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**Characterization of molecular forms of G protein-coupled receptor kinase 1
(rhodopsin kinase) in vertebrate retina and pineal gland**

by

Xinyu Zhao

A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

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Chairperson of Supervisory Committee

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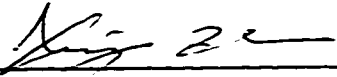
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Doctoral Dissertation

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University of Washington

Abstract

**Characterization of molecular forms of G protein-coupled receptor kinase 1
(rhodopsin kinase) in vertebrate retina and pineal gland**

by Xinyu Zhao

Chairperson of the Supervisory Committee: Professor Krzysztof Palczewski

Departments of Ophthalmology and Pharmacology

The purpose of my dissertation was to investigate the presence of molecular forms of G protein-coupled receptor kinase 1 (GRK1), and their functions in photoreceptor cells of the vertebrate retina and the pineal gland.

GRK1 is a critical enzyme in photopigments inactivation and recovery of the photoreceptors to the dark state. Only one photoreceptor kinase, GRK1 was cloned from rod-dominant rat retina, cone-enriched human fovea, and cone-dominant chicken retina using a combination of molecular cloning and RT-PCR. In addition, GRK1 was localized to the outer segments of both human rods and cones by two monoclonal antibodies specific for human GRK1. Strong immunolabeling of chicken photoreceptor outer segments provided further evidence that GRK1 is present in both rods and cones. GRK1 is also expressed in mammalian and chicken pineal gland as demonstrated using molecular cloning methods. We have also cloned rod opsin and blue cone opsin, putative substrates of GRK1 from rat pineal, suggesting that both rod and cone pigments are substrates for GRK1. This is consistent with the fact that cone pigments are good substrates for GRK1 *in vitro*. The colocalization of opsin and GRK1 in human pinealocytes suggests a novel role of GRK1 in an extraocular phototransduction system. In searching for different isoforms of GRK1, we have cloned splice variants of human

and chicken GRK1, GRK1b. Detailed characterization of human GRK1b indicates that GRK1b retained the last intron, intron 6. Although the mRNA of GRK1b is abundant and prevalent in humans and localized in photoreceptor cell cytosol, we only detected a low level of GRK1b protein in human retina, suggesting GRK1b is either unstable or is translated at low level. These data indicate the complex regulation of GRK1 at transcription and translational levels. In addition, a mutant form of GRK1 was found in a patient with Oguchi's disease. This patient was identified to have a deletion mutation in exon 5, which result in nonfunctional GRK1. The results obtained from psychophysical and electrophysiological studies indicate that GRK1 is a critical enzyme in the recovery of rod photoreceptor sensitivity, but play a minor role in the activation and adaptation to background illumination of photoreceptors. The small but definite delay of cone recovery in this Oguchi patient indicates that mechanisms other than phosphorylation by GRK1 may play predominant role in the inactivation of cone photoreceptors. Finally, biochemical data indicates that chicken GRK1 is likely to be geranylgeranylated, suggesting both geranylgeranylation and farnesylation of GRK1 are compatible with its function in the photoreceptor cells.

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LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
β ME	2-mercaptoethanol
bp	base pair
BTP	bis-tris propane
Ca ²⁺	calcium
CaM	calmodulin
cAMP	adenosine 3',5'-cyclic monophosphate
cDNA	complementary DNA
cGMP	guanosine 3',5'-cyclic monophosphate
cGMP-PDE	cyclic GMP-specific phosphodiesterase
CNG	cyclic GMP-gated channel
COS	cone outer segments
CPM	counts per minute
dCTP	deoxycytidine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate mixture
GCAP	guanylate cyclase activating protein
GDP	guanosine 5'-diphosphate
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GRK1	G protein-coupled receptor kinase 1 (rhodopsin kinase)
G _t	transducin
GTP	guanosine 5'-triphosphate
kb	kilobase, kilobase pair

kD _a	kilodalton
K _m	Michaelis constant
λ _{max}	maximal absorbance
Meta II	Metarhodopsin II
min	minute
mRNA	messenger RNA
Na	sodium
OS	outer segments
pAb	polyclonal antibody
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterase
Poly(A)	polyadenylated
RACE	rapid amplification of cDNA ends
RetGC	retinal GC
RGS	negative regulator of G protein signaling
Rho	rhodopsin.
Rho*	activated rhodopsin
RNA	ribonucleic acid
RNase	ribonuclease
ROS	rod outer segments
rpm	rotation per minute
RT-PCR	reverse transcription-PCR
Tris-HCl	Trizma, hydrochloric acid
V _{max}	maximal rate

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This dissertation is dedicated to my parents, Honju Zhao and Yurong Geng. Being scientists themselves, they always encourage me to pursue knowledge. Since I was a child, they have believed my ability to achieve and helped me to reach high. Thank you so much for all what you have done for me.

CHAPTER 1

INTRODUCTION

I. VISION

In vertebrates, eyes are specialized in sensing and perceiving light signals. Figure 1-1 illustrates a transverse section of the human eye (McDonnell, 1989). Light passes through and is focused by a series of transparent tissues including cornea, lens, and vitreous, before reaching the outer border of the retina where the photons are converted into neural signals by photoreceptor cells. The dark-pigmented retinal pigment epithelium (RPE) at the back of the retina not only reduces the refractive stray light, but also performs very important molecular functions in vision, such as serving as a molecular filter and nutrient supplier to the photoreceptor cells. It is also involved in phagocytosis of shed photoreceptor cells (Young and Droz, 1967).

The eye is an outgrowth of the brain, and a large amount of visual signal processing takes place in the eye. The retina is a thin sheet of neural tissue with a highly layered structure. This structural organization is closely related to its function (Figure 1-2) (Boycott and Dowling, 1969). The primary neurons are photoreceptor cells that transform the light signal into a neuronal signal. This signal is passed on to the secondary neurons (bipolar cells), and finally to the tertiary neurons (ganglion cells). The axons of ganglion cells form a bundle called the optic nerve, which projects to the visual cortex. There are also other neuronal cell types in retina. Horizontal cells in the outer plexiform layer and amacrine cells in the inner plexiform layer connect the signals between parallel pathways. Interplexiform cells have afferent contacts in the inner plexiform layer and efferent contacts in the outer plexiform layer. Müller cells are the major glial cells in

retina; they perform important functions in metabolism and ionic balance of neuronal cells and also maintain the framework of the retina.

The fovea is a small region of the retina specialized for high spatial resolution. The thinning of the inner neural tissues and the absence of blood vessels in the fovea region produce a pit where light travels through relatively fewer obstacles to reach the photoreceptor cells. Cone cells in the fovea are 4-5 times thinner, and their density is 20 times higher than that in the peripheral region. The retina surrounding the fovea has a yellow pigmentation and is called the "macula lutea"; the macula lutea filters short wavelength light to increase image acuity and to protect the retina from damage (reviewed by Rodieck, 1988). Foveae exist in every vertebrate class, but among mammals they are found only in primates.

II. VERTEBRATE PHOTORECEPTOR CELLS

From a single photon stimulus to bright illumination, vertebrate retinas can operate within a large range (10^8 -fold) of light intensities (Figure 1-3). This extraordinary function is initiated by the two types of photoreceptor cells in retina, the rods and cones, which mediate the two types of visual systems, scotopic (twilight) vision, and photopic (daylight) vision. Rod cells of a dark-adapted human eye can detect one photon captured by one rhodopsin molecule within one millisecond. On the other hand, cones are 100 times less sensitive to light, but have high visual acuity. There are three types of cones in human eyes, classified according to their sensitivity to different wavelength of light: blue ($\lambda_{\max}=420$ nm), green ($\lambda_{\max}=530$ nm) and red ($\lambda_{\max}=560$ nm) cones. Trichromatic color vision is based on the presence of the distinct photopigment molecules in each type of cone (Nathans et al., 1986).

The cellular organization of the two photoreceptor types is similar. Figure 1-3 is a schematic diagram of human rod and cone cells (Berman, 1991). About half of the space in the outer segments (OS) is a membrane structure, called the disk membrane, where phototransduction occurs. In rods, the disk membrane is separated from the plasma membrane. The inner segment (IS) contains cellular machinery for protein synthesis and energy production. Following synthesis in the rod IS, OS-specific transmembrane proteins, such as rhodopsin (Rho), are incorporated into the plasma membrane. The OS plasma membrane evaginates at its base, eventually pinching off to form a disk. The rod disk membrane differs in protein content from the plasma membrane. The older disks shed from the tip of the OS and are absorbed by the RPE. (Young and Droz, 1967). In cones, the disk membranes are formed by the invagination of the plasma membrane. The cone OS also shed, but the renewal process in cones is less clear.

Mammalian photoreceptor cells consist primarily of rods with less than 10% cones, while avian photoreceptor cells are primarily cones. Photopic vision, including color vision, is more important for our daily life than scotopic vision, but investigation of photopic vision has been hampered by the sparsity of cones.

III. VERTEBRATE PHOTOTRANSDUCTION

In vertebrate photoreceptor cells, a light stimulus is transformed into an electrical signal through a receptor/G protein/effector system (Figure 1-4). The rod visual pigment, Rho, constitutes more than 90% (about 3 mM or 120 mg/ml) of the total protein of the retinal rod outer segments (ROS). Absorbed photons isomerize the chromophore of Rho, 11-*cis*-retinal, leading to a series of rearrangements and to the formation of an active photobleaching intermediate, metarhodopsin II (Meta II). There is a tremendous amplification of signal in this process. A single Meta II molecule can activate hundreds of G proteins (G_T) by catalyzing the exchange of GTP for GDP on its α subunit ($G_T\alpha$). The

GTP-bound $G_t\alpha$ activates cGMP-specific phosphodiesterase (cGMP-PDE) by binding the regulatory subunits of cGMP-PDE (PDE γ). Activated cGMP-PDE hydrolyzes hundreds of cGMP molecules, leading to the closure of cGMP-gated channels (CNG) and subsequent hyperpolarization of the plasma membrane (reviewed by Pugh and Lamb, 1993; Lagnado and Baylor, 1992). Quenching of the cascade and restoration of the dark state starts with phosphorylation of the C-terminus of photolyzed rhodopsin (Rho*) by G protein-coupled receptor kinase 1 (GRK1, also called rhodopsin kinase or RK). Subsequently, arrestin prevents continuous G_t activation by binding to phosphorylated Rho* (Palczewski and Benovic, 1991). $G_t\alpha$ is inactivated through its intrinsic GTPase activity, resulting in the hydrolysis of GTP to GDP. The inactivation of $G_t\alpha$ terminates cGMP-PDE signaling. Activation of retinal guanylate cyclase (RetGC) at low intracellular calcium concentrations by a guanylate cyclase activating protein (GCAP) allows RetGC to restore cGMP to basal levels (Gorczyca et al., 1994; Palczewski et al., 1994). Retinol dehydrogenase reduces all-*trans*-retinal to all-*trans*-retinol, which is the final step in quenching and the first step in regeneration of the visual cycle. The resulting phosphorylated opsin is inactive. During regeneration, phosphorylated opsin becomes dephosphorylated by protein phosphatase 2A and subsequently binds 11-*cis*-retinal to reform Rho, which completes the phototransduction cycle (reviewed by Palczewski and Saari, 1997). A parallel mechanism also exists in cones, but because of the paucity of cones in most mammalian retinas, our knowledge of cone phototransduction is not as extensive as that of rod. For the following introduction, I will focus on the studies of rod phototransduction; the progress on cone phototransduction will be reviewed in part IV of this chapter.

PROTEINS INVOLVED IN THE ACTIVATION PATHWAY**(1) RHODOPSIN: THE LIGHT RECEPTOR IN ROD CELLS****Structure of vertebrate rhodopsin**

The trigger and essence of phototransduction is Rho. Vertebrate Rho is composed of a protein component, opsin, and a light sensitive chromophore, 11-*cis*-retinal. Opsins share high homology with cone pigments and other G protein-coupled receptors (reviewed in Hargrave and McDowell, 1992). 11-*cis*-retinal binds to opsin through a protonated Schiff base linkage to the ϵ -amino group of Lys-296 in transmembrane segment VII (Jackson, 1991).

Rho is present in both the disk and plasma membrane. Figure 1-5 shows the current model of Rho topography in the disk membrane (Hargrave and McDowell, 1992). This model illustrates that bovine Rho has seven α -helical transmembrane segments, with its amino terminus exposed to the lumen of the disk and its carboxyl terminus at the cytoplasmic surface. A variety of experimental observations have led to the development of this topographic model (Hargrave et al., 1983; Polans et al., 1986; Borjigin and Nathans, 1994;), including projection maps of Rho two-dimensional crystals to 9 Å resolution for bovine (Schertler et al., 1993), and 7 Å and 6 Å resolution for frog (Schertler and Hargrave, 1995) Rho. The helices 4, 6, and 7 were shown to be nearly perpendicular, while helices 1, 2, 3, and 5 are tilted relative to the plane of the membrane.

Rho is posttranslationally modified following its synthesis. The amino terminus of Rho is blocked with an acetyl group (Tsunasawa et al., 1980). Heterogeneous oligosaccharide chains are attached to Asn-2 and Asn-15 (Hargrave, 1977). Glycosylation of frog Rho is required for its incorporation into the ROS membrane (Fliesler and Basinger, 1985), while non-glycosylated Rho or Rho mutated at Asn-15 has been shown to have decreased light-dependent activation of G_t , inappropriate folding, and defective

posttranslational transport to the cell surface (Kaushal et al., 1994). Furthermore, there are ten cysteines in the Rho sequence, and the disulfide bond between Cys-110 and Cys-187 has been shown to be essential for both the formation of Rho (Karnik et al., 1988) and the stability of Meta II (Davidson et al., 1994). Cys-140 and Cys-316 are reactive with sulfhydryl reagents in the dark and are probably exposed to the cytoplasmic surface (Findlay et al., 1984). Cys-322 and Cys-323 are palmitoylated. These two cysteines are conserved among vertebrate opsins, but not in human red/green cone pigment and chicken iodopsin (Nathans, et al., 1986; Okano et al., 1989). Biochemical evidence suggests that the palmitoyl groups are anchored in the membrane and thus form the fourth cytoplasmic loop (Konig et al., 1989).

Activation of rhodopsin

Upon absorption of light, the initial event of photobleaching of Rho is the isomerization of 11-*cis*-retinal to all-*trans*-retinal. This change in retinal conformation is rapid at room temperature (~200 femtoseconds) and leads to the formation of the intermediates photorhodopsin, bathorhodopsin, lumirhodopsin, and metarhodopsin I (Meta I). Formation of the relatively stable photoproduct, Meta II, occurs in milliseconds and is accompanied by changes in the conformation of the protein (Farahbakhsh et al., 1995). In contrast to Rho, the cytoplasmic surface of Meta II offers binding sites for G_t, GRK1, arrestin, and p44. Meta II has a maximal absorption at 380 nm and an unprotonated Schiff base. Glu-113 has been identified as the counterion of the protonated Schiff base nitrogen of the chromophore (Sakmar et al., 1989). Mutation at either Lys-296 or Glu-113 results in Rho that can constitutively activate G_t in the absence of light. Mutant Rho with Glu-113 changed into glutamine, which lacks a negatively charged side chain necessary for the formation of a salt bridge with the chromophore, can stay inactive in the dark only in the presence of 11-*cis*-retinal. This suggests that 11-*cis*-retinal keeps Rho in the inactive conformation

(Zhukovsky et al., 1991; Buczylo et al., 1996). In addition, several other residues of Rho, such as Phe-115, Ala-117, Glu-122, Trp-126, Ser-127, Trp-265, and Tyr-268, are also involved in chromophore binding. Mutations in these residues lead to reduced regeneration, altered bleaching behavior, and reduced G_t activation (Nakayama and Khorana, 1990).

Recent work has shown that following a light stimulus, accumulated all-*trans*-retinal forms a noncovalent complex with opsin, which is 250 times less active than Rho* but at least 7.5 times more active than opsin. After illumination, this complex was shown to continuously activate phototransduction cascades to a lesser extent, and regulate the sensitivity of photoreceptors (Jager et al., 1996; Buczylo, et al., 1996).

Phosphorylation by receptor kinase

Photobleached rhodopsin (Rho*) can be phosphorylated by GRK1. This is the initial step in the quenching of the amplification cascade. The sites phosphorylated by GRK1 have been localized to the C-terminal region of Rho*, with the initial phosphorylation at Ser-338 and, subsequently, at Ser-343 and Thr-336 (Palczewski et al., 1991; Ohguro et al., 1993). The extent of phosphorylation was suggested to be controlled by two mechanisms: (1) the binding of arrestin to phosphorylated Rho* (P-Rho*) and (2) the reduction of all-*trans*-retinal to all-*trans*-retinol by retinol dehydrogenase (Ohguro, et al., 1994).

Mutations of Rho

Retinitis pigmentosa (RP) is a hereditary disease that causes retinal degeneration and occurs in both autosomal recessive and dominant forms. To date, more than eighty Rho mutations have been reported in RP patients. These mutations can be found in every functional domain of the Rho. Some autosomal dominant RP patients have mutations in the C-terminus of Rho molecule that may affect its phosphorylation by GRK1 and proper

inactivation. These patients have rod cell degeneration and suffer late onset (after the late teens) night blindness (Apfelstedt Sylla et al., 1993; Berson et al., 1991; Niemeyer et al., 1992). However, with the exception of a few specific cases (Kaushal, et al., 1994; Weitz and Nathans, 1992), it is not clear why these Rho mutations in RP patients lead to photoreceptor cell degeneration. To investigate the molecular mechanism of RP, transgenic mouse models have been generated (Naash et al., 1993; Huang et al., 1993). The photoreceptors of these mice show slow degeneration, which mimics human RP. Further molecular and biochemical studies that can detect structural and functional changes in these Rho mutants, are necessary.

(2) TRANSDUCIN

The heterotrimeric $G_t\alpha\beta\gamma$

Photoreceptor specific G_t is critical in controlling the amplification of the signal. Rod G_t is a heterotrimer that is composed of α , β , and γ subunits. The $G_t\alpha$ subunit has the binding site for GDP and GTP. This binding is regulated by $G_t\beta\gamma$ subunits, which are not separable under physiological conditions. Upon activation by Rho*, $G_t\alpha$ undergoes a conformational change (Dratz et al., 1993, and exchanges the bound GDP for GTP. The GTP-bound $G_t\alpha$ is capable of activating cGMP-PDE. Subsequent hydrolysis of GTP by its intrinsic GTPase activity leads to inactivation of G_t . It has been shown that the second, third, and fourth cytoplasmic loops of Meta II cooperate in the binding of G_t (Kuhn and Hargrave, 1981; Franke et al., 1992; Konig, et al., 1989). Mutant Rho, with an altered second or third cytoplasmic loop, has been shown to bind G_t in a light-dependent manner, but cannot activate G_t when GTP is added (Franke, et al., 1992; Ernst et al., 1995). This suggests that at least two sites on the Rho surface are implicated in the activation of G_t .

Crystal structures of $G_t\alpha$ -GTP γ S, $G_t\alpha$ -GDP, free $G_t\beta\gamma$, heterotrimeric G_t , and $G_t\beta\gamma$ -phosducin complex have been solved. The results confirmed previous conclusions from biochemical and mutagenesis studies and revealed the conformational changes during G_t activation, nucleotide exchange, and binding/release of $G_t\beta\gamma$ (Noel et al., 1993, Lambright et al., 1996 Sondek et al., 1996; Gaudet et al., 1996)

G_t subunits are posttranslationally modified by lipid. These modifications include heterogeneous N-myristoylation of $G_t\alpha$ (Yang and Wensel, 1992; Kokame et al., 1992). $G_t\gamma$, like other γ subunits of G proteins, has a CAAX motif (C: cysteine; A: aliphatic residues; X: any residues), and is modified by isoprenylation at the cysteine residue, proteolytic removal of the last three residues and α -carboxymethylation at the Cys residue (Fukada et al., 1990). The modifications of $G_t\alpha$ and $G_t\gamma$ might be important for $G_t\alpha$ and $G_t\beta\gamma$ interaction, because it has been shown that carboxymethylation and farnesylation of $G_t\gamma$ enhance the coupling of G_t to Meta II (Ohguro et al., 1991; reviewed by Fukada, et al., 1990).

Regulation of G_t activity

The GTPase activity of G_t measured *in vitro* or in diluted ROS preparations is much slower than *in vivo* or in concentrated ROS, which suggests the presence of GTPase activating proteins (GAPs) in OS. PDE γ was proposed to be one of the GAPs (Arshavsky and Bownds, 1992), and certain mutations in the C-terminal region of PDE γ reduce or abolish its ability to accelerate the GTPase activity (Slepek et al., 1995). Recent studies have shown that cGMP-PDE and its γ subunit contribute only a small part of the GTPase accelerating effect in native ROS (Otto-Bruc et al., 1994). Other protein factors are responsible for the fast inactivation kinetics of $G_t\alpha$ (Angleon and Wensel, 1993). A new family of proteins, called the negative regulators of G

protein signaling (RGS) was first discovered in yeast and later in the mammalian system. Several cloned RGSs were shown to accelerate GTPase activity of G_O , G_i and G_q (Berman et al., 1996; Hepler et al., 1997). Two of the newly found RGS proteins are proposed to be retinal-specific (Chen et al., 1996; Wieland et al., 1997; Faurobert and Hurley, 1997). More studies are needed to determine which RGS is responsible for the GAP activity in OS.

