A novel form of rhodopsin kinase from chicken retina and pineal gland[1]

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Abstract The G protein-coupled receptor kinases (GRKs) are important enzymes in the desensitization of activated G protein-coupled receptors (GPCR). Seven members of the GRK family have been identified to date. Among these enzymes, GRK1 is involved in phototransduction and is the most specialized kinase of the family. GRK1 phosphorylates photoactivated rhodopsin (Rho*), initiating steps in its deactivation. In this study, we found that chicken retina and pineal gland express a novel form of GRK that has sequence features characteristic of GRK1. However, unlike bovine GRK1 which is farnesylated, chicken GRK1 contains a consensus sequence for geranylgeranylation. Peptides corresponding to the C-terminal sequence of chicken GRK1 are geranylgeranylated by a cytosolic extract of chicken liver. Based on results of molecular cloning and immunolocalization, it appears that both rod and cone photoreceptors express this novel GRK1. These data indicate a larger sequence diversity of photoreceptor GRKs than anticipated previously.

Key words: Rhodopsin kinase; Rhodopsin; Phototransduction; Isoprenylation; Photoreceptor

1. Introduction

G protein-coupled receptor kinases (GRKs) comprise an important group of enzymes implicated in the desensitization of agonist-activated seven-transmembrane-helical receptors [1,2]. GRKs display broad and overlapping substrate specificity, restricted, however, to the agonist-activated forms of receptors. In vision, the duration of G protein activation is regulated by the inactivation of Rho*, a multiple-step process initiated by GRK1 [3]. After intense illumination, phosphorylation of Rho* by GRK1 is a critical step in the fast recovery of sensitivity during dark adaptation. Mutations in the GRK1 gene cause Oguchi disease, a stationary night blindness in humans [4], due to delayed inactivation of Rho* [5].

It is unclear whether a single GRK is expressed in both rod and cone photoreceptors. Recently, two different GRKs were cloned from medaka, or Japanese killifish, and called GRK1 and GRK7, respectively [6]. The deduced amino acid sequence for the two proteins revealed a C-terminal consensus sequence for geranylgeranylation (CALL) in the cone GRK7 (OlGRK-C) and a consensus sequence for farnesylation (CCAS) in the rod GRK1 (OlGRK-R). A search for a mammalian cone-specific homolog from rod-dominant retina has yet to reveal a novel kinase [7], but GRK7 was cloned from the cone-dominant ground squirrel retina [8]. The complete sequences of both GRK1 and GRK7 from the same mammalian species have not yet been reported. It is also possible that only one type of GRK is responsible for desensitization of rod and cone pigments in mammals. Indeed, targeted disruption of the GRK1 gene in mice leads to abnormally prolonged rod and cone responses [9,10], suggesting that mouse GRK1 is involved in deactivation of visual pigments in both rod and cone photoreceptors, even if another GRK is expressed in cones.

All GRKs have different methods of membrane attachment. GRK2 and GRK3, the β-adrenergic receptor kinases (βARK1 and βArk2), contain a pleckstrin homology domain for association with G protein βγ subunits. GRKs 4–6 contain either palmitoylation and/or highly charged regions involved in the membrane interaction [1]. GRK1 and GRK7 contain a C-terminal signal for farnesylation and geranylgeranylation, respectively [6,8,11]. It was speculated that farnesylation of GRK1 is specifically required for light-dependent translocation of the kinase from the cytosol to Rho*-containing membranes [11]. Furthermore, cloning of an invertebrate rhodopsin kinase (octopus RK) revealed a high homology to βARK, including a C-terminal pleckstrin homology domain rather than a signal for isoprenylation [12].

The analysis of other well-characterized vertebrate species could useful in understanding the diversity of retinal GRK. In this report, using cone-dominant chicken retina and chicken pineal gland, we identified a novel form of GRK that exhibits mixed sequence similarity to GRK1 and GRK7.

2. Materials and methods

2.1. Cloning of chicken GRK1

A *Nco*I/*Sac*I restriction fragment from bovine GRK1 (535 bp) containing the catalytic region [13] was isolated, labeled with [α-^{32}P]dCTP using random primer labeling of DNA (Megaprime DNA labeling system, Amersham), and used as a probe to screen 1×10^6 plaques from a chicken retinal cDNA library (obtained from Dr. S. Semple-Rowland, University of Florida). The filters were prehybridized and hybridized overnight at 40°C in buffer containing 50% formamide, 0.75 M NaCl, 0.075 M sodium citrate, 5×Denhardt's solution (1 g/l each of Ficoll-400, polyvinylpyrrolidone, and bovine serum albumin),

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[1] The chicken GRK1 nucleotide sequence reported in this manuscript has been submitted to the GenBank/EMBL Data Bank with the accession number AF019766.

