greatly between rods and cones. Direct measurements of G protein activation by chicken green-sensitive and human cone pigments (54,55), and *Xenopus* short-wavelength visual pigments (56,57) indicate that the efficiency of rod transducin activation by photoexcited cone visual pigment is only about two-fold lower than activation by rod Metarhodopsin II. In addition, the salamander SWS2 opsin was found in both green rods and blue-sensitive cones to couple with rod and cone transducins in two different photoreponses, respectively, with similar sensitivity and response kinetics (58). Furthermore, human rhodopsin expressed in *Xenopus* cones or human red cone pigment expressed in *Xenopus* rods produced similar photoreponses with identical amplification and kinetics compared to those produced by the endogenous pigments in the same photoreceptor cell (17). It has been suggested that the spontaneous thermal isomerization of 11-cis retinal in red cone pigment can be part of the cause for the dark noise and consequently contribute to the lower sensitivity and faster kinetics of cones (17). Investigation of the dark noise and spontaneous isomerization rate in gecko rods that have a “cone pigment functioning in the rod environment” situation will further delineate their roles in photoreceptor functions.

Because of the insufficient amounts of purified arrestin and visual pigment kinase from gecko ROS and bias of antibodies to proteins across species, we were unable to determine reliable concentrations and stoichiometry in gecko rods. Whether gecko arrestin and visual pigment kinase make important contributions to the rod-like photoreponse awaits further investigation.

In summary, our results not only support the transmutation theory by Walls (25), which hypothesizes that the gecko rods have evolved from cones, but also suggest morphological and physiological modifications of gecko photoreceptor cells have occurred much faster than the evolution of phototransduction proteins. Further studies of the rods of nocturnal geckos will likely shed additional light on what it takes at a molecular level to convert a cone into a rod.

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