Another proposed regulator of G_t is phosducin. Phosducin is reversibly phosphorylated by cAMP-dependent protein kinase. In retinal rods, phosducin was shown to be phosphorylated in the dark and dephosphorylated upon illumination. The dephosphorylated phosducin binds $G_t\beta\gamma$ and blocks its reassociation with $G_t\alpha$, and therefore inhibits $G_t\alpha$ activation by Rho*. The phosphorylation of phosducin abolishes this inhibitory effect (Yoshida et al., 1994). The precise function of phosducin is still not clear. The recently obtained crystal structure of the $G_t\beta\gamma$ -phosducin complex may provide more information concerning its possible function in G_t regulation (Willardson et al., 1996; Gaudet, et al., 1996).

Disease

Patients with the Nougaret form of congenital night blindness have mutations in rod $G_t\alpha$ that result in reduced GTPase activity and constitutively active $G_t\alpha$. This is believed to be the reason underlying the dominant phenotype of this type of disease (Dryja et al., 1996).

(3) CYCLIC GMP-SPECIFIC PHOSPHODIESTERASE

The subunits of cGMP-PDE

As the effector of photoactivation, rod photoreceptor cGMP-PDE holoenzyme is a tetramer containing catalytic α (90 kDa) and β (88 kDa) subunits and two identical inhibitory γ subunits (11 kDa). It has been suggested that the cGMP-PDE catalytic core is

the PDE $\alpha\beta$ heterodimer (Fung et al., 1990). Artemyev (1996) has shown that $\alpha_2\gamma_2$ and $\beta_2\gamma_2$ may also be minor cGMP-PDE holoenzymes. The inhibition by PDE γ is removed when it binds G_t -GTP during photoactivation or when it is selectively removed by trypsinization. Recently, a 17 kDa δ subunit of cGMP-PDE has been identified and shown to be associated with a soluble fraction of cGMP-PDE. The δ subunit may play an important role in binding and solubilizing membrane-bound cGMP-PDE, but the regulation and significance of its function under physiological conditions are still unknown (Florio et al., 1996).

Subunits of cGMP-PDE are posttranslationally modified. Both α and β subunits have a CAAX motif at their C-termini; the α subunit is farnesylated and the β subunit is geranylgeranylated (Anant et al., 1992). The modifications are thought to be important in membrane association, protein folding, catalytic activity, and dimerization. Mutation of Cys to Ser eliminates the membrane binding of cGMP-PDE (Qin et al., 1992). The γ subunit was shown to be reversibly phosphorylated by protein kinase C *in vitro* (Udovichenko et al., 1996). The function of this phosphorylation *in vivo* is unclear.

Function and regulation of cGMP-PDE

cGMP-PDE is activated by $G_t\alpha$ -GTP and inactivated when GTP is hydrolyzed to GDP by the intrinsic GTPase activity of $G_t\alpha$. Each α and β subunit contains one catalytic and one noncatalytic cGMP binding site (Charbonneau et al., 1990). There are two possible functions for these noncatalytic sites: one is to serve as a buffering system for intracellular cGMP (Gillespie and Beavo, 1989); the other is to regulate the interaction of PDE γ and PDE $\alpha\beta$, for when the noncatalytic sites are occupied by cGMP, G_t -PDE γ stays in complex with PDE $\alpha\beta$. This was thought to be important for regulating the rate of cGMP-PDE inactivation by G_t (Arshavsky and Bownds, 1992).

The PDE γ subunit is not only an inhibitor of PDE $\alpha\beta$, but also serves two other important functions: first, when bound to G α , it increases GTP hydrolysis of G α in association with the putative GAP activity (Arshavsky et al., 1994; Slepak, et al., 1995); second, the interaction between PDE γ and PDE $\alpha\beta$ may have chaperon-like activity. This activity is important to the integrity and function of the holoenzyme *in vivo*, as demonstrated by PDE γ knock-out mice, where the PDE $\alpha\beta$ catalytic core is formed but lacks activity, and photoreceptor cells degenerate. (Tsang et al., 1996)

Disease

Mutations in PDE β result in photoreceptor degeneration in both *rd* (retinal degeneration) mice (Bowes et al., 1990; Pittler and Baehr, 1991) and *rcd* (rod/cone dysplasia) Irish setter dogs (Farber et al., 1992; Suber et al., 1993). Recently, several types of mutations in the PDE β gene were also found in certain human RP patients (McLaughlin et al., 1995). Mutation in PDE α was also found in some RP patients (Huang et al., 1995). In all these cases, decreased cGMP-PDE activity in the rod outer segment results in an abnormally high cGMP level and leads to cell degeneration. This also suggests that neither the $\alpha_2\gamma_2$ nor the $\beta_2\gamma_2$ tetramer can compensate for the $\alpha\beta\gamma_2$ holoenzyme *in vivo*.

(4) CYCLIC GMP-GATED CHANNEL ON THE PLASMA MEMBRANE

The rod CNG consists of two subunits, α and β , which form a heterotetramer, $\alpha_2\beta_2$. The initially cloned 63 kDa α subunit has a cGMP binding domain and a six-transmembrane structure. The topology is similar to the voltage-gated ion channels (reviewed by Molday, 1996). When expressed in *Xenopus* oocytes, the α subunit can form functional channels by itself, but these channels have different features than the native CNG (Kaupp et al., 1989). The latter cloned homologous β subunit is 155 kDa, but it migrates as 240 kDa on SDS-PAGE because of a Glu rich sequence in its N-terminal

region. It has a unique bipartite structure. The N-terminal region is identical to a previously cloned Glu-rich protein (GARP) (Sugimoto et al., 1991), and the C-terminal region contains similar sequences to CNG α and other CNGs, including six transmembrane domains and cGMP binding sites. CNG β also has a calmodulin (CaM) binding site. The CNG β subunit cannot form a functional channel by itself, but, when coexpressed with an α subunit in HEK293 cells, the units form a channel that has characteristics similar to the native channel, including Ca²⁺/CaM sensitivity (Chen et al., 1993; Korschen et al., 1995).

CNG is regulated by Ca²⁺. Ca²⁺ decreases the binding affinity of CNG to cGMP; therefore, when the concentration of intracellular Ca²⁺ falls in light, some of the channels tend to reopen despite the decrease in cGMP level. The Ca²⁺ sensitive mediator in this process has not been identified. Since the Ca²⁺ sensitivity has been localized to the β subunit that has a CaM binding site (Hsu and Molday, 1994), it has been proposed that CaM or its close homologue is the physiological Ca²⁺ sensor for CNG. At low cGMP levels, when the concentration of intracellular Ca²⁺ increases from 50 to 500 nM, Ca²⁺/CaM decreases CNG conductance two- to six- fold. This regulation is within the physiological range and can be blocked by the CaM inhibitor mastoparan (Hsu and Molday, 1994).

PROTEINS INVOLVED IN RECOVERY AFTER ILLUMINATION

(5) G PROTEIN-COUPLED RECEPTOR KINASE 1

To preserve the sensitivity of rod photoreceptors, Rho* needs to be inactivated. The research to date supports the idea that phosphorylation by GRK1 plays a critical role in the inactivation of Rho*. However, the precise contribution of GRK1 in vision and photoreceptor cell functions *in vivo*, including activation, recovery, adaptation and sensitivity is still not clear.

GRK1 was initially cloned from bovine retina, though its activity has also been shown in the pineal gland. It is unclear if this activity represents an identical kinase to the retinal GRK1 (Ho et al., 1986; Palczewski et al., 1990). GRK1-like activity has also been purified from *Musca domestica* (Doza et al., 1992), which suggests that inactivation of photoreceptors by GRK1 is a common mechanism among both vertebrates and invertebrates.

Primary structure

The primary sequence of bovine GRK1 was solved in 1991 (Lorenz, 1991). Bovine GRK1 has 561 amino acids and a molecular weight of 62,934 Da. It was the second member cloned in the GRK family.

The N-terminal region (residues 1-184) of GRK1 is the site that interacts with Rho* (Figure 1-6) (Palczewski et al., 1993). The catalytic region is located in the middle of the molecule. This region has conserved features among all the Ser/Thr kinases, such as the GXGXXG sequence in the ATP binding site and Asn-332 and Lys-216, which are important for phosphate transfer (Zhao et al., 1995). The C-terminal region of the kinase (105 residues) contains an autophosphorylation domain that is well conserved among all the GRKs. Ser-488 and Thr-489 are the autophosphorylated residues; mutations in this region affect autophosphorylation, ATP binding, substrate selectivity, degree of phosphorylation of rhodopsin in dark, and changes in the initial phosphorylation sites on Rho* (Buczylko et al., 1991; Palczewski et al., 1995). Autophosphorylated GRK1 has lower affinity for phosphorylated Rho* (P-Rho*).

GRK1 is posttranslationally modified at several sites. The N-terminus of GRK1 is blocked, although the type of modification is unknown. The C-terminus has a C⁵⁵⁸AAX motif and is farnesylated, proteolyzed, and carboxymethylated at Cys-558. The function of these modifications in GRK1 is not clear. It has been shown that farnesylation might

be important for light-dependent membrane translocation. GRK1 lacking the farnesyl group (C15) was shown to be four-fold less active and had an increased dissociation rate from Rho*-containing membrane. When GRK1 was modified by geranylgeranylation (C20), it seemed permanently associated with the membrane (Inglese et al., 1992). However, previous results have shown that GRK1 without farnesylation is significantly active when transfected into COS-7 cells (Palczewski, K., unpublished data). Since ROS has a 50% membrane content, it is very likely that GRK1 is permanently associated with the membrane and close to its substrate, Rho. Indeed, Tween 80 is needed to extract GRK1 from ROS. The C-terminal modification of GRK1 might be important for its transport to the ROS after synthesis (reviewed by Palczewski, 1997).

Enzymology:

In vitro, GRK1 phosphorylates Rho* with the $K_m=4 \mu\text{M}$ and the $V_{\text{max}}=700$ nmole phosphate/min/mg (Palczewski et al., 1988b). Activation is due primarily to a change of V_{max} . *In vitro*, the mean stoichiometry is 6.2 phosphates per receptor; however, *in vivo*, GRK1 phosphorylates rhodopsin at mainly two to three sites (Ohguro, et al., 1994).

GRK1 recognizes and phosphorylates several forms of photolyzed Rho, such as Meta I, Meta II, Meta III, and opsin/all-*trans*-retinal complex (Buczylko, et al., 1996). The phosphorylation sites are exclusively at the C-terminal region of the molecules. In addition, GRK1 can phosphorylate agonist-activated βAR and m_2AChR (reviewed by Premont et al., 1995), suggesting that the conformation of the active receptors is more potent than their primary sequences in activating GRK1. GRK1 can phosphorylate synthetic peptides from red/green cone pigments, βAR , and m_2AChR , as well as Rho (Palczewski et al., 1989). For peptide substrates, acidic residues flanking Ser or Thr are preferred over basic residues (Palczewski, et al., 1989). However, peptides are much poorer substrates for GRK1 than either intact receptor or Rho* lacking the C-terminal

phosphorylation region (Palczewski et al., 1991), suggesting a multisite interaction between GRK1 and Rho*.

GRK1 is activated by polycations, such as spermine or spermidine, and inhibited by polyanions, such as poly(Asp)_n or dextran sulfate. Mastoparan, a peptide toxin from wasp venom, can mimic G protein-coupled receptor (GPCR) to activate G proteins and can also activate GRK1, which suggests GRK1 and G_t interact with a similar region of Rho* (Palczewski, et al., 1991). Magnesium-ATP (Mg-ATP) complex is essential for GRK1 activity, but the complex Mg-ATP-Mg inhibits GRK1 activity (Palczewski et al., 1988).

Regulation

In vivo, GRK1 is activated by Rho*, and its binding affinity to the receptor is regulated by the autophosphorylation state of the kinase. Fully autophosphorylated GRK1 binds tightly to Rho*, but not to P-Rho*, while unphosphorylated GRK1 binds tightly to both Rho* and P-Rho*. This ensures that arrestin can displace GRK1 from P-Rho* after the initial quenching (Palczewski, 1994).

Dean and Akhtar (1996) have proposed that interaction of the GRK1-ATP complex with Rho* results in a soluble active kinase species that can phosphorylate Rho and peptide substrates in the dark. This is called "high gain phosphorylation" and is presumably important in preventing Rho from being activated after a light stimulus (Binder et al., 1990;). GRK1 might be regulated by recoverin or a recoverin-like factor at high Ca²⁺ concentrations. Chen, et al. (1995) have shown that recoverin inhibits GRK1 activity in a Ca²⁺-dependent manner, with half inhibition of GRK1 at 1.5-3.0 μM Ca²⁺. Furthermore, Senin et al. (1997) showed that recoverin inhibits the phosphorylation of dark-adapted Rho more than bleached Rho. These results suggest that recoverin inhibits

"high gain phosphorylation" of Rho. More work is needed to clarify its physiological basis and significance.

Disease:

Oguchi's Disease is a type of congenital stationary night blindness. The patients suffer delayed rod and, in some cases, also cone dark adaptation after a bleaching illumination (Carr et al., 1967; Krill, 1972; Yamanaka, 1969). Recently, it has been shown that mutations in the GRK1 gene give rise to the disease. One Oguchi patient was shown to have a homozygous point mutation in the catalytic region and a deletion at the C-terminal region of GRK1. Two other patients have a deletion of exon 5 of GRK1. According to biochemical studies, all three mutations will result in the loss of catalytic activity of GRK1 (Yamamoto et al., 1997). Unlike the GRK1 gene-knockout mice, which suffer retinal degeneration, Oguchi patients have no observed degeneration until very late in their lives. These patients, together with gene-knockout animal models, will help us to understand the function of GRK1 and its role in photoreceptor cell adaptation.

(6) ARRESTIN AND P44

Retinal arrestin, also called S-antigen, is a monomeric protein with an apparent molecular weight of 48 kDa. Circular dichroism analysis has shown that it has no α -helical structure, approximately 40% β -structure, 18% β -turns, and 40% other structure (Palczewski et al., 1992). The central region of all cloned arrestins is highly conserved, and this region is less exposed to external environment than the N- and C-termini. Arrestin is acetylated at its N-terminus, as revealed by mass spectrometry analysis (Ohguro et al., 1994).

Arrestin has no enzymatic activity; its function is to bind P-Rho* and quench its activity. Under physiological conditions, the apparent dissociation constant for Meta II-

arrestin complex is about 50 nM, and binding requires < 200 ms. The C-terminal region (20-30 residues) may play a regulatory role in arrestin's function, since truncated arrestin lacking the C-terminal region can bind P-Rho* independently of light (Palczewski, et al., 1991). A model for arrestin activation was suggested by Ohguro et al. (Ohguro, et al., 1994): when arrestin is inactive, the negatively charged C-terminus forms an ion pair with a highly basic region in the central region of arrestin. When arrestin interacts with P-Rho* or heparin, the C-terminus is replaced with a negatively charged phosphorylated receptor. The binding of arrestin to P-Rho* is resistant to dephosphorylation. Only when the active ligand of the Rho*, all-*trans*-retinal, is reduced to all-*trans*-retinol does arrestin dissociate from phosphorylated opsin (P-opsin) (Palczewski et al., 1989; Hofmann et al., 1992).

The picture of arrestin's function is further enriched by the isolation of its splice variant, p44, which has the last 35 residues replaced by an Ala (Palczewski et al., 1994). In contrast to arrestin, which is cytosolic and also present in other cell types of retina, p44 is tightly associated with membranes and is ROS-specific. p44 can bind not only P-Rho*, but also Rho*, to prevent them from binding G_t. *In vitro*, p44 is more potent than arrestin in Rho* quenching (Palczewski, et al., 1994). These data suggest p44 and arrestin play different roles in quenching Rho*, but their precise functions *in vivo* remain to be clarified.

Disease

Arrestin is a highly immunogenic protein. The autoantibody against arrestin was initially found in patients suffering autoimmune uveitis (Shinohara et al., 1987), and hence arrestin is called S-Antigen. The same antibody was also found in the sera of patients suffering multiple sclerosis, together with anti β -arrestin autoantibody. (Ohguro et al., 1993).

The importance of arrestin in quenching phototransduction is best elucidated by its involvement in Oguchi's disease. Certain patients with Oguchi's disease have been shown to have a homozygous deletion of nucleotide 1147 in codon 309 that results in a frameshift and premature termination of translation (Fuchs et al., 1995). These patients suffer from delayed rod dark adaptation and night blindness, similar to patients who lack a functional GRK1 gene (Yamamoto, et al., 1997). This also suggests that the complex of P-Rho* and GRK1 forms rapidly but is unstable and arrestin is needed to replace GRK1 and form a stable complex with P-Rho*.

(7) GUANYLATE CYCLASES AND GUANYLATE CYCLASE ACTIVATING PROTEINS

Retinal guanylate cyclases (RetGC) belong to a family of membrane-bound guanylate cyclases (Shyjan et al., 1992). These proteins are characterized by four functional domains: an N-terminal extracellular domain, a transmembrane region, an intracellular kinase homology domain, and a C-terminal cyclase catalytic domain (Chinkers and Garbers, 1991). Unlike other members of this family that are regulated by peptide ligands binding to the extracellular domain of the cyclase, the RetGCs are regulated through their intracellular domains (Laura et al., 1996). Electrophysiological studies on single ROS have confirmed the importance of RetGC1 in phototransduction (Koutalos et al., 1995). A newly cloned RetGC2 is very similar to RetGC1 with respect to sequence and, possibly, regulation (Lowe et al., 1995). RetGC2 is proposed to be localized in ROS (Yang and Garbers, 1997), but its precise localization and function need further investigation.

Regulation

RetGC1 is regulated in a Ca²⁺-dependent manner. In the dark, when the Ca²⁺ level is high (about 500 nM), RetGC activity is low. The Ca²⁺ level drops in response to light and RetGC is activated by Ca²⁺-sensitive proteins, GCAPs, to restore the intracellular

cGMP level. GCAP1 (Gorczyca et al., 1994) and GCAP2 (Dizhoor et al., 1995) are EF-hand family Ca^{2+} -binding proteins. Recombinant RetGC1 was shown to be activated by both GCAP1 and GCAP2, and recombinant RetGC2 was shown to be activated by GCAP2, with an EC50 of approximately 200 nM (Dizhoor, et al., 1995; Gorczyca et al., 1995; Lowe, 1995), which is consistent with the characteristics of native RetGC. It is clear that GCAP regulates RetGC through its intracellular domain, probably the kinase homology domain, since the truncated RetGC lacking the extracellular domain responds equally well to GCAP (Laura, et al., 1996; Duda et al., 1996). The specific localizations of RetGC1 and GCAP1 in the ROS and COS suggest that GCAP1 is more likely to be the physiological activator of RetGC1 in phototransduction (Gorczyca, et al., 1994; Hallett et al., 1996). GCAP2 is localized primarily to the inner segment, somata and synapses of cones and to a lesser extent to rods (Otto-Bruc et al., 1997). This result, together with the finding that GCAP1 and GCAP2 interact with RetGC at different regions, suggests the two GCAPs affect RetGC through different mechanisms for different physiological purposes (Duda et al., 1997).

The importance of RetGC and Ca^{2+} regulation in phototransduction has attracted increasing attentions. Recently, other protein factors have been suggested to activate RetGC in a Ca^{2+} -dependent manner. One of them is the S100A/S100B protein (or CD-GCAP), which activate RetGC at high Ca^{2+} levels, with an EC50 at 35-40 μM Ca^{2+} (Duda et al., 1996; Pozdnyakov et al., 1995). S100 proteins and GCAPs interact with RetGC at different sites. Since it is hard to comprehend the relevance of this process to phototransduction, the physiological significance of these factors in retina needs further research.

Interestingly, RetGC has been found to have kinase activity. It autophosphorylates and also phosphorylates a myelin basic protein (Aparicio and Applebury, 1996). This is

consistent with the finding that ATP can stimulate RetGC activity to two-fold (Gorczyca, et al., 1994). This kinase activity seems to be Ca^{2+} -independent but its role in phototransduction is not clear.

Disease

Leber's congenital amaurosis (LCA) patients suffer severe retinopathy. Recently, it has been shown that mutations in RetGC1 gene are associated with this disease (Perrault et al., 1996). This phenotype closely resembles that of the *rd/rd* chicken (Semple-Rowland et al., 1997). In the retinas of both LCA patients and the *rd/rd* chicken, cGMP synthesis is abolished and the CNG is permanently closed. This simulates the effect of continuous light stimuli on the photoreceptor cells which leads to their degeneration. This phenotype also demonstrates that RetGC2 cannot replace RetGC1 in phototransduction.

IV. DIFFERENCES BETWEEN ROD AND CONE PHOTOTRANSDUCTION

(1) THE DIFFERENCES IN PHOTOTRANSDUCTION KINETICS

It was demonstrated in early experiments that rods and cones behave differently in photoresponse (Baylor and Hodgkin, 1974). The major difference is that, in any given species, the suppression of the photocurrent in rods is more sensitive to light and slower in response than in cones. The light sensitivity of the striped bass cones is approximately 100 times less than that of rods and the time to peak is 5 times faster than in rods. (Miller and Korenbrot, 1993); The second major difference is that light adaptation range of cones is much larger: The striped bass cones adapt within a 1000-fold larger range of light than rods. Cones can therefore operate under bright light that would saturate the rod responses (Miller and Korenbrot, 1993)

(2) THE MECHANISM UNDERLYING THESE DIFFERENCES

Despite the progress in molecular, biochemical and electrophysiological techniques, the understanding of the differences in the kinetics of rod and cone light response is still in a very early stage. Upon saturating light stimulus, two parameters are similar between rods and cones: (1) the delay between the flash and the onset of photocurrent, and (2) the exponential time constant with which the photocurrent reaches maximum. This suggests that the kinetic differences between rods and cones may not be due to differences in the activation phase, but rather due to the differences in the recovery to dark level (Hestrin and Korenbrot, 1990).