Abbreviations: GGPP, geranylgeranyl pyrophosphate; GRK, G protein-coupled receptor kinase; PFT, protein farnesyltransferase; PGGT-I, protein geranylgeranyltransferase type I; Rho, rhodopsin; Rho*, photolysed rhodopsin; ROS, rod outer segments

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0.5% SDS, 5 mM phosphate, pH 7.4, and 100 μg/ml of denatured salmon sperm DNA. The filters were washed with 0.3 M NaCl, 0.03 M sodium citrate, and 0.5% SDS at 55°C for 30 min. Three positive clones on duplicate filters were replated, rescreened, and sequenced.

The 5′- and 3′-coding regions of chicken GRK1 were cloned by RACE-PCR using a Marathon cDNA Amplification kit (Clontech Laboratories, Inc.). For cloning the 5′-region, the two nested gene-specific primers used were XZ-26 (5′-AAGTCATCCGGAGCGCCG-3′) and XZ-28 (5′-TCCAAGTGGGCCAACAGC-3′). For cloning the 3′-region, the three nested gene-specific primers used were XZ-23 (5′-CAAAGTGTGCGCCTGCCA-3′), XZ-27 (5′-GGACACCCGGTTT-CATGG-3′), and XZ-41 (5′-GGCGTTTTCCACCGTCCGTGG-3′). The reactions were cycled 40 times at 94°C for 1 min and 68°C for 4 min. The PCR products were cloned into pCR2.1 (Invitrogen) and sequenced. The 3′-coding region of the chicken GRK1 was independently amplified from chicken retinal cDNA using primer pairs XZ-31 (catalytic region: 5′-TCAGCGCCGCTTCGCGG-3′) and XZ-60 (at 3′-end from stop codon: 5′-CCCCTCTGTACCCCCCATCCAT-3′); XZ-41 (autophosphorylation region: 5′-GGCGTTTTCCACCG-TCCGTGG-3′) and XZ-60. The PCR products were cloned into pCR2.1 and sequenced both manually and automatically (ABI-Prism). The PCR product using XZ-27 (catalytic region) and XZ-66 (C-terminal coding region: 5′-TCACAGCACCCCACAAGTAG-3′) was verified by restriction mapping.

Chicken mRNA was isolated from young and adult chicken pineal gland using the guanidinium isothiocyanate method [7]. The primers used to clone chicken GRK1 from pineal are XZ-23 and XZ-48 (AGAGTGCGCCGCGTCACCTCTTT). The reactions were cycled 40 times (94°C for 1 min and 68°C for 4 min), and the PCR products were cloned into the pCR2.1 vector and sequenced (ABI-Prism, Perkin Elmer).

2.2. Immunocytochemistry

Chicken retinal sections were processed as described previously for human retinal sections [7]. Sections were incubated with monoclonal primary antibody to bacterially expressed human GRK1, G8 [7], and secondary Cy-2-conjugated goat anti-mouse IgG (Jackson Immuno-Research Laboratories, Inc.).

2.3. Purification of chicken GRK1

Chicken GRK1 was purified from whole chicken retina with modifications to the procedure for the purification of bovine GRK1 [14]. All procedures were performed at 4°C. Chicken retinas (ACME Poultry), previously frozen at −80°C, were homogenized in 20 mM BTP, pH 7.5, containing 5 mM $MgCl_2$ and 1 mM benzamidine in a Dounce homogenizer with a motor-driven Teflon pestle (20 ml/100 retinas). The suspension was bleached on ice for 2 min under white light, then centrifuged at $37\,000\times g$ (JA-20 rotor, Beckman) for 10 min. The extraction of soluble contaminating proteins was repeated once more, without bleaching. To extract GRK1, the pellets were suspended in 25 ml NH_2OH extraction buffer (20 mM BTP, pH 7.5, containing 10 mM NH_2OH, 1 mM EDTA, 240 mM KCl, 1 mM benzamidine, 0.4% Tween-80) and further bleached on ice for 10 min under bright white light. The sample was centrifuged at $37\,000\times g$ for 10 min. The resulting pellet was resuspended in 25 ml NH_2OH extraction buffer, bleached on ice for 5 min, then centrifuged for 10 min at $37\,000\times g$.

The combined supernatants from the NH_2OH extractions were diluted 2-fold with buffer A (20 mM Bis-Tris-propane, pH 7.5, 0.4% Tween-80, 1 mM benzamidine) and loaded onto 5 ml heparin-Sepharose (Pharmacia) topped with a 1 ml bed of DEAE Sephacel (Pharmacia). The column was washed with buffer A until $A_{280}\leq 0.01$ ($\sim$30 ml) using a Pharmacia FPLC system at 4°C. A linear gradient from 0 to 500 mM KCl in buffer A over 40 min was developed at 1.0 ml/min, and 40×1 ml fractions were collected. Kinase activity-containing fractions, eluting at 250 mM KCl, were combined and diluted 2-fold with buffer B (20 mM Bis-Tris-propane, pH 7.5, containing 1 mM *n*-dodecyl-β-D-maltoside), then loaded onto a 1 ml heparin-Sepharose column. After washing with 10 ml buffer B, a linear salt gradient was developed from 0 to 500 mM KCl in buffer B over 20 min at 0.5 ml/min, and 20×0.5 ml fractions were collected. Activity-containing fractions, eluting at 350 mM KCl, were pooled and diluted 3-fold for Q Sepharose chromatography. After washing the loaded 1 ml column (Pharmacia HR 5/5) with 5 ml buffer B, GRK1 was eluted with a salt gradient from 0 to 500 mM NaCl in buffer B over 20 min at 0.5 ml/min, collecting 20×0.5 ml fractions. GRK1 activity was assayed using bovine Rho* as described previously [14].

2.4. Prenylation studies

RSATCVLS and RSATCVLL peptides, and the derivatives containing an N-terminal biotin-CONH-$(CH_2)_5$-CO- group, were synthesized by SynPep (Dublin, CA). Peptides were purified by reversed phase HPLC using a water/CF_3COOH-acetonitrile/CF_3COOH gradient, and their structures were confirmed by electrospray mass spectrometry. The concentrations of peptides in stock solutions were determined by thiol analysis using Ellman's reagent.

Fresh liver (42.6 g) from 12 3-week-old chickens was rinsed briefly with ice cold 0.9% KCl and homogenized using a Potter-Elvehjem homogenizer (4 strokes) in 170 ml of 20 mM HEPES-NaOH buffer, pH 8.0, containing 1 mM DTT, 0.2 mM EDTA, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 30 μM tosyl-lysine-chloromethyl ketone, 30 μM tosyl-phenylalanine-chloromethyl ketone, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin A; Sigma Chemical Co.) at 4°C. The homogenate was centrifuged at $20\,000\times g$ for 20 min, and the supernatant was centrifuged at $140\,000\times g$ for 1 h at 4°C. The resulting supernatant (cytosol fraction) was subjected to ammonium sulfate precipitation at 0°C. The protein pellet, precipitated by 30–50% saturated ammonium sulfate, was dissolved in about 40 ml of dialysis buffer (20 mM Tris-HCl, 50 mM NaCl, 1 mM DTT, pH 8.0) and dialyzed overnight against 2 liters of the same buffer (3 exchanges) at 4°C. The dialyzed 30–50% ammonium sulfate fraction of chicken liver cytosol contained 19.5 mg/ml of protein and was used as a source of chicken protein prenyltransferases.

To assay protein prenyltransferase activity, 10 μM biotinylated RSATCGVL or RSATCVLS was incubated with the 30–50% ammonium sulfate fraction (38 μg of protein) of chicken liver cytosol and either 1 μM [^{3}H]GGPP (geranylgeranyl pyrophosphate) or 1 μM [^{3}H]FPP (farnesyl pyrophosphate) (both 15 Ci/mmol, American Radiolabeled Chemicals) in a total volume of 20 μl containing 30 mM potassium phosphate, 5 mM DTT, 0.5 mM $MgCl_2$, 20 μM $ZnCl_2$, pH 7.7, and 1 μl of 0.25 M NH_4HCO_3/EtOH (3:7 v/v). After 1 h at 30°C, the reaction was terminated by boiling for 3 min, and 40 μl of avidin agarose suspension (50% aqueous suspension, Pierce) was added to the mixture to measure radioactivity transferred to the biotinylated peptide as described previously [15]. Recombinant rat protein farnesyltransferase (PFT) and protein geranylgeranyltransferase type I (PGGT-I) were produced using a baculovirus/Sf9 cell expression system and purified as described previously [16]. Assays using recombinant rat PFT or PGGT-I (0.1 μg protein/assay) were carried out at 30°C for 5 min under the same conditions as described above, except that 0.25 M NH_4HCO_3/ethanol was omitted.

The amounts of radioactivity transferred to the biotinylated peptide represent the levels above minus peptide controls (4230 cpm and 3756 cpm for the assays using chicken liver crude extract and either [^{3}H]GGPP or [^{3}H]FPP, respectively, and 2734 cpm and 2501 cpm for the assays using rat PGGT-I/[^{3}H]GGPP and rat PFT/[^{3}H]FPP, respectively).

Geranylgeranylated bovine GRK1 (GRK1-GG), farnesylated GRK1 (GRK1-F), and non-prenylated GRK1 mutant (GRK1-C588S) were generated as described previously [11]. GRK1 forms were isolated and analyzed as described by Palczewski et al. [17].