Differences between rod and cone phototransduction proteins

For most of the critical phototransduction proteins, rods and cones have homologous but distinct counterparts. The homology of amino acid sequences between the two sets varies from 30% to 90%. Table 1 lists the phototransduction proteins that have been identified in rods and cones and the major differences that have been demonstrated in their kinetics and functions.

Because of the difficulty in isolating COS and cone-specific proteins, few studies have been done examining the kinetic, enzymatic differences between rod and cone-specific proteins. Gillespie and Beavo (1988) have shown that cone cGMP-PDE has higher basal activity and is activated by lower concentrations of rod G_t . Cone cGMP-PDE has lower affinity for bacterially expressed cone PDE γ than for rod PDE γ (Hamilton et al., 1993). These might contribute to the fast activation in cones. CNG in cones have been suggested to have twice the conductance of rod CNG, therefore cone CNG might have higher Ca^{2+} flux rate than that of rods (Picones and Korenbrot, 1995; Miller et al., 1994). Whereas, the moderate differences are not enough to be responsible for the differences of phototransduction kinetics. In fact, the key players in

the inactivation pathway, RetGC (Shyjan, et al., 1992) and GCAP (Gorczyca, et al., 1994; Otto-Bruc, et al., 1997) were shown to be the same in rods and cones. It was unknown if GRK1 has its distinct homologue in cones (Palczewski, et al., 1993).

Differences in Ca²⁺ homeostasis:

Ca²⁺ homeostasis is significantly different between rods and cones. The changes in intracellular Ca²⁺ in response to light are faster and larger in cones than in rods. Several factors have been suggested to be responsible for this (reviewed by Miller, et al., 1994): first, the Na⁺/Ca²⁺ exchanger of cones is 5-8 times faster than that of rods, which leads to high rate of Ca²⁺ clearance; second, the cytoplasmic Ca²⁺ buffering of rods is slightly higher than that of cones; third, the CNG of cones is twice as permeable to Ca²⁺ as that of rods, so that the Ca²⁺ influx rate is higher in cones; fourth, the surface to volume ratio is larger in cones than in rods, so that both the Ca²⁺ influx and efflux rates are higher in cones.

The mechanism underlying the different photoresponse kinetics in rods and cones might be in the photoreceptor molecules, Rho and cone pigments, themselves. It is possible that the trigger of the cascade also control the sensitivity and kinetics of the response. This is supported by several lines of evidence, including the fact that a single mutation of Glu-122 in Rho into the corresponding residue in red/green pigments converts Rho regeneration and Meta II decay kinetics into those of red/green pigments, and the opposite association is also observed (Imai et al., 1997). This suggests that the characteristics of regeneration and decay of the active receptors are, in part, intrinsic to the opsin protein structure.

V. THE PINEAL GLAND AND EXTRAOCULAR PHOTORECEPTIVE SYSTEM

In lower vertebrates, pineal photoreceptors have a similar structure to retinal cone photoreceptors and they hyperpolarize in response to light stimuli. Pineals in birds and mammals have evolved into secretory glands and contain predominantly melatonin-secreting pinealocytes. Pineals of certain fish, birds and neonatal rats are directly light sensitive, whereas pineals of adult mammals receive light stimuli from retina via a sympathetic input. Studies have shown that chicken and mammalian pinealocytes retain critical phototransduction proteins, such as opsin, GRK1, G_t , cGMP-PDE, arrestin, recoverin, phosducin, and others (Table 1-2). However, most of these results have been obtained primarily from immunocytochemistry studies and the evidence is conflicting. Clear identification of these phototransduction components in mammalian and chicken pineal glands will help to clarify if they form a nonvisual phototransduction pathway in circadian regulation and melatonin synthesis, and in addition will provide insight into pineal and retinal photoreceptor evolution.

(1) THE PINEAL GLAND

Histology

The pineal gland is believed to develop from the same stem cells as retinal photoreceptors but with a differentiation lineage closer to cones than to rods (Zimmerman and Tso, 1975). Schomerus et al., 1994) has classified pineal parenchymal cells into three types:

Type I classical pineal photoreceptors, such as those in anaminotes. These cells resemble retinal photoreceptors, for they display inner and regularly lamellated outer segments, and their basal processes contact secondary neurons. These cells also hyperpolarize in response to direct light stimuli.

Type II modified photoreceptors, such as the pinealocytes in reptiles and birds.

The OS of these cells are not as regularly organized as the OS of Type I cells, and the basal processes do not establish synaptic contacts with secondary neurons. These cells secrete melatonin and light triggers the inhibition of melatonin secretion. The chicken pineal receives both sympathetic control from the avian superior cervical nucleus (SCN) (norepinephrine inhibits melatonin secretion), and direct light stimuli.

Type III neuroendocrine pinealocytes, such as the pinealocytes of mammals.

These cells have no inner or OS. Except in young rats (Zweig et al., 1966), the mammalian pineal gland is believed to receive light stimuli through output from the SCN. The SCN signal is transmitted to the pineal by a multineural pathway terminating in adrenergic fibers that release norepinephrine at night to stimulate melatonin synthesis. The mammalian pineal consists of two cell types: 95% of the cells are pinealocytes and 5% of the cells are glial cells (interstitial cells).

Function

The function of the pineal is not fully understood. The pineal in any species secretes melatonin under circadian control. The critical enzyme in melatonin synthesis, arylalkylamine-N-acetyltransferase, has been recently cloned from both mammals and chickens and its mRNA level has been shown to be controlled by circadian oscillators (Coon et al., 1995; Bernard et al., 1997). Melatonin has been proposed to participate in reproductive control, metabolism, sleeping patterns, aging and other physiological processes (reviewed by McDonnell, 1989). The preliminary research in this area has generated great public attention and the results are vast and intriguing, but further confirmation is needed.

(2) PHOTOTRANSDUCTION AND THE PINEAL

There are common characteristics between retina and pineal: first, both organs develop as evaginations from the diencephalon; second, they primarily serve photoreceptive functions; and third, they are both capable of producing melatonin, and the level of melatonin production is affected by light.

The major difference between retina and pineal is that the pineal has undergone a major change during evolution and, as a result, pinealocytes differ in their fine structure and function among vertebrates, while the structure of retina and basic phototransduction mechanism are well conserved through out species.

Table 1-2 shows the retinal phototransduction proteins that have been found in the pineal. Studies of the chicken pineal have been more fruitful than those in mammalian pineal. Pinopsin, a homologue of retinal Rho with $\lambda_{\max}=470$ nm (blue sensitive) has been cloned from chicken (Okano et al., 1994; Max et al., 1995). The blue sensitivity of this opsin is consistent with the fact that the pineal gland is sensitive to short wave length light. A G_t -like protein was also found in chicken pineal (Okano et al., 1997). Two alternatively spliced forms of the cone CNG have been cloned from chicken pineal but no CNG has been detected in either bovine or rat pineal (Dryer and Henderson, 1991; Bonigk et al., 1996).

The presence of opsin in mammalian pineal glands is controversial. Immunocytochemistry studies have yielded different results depending on the antibodies used and the species studied (reviewed by Schomerus, et al., 1994). Both rod and cone arrestin have been cloned from the rat pineal (Abe and Shinohara, 1990). Pineal also expresses cone cGMP-PDE, or its homologue, but not rod cGMP-PDE (Carcamo et al., 1995). Pineal has been shown to have GRK1-like activity (Ho, et al., 1986; Palczewski, et al., 1990), but it is not clear if this is an identical kinase to the retinal GRK1 .

(3) OTHER EXTRAOCULAR PHOTORECEPTIVE SYSTEM

It has been proposed that the central nervous system contains photosensitive neurons, called extraocular photoreceptive system (EOP). EOP has been found in the abdominal ganglia of various crustacea, including crayfish, lobster, and shrimp, and it functions to protect their tails from exposure to predators (Czeisler et al., 1995). EOP neurons have also been found in other lower vertebrates but their function is not very clear (Baines and Bacon, 1994). It has been suggested that higher mammals also have EOP. For example, enucleated rats can still sense light (Zweig, et al., 1966), which may be due to the light sensitivity of the young rat pineal. Furthermore, some blind patients show altered blood melatonin concentrations and circadian rhythms in response to light. But, in the studies of Czeisler, et al. (Czeisler, et al., 1995), the patients lost light sensitivity when their eyes were covered. Adult *rd/rd* mice with complete rod degeneration can still sense light through their eyes, and experience altered circadian rhythms (Mrosovsky and Hampton, 1997). The light-sensing mechanism in blind human and mouse eyes may depend upon a distinct pathway from that in the photoreceptor OS.

VI. OTHER G PROTEIN-COUPLED SYSTEMS

Many cellular signals, including peptides, nonpeptide hormones, neurotransmitters, cytokines, prostanoids, and proteinases are transduced by G protein-coupled receptor (GPCR) system. The best and most studied examples are the Rho-mediated phototransduction cascade and the β AR-mediated hormonal responses. The studies of these two systems serve as a framework for understanding other GPCR signaling mechanisms.

(1) G PROTEIN-COUPLED RECEPTOR SYSTEMS

The GPCRs are 7-helix transmembrane proteins. This information, originally proposed from sequence analysis, was later confirmed by studying the two dimensional

structure of bovine and frog Rho using electron cryomicroscopy (Unger and Schertler, 1995; Schertler and Hargrave, 1995). The principle function of the GPCR is to transmit the extracellular stimulus to the interior of the cells by interacting with heterotrimeric G protein. The extracellular loops of GPCR contain the ligand-binding sites, with the exception of Rho, whose ligand is in the transmembrane chromophore-binding pocket. The intracellular loops have sites to interact with heterotrimeric G protein, receptor kinases and arrestins.

To maintain the sensitivity, GPCRs undergo rapid adaptation or desensitization upon agonist binding. This is mediated by several mechanisms: (1) phosphorylation by GRKs followed by arrestins/ β -arrestins binding, as in the phototransduction cascade (reviewed by Zhao, et al., 1995); (2) phosphorylation by second messenger-dependent kinases, as in the case of β AR (Lohse et al., 1990); (3) agonist induced endocytosis, as in the case of the gastrin releasing peptide receptor (Grady et al., 1995) and the neurokinin 1 receptor (Grady et al., 1996); (4) down regulation of the receptor after prolonged exposure to a stimulus, mediated by either increased degradation or decreased synthesis of the GPCR, as in the case of β_2 AR (Collins et al., 1991; Collins et al., 1990).

(2) G PROTEIN-COUPLED RECEPTOR KINASES

Desensitization of GPCRs by phosphorylation is primarily mediated by a family of Ser/Thr kinases called G protein-coupled receptor kinases (GRKs). The characteristics of GRKs are: (1) they only phosphorylate active forms of GPCRs; (2) they have broad and overlapping substrate specificities; and (3) the phosphorylation sites are separated from the activation sites. To date, six members of the GRK family have been cloned from mammals and *Drosophila*. Based on sequence homology, they can be divided into three subgroups: group 1 is GRK1; group 2 includes GRK2 and 3 (also known as β ARK1 and 2) and *Drosophila* GPRK1; group three contains the newly identified members, GRK4, 5,

6 and *Drosophila* GPRK2 (reviewed by Premont, et al., 1995). The overall sequence similarity among these kinases is 53-93%, with the lowest sequence homology between group 1 and 2 (Figure 1-6) (Zhao, et al., 1995). To date, only GRK4 has been shown to have splice variants (α , β , γ , and δ forms), which differ in either the N- or C-terminal region. All the splice variants are found predominantly in the testis (Premont et al., 1996; Sallese et al., 1997).

Primary structure

As shown in Table 1-3 (reviewed by Palczewski, K. 1997), GRKs have similar molecular organizations. The catalytic region (~270 residues) is located in the middle portion of the molecule and this region is highly conserved among all GRKs, including the "GXGXXGX" for the ATP binding site and a "D(L/M)G" signature which is specific to GRKs (all other kinases have "DFG" signature). The N-terminal region of the GRKs is believed to be involved in receptor binding. The C-terminal region is the most diverse region among the GRKs. Both GRK1 and 5 are autophosphorylated at conserved Ser and Thr residues (Ser 488, Thr 489 in GRK1). Mutations in either Ser/Thr or in other conserved sites within the autophosphorylation domain result in reduced kinase activity and altered substrate binding (Palczewski, et al., 1995; Kunapuli et al., 1994). GRK6 is not extensively autophosphorylated, even though it has the conserved sequence for autophosphorylation (Loudon and Benovic, 1994). GRK2 and 3 do not have the conserved Ser and Thr; instead, they have a negatively charged "DEED" in the corresponding position (reviewed by Zhao, et al., 1995). The C-terminal region is also believed to be responsible for membrane and lipid interaction. GRK2 and 3 have pleckstrin homology (PH) domain for binding $G_t\beta\gamma$ (Pitcher et al., 1992). GRK1, with a CAAX box in the C-terminus, is farnesylated and carboxymethylated. GRK 4 and 6 are palmitoylated on one or more cysteines at the C-terminus (Stoffel et al., 1994; Premont, et al., 1996). The lipid modifications were thought to be responsible for membrane

association (Inglese, et al., 1992). The C-terminal region of GRK5 is not modified by any lipid but has been shown to interact with lipid (Kunapuli, et al., 1994).

Substrate:

Except for GRK1 found only in photoreceptors, and GRK4 found primarily in testis, all the other GRKs have wide tissue distributions (Table 1-3). The most striking feature of the GRKs is that they phosphorylate only activated receptors. The conformation of the active receptor, but not the primary sequence is potent activator of GRKs. *In vitro*, all GRKs have broad receptor specificities (Table 1-3). This is consistent with the fact that a large number of G protein-coupled receptors have been identified thus far, but only six GRKs are responsible for their phosphorylation, which also makes it difficult to assign each GRK to individual G proteins *in vivo*. GRK2 and 3 are probably involved not only in β ARs but also in m_2 AChR and A1 adenosine receptor phosphorylation *in vivo* (Kameyama et al., 1993; Ramkumar et al., 1993). GRK5 appears to be involved in phosphorylation and desensitization of the thyrotropin receptor (Nagayama et al., 1996). Because of the low abundance of most GPCRs and the difficulty in isolating them, the phosphorylation sites of GPCRs except for those in Rho (C-terminus), β AR (C-terminus) and m_2 AChR (third intracellular loop), have not been characterized.

As in the case of GRK1, peptides are much poorer substrates for GRKs than the intact receptors, suggesting that multisite interactions between receptors and kinase are the common mechanism for GRK activation. GRK1 and 5 prefer acidic peptides or proteins, while neutral peptides are better substrates for GRK6 (reviewed by Premont, et al., 1995).

Regulation

Because their substrates are transmembrane receptors, GRKs interact extensively with lipids and membranes. It is very likely that all GRKs are membrane-associated proteins. Agonist-induced translocation of GRK to GPCR has been proposed to be the mechanism of GRK1, 2, and 3 activation (Inglese, et al., 1992). Recent studies have shown that GRK2 and 3 are primarily localized in membrane, close to β AR (Ruiz-Gomez and Mayor, 1997). Our experience with GRK1 demonstrates that GRK1 is also membrane associated even in the dark (Palczewski, unpublished data). GRK2 activity is stimulated by membrane lipid (Onorato et al., 1995). GRK5 contains neither a PH domain (found in GRK2 and 3), nor CAAX motif (found in GRK1); however, it has been shown to be membrane associated through its basic sequence at the C-terminal region (Kunapuli, et al., 1994). and phospholipid stimulates GRK5 auto-phosphorylation and enhances its kinase activity 15-20 fold (Kunapuli, et al., 1994). GRK4 α , the only spliced form GRK4 that can phosphorylate Rho*, is inhibited by Ca²⁺/CaM (Sallese, et al., 1997).

There is possibly cross-talk between GRKs and other types of kinases, or among GRKs. Both GRKs and cAMP-dependent protein kinase phosphorylate β_2 AR. GRK2 is phosphorylated by protein kinase C within its PH domain, which increases kinase activity and its interaction with lipid (Chuang et al., 1995; Winstel et al., 1996). GRK 5 is also phosphorylated by protein kinase C at its C-terminal region, but this phosphorylation leads to decreased kinase activity with little influence on its interaction with lipid (Pronin and Benovic, 1997). Phosphoinositol enhances the kinase activity of GRK2, 3, 4, 5, and 6 (Pitcher et al., 1996). More work needs to be done to clarify the complex picture of GRK in signal transduction.

(3) ARRESTIN AND β -ARRESTIN:

The rod and cone arrestins also have their homologues in other GPCR systems. Six distinct members of the arrestin family have been identified so far: rod and cone arrestins (Yamaki et al., 1987; Craft et al., 1994), β -arrestin1 and β -arrestin2 (Lohse et al., 1990; Attramadal et al., 1992), D and E arrestins (Craft, et al., 1994). Following the retinal arrestins, the most characterized are β -arrestin1 and β -arrestin2. Both of them have wide tissue distribution. Their significance in GPCR desensitization in conjunction with GRKs have been clearly demonstrated (reviewed by Ferguson et al., 1996).

Figure 1-1. Sagittal section of the human eye.

Major structures are indicated. (Modified from McDonnell, 1995)

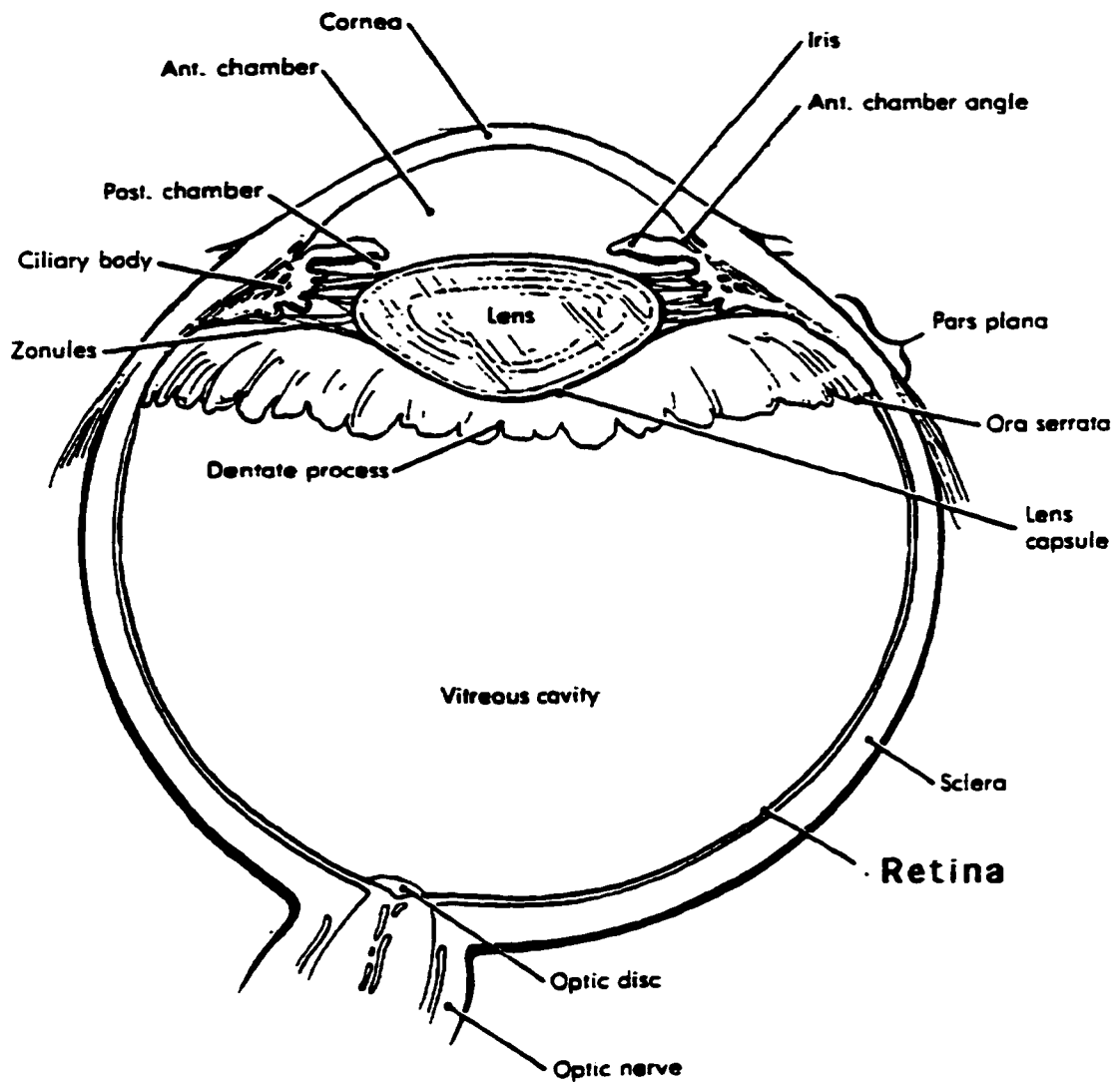
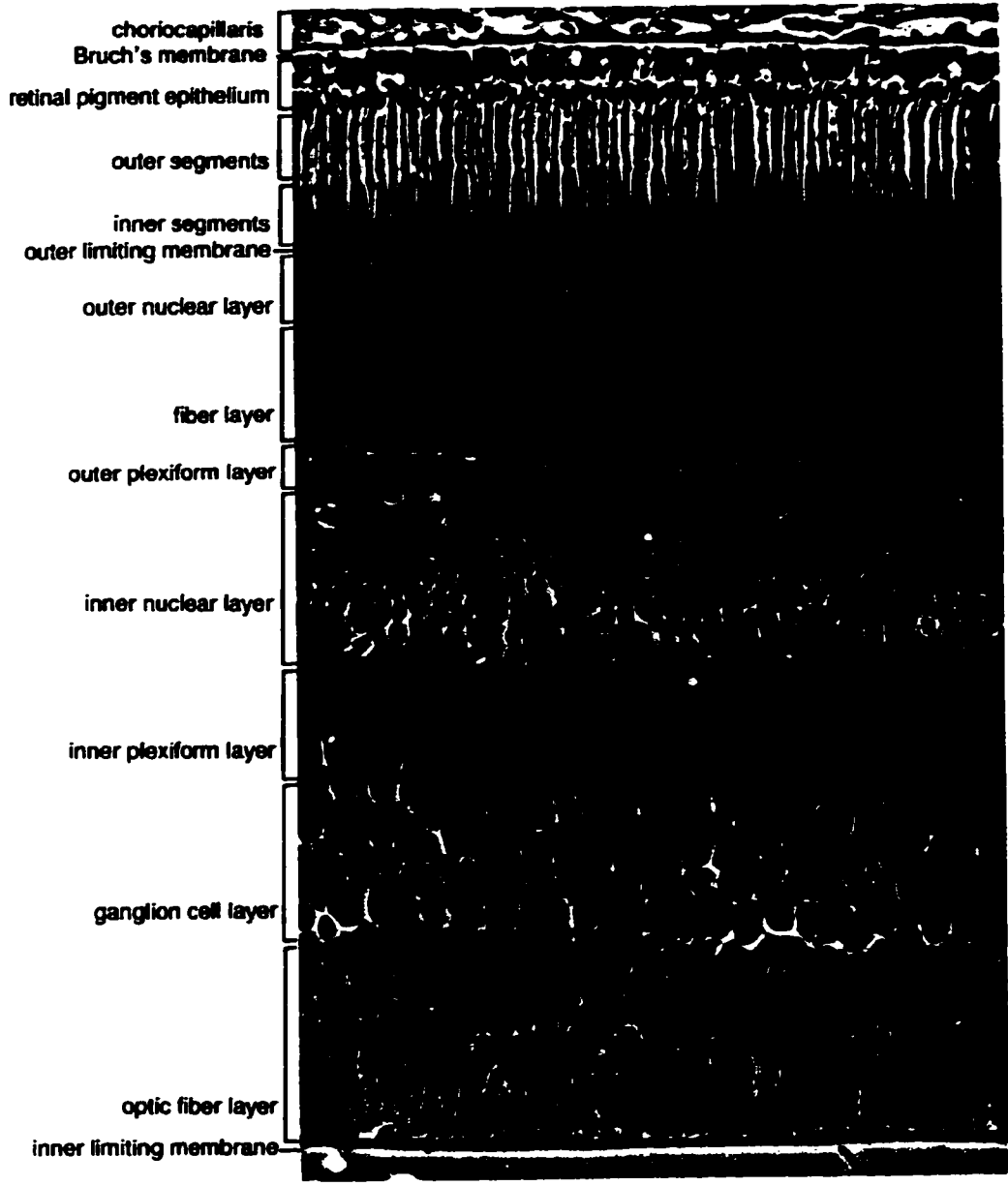