3. Results

3.1. Cloning of chicken photoreceptor GRK1

The cone-dominant chicken retina was employed in a search for cone GRK. This approach has been successfully used to clone the cone-specific α-subunit of the cGMP-gated channel [18]. Using a probe containing the catalytic region of bovine GRK1, we screened a chicken retinal cDNA library at low stringency. From $\sim 10^6$ clones, four positives were obtained. Three identical clones represent GRK1, because they had the highest homology with bovine and human GRK1 (74% at the DNA level and 81% at the amino acid level). In addition, the functionally important residues in bovine and human GRK1 were also conserved in chicken GRK1, including the 'GXGXXG' and the 'DL(M)G' motifs of the ATP

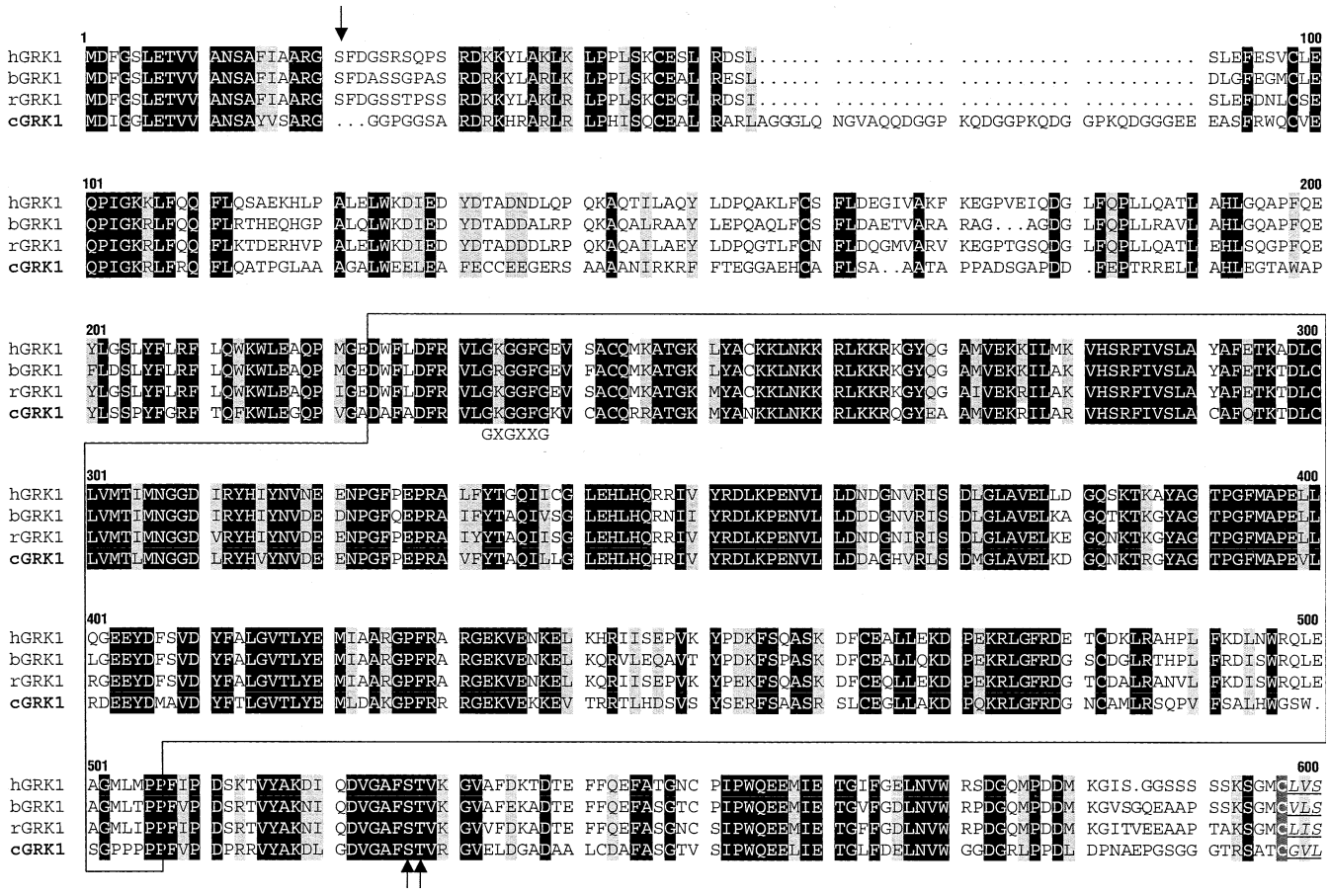

Fig. 1. Sequence alignment of GRK1 from different species. The sequence of chicken GRK1 was determined in the present study, and the sequences of bovine, human, and rat GRK1 were published previously [13,20,28]. The identical amino acids are shown as white letters on black background, the conservative amino acids (R = K, L = I = M = V, E = Q = D = N, S = T = A, and W = F = Y) are on gray background; the box shows the catalytic region with a protein kinase signature GXGXXG. Arrows indicate the autophosphorylated residues [2]. Note that Ser^{21}, a minor autophosphorylation residue, is conserved among mammalian photoreceptor kinase, but not in chicken GRK1 [2]. The C-terminal CaaX motif dictates posttranslational prenylation at Cys, proteolysis to remove GVL, and carboxymethylation at the prenylated Cys residue.

domain, which are conserved only among GRKs [2]. These results suggest that the sequenced kinase is a chicken homolog of mammalian GRK1 (Figs. 1 and 2). Unexpectedly, the cloned GRK1 exhibits lower sequence homology with mammalian GRK1s than does medaka GRK1 with mammalian GRK1 (Fig. 2). The fourth clone had the highest homology (71–73%) with an SNF-related protein kinase (SNRK) [19], but only 57% homology with bovine GRK1 (data not shown).

Chicken GRK1 clones were truncated at the 5′-region that corresponded to residue 105. To complete the sequence, the 5′-RACE method was used to amplify this region. The deduced N-terminal sequence of chicken GRK1 had only 39–44% similarity with mammalian GRK1 proteins, although it contained several regions that are conserved among GRK1s. An 'insert' in the chicken GRK1 sequence (Fig. 1) was found in all RT-PCR and RACE-PCR products using different sets of primers. This insert would increase the molecular mass of chicken GRK1. This prediction was borne out by immnunoblot analysis using anti-GRK1 antibody (Fig. 3), which showed that chicken GRK1 had a slightly slower electrophoretic mobility than mammalian GRK1.

The 3′-coding sequences of all three chicken GRK1 clones are truncated at the end of the catalytic domain, and this is followed by short and repetitive sequences with poly(A) tails (data not shown). The 3′-coding region of chicken GRK1 was also investigated by RACE PCR on chicken retinal cDNA. About 30% of the clones (chicken GRK1) obtained encoded a sequence homologous with the C-terminal region of mammalian GRK1 (60–70% similarity at the amino acid level), including the conserved major sites of autophosphorylation within the DVGAFSTVRGV sequence and C-terminal isoprenylation CaaX motif (CGVL) (Fig. 1). This CaaX motif suggests that chicken GRK1 is geranylgeranylated rather than like mammalian GRK1s which contain a C-terminus motif (CVLS) and is farnesylated [11,13]. Approximately 70% of the clones had short and repetitive sequences similar to the chicken GRK1 clones obtained from the cDNA library (data not shown). The junctions between the catalytic region and divergent C-termini are in the same position as that found for a splice form of human GRK1b [7]. Thus, those forms were likely produced by alternative splicing of the chicken GRK1 gene, either by exon deletion or intron retention, as in the case of human GRK1b [7].

Despite the low stringency used in cloning, only one mammalian GRK1 homolog was found in the chicken retina. Given the high abundance of cones in chicken, chicken GRK1 is likely to be the photoreceptor kinase of rods and cones. This hypothesis is further supported by Western blot analysis of

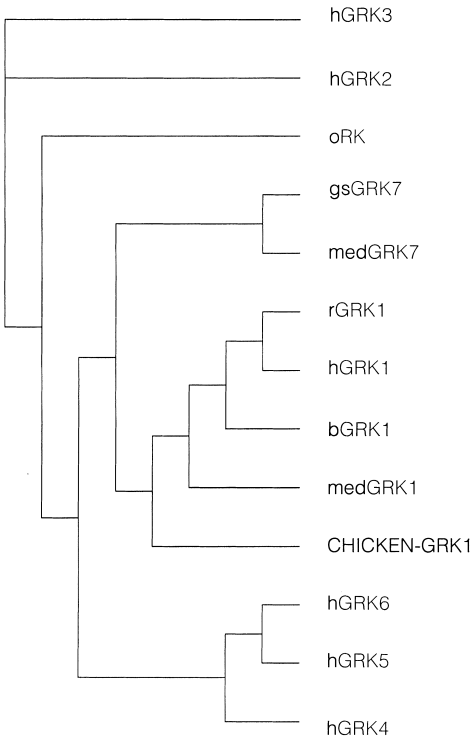

Fig. 2. Phylogenetic tree. The tree was built with a bootstrap analysis of neighbor-joining distance using PAUPSearch in GCG (University of Wisconsin-Genetics Computer Group). The accession numbers are: **h**uman GRK1, Q15835; **b**ovine GRK1, P28327; **r**at GRK1, Q63651; CHICKEN GRK1, AF019766; **med**aka GRK1, AB009569; **h**uman GRK2, X61157; **h**uman GRK3, P35626; **h**uman GRK4, P32298; **h**uman GRK5, P34947; **h**uman GRK6, P43250; **med**aka GRK7, AB009568; **g**round **s**quirrel GRK7, AF063016, and **o**ctopus RK, AB009875.

highly enriched fractions of RK (Fig. 3). Western blot analysis of fractions during purification indicated a single form of GRK1 (data not shown). Immunocytochemical data using G8 monoclonal antibody showed also that chicken GRK1 is specifically expressed in all photoreceptor cells of the retina (Fig. 4).

GRK1 has been shown to be present in mammalian pineal glands, which express both Rho and blue cone pigment [20]. A pineal-specific opsin, pinopsin, as well as cone opsins, but not Rho, have also been found in chicken pineal gland [21,22]. We investigated the presence of GRK1 in the chicken pineal gland by RT-PCR. Amplification from both young and adult chicken pineal gland cDNA using primers from different exons and specific for chicken GRK1 (Section 2) generated products of the predicted size (data not shown). The sequences of these products were identical to that of the chicken retinal kinase. Therefore, despite lacking Rho, chicken pineal gland expresses retinal GRK1.