Figure 1-2. Vertical section of the human retina.

The tissue was taken approximately 1.25 mm from the center of the fovea. The material was fixed in osmium tetroxide, embedded in Araldite, sectioned at approximately 2-3 μm thickness, and photographed by phase-contrast microscopy. (Modified from Boycott and Dowling, 1969)



100µm

Figure 1-3. Diagram of human rod and cone photoreceptors.

Upper panel: Structure of human rod and cone photoreceptors (modified from Berman, 1991). *Lower panel:* Range of sensitivity of human rod and cone photoreceptors (modified from Rodieck, 1997).

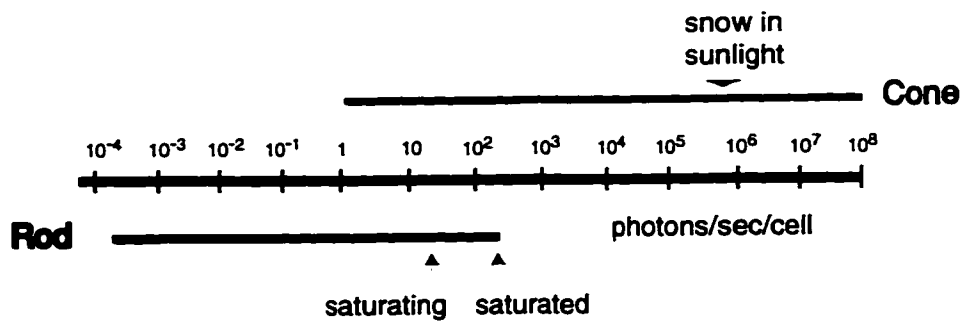
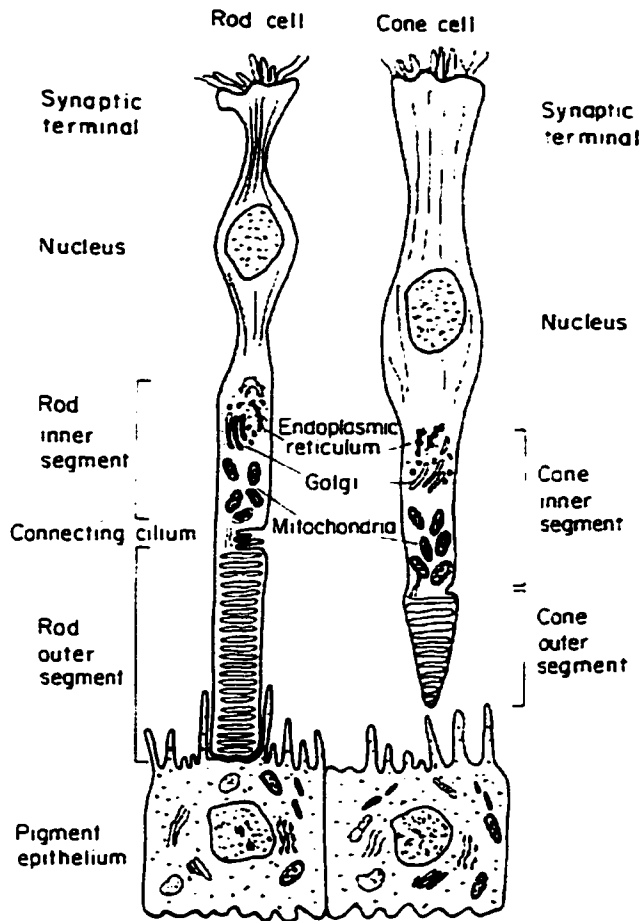


Figure 1-4. Phototransduction pathway in vertebrate rod photoreceptors.

R: rhodopsin; O: opsin; GRK1: G-protein coupled receptor kinase 1; Arr: arrestin; Gt: transducin; PDE: cGMP-specific phosphodiesterase; GC: guanylate cyclase; GCAP: guanylate cyclase activating protein; E: ion exchanger; CNG: cGMP-gated channel. (For detail, please see text.)

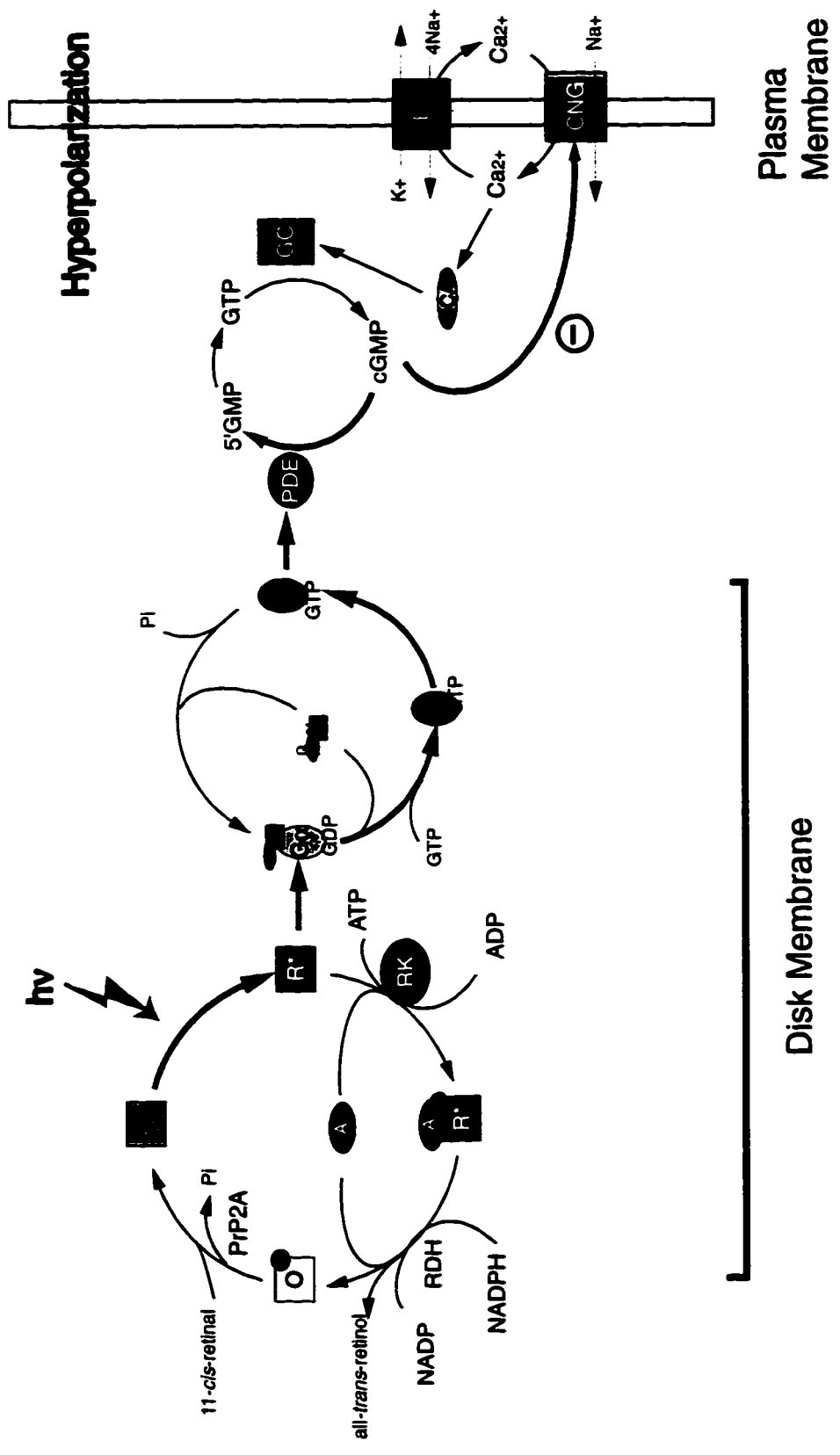


Figure 1-5. A topographic model for bovine rhodopsin in the rod disk membrane.

About 50 % of the molecule is embedded in the membrane. The loops i1, i2, i3 and i4 and the C-terminus face the cytoplasmic surface. The loops e1, e2 and e3 and the N-terminus are in the disk lumen. (Modified from Hargrave, 1992).

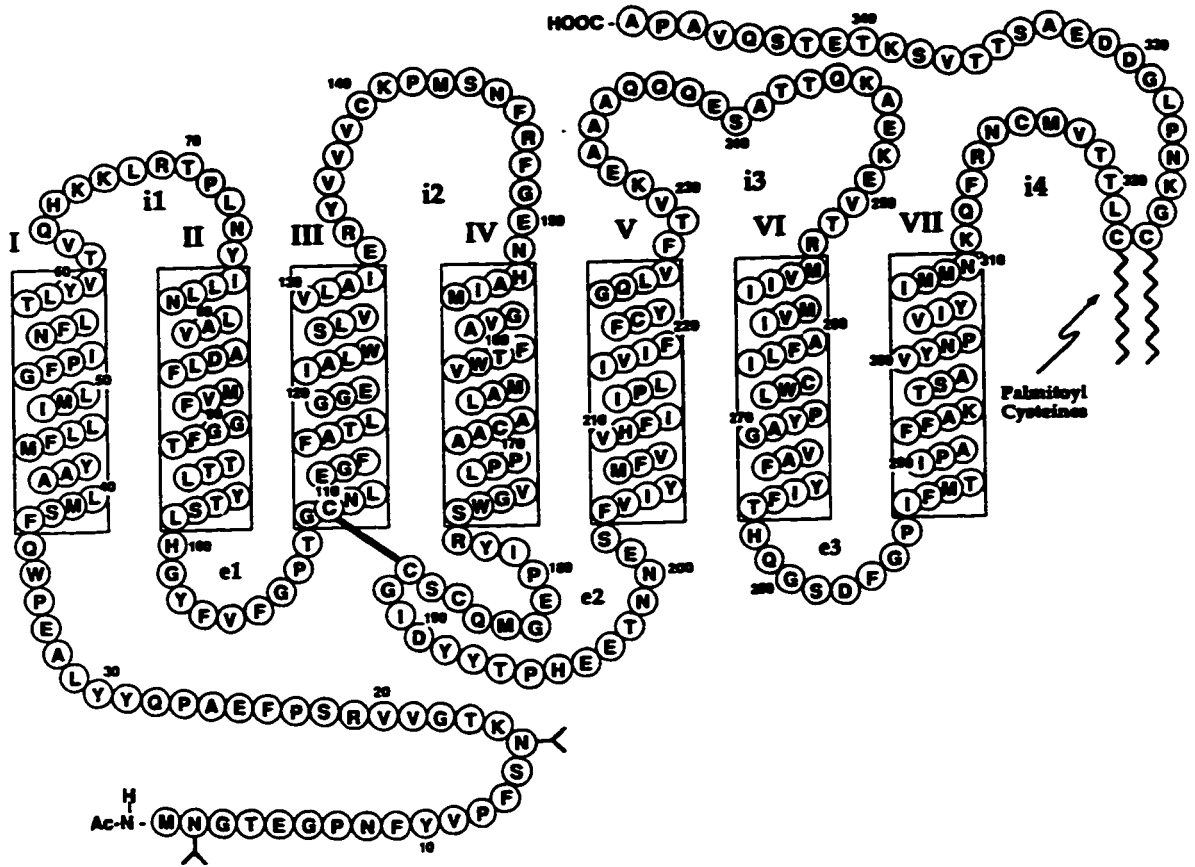


Figure 1-6. Molecular structure of bovine G protein-coupled receptor kinase 1.

The green box indicates the autophosphorylated residues. The red box shows the farnesylated C-terminus. The enclosed percentages indicate the amino acid sequence identity/similarity among the catalytic regions of GRK family members.

GRK1



	GRK2	GRK3	GRK4	GRK5	GRK6
GRK1 (RK)	58/68%	58/68%	68/78%	68/77%	69/77%
GRK2 (SARK1)		93/96%	58/69%	58/68%	59/71%
GRK3 (SARK2)			56/70%	57/68%	58/70%
GRK4 (IT-11)				82/90%	83/90%
GRK5					83/88%

Table 1-1. Phototransduction Proteins in Rods and Cones

Phototransduction Proteins	Rho	many species	red/green/blue pigment	hu/bo/mu/ra	1. Meta II decay kinetics 2. pigment regeneration kinetics	References
Pigment	Rho	many species	red/green/blue pigment	hu/bo/mu/ra	1. Meta II decay kinetics 2. pigment regeneration kinetics	Imai, H., 1997 Nathans, 1986
G α β γ	α β γ	hu/bo	α β γ	hu/bo/mu	?	G α : Lerea, 1986 G β : Ong, 1995 G γ : Lee, 1992
cGMP-PDE	α β γ δ	hu/bo	α ? ? γ	hu/bo/lizard	1. cone PDE has higher basal activity activated by lower concentration of rodG α ; 2. cone PDE has lower affinity to cone PDE γ	PDE α : Li., 1990 PDE γ : Hamilton, 1993 1. Gillespie, 1988 2. Hamilton, 1993
CNG	α β	hu/bo/ch	α ?	ch	cone CNG is more permeable to Ca $^{2+}$	CNG α : Bonigk., 1993 Miller, 1993 Picones, 1995
Receptor Kinase	GRK1	hu/bo/ra/ch	?	?	immunocytochemistry shows staining in both rods and cones	Palczewski, 1993 Zhao, in prep
Arrestin	rod Arr	hu/bo/fg	cone Arr	ra/fg	?	Craft, 1994; Abdulaeva, 1995
RetGCI	rod RetGCI	hu/bo	?	?	immunocytochemistry shows staining in both rods and cones	Shyjan, 1992 Dizhoor, 1994 Margulis, 1993
GCAP1	rod GCAP1	hu/bo/mu/fg	?	?	immunocytochemistry shows staining in both rods and cones	Gorczyca, 1994 Palczewski, 1994

Abbreviations: hu: Human; bo: bovine; mu: mouse; ra: rat; fg: frog; ch: chicken.

For all other abbreviations: please see the List of Abbreviation.

?: Data not available.

Table 1-2. Retinal Phototransduction Proteins Identified in Pineal

	yes: in hu/bo/ra#; <i>Anolis.C.</i>	hu/bo/ra <i>Anolis.C.</i>	yes: in hu/bo/ra#, No: in <i>Anolis.C.</i>	Zhao, 1997 Kawamura, 1994
Goat rhodopsin	yes	ch	yes	Okano, 1994;
Goat blue cone	yes	ch	yes	Max, 1995
Goat blue cone	yes#	ra#	yes??#	Zhao, 1997
Goat pinopsin	pinopsin	ch	No	Okano' 1994; Max 1995
Goat rhodopsin	No*	-	-	Schomerus, 1994
Goat cone ops	No*	-	-	Schomerus,, 1994
Goat rod ops	No	-	-	Carcamo, 1995
Goat cone ops a b c d	yes	bo/ra	?	Carcamo, 1995
Goat cone ops e f	yes ?	ch	yes	Bonigk, 1996
Goat cone ops	yes	ch	No: Two spliced forms	Bonigk, 1996
Goat cone ops	yes#	hu/bo/ra/ch	yes#	Zhao, 1997
Goat cone ops III	yes	ra	yes	Abe and Shinohara, 1990
Goat cone ops	yes	ra	yes	Craft, 1994
Goat cone ops	?	-	-	-
Goat cone ops	?	-	-	-

*: Results were obtained from immunocytochemistry studies only.

#: Studies reported in this dissertation.

hu: human; bo: bovine; ra: rat; *Anolis C.*: *Anolis Carolinasis*.

Table 1-3. G Protein-Coupled Receptor Kinases (GRKs)
(Modified from Palczewski, 1997)

Kinase	MW/aa [*] ; Gene Structure; Chromosomal Localization (human)	Major Occurrence of Expression ^{**}	Ce- and Post-translational Modifications	Selected <i>in vitro</i> Substrates ^{***}	Comments; General Ref. ^{****} ; Accession # ^{*****}
GRK1 (rhodopsin kinase)	62,934/561; additional splice variants; 7 exons; 13q34	Retinal photo-receptors, pinealocytes	Farnesylated and methylated at Cys ⁵⁵⁸ ; auto-phosphorylated at Ser ²¹ , Ser ⁴⁸⁸ , and Thr ²⁸⁹	Photolyzed rhodopsin; constitutively active mutants of opsin, opsin with all-trans-retinal; β 1-, β 2-adrenergic receptors, acidic peptides and proteins	The most specialized of all GRKs (Zhao <i>et al.</i> , 1995); P26327
GRK2 (β -adrenergic receptor kinase 1)	79,646/689; 21 exons; 11q13	Neurons of the CNS, associated with postsynaptic densities and in axons; olfactory epithelia	Unknown; phosphorylated by PKC	α 1-, α 2-, β 1-, β 2-adrenergic receptors; thrombin receptor, substance P-receptor; olfactory receptor photolyzed rhodopsin; N-formyl-peptide receptor; m3-muscarinic receptor	Activated by lipids, β -subunits of G-protein and PKC. (Benovic <i>et al.</i> , 1987); P2146
GRK3 (β -adrenergic receptor kinase 2)	79,803/688; 22q11	Neurons of the CNS, associated with postsynaptic densities and in axons; olfactory epithelia	Unknown	α 1-, α 2-, β 1-, β 2-adrenergic receptors; thrombin receptor, the substance P-receptor; olfactory receptor photolyzed rhodopsin; N-formyl-peptide receptor; m3-muscarinic receptor	Activated by β -subunits of G-protein; expression of GRK3 is ~10% of GRK2. (Benovic <i>et al.</i> , 1991); P26818
GRK4 (IT11)	57,693/500; three additional splice variants; 16 exons; 4p16.3	Testis	Palmitoylated	β 2-adrenergic receptors; chorionic gonadotropin receptor	Premont <i>et al.</i> (1996); P32296
GRK5	67,716/590; 10q24	Lung, heart, and retina	Unknown to be modified by a lipophilic group but regulated by lipids; autophosphorylated at Ser ⁴⁸⁴ and Thr ⁴⁸⁵	β 2-adrenergic receptor; thyrotropin receptor; photolyzed rhodopsin; m2-muscarinic receptor; neutral peptides; acidic proteins	Associated with membranes. (Kunapuli <i>et al.</i> , 1994; Premont <i>et al.</i> , 1994); P43249
GRK6	65,969/576; 5q35	Brain, skeletal muscle, pancreas, heart, and lung	Palmitoylated at the C-terminal region on one or more Cys residues (561, 562, and 565)	β 2-adrenergic receptor; m2-muscarinic receptor; acidic proteins; photolyzed rhodopsin; neutral peptides	Lower activity than other GRKs toward standard <i>in vitro</i> substrate; (Loudan & Benovic, 1994; Stoffel <i>et al.</i> , 1994); P43250

- * Posttranslational modifications were not considered.
- ** For most GRKs, the cell type expression is unknown.
- *** Agonist-stimulated receptors.
- **** Review articles or papers related to the enzymological characterization of GRKs.
- ***** EMBL/GenBank Accession Number.