3.2. Prenylation of chicken GRK1

GRK1 attaches to membranes by the insertion of the hydrophobic C-terminal prenyl group into the membrane. Native farnesylated GRK1 was found in the aqueous phase in the dark and associated with membranes after bleaching [11]. Without the farnesyl group (15-carbon isoprenoid), GRK1 is ~30% active and mostly soluble [11], while replacing the C-terminal CVLS of GRK1 with CVLL caused it to become modified by geranylgeranylation (20-carbon isoprenoid) in transfected COS-7 cells independently of receptor activation [11]. To investigate whether chicken GRK1 is modified with a geranylgeranyl or a farnesyl group, protein prenyltransferase assays were carried out using biotinylated RSATCGVL peptide, encompassing the C-terminal sequence of chicken GRK1, as a prenyl acceptor substrate, and the 30–50% ammonium sulfate fraction of chicken liver cytosol as a source of protein prenyltransferases. In control experiments, biotinylated RSATCVLS peptide in which the CaaX motif is replaced with bovine GRK1 CaaX sequence was used. Native bovine GRK1 has been shown to be farnesylated in vitro and in vivo [11,23]. When biotinylated RSATCGVL was incubated with the chicken liver crude extract and either [^{3}H]GGPP or [^{3}H]FPP, geranylgeranylation of the peptide was detected, and farnesylation was barely detectable (Table 1). Addition of unlabeled FPP at the same concentration as [^{3}H]GGPP did not significantly affect geranylgeranylation of the peptide, while addition of unlabeled GGPP completely abolished the farnesylation, indicating that geranylgeranyl transfer to biotinylated RSATCGVL and the low level of the farnesylation are mostly due to the action of PGGT-I present in chicken liver but not PFT. Mammalian PFT and PGGT-I have been shown to have differential binding affinities to prenyl pyrophosphates: PFT binds FPP 15-fold more tightly than GGPP, and PGGT-I binds GGPP 330-fold more tightly than FPP [24]. Therefore, although these two enzymes can transfer both farnesyl and geranylgeranyl groups in vitro, PFT and PGGT-I preferentially utilize FPP and GGPP, respectively, when these two prenylpyrophosphates are present at the same concentration. In contrast to biotinylated

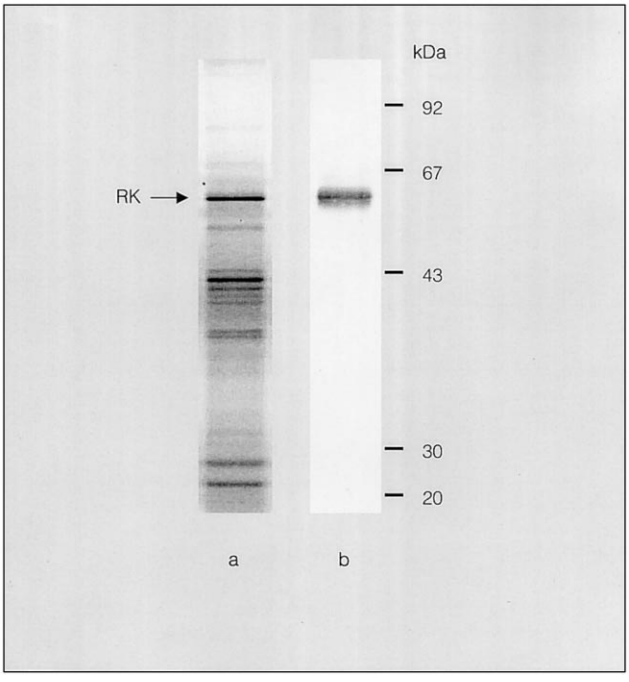

Fig. 3. Purification of chicken GRK1 from whole retina. a: Silver stain of a 12% SDS-PAGE gel after purification of GRK1 from whole chicken retina as described in Section 2. b: Western blot analysis of partially purified chicken GRK1 using monoclonal anti-GRK1 antibody G8 and secondary alkaline phosphatase-linked anti-mouse IgG (Promega). Positions of molecular weight markers and chicken GRK1 are indicated.

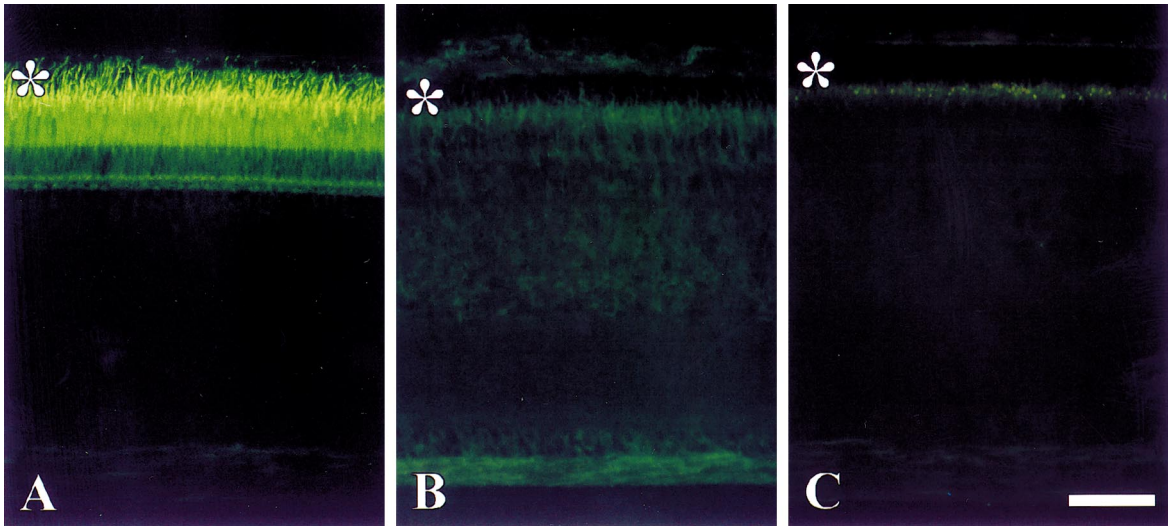

Fig. 4. Immunofluorescence localization of GRK1 in the chicken retina. Chicken retina sections were incubated (A) with G8 monoclonal antibody raised against bacterially expressed human GRK1 [7], (B) with G8 antibody pre-incubated with bacterially expressed human GRK1 (4 μg), or (C) without primary antibody. Immunolabeling was strongest in cone and rod outer segments and was also seen in cone and rod inner segments (A). Pre-incubation of human GRK1 with G8 monoclonal antibody resulted in loss of GRK1 immunoreactivity (B). Without addition of anti-GRK1 antibody, weak autofluorescence was detected in the photoreceptor layer. The asterisks represent the photoreceptor layer. (Bar = 50 μm).

RSATCGVL, biotinylated RSATCVLS was selectively farnesylated by the chicken enzyme (Table 1). In addition, biotinylated RSATCGVL is a preferred substrate for recombinant rat PGGT-I over PFT, whereas biotinylated RSATCVLS is a preferred substrate for rat PFT (Table 1). These results clearly indicate that the C-terminal sequence of chicken GRK1 has a signal for geranylgeranylation by chicken PGGT-I.

In agreement with previous studies [11], farnesylated GRK1, geranylgeranylated GRK1, and unprenylated mutant GRK1-C588S, all display high activities in in vitro phosphorylation of Rho* (data not shown). The initial velocity for the incorporation of ^{32}P from γ-[^{32}P]ATP into Rho* was plotted as a function of the amount of Rho-containing membranes. The data was fit to the Michaelis-Menten equation by nonlinear regression. The kinetic parameters are: farnesylated GRK1, $V_{max} = 260 \pm 30$ nmol/min/mg and $K_m = 6 \pm 2$ mM; geranylgeranylated GRK1, $V_{max} = 700 \pm 70$ nmol/min/mg and $K_m = 30 \pm 4$ mM; GRK1-C588S, $V_{max} = 100 \pm 10$ nmol/min/mg and $K_m = 9 \pm 2$ mM (data not shown). Thus, the catalytic efficiencies (V_{max}/K_m) are 43 for farnesylated GRK1, 23 for geranylgeranylated GRK1, and 11 for GRK1-C588S.

4. Discussion

4.1. One receptor kinase or two receptor kinases in photoreceptors?

Phototransduction in both rods and cones proceeds through similar, if not identical, steps that include absorption of light, an amplification cascade of reactions and recovery of dark conditions. Large differences in sensitivity to light, spectral sensitivity, and light and dark adaptation of rods and cones are attributed to specific properties of proteins comprising both transduction systems. We have hypothesized, based on cloning and biochemical results, that human retina contains one form of Rho kinase, GRK1 [7]. The lack of the requirement for cone- or rod-specific kinase was also supported by a broad substrate specificity of GRKs, where a

Table 1
Prenylation of the C-terminal peptide of chicken GRK1

Enzyme	Biotinylated (Bi) peptide	Prenyl donor(s)	Radioactivity transferred (pmol)
Chicken liver crude extract	Bi-RSATCGVL	[^{3}H]GGPP	0.298
		[^{3}H]GGPP, FPP	0.288
		[^{3}H]GGPP, GGPP	0.083
		[^{3}H]FPP	0.010
		[^{3}H]FPP, GGPP	0.000
		[^{3}H]FPP, FPP	0.009
	Bi-RSATCVLS	[^{3}H]GGPP	0.021
		[^{3}H]FPP	0.557
Rat PGGT-I	Bi-RSATCGVL	[^{3}H]GGPP	0.416
Rat PFT		[^{3}H]FPP	0.031
Rat PGGT-I	Bi-RSATCVLS	[^{3}H]GGPP	0.046
Rat PFT		[^{3}H]FPP	2.467

Chicken liver crude extract (38 μg of protein, 30–50% ammonium sulfate fraction of the cytosol) and either 10 μM biotinylated RSATCGVL or biotinylated RSATCVLS were incubated at 30°C for 1 h with either 1 μM [^{3}H]GGPP or [^{3}H]FPP (15 Ci/mmol) in the absence or presence of 1 μM unlabeled FPP or GGPP. Incubation of recombinant rat PGGT-I or PFT (0.1 mg of protein) with 5 μM biotinylated peptide and either 1 μM [^{3}H]GGPP or [^{3}H]FPP was carried out at 30°C for 5 min under the same conditions.

small number of GRKs desensitize thousands of G protein-coupled receptors due to their unique mechanism of substrate recognition [2]. Similarly, the studies of the mouse visual system suggest that GRK1 is involved in desensitization of visual pigments in both photoreceptor cell types [9,10].