CHAPTER 2

G PROTEIN-COUPLED RECEPTOR KINASE 1 IS PRESENT IN BOTH ROD AND CONE PHOTORECEPTORS

INTRODUCTION

Rod and cone photoreceptors have distinct sensitivities and phototransduction kinetics. Rods are more sensitive to light but respond in a slower time course, and are saturated easily, while cones are less sensitive but have a faster photoresponse, and can operate under bright background illumination (Chapter 1). These features enable our eyes to sense light within a large range of illumination intensity, while still maintaining high visual acuity. One of the fundamental questions in vision research is to understand cone photoresponses. However, because of the paucity of cones and the difficulties in their isolation from mammalian retina, cone phototransduction is less well-understood at the biochemical level.

Many lines of evidence have suggested that cone phototransduction is similar to the mechanism found in rods. The differences in electrophysiological response kinetics and sensitivities between rods and cones are partially due to the differences in cell-specific subsets of phototransduction proteins. Molecular identification of cone phototransduction proteins has been partially successful, including cloning of red/green/blue color pigments (Nathans et al., 1986), cone G_tβ subunits (Lerea et al., 1986; Ong et al., 1995; Lee et al., 1992), cone PDE α- and γ-subunits (Li et al., 1990; Hamilton et al., 1993), cone arrestin (Craft et al., 1994; Abdulaeva et al., 1995), and the α-subunit of the cGMP-gated cation channel (Bonigk et al., 1993). Several critical rod

phototransduction proteins are present in both rods and cones, including retinal guanylate cyclase-1 (Dizhoor et al., 1995), guanylate cyclase activating proteins (GCAP1 and GCAP2)(Gorczyca et al., 1994; Otto Bruc et al., 1997), and recoverin (Polans et al., 1993). GRK1 has also been localized in both bovine rods and cones using polyclonal antibodies, suggesting that cones may contain either GRK1 or its closely related homologue (Palczewski et al., 1993).

The C-terminus of bovine GRK1 has a CVLS motif, a consensus sequence for posttranslational modifications that result in farnesylation of the Cys residue, removal of VLS by proteolysis, and methylation of the farnesylated Cys residue on its α -carboxyl group. The role of these modifications in supporting GRK1 function is not clear, although it has been proposed that farnesylation is important for light-dependent translocation of GRK1 from the aqueous phase to the photoreceptor membrane in *in vitro* reconstitution assays. Without the farnesyl (15-carbon isoprenoid), GRK1 is fourfold less active and mostly soluble. Replacing the C-terminal CVLS of GRK1 with CVLL results in its modification by geranylgeranylation in transfected COS-7 cells (20-carbon isoprenoid), and this causes GRK1 to associate with photoreceptor membranes in their *in vitro* reconstitution system independently of receptor activation (Inglese et al., 1992).

In this study, we have used a combination of molecular cloning and immunocytochemistry methods to show that GRK1 is localized in both rods and cones of bovine, human and chicken retinas. We also discovered that chicken GRK1 is modified by geranylgeranylation at its C-terminus.

MATERIALS AND METHODS

Materials

A chicken retinal cDNA library was provided by Dr. S. Semple-Rowland (University of Florida, Gainesville). Human retinas were obtained from the Lions' Eye Bank (University of Washington, Seattle), chicken retinas were obtained from Mr. Wendell Luse of ACME Poultry Co., Inc. (Seattle, WA), and fresh bovine eyes were obtained from a local slaughterhouse (Schenk Packing Co. Inc., Stanwood, WA). The retinas were dissected under dim red light and ROS were prepared according to Papermaster (Papermaster, 1982). Oligonucleotides were purchased from Oligos Etc., Inc.

Standard molecular biology techniques

The basic molecular biology techniques used in this dissertation are according to the books entitled "Molecular cloning: a laboratory manual" (Sambrook, 1989), and "Current Protocols in Molecular Biology" (Ausubel et al., 1996).

The automated DNA sequencing was done using a *Taq* dye-deoxy terminator cycle sequencing kit (ABI-prism, Perkin Elmer), at two facilities at the University of Washington: the Molecular Pharmacology Facility and the Center for AIDS Research, DNA Sequencing Facility.

List of primers

Name	Sequence (5'---3')	Position
XZ-1*(f)	ACGAATTCACNGGNAA(A/G)CTNTA(T/C)GC	GRK: catalytic
XZ-2*(r)	ATCAAGCTT(T/C)TCNGGNGCCAT(A/G)AANC	GRK: catalytic
XZ-3(f)	GGNGGNTT(C/T)GGNGA(A/G)GT	GRK: catalytic

XZ-4(r)	AGNCC(A/C)AGGTCNGA(A/T/G)AT	GRK catalytic
XZ-5*(f)	ACT(C/G)GA(A/G) AC(C/G)GTGGTGGC	GRK1: N-terminus
O-64*(r)	CTAGGAAAGCACCGCCATGCCCGACTTGGATGAGGG	
		bRK: C-terminus
hRK3*(f)	ACAGGCCACAGGCCAAGGGC	bRK: 5' UTR
PA10*(r)	GGAAGCTGCAGAAGAGTTTGC	bRK: bp376-354
hRK4*(f)	CTGTCTACGCAAAGGATATTCAGGAC	bRK: bp1432-1458
hRK5*(f)	GTGGCCTTTGACAAAACAGACACAG	bRK: bp1486-1507
XZ-23(f)	CAAAGTGTGCGCCTGCCA	cRK: bp 707-726
XZ-26(r)	AAGTCATCCGGAGCGCCG	cRK: bp 531-548
XZ-27(f)	GGACACCCGGTTTCATGG	cRK: bp 1173-1190
XZ-28(r)	TCCAAGTGGGCCAACAGC	cRK: bp 567-588
XZ-41(f)	GGCGTTTTCCACCGTCCGTGG	cRK: bp 1573-1593

N: A/T/G/C; UTR: untranslated region

*: Named differently in Zhao et al. (1997). (see Methods)

(f) and (r): forward (sense) and reverse (antisense) primers

All primers were synthesized by Oligos Etc. Inc., Oregon.

hRK: human GRK1; bRK: bovine GRK1; cRK: chicken GRK1

Cloning of GRKs from the fovea region of the human retina.

Human fovea tissue punches were taken from 22 human retinas (Dr. Ann H. Milam, Department of Ophthalmology, University of Washington) using an 18-gauge needle. Messenger RNA was isolated (FastTrack™, Invitrogen), and RT-PCR was performed as described (Zhao et al., 1997). The degenerate oligonucleotide primers were designed according to the conserved sequences in the catalytic regions of GRKs (Zhao et al., 1995). The primer pairs used in the first-round of PCR were as follows: XZ-1 and XZ-2; XZ-3 and XZ-4; XZ-1 and XZ-4; or XZ-3 and XZ-2. The first-round of PCR contained

10 mM Tris/HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl₂, 0.2 mM dNTP, ~10 ng cDNA, and 1 μM primers. The samples were heated to 94°C for 5 min, followed by the addition of 5 U *Taq* DNA polymerase (Promega). The reactions were first cycled 5 times at low stringency (94°C for 1 min, 40°C or 50°C for 2 min, and 72°C for 3 min), and then cycled 35 times at high stringency (94°C for 1 min, 60°C or 65°C for 2 min, and 72°C for 3 min). The PCR products were separated on a 1% agarose gel, and DNA bands corresponding to the predicted size were excised and extracted using a Qiax™ Gel Extraction kit (Qiagen). This DNA was then used as a template in the second-round of the amplification reactions using XZ-1 and XZ-4 primers. The PCR conditions were similar to those described above, but the initial 5 cycles at lower annealing temperature were omitted. The products from the first and the second rounds of PCR were cloned into the pCR™II vector (Invitrogen) and sequenced either manually (Sequenase 2.0, United States Biochemical) or using an automated *Taq* dye-deoxy terminator cycle sequencing kit (ABI-prism, Perkin Elmer) at the University of Washington Molecular Pharmacology Facility.

Cloning human photoreceptor kinase

Messenger RNA was isolated from retinal tissue using a FastTrack™ mRNA Isolation Kit (Invitrogen). cDNA used in PCR was prepared by reverse transcription with oligo (dT) from 1 μg of mRNA in a 20 μl reaction (Gibco BRL). Two pairs of degenerate primers, XZ-5 and XZ-2, and XZ-1 and O-64 (named as XZ-1, XZ-5, XZ-6 and XZ-7, respectively in Zhao et al., 1997), were designed based on conserved regions in the GRKs (Zhao et al., 1997), and used to amplify human GRK1 in two overlapping fragments of 1666 bp and 1060 bp. The conditions for PCR were the same as described above. Three amplification products from two different PCR procedures were sequenced by the dye-deoxy terminator method (ABI-prism Perkin Elmer).

Cloning the 5' and 3' ends of human GRK1:

(In collaboration with Dr. Françoise Haeseleer, Zhao et al., 1997). The 5' end of the human rhodopsin kinase DNA was isolated by amplification between two primers. hRK3 (XZ-8 in Zhao et al., 1997) was designed based on a conserved sequence in the 5'-end of bovine and rat GRK1 cDNA (for the sequence of rat GRK1, see Chapter 3). PA10 was derived from the 5' region of human GRK1 (XZ-9 in Zhao et al., 1997). Two PCR products cloned into pCRTM2.1 vector (Invitrogen) were sequenced (ABI-prism, Perkin Elmer).

Rapid Amplification of cDNA ends (3'-RACE) method was performed to amplify the 3' end of the human GRK1 cDNA using the MarathonTM cDNA Amplification Kit (Clontech Laboratories, Inc.). cDNA synthesis was performed according to the manufacturer's protocol but starting from 20 µg of total RNA. 3'-RACE-PCR, containing 1 µl (~10 ng) of undiluted double-stranded cDNA, 1X reaction buffer (Boehringer Mannheim), 1.75 mM MgCl₂, 0.4 mM dNTP, 2.5 *Taq/Pwo* polymerase (ExpandTM Long Template PCR System, Boehringer Mannheim), was primed with an internal gene-specific primer hRK4 (XZ-10 in Zhao et al., 1997) and the Marathon adapter primer (AP1) (Clontech Laboratories, Inc.), 0.2 µM each. After heating at 95°C for 5 min, the reactions were cycled 40 times (94°C for 30 sec, and 68°C for 3 min). A second PCR reaction was carried out using the AP2 primer (Clontech Laboratories, Inc.) and a nested gene-specific primer, hRK5 (XZ-11 in Zhao et al., 1997) using the same PCR condition. Four PCR products were cloned into the pCRTM2.1 vector (Invitrogen) and sequenced (ABI-prism Perkin Elmer).

Cloning of chicken GRK1.

A *NcoI/SacI* restriction fragment from bovine GRK1 (535 bp) containing the catalytic region (Lorenz et al., 1991) 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. The filters were prehybridized and hybridized overnight in buffer containing 50% formamide, 5X SSC (0.75 M NaCl, 0.075 M sodium citrate), 5X Denhardt's (1g/1L each: Ficoll, polyvinylpyrrolidone, bovine serum albumin), 0.5% SDS, 5 mM phosphate, pH 7.4, and 100 μ g/ml of denatured salmon sperm DNA at 40°C. The filters were washed with 2X SSC (0.30 M NaCl, 0.03 M sodium citrate), 0.5% SDS at 55°C for 30 min. Three positive clones on duplicate filters were replated, rescreened, and sequenced (ABI-prism, Perkin Elmer).

The 5'- and 3'- coding regions of chicken GRK1 were cloned by RACE-PCR using a Marathon™ DNA Amplification kit (Clontech Laboratories, Inc.) as described above. For cloning the 5'-region, the two nested gene-specific primers used were XZ-26 and XZ-28. For cloning the 3'-region, the three nested gene-specific primers used were XZ-23, XZ-27, and XZ-41. The reactions were cycled 40 times (94°C for 1 min and 68°C for 4 min). The PCR products were cloned into the pCR™2.1 vector (Invitrogen) and sequenced (ABI-prism, Perkin Elmer).

Expression and purification of human GRK1 in bacteria:

The bacteria expression vector, pFR31 was derived from pQE40 (Qiagen) by excising out the sequence encoding dihydrofolate reductase (DHFR) using *Bgl*II and *Bam*HI digest, followed by religation of the vector. Full length or partial human GRK1 cDNAs were cloned into pFR31 (for details in constructing expression vectors for different regions of human GRK1, see Chapter 4). The plasmid DNA was transformed into the M15 *E. coli*

strain (Qiagen) for protein expression. Protein expression and purification were carried out according to the protocol provided by the manufacturer (Qiagen). Briefly, an overnight culture of M15 bacteria (150 ml) containing the expression plasmid was diluted 50 times and grown to $OD_{600\text{ nm}} = 0.7$. Isopropyl-D-galactopyranoside (1 mM) was added to induce protein expression. The cells were harvested after five hours of induction and lysed in either ~30 ml of buffer A (6 M guanidine hydrochloride, 100 mM sodium phosphate, 10 mM Tris/HCl, pH 8.0) or ~30 ml of buffer B (8 M urea, 100 mM sodium phosphate, 10 mM Tris/HCl, pH 8.0). Insoluble particles were removed by centrifugation at 80,000g for 15 min. The supernatant was mixed with 2 ml of Ni-NTA resin (Qiagen) preequilibrated with lysis buffer, washed with buffer C (8 M urea, 100 mM sodium phosphate, 10 mM Tris/HCl, pH 6.3) until $OD_{280\text{ nm}} < 0.01$, and eluted with 200 mM imidazole in buffer C. The purity of His-tagged recombinant proteins was greater than 80% (SDS-PAGE).

Generation of anti-human GRK1 monoclonal antibodies

The bacterially expressed, full length human GRK1a was dialyzed against PBS (140 mM NaCl, 2.7 mM KCl, 8.1 mM sodium phosphate, 1.5 mM potassium phosphate, 0.9 mM CaCl_2 and 0.4 mM MgCl_2), and injected into mice with Ribi adjuvant (Ribi Immunochem Research, Inc.). Two monoclonal antibodies were produced according to a standard procedure (Campbell, 1984): G8 (C-terminal specificity, Figure 2-5) and D11 (N-terminal specificity, Figure 2-5). Monoclonal antibodies were purified using protein A-Sepharose (Pharmacia).

Partial purification of human and chicken GRK1

Human GRK1 was partially purified as described in Ohguro et al. (1996). Briefly, 18 retinas were homogenized in 10 mM BTP, pH 7.5, containing 0.4% Tween 80, and 5 mM

benzamidine, and the suspension was loaded on a DEAE cellulose column, washed with the same buffer, and eluted with 100 mM NaCl.

Partial purification of chicken kinase was achieved using an immunoaffinity column, according to the manufacturer's protocol (Pharmacia). Briefly, 185 chicken retinas were homogenized in 90 ml of 10 mM BTP, pH 7.5, containing 5 mM EGTA, 5 mM benzamidine and 5 µg/ml leupeptin. After centrifugation for 10 min at 80,000g, the supernatant was loaded on mAb G8-coupled CNBr-activated sepharose column (Pharmacia), washed with the same buffer containing up to 300 mM NaCl, and eluted by 0.1 M glycine buffer at pH 2.5. The elute (1 ml fraction) was immediately mixed with 0.2 ml of 1 M Tris, pH 8.6, and stored at -80°C prior to analysis.

SDS-PAGE and immunoblotting

SDS-PAGE was performed according to Laemmli et al. (1970), using 12% acrylamide gels in a Hoefer minigel apparatus and low molecular weight markers from Pharmacia. The gels were stained with Coomassie Brilliant Blue R-250 and destained with 50% methanol and 7% acetic acid. The immunoblotting was performed essentially as described by Burnette (Burnette, 1981), using the primary antibody at 1:10,000 and an alkaline phosphatase-conjugated secondary antibody at 1:5,000 dilution (Promega).

Immunocytochemistry

(In collaboration with Dr. Jing Huang). Fresh human and chicken eyecups were fixed at 4°C overnight in 4% paraformaldehyde and infiltrated overnight in 30% sucrose. The infiltrated tissues were immersed in Tissue-Tec O.C.T. compound (Miles, Inc.), frozen on dry ice, and cryosectioned at 10 µm thickness. The sections were preincubated for 30 min at room temperature in blocking buffer (0.5% bovine serum albumin, 0.3% Triton X-100, and 5% horse serum in PBS), followed by incubation for 1 hour at room temperature in

hybridoma cell supernatant containing monoclonal antibodies to GRK1 (G8 or D11) and 0.3% Triton X-100. Controls were prepared by omitting primary antibodies from the incubation buffer or by preabsorbing antibodies with antigen (12.5 mg/ml bacterially expressed, full length human GRK1a). The sections were rinsed with PBS (140 mM NaCl, 2.7 mM KCl, 8.1 mM sodium phosphate, 1.5 mM potassium phosphate, 0.9 mM CaCl₂ and 0.4 mM MgCl₂) and incubated overnight at 4°C in Cy-2-conjugated anti-mouse IgG (1:100 in PBS) (Jackson ImmunoResearch Laboratories, Inc.). Sections were rinsed in PBS, mounted in 5% n-propyl gallate in glycerol, and coverslipped. For double labeling, tissue sections were first incubated with primary antibodies to GRK1 (G8) and red/green cone opsin (JH492) or blue cone opsin (JH455) (Chiu and Nathans, 1994), followed by secondary Cy-3-conjugated goat anti-rabbit IgG (1:100 in PBS) and Cy-2-conjugated goat anti-mouse IgG (1:500 in PBS) (Jackson ImmunoResearch Laboratories, Inc.). The sections were further processed as in the single labeling experiments.

Retinal flat mount immunocytochemistry was performed as described previously (Milam et al., 1997). Briefly, free floating samples from human retinas were treated with a mixture of methanol, dimethylsulphoxide, and 30% hydrogen peroxide (4:1:1, v/v) for 4 hours at room temperature. Samples were rehydrated through a methanol series and treated with blocking buffer (0.5% BSA, 0.3% Triton X-100, and 5% horse serum in PBS). The samples were incubated with G8 anti-GRK1 monoclonal antibody (~1.5 mg/ml diluted 1:5,000) at 4°C overnight in PBS containing 0.3% Triton X-100. The retinas were processed with a Vectastain Elite avidin-biotin-peroxidase complex (ABC) kit (Vector Laboratories, Inc.).

Prenylation of chicken kinase

(In collaboration with Dr. Kohei Yokoyama and Dr. Michael H. Gelb). RSATCVLS and RSATCVLL peptides and the derivatives containing an N-terminal biotin-CONH-(CH₂)₅-

CO- group were synthesized by SynPep (Dublin, CA). Peptides were purified by reverse-phase HPLC using a water/CF₃COOH-acetonitrile/ CF₃COOH gradient, and their structures were confirmed by electrospray mass spectrometry. The concentrations of peptides in stock solutions were determined by analysis of -SH using Ellman's reagent.

Fresh liver (42.6 g) from 12, three-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 mM tosyl-lysine-chloromethyl ketone, 30 mM tosyl-phenylalanine-chloromethyl ketone, and 10 mg/ml each of aprotinin, leupeptin, and pepstatin A; Sigma Chemical Co.). The homogenate was centrifuged at 20,000 x g for 20 min, and the supernatant was centrifuged at 140,000 x g for 1 h. The resulting supernatant (cytosol fraction) was subjected to ammonium sulfate precipitation at 0°C. The protein pellet, precipitated by 30 to 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). 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 mM biotin-RSATCGVL or biotin-RSATCVLS was incubated with the 30-50% ammonium sulfate fraction (38 mg of protein) of chicken liver cytosol and either 1 mM [³H]GGPP (geranylgeranyl pyrophosphate) or 1 mM [³H]FPP (farnesyl pyrophosphate) (both 15 Ci/mmol, American Radiolabeled Chemicals) in a total volume of 20 ml containing 30 mM potassium phosphate, 5 mM DTT, 0.5 mM MgCl₂, 20 mM ZnCl₂, pH 7.7, and 1 ml of 0.25 M NH₄HCO₃/ethanol (3:7, by volume). After 1 h at 30°C, the reaction was terminated by

boiling for 3 min, and 40 ml of avidin agarose suspension (50% aqueous suspension, Pierce) was added to the mixture to measure radioactivity transferred to the biotinylated peptide as described previously (46). 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 (47). Assays using recombinant rat PFT or PGGT-I (0.1 mg 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 (4,230 cpm and 3,756 cpm for the assays using chicken liver crude extract and either of [^3H]GGPP and [^3H]FPP, respectively, and 2,734 cpm and 2,501 cpm for the assays using rat PGGT-I/[^3H]GGPP and rat PFT/[^3H]FPP, respectively).

RESULTS

GRK in the human retinal fovea

Several approaches were employed to test for the presence of different forms of GRKs in the human retina, especially the existence of a cone-specific kinase. For example, a combination of oligonucleotide primers and PCR using freshly prepared cDNA, as well as screening of the human and bovine retinal cDNA libraries with the bovine GRK1 probe yielded only GRK1 (rhodopsin kinase). Because the human retina is rod-dominant with 95% rod and 5% cone photoreceptors (Curcio et al., 1990), these methods could have inherent problems with the greater expression of GRK1 over the putative cone kinase. To enrich with cDNA encoding putative cone kinase, 18-gauge

punches were taken from human retinas around the fovea which contains a higher ratio of cone to rod cells in addition to the cells of the neuronal retina. mRNA was isolated and reverse-transcribed, and degenerate oligonucleotide primers and PCR were used to amplify the GRKs' highly conserved catalytic regions. Among the 41 clones sequenced, 22 encoded GRK1 (Lorenz et al., 1991; Khani et al., 1996; Zhao et al., 1997), 15 encoded GRK2/3 (in this region the sequences of both kinases are identical) (Benovic et al., 1987, Benovic et al., 1991), 2 contained sequences identical to GRK5 (Kunapuli and Benovic, 1993; Premont et al., 1994), and 2 encoded GRK6 (Benovic and Gomez, 1993). No GRK4 sequence (Ambrose et al., 1992; Premont et al., 1996) was identified in this cDNA (Figure 2-1). Despite the fact that different GRKs could be amplified from this cDNA, we were unable to detect any homologue of GRK1, suggesting that the human retina contains one visual pigment kinase. Alternatively, rod and putative cone kinases are identical in the catalytic regions.

Cloning photoreceptor kinase from the human retina

The coding region of human GRK1 was obtained by PCR using primers homologous to bovine GRK1. The missing 5' end of the cDNA was obtained by PCR using primers hRK3 derived from the homologous regions between bovine and rat GRK1 (for the sequence of rat GRK1, see Chapter 3) and PA10 derived from the 5' region of bovine GRK1. The 3' end of the coding region was obtained by 3'-RACE PCR using two gene-specific forward primers in the 3' region of human GRK1 (Figure 2-2). Human GRK1 shows 84% homology with bovine GRK1 at the DNA level and 92% similarity at the amino acid level. In the catalytic region, the homology increases to 85% and 94%, respectively. Recently, Khani et al (1996) published the amino acid sequence of human GRK1 that is identical to the sequence reported in this study. The high homology of

human kinase with bovine GRK1 strongly suggests it is the human homologue of bovine GRK1.

Cloning photoreceptor kinase from the chicken retina

The cone-dominant chicken retina has been successfully used to clone the cone-specific α -subunit of cGMP-gated channel (Bonigk et al., 1993). We used this strategy to clone GRK1s present in the chicken retina. Using a probe containing the catalytic region of bovine GRK1, we screened a chicken retinal cDNA library at low stringency. From one million clones, four positives were obtained. One of them, clone 7, had the highest homology (71-73%) with a SNF-related protein kinase (SNRK) (Becker et al., 1996), but only 57% homology with bovine GRK1 cDNA. The other three identical clones (clones 10, 12, and 16) 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" motif of the ATP domain, which is conserved only among GRKs (Zhao et al., 1995). These results suggest that the sequenced kinase is a chicken homologue of bovine and human GRK1 (Figures 2-3, 2-4).

Chicken GRK1 clones were truncated at the 5' region and 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 protein, although it contained several regions that are conserved among GRK1s. An "insert" found in the chicken GRK1 sequence (Figure 2-4) should increase the molecular mass of chicken GRK1. This prediction was verified by the immunoblot using the anti-GRK1 antibody (Figure 2-5), which showed that chicken GRK1 had a slower mobility than mammalian GRK1. This insert and a shorter version of it have been observed in several

PCR amplification products using different sets of primers. cDNAs lacking this insert have not been detected by library screening, RACE-PCR, or RT-PCR.

The 3' region of chicken GRK1 clones had short and repetitive sequences followed by poly(A) tails. The sequence in this region is not homologous with any known gene, suggesting that these forms were produced by truncation or alternative splicing (Figure 2-6). We also investigated the 3'-coding regions of chicken GRK1 by RACE PCR. 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 CaaX motif (Figure 2-6). Approximately 70% of the clones had short and repetitive sequences similar to the chicken GRK1 clones obtained from the cDNA library. The divergent C-terminal sequence is in the same region as found for human GRK1b. 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. Therefore, despite the low stringency used in cloning, only one mammalian GRK1 homologue was found in the chicken retina. Given the high abundance of cones in the chicken retina, chicken GRK1 is likely to be the photoreceptor kinase of rods and cones. This hypothesis is further supported by immunocytochemical data using the G8 monoclonal antibody, which showed that chicken GRK1 is present in all photoreceptor cells of the retina (Figure 2-7).

GRK1 is localized to both rods and cones

To localize GRK1 in human retina, monoclonal antibodies were raised against bacterially expressed kinase. Two antibodies were selected for their recognition of the N- (D11) and C- (G8) terminal sites (Figure 2-5). Retinal flat mount immunolocalization with G8 monoclonal antibodies showed intense staining of cone and rod outer segments

throughout the retina (Figure 2-8). The immunostaining was blocked by preincubation of the antibody with recombinant kinase. Immunofluorescence microscopy of the human retina reacted with the monoclonal antibody against the C-terminal domain of the kinase revealed that GRK1 was present mainly in the cone outer segments, and to a lesser degree in the rod outer segments. Weak labeling was found in somata and synaptic terminals of the cones and the inner segments of rods (Figure 2-9). The immunolabeling was abolished by preincubation of the antibody with bacterially expressed GRK1 (Figure 2-9D). In double labeled sections of human retina, GRK1 was localized to cone outer segments (Figure 2-9 A, D), including those whose outer segments were reactive with anti-red/green (Figure 2-9 B, C) and -blue (Figure 2-9 E, F) cone opsins. Identical localization of GRK1 was obtained using another monoclonal antibody with a specificity toward the N-terminal GRK1 (data not shown), polyclonal antibodies raised against recombinant GRK1 (data not shown), and native GRK1 (Palczewski et al., 1993). Thus, the immunostaining was indistinguishable using antibodies of different specificity. These results support the idea that the same kinase may be present in rod cells and all classes of cone cells.

Prenylation of chicken GRK1

Chicken GRK1 has a C-terminal CaaX motif, which is the indication of a series of posttranslational modifications: prenylation of the Cysteine, proteolytic removal of aaX, and methylation of the α -carboxyl group of the S-prenyl-Cysteine (see Chapter 1). The identity of the last amino acid. "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 (reviewed by Clarke, 1992; Casey and Seabra, 1996). The C-terminal "CGVL" sequence in chicken GRK1 suggests that it is modified by

geranylgeranylation instead of farnesylation. Bovine GRK1 contains CVLS at its C-terminus and reconstitution studies have shown that farnesylated bovine GRK1 behaves differently than geranylgeranylated bovine GRK1 (Inglese et al., 1992). Thus, we decided to investigate the C-terminal modification of chicken GRK1.

We were unable to isolate chicken GRK1 for chemical analysis of the C-terminus. The chicken retina contains oil droplets which interfere with the developed GRK1 isolation procedure, in spite of the fact that similar methods were suitable for isolation of bovine GRK1 from whole retina extract (Palczewski et al., 1993; Ohguro et al., 1996). Because the last amino acid in the CaaX motifs dictates the type of prenylation in bovine GRK1 (Inglese et al., 1992), we employed model peptides to study the type of prenylation of chicken GRK1.

To determine the type of prenylation of chicken GRK1, biotin-RSATCGVL peptide corresponding to the C-terminal sequence of chicken GRK1 was used as prenyl acceptor substrates in protein prenyltransferase assays. The 30-50 % ammonium sulfate fraction of the chicken liver cytosol was used as a source of protein prenyltransferase. In control experiments, biotin-RSATCVLS, in which the CaaX motif is replaced with bovine GRK1 C-terminal sequence was used. Bovine GRK1 was shown to be farnesylated *in vitro* and *in vivo* (Inglese et al., 1992; Anant and Fung, 1992). When the biotin-RSATCGVL was incubated with chicken liver crude extract and either [³H]GGPP or [³H]FPP, geranylgeranylation of the peptide was detected in a level much higher than the farnesylation (Table 2-1). Addition of unlabeled FPP at the same concentration as [³H]GGPP did not significantly affect the geranylgeranylation of the peptide while addition of unlabeled GGPP completely abolished the farnesylation, indicating that geranylgeranyl transfer to biotin-RSATCGVL and the low level of the farnesylation are mostly due to the action of PGGT-1 present in chicken liver but not PFT. Mammalian

PFT and PGGT-1 have been shown to have different binding affinities to prenylpyrophosphates: PFT binds FPP 15-fold tighter than GGPP, and PGGT-1 binds GGPP 330-fold tighter than FPP (Yokoyama et al., 1997) Therefore, although these two enzymes can transfer both farnesyl and geranylgeranyl groups *in vitro*, PFT and PGGT-1 preferentially utilize FPP and GGPP, respectively, when these two prenylpyrophosphates present at the same concentration. In contrast to biotin-RSATCGVL, biotin-RSATCVLS is selectively farnesylated by chicken enzyme (Table 2-1). In addition, biotin-RSATCGVL is a preferred substrate for the recombinant rat PGGT-1 over PFT, whereas biotin-RSATCVLS is a preferred substrate for rat PFT (Table 2-1). These results clearly indicate that the C-terminal sequence of chicken GRK1 has a signal for geranylgeranylation by chicken PGGT-1.

DISCUSSION

Several independent lines of evidence suggest that the GRK1s cloned from the human and chicken retinas represent species-specific GRK homologues: (1) In the catalytic region, the sequence identities between the bovine and human enzyme are 85% at DNA level and 88% at the amino acid level. The identities between chicken and mammalian kinases is 73-74% and 72-75% at the DNA and protein levels, respectively. The sequence identity with other related kinases is much lower (for example, human GRK2 and GRK1 are only 58% identical in this region at the protein level). (2) The N-terminal domain is highly conserved among mammalian kinases (76-81% identical), and less conserved between the mammalian and chicken kinases (39-44% identical). This might be due to a species difference because chicken kinase contains several regions that are highly conserved only among GRK1 sequences, but not between GRK1 and other GRKs (Figure 2-4). (3) The C-terminal region of GRK1 has important functions and as a

result, the homology in this region is relatively high (81-86% identical among mammalian GRK1s and 51-62% identical between mammalian and chicken kinases). The canonical sequences for all regulatory posttranslational modifications are conserved, such as the autophosphorylation domain and C-terminal CaaX motif, found only in GRK1s, and not in other GRKs (Figure 2-4).

Rods and cones share GRK1

We concluded that the human and chicken retinas have one photoreceptor-specific kinase based on the following facts: (1) In order to investigate the putative cone-specific receptor kinase, we have explored the rod-dominant bovine retina, the cone-enriched human fovea region and the cone-dominant chicken retina by low stringency cloning and only GRK1 was found in each species. (2) PCR using degenerate oligonucleotides derived from the catalytic region of GRKs was able to amplify even weakly related GRK2/3. The relatively large number of GRK2/3 clones obtained is consistent with the adrenergic innervation of neural retina. But no new GRK was isolated during this process. (3) We generated two GRK1-specific monoclonal antibodies with different specificities (G8 and D11). Both antibodies stained the outer segments of human rods and cones. The G8 antibody also strongly labeled outer segments of the cone-dominant chicken retina, suggesting that GRK1 is present in both rods and cones. (4) The presence of GRK1 in the chicken and mammalian pineal glands has been demonstrated (Zhao et al., 1997; Chapter 3). (5) The absence of deposited homologous EST sequences derived from human retina (data not shown).

The reasons why rods and cones may share GRK1

First, the GRK family of kinases recognizes the conformations of the active receptors rather than the primary amino acid sequences (Premont et al., 1995). It has been

shown that many G proteins share common GRKs. GRK1 has been shown to phosphorylate C-terminal peptides from rhodopsin and cone pigments equally well (Palczewski et al., 1989). Therefore, it is possible, on both a molecular and enzymatic level, that cones and rods share a common kinase.

Second, if phosphorylation by a receptor kinase is the only mechanism in photoreceptor shut off, then a common kinase would produce an identical inactivation kinetics in rods and cones. However, according to the reproducibility of single photon response, rhodopsin shut-off results from a combination of several different factors (Baylor, 1996). Receptor phosphorylation by GRK1 is only one of the mechanisms, therefore other pathways might determine the differences in kinetics between rod and cone responses.

Third, as discussed in Chapter 1, the sequence difference between rhodopsin and cone visual pigments may contribute to the kinetic differences of rod and cone responses to a large extent. The regeneration rate of cone visual pigment is about five times faster than Rho (cite?). Cones also do not saturate. Therefore, cones regain their light sensitivity much faster than rods, independent of receptor phosphorylation.

Fourth, an Oguchi patient who lacks functional GRK1 has delayed recovery kinetics in both rod and cone phototransduction (Chapter 5).

Finally, GRK1 antibody (G8) was used on the retinal tissue of mice lacking GRK1 gene (generated by Dr. Melvin Simon's laboratory). No positive signal was detected from either rod or cone photoreceptors (Dr. C. K. Chen, personal communication).

In summary, GRK1 is important for quenching the active rhodopsin. If this kinase is the same in both cell types, then the factors underlying shorter and faster kinetics of the

cone response should depend on other determinants of phototransduction (Chapter 1 and 5). The results of this study will provide further understanding in cone phototransduction.

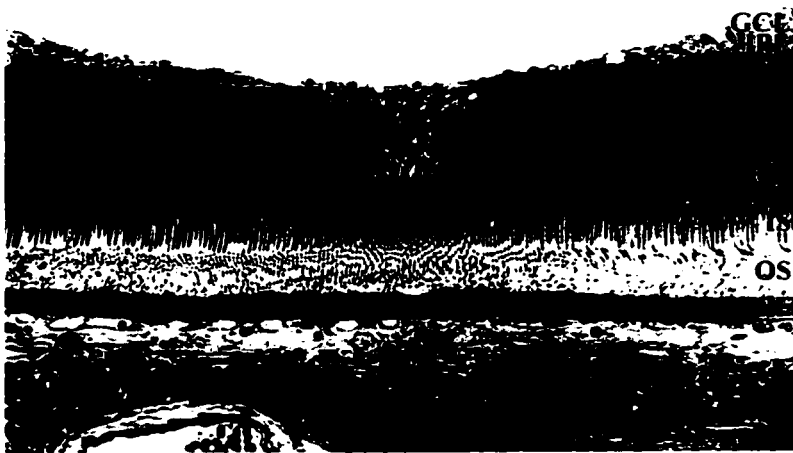
Prenylation of chicken GRK1

As discussed in Chapter 1, GRKs are likely to be associated with cell membranes where their substrates are found. Native GRK1, which is hydrophobic, is tethered to membranes and requires detergent-containing buffers for efficient extraction from rod outer segments (Palczewski, 1993). A previous conclusion has been drawn from experimental results based on a diluted reconstituted system (~0.7 mg/ml rhodopsin): only farnesylated, not geranylgeranylated bovine GRK1 is soluble in the dark but translocates to the photoreceptor cell membrane upon light stimulus (Inglese et al., 1992). However, other observations suggest that *in vivo*, native GRK1 likely resides on membranes under all light conditions, particularly if one takes into account the fact that the concentration of rhodopsin in rod outer segments is ~100 mg/ml. This concentration is much higher than the rhodopsin concentration used in the previous experiments. The likelihood that GRK1 always resides in membranes is underscored by the present results with chicken GRK1. As shown in this study, chicken GRK1 is almost certainly geranylgeranylated indicating that the length of the GRK1 prenyl group is not evolutionarily conserved and both farnesylation and geranylgeranylation are compatible with the function of GRK1. These results demonstrate that the prenyl group of GRK1 anchors it in membrane under all light conditions. The colocalization of GRK1 and rhodopsin in membranes may facilitate their specific interactions.

Figure 2-1. Different GRK isoforms in the human retinal fovea.

Upper panel: Cross section of the human retinal fovea (please see chapter 1 for detail description). The retina layers are indicated as follows: OS, outer segments; IS, inner segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. The section was stained with Richardson Blue. *Lower panel:* The GRK clones were obtained from the human fovea region by RT-PCR. Degenerate oligo primers were designed based on the highly conserved sequences in the catalytic regions of GRKs.

Fovea



GRK1 (rhodopsin kinase)	22
GRK2 (β -ARK1); GRK3 (β -ARK2)	15
GRK4	0
GRK5	2
GRK6	2
Total	41

Figure 2-2. Nucleotide and deduced amino acid sequence of human GRK1.

Lower case letters indicate the DNA sequence. Upper case letters indicate the amino acid sequence. Asterisk (*) represents stop codon.

acaggccacaggccaaggcagcagtcaggcctgctctgtctgtgaaacgctcccggcttg
gcctcggctgatgggcccctcacgcctgaagcgggcaggaagctccgggatggatttcggg
M D F G
tctttggagaccgtggtggccaactctgccttcacgcgcccaggcagctttgacggc
S L E T V V A N S A F I A A R G S F D G
agcagctcccaaccctcccgggacaagaagtacctggccaagctcaagctgccccgctg
S S S Q P S R D K K Y L A K L K L P P L
tccaagtgtgagtcctccgcgacagcctcagcctggagtttgagagtggtgcttgag
S K C E S L R D S L S L E F E S V C L E
cagcccacggcaagaagctctttcagcagttcctacaatcggcagagaagcacctgccc
Q P I G K K L F Q Q F L Q S A E K H L P
gccctggagctctggaaagacatcgaggactatgacacggcagacaatgacctccagcca
A L E L W K D I E D Y D T A D N D L Q P
cagaaggcccagaccatcctggcccagctacctggaccccaggccaactcttctgcagc
Q K A Q T I L A Q Y L D P Q A K L F C S
ttcctggatgaggggatagtggcgaagtttaaggaggggctgtggagatccaggcagg
F L D E G I V A K F K E G P V E I Q D G
ctctccagcccctgctgcaggccaccctggcacacctgggccaagccccttccaggag
L F Q P L L Q A T L A H L G Q A P F Q E
tacctgggcagcctgtacttctgaggttctctgcagtggaagtggctggaagcccagccc
Y L G S L Y F L R F L Q W K W L E A Q P
atgggggaggactggttctggaactcagggtcctggggaagggggcttcggggagggtg
M G E D W F L D F R V L G K G G F G E V
tcggcctgccagatgaaggcgaccggcaagctgtatgcctgcaagaagctgaacaagaag
S A C Q M K A T G K L Y A C K K L N K K
cggctgaagaagaggaagggtaccagggtgctatggtggagaagaagattctgatgaaa
R L K K R K G Y Q G A M V E K K I L M K
gtacacagcaggttcatcgtgtctctggcctatgcgtttgaaaccaagccagcctctgt
V H S R F I V S L A Y A F E T K A D L C
ctggtgatgaccatcatgaacggaggtgacatcaggtaccacatctacaacgtgaatgag
L V M T I M N G G D I R Y H I Y N V N E
gagaaccctggcttcccggagccgcgcgccctcttctacacggcgcagatcatctgcggc
E N P G F P E P R A L F Y T A Q I I C G
ctggagcactgcaccagaggcggatcgtctaccgcgacctcaagcccagagaagcctgctg
L E H L H Q R R I V Y R D L K P E N V L
ctggacaatgacggcaatgtccggatctctgacctgggctggcctggagctgctggac
L D N D G N V R I S D L G L A V E L L D
ggacagagcaagaccaagggtacgcagggaccaccaggttcatggccccgagctcctg
G Q S K T K G Y A G T P G F M A P E L L
caggggcaggagtagcacttctcgtggactttgcccctgggggtcaccctgtatgag
Q G E E Y D F S V D Y F A L G V T L Y E
atgattgcccagaggacccttccgagcccgtggagagaaggtggagaacaaggagctg
M I A A R G P F R A R G E K V E N K E L
aagcaccggatcatctcagagcccgtgaagtacctgataagttcagccaggccagcaag
K H R I I S E P V K Y P D K F S Q A S K
gacttctgcgaggcgtgctggagaaggaccggagaagcgcctgggggtcagagatgag
D F C E A L L E K D P E K R L G F R D E
acctggacaagctccgtgccaccctcttcaaggaccttaactggaggcagctggag
T C D K L R A H P L F K D L N W R Q L E
gctgggatgctgatgcccccttcatcccagactccaaaactgtctacgcaaaggatatt
A G M L M P P F I P D S K T V Y A K D I
caggacgtgggtgcctttccaccgtcaaagggtggcctttgacaaaacagacacagaa
Q D V G A F S T V K G V A F D K T D T E
ttctttcaggaatttgccactggcaactgccccatcccctggcaggaggagatgatcgag
F F Q E F A T G N C P I P W Q E E M I E
acgggcatctttggcgagctgaacgtgtggcgctcggacggtcagatgccggacgacatg
T G I F G E L N V W R S D G Q M P D D M
aagggcatctccggggctccagctcctcgtccaagtccaggatgtgtctggttctcctag
K G I S G S S S S S K S G M C L V S *
gtgacgcccagagctccacgtggaggaaaaggaccatacggctcgatgggggcccgcctg
cctccgtggtgccagcctggggctgctagcaaggggacacgtggttccctccaccagg
tccccatcacgccatctccttgcggccaagg. .

Figure 2-3. Nucleotide and deduced amino acid sequence of chicken GRK1.