Chicken GRK1 has a C-terminal CaaX motif, which is the hallmark sequence for directing a series of posttranslational modifications: prenylation of the Cys residue, proteolytic removal of aaX, and methylation of the α-carboxyl group of the S-prenyl-cysteine (for review, see [25–27]). In the CaaX motif, 'a' is usually but not necessarily an aliphatic residue and X is a variety of residues. The identity of X is thought to be the major determinant for directing which prenyl group, 15-carbon farnesyl or 20-carbon geranylgeranyl, is attached to the protein. When 'X' is Met, Ser, Ala, or Gln, the protein is farnesylated; when 'X' is Leu, the protein is geranylgeranylated. Other X residues may also exist. The C-terminal 'CGVL' sequence in chicken GRK1 suggests that it is modified by geranylgeranylation instead of farnesylation. Human and mouse retina is rod-dominant and the cloned GRK1 is farnesylated. Medaka and ground squirrel retinas contain high levels of cones and they express GRK7, a novel cone kinase. GRK7 shares sequence homology at the same level with GRK2/3 as well as with GRK1, and contains a consensus sequence for geranylgeranylation. In cone-dominant chicken retina and in pineal gland, which expressed only cone pigments, only GRK1 has been cloned, isolated, and localized in rods and cones using GRK1-specific monoclonal antibodies. This chicken GRK1 has higher sequence similarity to mammalian GRK1 than GRK7. Furthermore, the sequence similarity between chicken GRK1 and mammalian GRK1 is lower than between medaka GRK1 and mammalian GRK1. These data describe a novel form of photoreceptor GRK1 in chicken, and show a larger variation in GRKs between species than anticipated based on earlier studies. These findings suggest that in depth analysis of photoreceptor GRKs from different species may provide additional insights on the evolution of the vertebrate visual system.

4.2. Prenylation of GRK1

All GRKs have a mechanism of attaching to membranes where their substrates are found (for review, see [1,2]). For GRK1s membrane attachment is thought to occur with the insertion of the hydrophobic C-terminal prenyl group into the membrane. Based on experiments in a diluted reconstituted system (0.5 mg/ml of Rho), native farnesylated GRK1 was found in the aqueous phase in the dark and associated with membranes after bleaching ('light-dependent translocation') [11]. Without the farnesyl group (15-carbon isoprenoid), GRK1 is ~30% active and mostly soluble [11]. Replacing the C-terminal CVL**S** of GRK1 with CVL**L** caused it to become modified by geranylgeranylation (20-carbon isoprenoid) in transfected COS-7 cells. This modification caused GRK1 to associate with photoreceptor membranes independently of receptor activation [11]. Geranylgeranylated bovine GRK1 mutant was constitutively bound to membranes, while non-prenylated GRK1 mutant was mostly soluble under all conditions tested. These data have been interpreted to mean that native GRK1 translocates from the cytosol to membranes and specifically anchors to Rho* [11].

However, it is puzzling that bovine farnesylated GRK1, geranylgeranylated GRK1, and unprenylated GRK1-C588S display similar catalytic efficiencies for Rho* phosphorylation ([11], see Section 3). These results and the fact that the structure of the prenyl group has not been conserved during evolution (native chicken GRK1 is likely to be geranylgeranylated), leave the significance of cytosol-to-membrane translocation of GRK1 in phototransduction open to discussion. Native GRK1, which is hydrophobic, is tethered to membranes and requires detergent-containing buffers for efficient extraction from rod outer segments [14]. In vivo, native GRK1 likely resides on membranes under all light conditions, i.e. without translocation from the cytosol, particularly if one takes into account the fact that the concentration of Rho in ROS is ~100 mg/ml, which is much higher than the Rho concentration used in the previous reconstitution experiments.

It is intriguing that values of K_m for the lipidated and non-lipidated forms of bovine GRK1 for Rho* phosphorylation are similar. This suggests that the degree of partitioning of GRK1 into membranes is not important for its action toward Rho*, which further implies that GRK1 does not remain anchored via its prenyl group to membranes while bound to Rho* in the catalytically productive complex. A full analysis of the action of various lipidated forms of GRK1 requires not only kinetic but also equilibrium membrane binding measurements. At the present time, the role of the prenyl group of GRK1 in supporting its function in visual transduction remains unclear.

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