Lower case letters indicate the DNA sequence. Upper case letters indicate the amino acid sequence. Asterisk (*) represents stop codon.

gaacggggagcagctgtaggccatggacatcggeggcttggagacgggtggtggccaactc
M D I G G L E T V V A N S
tgcttacgtgtcggcacggggtggagggccgggggctccgcccgggaccggaagcatcg
A Y V S A R G G G P G G S A R D R K H R
cgcccgccttcgcctgcccacatctccagtgagaagcccttcgcccgcctggccgg
A R L R L P H I S Q C E A L R A R L A G
cggggggctgcagaacggcgtcgcacagcaagatggcggccccaagcaagatggcggccc
G G L Q N G V A Q Q D G G P K Q D G G P
caagcaagatggcggccccaagcaagatggcggggggaggaggaagcgtccttccggtg
K Q D G G P K Q D G G G E E E A S F R W
gcagtgcggtggagcagccaatcgggaagcggctttccggcagttcctgcaagccacgcc
Q C V E Q P I G K R L F R Q F L Q A T P
gggattggcccgccggggcgctgtgggaggagctcgaggccttcgagtgctgcgagga
G L A A A G A L W E E L E A F E C E E
gggagagaggagcgcggcccgcccaacatccggaagcggttcctcaccgaggaggggc
G E R S A A A A N I R K R F F T E G G A
cgaacactgcgccttctgagcgcgcgcccacagcgcaccagcagattccggcgctcc
E H C A F L S A A A T A P P A D S G A P
ggatgactttgagccgaccgcccgcgagctgttggcccacttggaggggacggcctggg
D D F E P T R R E L L A H L E G T A W A
tccgtacctctcgtctccgtacttcggctcgttccactcagttcaaaggttggaggggca
P Y L S S P Y F G R F T Q F K W L E G Q
gccggtggggggcgacgccttcgcccagttccgagtcctgggcaaagggggcttcggcaa
P V G A D A F A D F R V L G K G G F G K
agtgtgcgcctgccaacgcgcgcccacggcaagatgtacgccaacaagaagctcaaca
V C A C Q R R A T G K M Y A N K L N K
gaagaggctcaagaagaggcagggctacgaggcggccatggtggagaagcgcacctggc
K R L K K R Q G Y E A A M V E K R I L A
gcgtgtgcacagccgcttcatcgtctctcggcctgcgccttcagaccaagaccgacct
R V H S R F I V S L A C A F Q T K T D L
ctgcctcgtatgacgctgatgaacggcggcgcacctgcggatcacgtgtacaacgtgga
C L V M T L M N G G D L R Y H V Y N D
cgaggagaacccccgggttccccggagccgagggcgtattctacacagctcagatcctatt
E E N P G F P E P R A V F Y T A Q I L L
ggcctggaacacctccaccaacaccgcatcgtctacagagacctcaaacctgagaacgt
G L E H L H Q H R I V Y R D L K P E N V
cctattggatgacgctgggcacgtccgtctgtccgacatggggttggccgtggagctgaa
L L D D A G H V R L S D M G L A V E T K
ggacggacagaaacaaaaccagggggtagcggggacacccgggttcatggccccgaggt
D G Q N K T R G Y A G T P G F M A P E V
gctgcgggacgaggagtagatattggcgggtggattacttcacgttgggggtgacctcta
L R D E E Y D M A V D Y F T L G V T L Y
tgamatgttggacgccaagggcccttccggcgcggtggggagaaggtggagaagaaaga
E M L D A K G P F R R R G E K V E K K E
ggtgacgcggcgcactctccacgactccgtgtcgtattcggagcgttcagcgcgccttc
V T R R T L H D S V S Y S E R F S A A S
gcggtctcttgcgaggggcttttggccaaagacccccaaaaacgttgggggtccggga
R S L C E G L L A K D P Q K R L G F R D
tgggaactgcgcaatgctccgatcccaaccgcttctctgcctgcatgggggagctg
G N C A M L R S Q P V F S A L H W G S W
gagcgggctcctccccccattcgttccggacccccggtcgggtttacgccaagattt
S G P P P P P F V P D P R R V Y A K D L
gggggacgtgggggctttccaccgtccgtggggtggaattggatggggccgacgcggc
G D V G A F S T V R G V E L D G A D A A
gctgtgcgacgccttcgcttcggggacggtgtccatcccgtggcaggaagagctgatga
L C D A F A S G T V S I P W Q E E L I E
gacggggctgttcgatgagctcaacgtatgggggggggacgggcggctgcccccgattt
T G L F D E L N V W G G D G R L P P D L
ggatcccaacgcccgaaccggatcggggggggggacgagatcggctacttgtgggggtgct
D P N A E P G S G G G T R S A T C G V L
gtgatggatgggggggtacagagggg

*

Figure 2-4. Sequence alignment of GRK1 from different species.

The sequences of human, rat and chicken GRK1 were determined in this dissertation (Chapter 2; Chapter 3; Zhao *et al.*, 1997). The bovine GRK1 sequence is as published (Lorenz *et al.*, 1991). The red letters indicate the non-conservative substitutions (other than R=K, L=I=M=V, D=E, S=T=A, and W=Y); the yellow box shows the catalytic region with a protein kinase signature GXGGXG; the red box demonstrates the autophosphorylation domain, with white letters indicating the autophosphorylated residues. Note that Ser-21, a minor autophosphorylation residue, is conserved among mammalian GRK1, but not in chicken GRK1. The C-terminal CAAX motif (green box) indicates posttranslational isoprenylation and carboxymethylation at the Cys residue

MDFGSLETVV	ANSAPFAARG	SFDGSSSTPSS	RDKKYLAKLR	LPPLSKCEGL	RDSI.....	Rat
MDFGSLETVV	ANSAPFAARG	SFDGSSRSQPS	RDKKYLAKLK	LPPLSKCESL	RDSL.....	Hum
MDFGSLETVV	ANSAPFAARG	SFDASSGPAS	RDKKYLARKL	LPPLSKCEAL	RESL.....	Bov
MDIGGLETVV	ANSAYVSARG	...GGPGGSA	RDKKRRARLR	LPHISQCEAL	RARLAGGGLQ	NVAQQDGGP	Chk

.....	SLEFDNLCS	QPIGKRLPQQ	FLKTDERRVP	ALQLWKDIED	YDTADDDLRP
.....	SLEFESVCL	QPIGKRLPQQ	FLQSAEKELP	ALQLWKDIED	YDTADNDLQP
.....	DLGFEGMCL	QPIGKRLPQQ	FLRTEHQHGP	ALQLWKDIED	YDTADDLRP
KQDGGPKQDG	GPKQDGGGEE	EASFRWQCV	QPIGKRLPQQ	FLQATPGLAA	AGALWEELEA	FECCEEGERS

QKAQAILAEY	LDPQGTLCFN	FLDQGMVARV	KEGPTGSQDG	LPQPLLQATL	EHLGQFPQE	YLGSLYFLRF
QKAQITLAQY	LDPQAKLPCS	FLDEGIVAKP	KEGPVEIQDG	LPQPLLQATL	AHLGQAPFQE	YLGSLYFLRF
QKAQALRAAY	LEPQAQLPCS	FLDAETVARA	RAGAG...DG	LPQPLLRAVL	AHLGQAPFQE	FLDSLIFLRF
AAAANIRKRF	FTEGGAEHCA	FLSAAATAPP	ADSGAPDD..	.FEPTRRELL	AHLEGTAWAP	YLSSTYFGRF

LQWKNLEAQP	IGEDWFLDFR	VLGRGGFGEV	SACQKQATGK	MYACKKLNKK	RLKRRKGYQG	AIVEKRILAK
LQWKNLEAQP	MGEDWFLDFR	VLGRGGFGEV	SACQKQATGK	LYACKKLNKK	RLKRRKGYQG	AMVEKRILMI
LQWKNLEAQP	MGEDWFLDFR	VLGRGGFGEV	FACQKQATGK	LYACKKLNKK	RLKRRKGYQG	AMVEKRILAI
TQFKWLEGGP	VGADAFADFR	VLGRGGFGEV	CACQKQATGK	MYANKKLNKK	RLKRRKGYQA	AMVEKRILAR

VHSRFIVSLA	YAFETKTDLC	LVMTIMGGD	VRYHIYNVDE	ENPGFPEPRA	IYYTAQIISG	LEHLQRRIV
VHSRFIVSLA	YAFETKADLC	LVMTIMGGD	IRYHIYNVNE	ENPGFPEPRA	LYTTGQIICG	LEHLQRRIV
VHSRFIVSLA	YAFETKTDLC	LVMTIMGGD	IRYHIYNVDE	ENPGFPEPRA	IFYTAQIVSG	LEHLQRRNII
VHSRFIVSLA	CAFQTKTDLC	LVMTIMGGD	LYRHYYNVDE	ENPGFPEPRA	VFYTAQIILG	LEHLQRRIV

YRDLKPEVNL	LDDGNVRIS	DLGLAVELKE	GQNKTRGYAG	TPGFMAPELL	RGEYDFSV	YFALGVTLYE
YRDLKPEVNL	LDDGNVRIS	DLGLAVELLD	GQSKTRAYAG	TPGFMAPELL	QGEYDFSV	YFALGVTLYE
YRDLKPEVNL	LDDGNVRIS	DLGLAVELKA	GQNKTRGYAG	TPGFMAPELL	LGEYDFSV	YFALGVTLYE
YRDLKPEVNL	LDDAGVRLS	DMGLAVELKD	GQNKTRGYAG	TPGFMAPEVL	RDEYDMAVD	YFALGVTLYE

MIAARGPFRA	RGEKVENKEL	KQRIISEPVK	YPKKFSQASK	DFCEQLLEKD	PEKRLGFRDG	TCDALRANVL
MIAARGPFRA	RGEKVENKEL	KHRIISEPVK	YPKKFSQASK	DFCEALLEKD	PEKRLGFRDE	TCDKLRAPHL
MIAARGPFRA	RGEKVENKEL	KQRVLEQAVT	YPKKFSQASK	DFCEALLEKD	PEKRLGFRDG	SCDGLRTHPL
MIDARGPFRR	RGEKVERKEV	TRRTLEDSVS	YSKRFSAASR	SLCEGLLEKD	PEKRLGFRDG	NCAMLRSQPV

FRDISWRQLE	AGMLIPFFIP	DSRTVYAKNI	Q	ST	VYDKADTE	FFQEFASGNC	SIPWQEMIE
FRDLNWRQLE	AGMLIPFFIP	DSRTVYAKDI	Q	ST	YAFDKADTE	FFQEFATGNC	PIPWQEMIE
FRDISWRQLE	AGMLIPFFVP	DSRTVYAKNI	Q	ST	YAFERADTE	FFQEFASGTC	PIPWQEMIE
FSALHWGSW.	SGPPEPFFVP	DKRVYAKDL	Q	ST	VELDGADA	LCDAFASGTV	SIPWQELIE

TGPFGLNVW	RPDGQMPDDM	KGITVEEAP	TAKSG	
TGIFGLNVW	RSDGQMPDDM	KGIS.GGSSS	SSKSG	
TGVFGLNVW	RPDGQMPDDM	KGVSQGEAAP	SSKSG	
TGLFDELNVW	GGDGRLEPDL	DPNAEPGSGG	GTRSAT	

Figure 2-5. Specificities of anti-GRK1 monoclonal antibodies.

Panel A. An immunoblot was probed with the G8 monoclonal antibody using partially purified GRK1 from bovine ROS (lane a), chicken (lane b) and human retinas (lane c), and bacterially expressed C- (lane d), and N- (lane e) terminal fragments of human GRK1. The G8 antibody displayed C-terminal specificity and reacted with bovine (two autophosphorylation forms), human, and chicken (two autophosphorylation forms) GRK1. *Panel B.* An immunoblot was probed with the D11 monoclonal antibody using partially purified GRK1 from bovine ROS (lane a), chicken retina (lane b), human retina (lane c), bacterially expressed C- (lane d), and N-(lane e) terminal fragments of human GRK1. The D11 antibody displayed N-terminal specificity and reacted strongly with human GRK1.

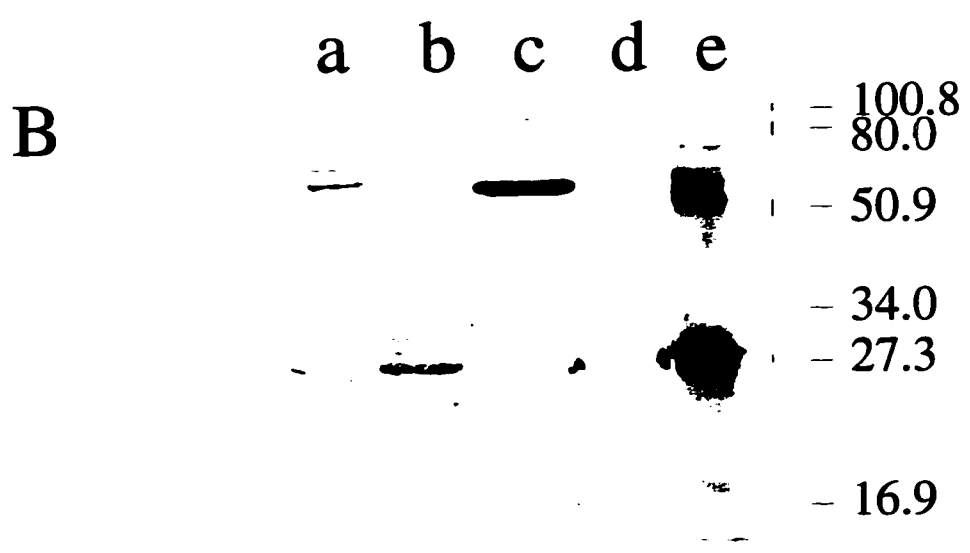
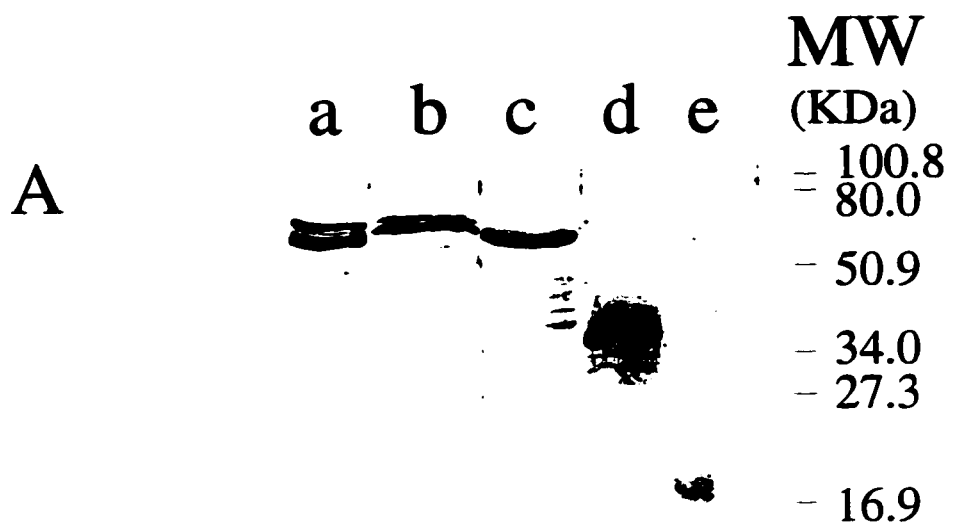


Figure 2-6. Splice variants of the chicken photoreceptor kinase.
Chicken GRK1 clones obtained by library screening (indicated by arrow) and 3'-RACE PCR have either a C-terminal sequence containing CAAX box (30%, underlined), or short and distinct C-terminal regions (70%).

Figure 2-7. Localization of GRK1 in the chicken retina.

Chicken retinal sections were incubated (A) with human GRK1a monoclonal antibody (G8); (B) antibody was preblocked by bacterially expressed human GRK1a protein; (C) without primary antibody. Scale bar = 50 μm .

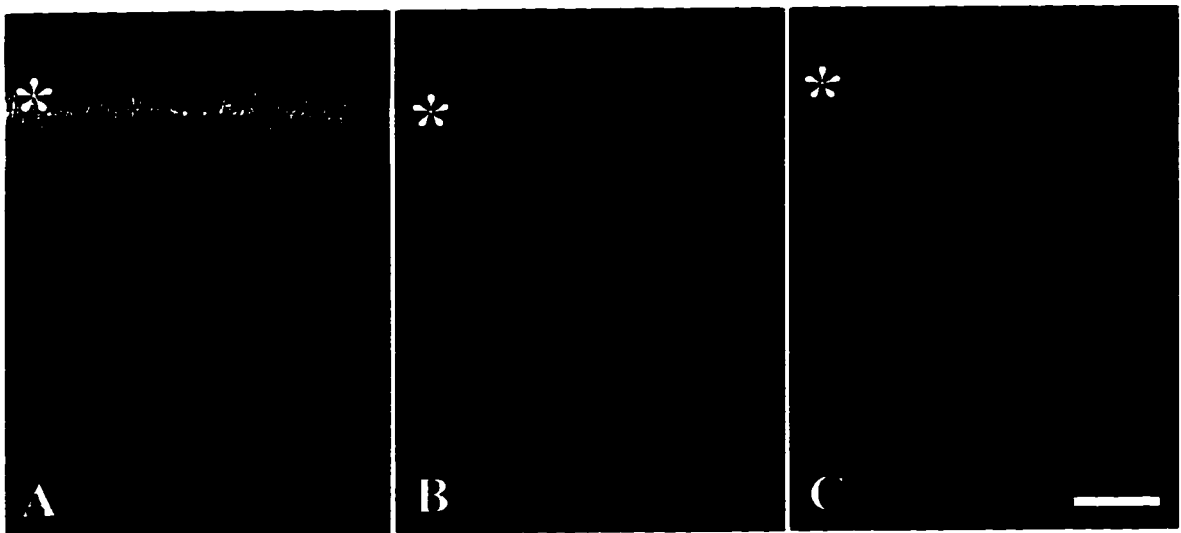


Figure 2-8. Distribution of GRK1 positive cells in the human retina.
Flat mount immunocytochemistry showing the localization of GRK1 in human rod and cone outer segments. Floating samples from human retinas were incubated (A) with human GRK1a monoclonal antibody (G8); (B) without primary antibody; (C) antibody was preblocked by bacteria expressed human GRK1a protein. Arrow heads indicate rod and cone outer segments. Scale bar = 50 μ m

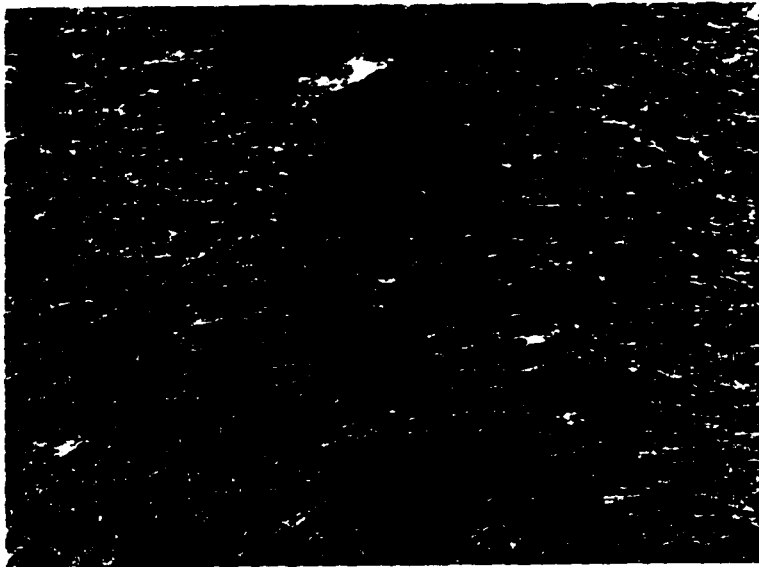
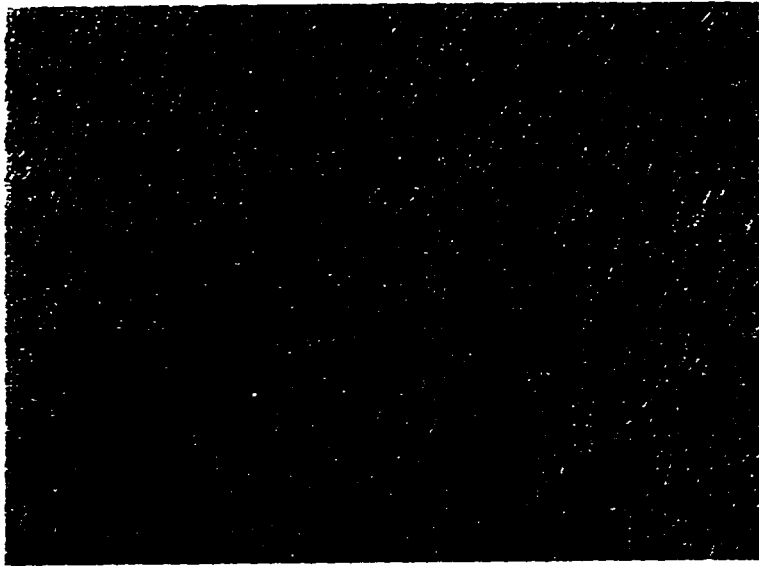
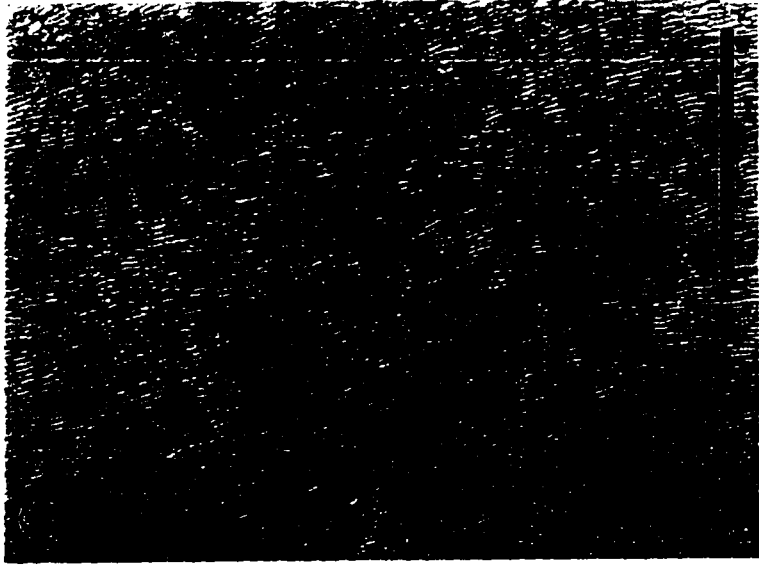


Figure 2-9. Immunofluorescence localization of GRK1 in the human retina.

(A) GRK1 immunolabeling using G8 monoclonal antibody was strongest in cone (arrows pointing down) and rod (arrowheads pointing down) outer segments. Immunolabeling was also present in cones (arrows pointing up) and rods (arrowheads pointing up) somata. (B) Addition of bacterially expressed GRK1 (20 $\mu\text{g/ml}$) to anti-GRK monoclonal antibodies abolishes GRK1 immunoreactivity. (C) Sections preincubated with buffer without anti-GRK1 showed weak autofluorescence. (Panels D, E, and F) Localization of GRK1 and red/green cone opsin (anti-red/green cone opsin polyclonal antibodies, JH492). (D) The cones and rods were immunolabeled with anti-GRK1, with the strongest labeling in the cone outer segments. (E) Anti-red/green cone opsin labeled a majority of cones. (F) Double labeling with anti-GRK1 (green) and anti-red/green cone opsin (red) showed that red/green cones are immunopositive for GRK1. (Panels G, H, and I) Localization of GRK1 and blue cone opsin (blue cone opsin polyclonal antibody JH455, from Dr. Jeremy Nathans). (G) The cones and rods were immunolabeled with anti-GRK1. (H) Anti-blue cone opsin labeled a single cone. (I) Double labeling with anti-GRK1 (green) and anti-red/green cone opsin (red) showed that the blue cone is immunopositive for GRK1. (Bar = 50 μm .)

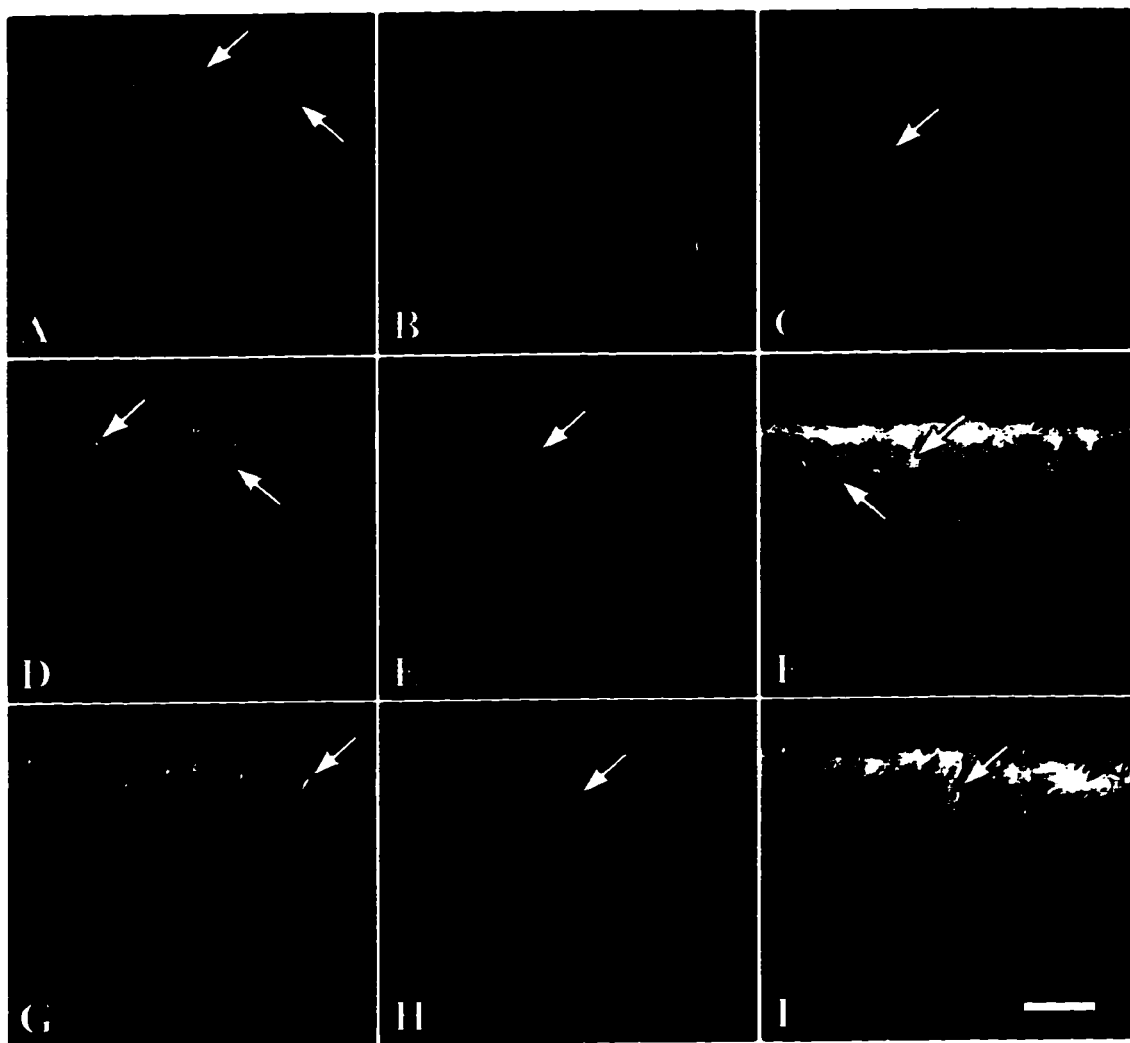


Table 2-1. Prenylation of the C-terminal peptides of chicken GRK1.

Enzyme	Peptide	Prenyl donor(s)	Radioactivity transferred
			pmole
Chicken liver crude extract	Biotin-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
	Biotin-RSATCVLS	[³ H]GGPP	0.021
		[³ H]FPP	0.557
Rat PGGT-I	Biotin-RSATCGVL	[³ H]GGPP	0.416
Rat PFT		[³ H]FPP	0.031
Rat PGGT-I	Biotin-RSATCVLS	[³ H]GGPP	0.046
Rat PFT		[³ H]FPP	2.467

Chicken liver crude extract (38 mg of protein, 30-50% ammonium sulfate fraction of the cytosol) and either 10 mM biotin-RSATCGVL or biotin-RSATCVLS were incubated at 30°C for 1 h with either 1 mM [³H]GGPP or [³H]FPP (250.3 mCi) in the absence and presence of 1 mM unlabeled FPP or GGPP. Incubation of recombinant rat PGGT-I or PFT (0.1 mg of protein) with 5 mM biotinylated peptide and either 1 mM [³H]GGPP or [³H]FPP was carried out at 30°C for 5 min under the same conditions.

CHAPTER 3

G PROTEIN-COUPLED RECEPTOR KINASE 1 IN THE PINEAL GLAND

INTRODUCTION

Vertebrate pineal glands receive light signals through sympathetic input, and regulate melatonin production (Deguchi, 1979). Bilateral enucleation in adult mammals abolishes the light effect on melatonin rhythm. (Zweig et al., 1966; Klein and Moore, 1979). However, in newborn rats, the light effects persist even after enucleation, suggesting the existence of an extraretinal pathway involving direct stimulation of light-sensitive receptors. Morphological evidence has shown that pinealocytes of neonatal rats undergo transient photoreceptor-like differentiation, indicating a close relationship between retina and pineal gland (Zimmerman and Tso, 1975; Araki et al., 1994). It is important to determine if proteins involved in perceiving light response are expressed in this tissue.

Retinal phototransduction proteins have been found in mammalian and chicken pineal gland (Chapter 1, Table 1-2). The light sensitive receptor in chicken pineal gland was identified as a pineal-specific opsin (Okano et al., 1994; Max et al., 1995). In addition, retinal red (Okano et al., 1994; Max et al., 1995) and green (Max et al., 1995) cone opsins were also found in chicken pineal (Okano et al., 1994). In rats, a PCR product appeared to be related to rod opsin, although sequence information was not presented (Araki and Taketani, 1992). These results are also in agreement with immunocytochemical data that showed opsin localization to a subset of pinealocytes in

mammals (Schomerus et al, 1994). In contrast, Gonzalez-Fernandez et al. (1993) concluded that rat pineal does not express rod opsin.

GRK1 activity has been detected in both bovine and rat pineal gland (Somers and Klein, 1984), and its expression appears to be regulated during the light/dark cycle (Ho et al., 1986). The enzymatic and functional properties of GRK1 from pineal gland were indistinguishable from those of the retinal enzyme (Palczewski et al., 1990); however, it was not known if the same enzyme was expressed in both retina and pineal, and what the substrate of this kinase was in pineal.

In this study, we have investigated the opsin and GRK1 in retina and pineal gland of human, rat and chicken using a combination of cDNA library screening, RT-PCR and immunocytochemistry methods. We have shown that retina and pineal gland have identical GRK1. We also cloned rhodopsin and blue cone opsin from rat pineal, and showed the colocalization of GRK1 and rhodopsin immunoreactivity in many of the same pineal neurons. The possible function of these proteins in pineal will be discussed.

MATERIALS AND METHODS

Materials

Retinal tissues for immunocytochemistry were prepared as in Chapter 2. A rat LL pineal cDNA library was provided by Dr. David C. Klein (National Institutes of Health). A chicken pineal cDNA library was obtained from Dr. Yoshitaka Fukada (Tokyo, Japan). Postmortem samples of formalin-fixed adult human pineal glands were obtained from Dr. C. M. Shaw, Departments of Pathology and Neuropathology, University of Washington.

List of primers

Name	Sequence (5'—3')	Position
XZ-1*(f)	TACGAATTCACNGGNA(A/G)CTNTA(T/C)GC	GRK: catalytic
XZ-2*(r)	ATCAAGCTT(T/C)TCNGGNGCCAT(A/G)AANC	GRK: catalytic
XZ-3(f)	GGNGGNTT(C/T)GGNGA(A/G)GT	GRK: catalytic
XZ-4(r)	AGNCC(A/C)AGGTCNGA(A/T/G)AT	GRK: catalytic
XZ-5*(f)	ACT(C/G)GA(A/G) AC(C/G)GTGGTGGC	GRK1: N-terminus
O-64*(r)	CTAGGAAAGCACCGCCATGCCCGACTTGGATGAGGG	bRK: C-terminus
hRK3*(f)	ACAGGCCACAGGCCAAGGGC	bRK: 5' UTR
PA10*(r)	GGAAGCTGCAGAAGAGTTTGC	bRK: bp 376-354
hRK4*(f)	CTGTCTACGCAAAGGATATTCAGGAC	bRK: bp 1432-1458
hRK5*(f)	GTGGCCTTTGACAAAACAGACACAG	bRK: bp1486-1507
XZ-14*(r)	ATAGGAGATGAGACACAT	rRK: C-terminus
XZ-15*(f)	ACTGCCAAATGAAGGCGA	rRK: bp 802-818
XZ-23(f)	CAAAGTGTGCGCCTGCCA	cRK: bp 707-726
XZ-48B(r)	AGAGTGCGCCGCGTCACCTCTTT	cRK: bp 1316-1338
PA8(r)	TTCTCTCCACGGGCTCGGAA	hRK: bp 1282-1301
XZ-19(f)	CAGCACAAGAAGCTGCGC	ropsin: bp 190-207
XZ-20(r)	TGCCTTCTGAGTGGTAGC.	ropsin: bp 721-73

N: A/T/G/C

*: Named differently in (Zhao et al., 1997). (see Methods)

(f) and (r): forward (sense) and reverse (antisense) primers

All primers were synthesized by Oligos Etc., Inc..

hRK: human GRK1; bRK: bovine GRK1; cRK: chicken GRK1; rRK: rat GRK1

ropsin: rat opsin

cDNA Cloning of rat pineal GRK1

A *NcoI/SacI* restriction fragment from bovine GRK1 (537 bp long encompassing the catalytic region) (Lorenz et al., 1991) was isolated and labeled with [α - 32 P]dCTP using a random primer kit (Amersham). This fragment was employed as a probe to screen 1×10^6 plaques from rat LL pineal cDNA library. First, the filters were prehybridized, and then hybridized overnight with the probe in buffer containing 50% formamide, 5X SSC, 5X Denhardt's, 0.5% SDS, 5 mM phosphate, pH 7.4, 100 μ g/ml of denatured salmon sperm DNA at 40°C, washed with buffer containing 2X SSC, 0.5% SDS, 0.1% sodium pyrophosphate, 5 mM phosphate buffer, pH 7.4 at 60°C for 40 min, and washed twice for 1 hour at 65°C in the same buffer. The positive clones were isolated by replating and rescreening, followed by dye-deoxy terminator sequencing (ABI-Prism; Perkin Elmer) on both strands.

Cloning rat retinal GRK1 and pineal rhodopsin by RT-PCR

Eyes and pineal glands from 18 Sprague-Dawley rats were removed from freshly decapitated heads, frozen in liquid nitrogen, and stored in -80°C until needed. Total RNA was isolated using UltraspecTM RNA Isolation system (Biotex Laboratories, Inc.). cDNA used in PCR was prepared by reverse transcription with oligo (dT) (Gibco BRL). PCR reaction was carried out using 1 μ l (~10 ng) of cDNA, 1x reaction buffer (Promega), 1.5 mM MgCl₂, 0.2 mM dNTP, 1 mM each primer in 50 μ l. The samples were first heated to 95°C for 5 min, followed by the addition of 2.5 U *Taq* polymerase (Promega). The reactions were cycled 35 times (95°C for 1 min, 45-60°C for 2 min, and 70°C for 3 min). The PCR products were cloned into pCRTM2.1 vector (Invitrogen), and sequenced (ABI-prism Perkin Elmer) on both strands. The PCR primers used to clone rat retinal GRK1 are XZ-5, XZ-14, XZ-15 and PA8 (named as XZ-1, XZ-2, XZ-3 and XZ-4 in Zhao, 1997, respectively). The PCR primers used to clone rat pineal rhodopsin are: XZ-19 and XZ-20.

The RT-PCR for pineal opsin was carried out independently three times. Each time, an intense PCR product of the correct size were formed. No comparable product was observed in the control PCR that lacked pineal cDNA.

Cloning human and chicken pineal GRK1 by RT-PCR

Chicken mRNA was isolated from young and adult chicken pineal gland using the guanidium isothiocyanate method as described previously (Sambrook, 1989). Human retinal cDNA was made as described in Chapter 2. The RT-PCR conditions were performed as described (Zhao et al., 1997, see also Chapter 2). The primers used to clone human GRK1 from pineal glands are XZ-1 and XZ-4. The primers used to clone chicken GRK1 from pineal are XZ-23 and XZ-48. The PCR products were cloned into pCRTM2.1 vector and sequenced (ABI-prism Perkin Elmer).

Immunocytochemistry of human pineal

(In collaboration with Dr. Jing Huang) Pineal samples were rinsed in chilled phosphate-buffered saline (as described in Chapter 2), pH 7.3, and cryoprotected by immersion in a 30% sucrose solution for 12 h at 4°C. Samples were embedded in OCT Tissue-TekTM (Miles), frozen, and cryosectioned at a thickness of 20 or 40 µm.

The 20-µm cryosections of human pineal were transferred to PBS and processed as free-floating sections. The immunocytochemical procedure is as described in Zhao et al. (1997). To detect rhodopsin, two antibodies were used: mouse monoclonal antibody 4D2 (Dr. Robert Molday, University of British Columbia), diluted 1:20 in PBS and rabbit polyclonal antibody (Dr. Edward Kean, Case Western Reserve University), diluted 1:10,000 in PBS. To detect GRK1, rabbit polyclonal antibody, Rho-RK (Palczewski et al., 1993), diluted 1:350 in PBS was used. Controls were prepared by omitting primary antibodies from the incubation buffer. An additional control for GRK1 was prepared by

absorbing the immune serum with excess (5 µg/ml) antigen produced from an insect cell expression system (Ohguro et al., 1996). The sections were further processed using an avidin-biotin amplification technique (Vectastain ABC system, Vector Laboratories, Inc.) with or without counterstaining with Richardson's methylene blue/azure II mixture.

Confocal microscopy

(In collaboration with Dr. Jing Huang) For immunofluorescence, 40 µm free-floating cryosections of human pineal were double-labeled with antibodies to rhodopsin (mouse, monoclonal, 4D2, diluted 1:20 in PBS), and GRK1 (rabbit polyclonal, Rho-RK , 1:350 in PBS). Triton X-100 (0.3%) was added to all PBS solutions. Nonspecific labeling was blocked by preincubating sections in normal serum diluted in PBS. Following overnight incubation in primary antibodies, sections were rinsed with PBS, and incubated overnight at 4°C in FITC-conjugated goat anti-rabbit (1:200 in PBS) and Cy-3-conjugated goat anti-mouse (1:500 in PBS) antibodies (Jackson ImmnoResearch Laboratories, Inc.). Sections were rinsed in PBS, mounted in 5% n-propyl gallate in glycerol and coverslipped.

(In collaboration with Dr. Robert N Fariss) Immunolabeling was analyzed with a BioRad MRC 600™ laser scanning confocal microscope using a dual channel scan mode. Single scan and z series images were collected and stored as unprocessed files. To evaluate signal overlap, double-labeled samples were examined sequentially under excitation at 488 nm and 568 nm. Images files selected for publication were imported into Adobe Photoshop version 3.0. Dye sublimation prints of these files were generated by the Health Science Imaging Services Bureau, University of Washington.

RESULTS

Cloning photoreceptor kinase from rat, human, and chicken pineal

A cDNA probe encoding the catalytic region of bovine GRK1 was used to screen a rat pineal cDNA library at moderate stringency. In addition, PCR amplification with specific primers was employed to clone the corresponding cDNAs from the rat retina and human pineal. Approximately 140 positive clones from 10^6 pfu of rat pineal LL cDNA library were identified, from which, 20 were sequenced. Based on sequence similarity, these clones were tentatively identified as partial or full length cDNAs encoding rat GRK1 (Figure 3-1). This identity was unambiguously established by cloning and sequencing the corresponding rat retinal GRK1. The human pineal also contained the identical kinase as human retinal GRK1. The amino acid sequence similarity among the cloned bovine, rat and human GRK1 was approximately 85-90%. The sequences between mammalian and chicken GRK1 are only 67-70% (see chapter 2, Figure 2-4). The results demonstrate, at least in rat and human, that the corresponding GRK1 is expressed in both retina and pineal. The high frequency of GRK1 clones in the rat pineal cDNA library suggest a high level of the kinase mRNA in this tissue, consistent with the high kinase activity (Ho et al., 1986, Palczewski et al., 1990).

GRK1 has been shown to be present in mammalian pineal gland, which expresses both rhodopsin and blue cone pigment (Zhao et al., 1997). A pineal specific opsin, pinopsin, as well as cone opsins, but not rhodopsin, has been also found in chicken pineal (Okano et al., 1994; Max et al., 1995). We investigated the presence of GRK1 in the chicken pineal by RT-PCR. Amplification from both young and adult chicken pineal cDNA, using primers specific for chicken GRK1, generated products of the predicted

size, while no product was seen in a control PCR reaction (data not shown). The sequences of those products were identical to that of the chicken retinal kinase. Therefore, chicken pineal gland expresses the chicken retinal GRK1, even though rhodopsin is not expressed.

Cloning of rat opsin and rat blue cone opsin in rat pineal

To determine if opsin, the substrate for GRK1, is expressed in rat pineal, a PCR product (548 bp) encoding the first to third intracellular loops of rat opsin was amplified from retinal cDNA. A product identical in size and sequence was obtained in rat pineal cDNA suggesting that rod opsin is expressed in these tissues (additional immunocytochemical evidence is shown in Figure 3-4). The PCR product encoding opsin was radiolabeled and used for screening a rat pineal cDNA library (10^6 plaque) under relaxed stringency and yielded 38 unidentified clones. Two of these clones (RO2.5 and RO9.1) were completely sequenced and shown to encode a protein that was 96% identical to mouse blue cone opsin, with 93% similarity to human blue opsin (Figure 3-2). Rat and mouse rod opsins (Barnstable and Morabito, 1994) have previously been shown to be 97% similar. The sequence similarity of rat blue opsin and chicken violet cone opsin (Okano et al., 1992), the chicken homologue of mammalian blue cone opsin, is 89%. This high homology score suggests that RO clones encode the putative rat blue opsin (Figure 3-3).

Localization of opsin and GRK1 in mammalian pineal

Immunocytochemistry studies were performed to determine which cell type in the pineal express GRK1 and rhodopsin. As shown in Figure 3-4, sections of human pineal gland contain numerous rhodopsin positive cells. Many of these cells have a bipolar or

stellate morphology typical of neurons. Identical patterns of immunolabeling were found in sections of pineal immunolabeled with either monoclonal or polyclonal antibodies to rhodopsin. In control experiments, sections of human hippocampus were not immunoreactive with anti-rhodopsin or anti-GRK1 .

The distribution and morphology of the GRK1 positive and rhodopsin positive cells in the human pineal were very similar, suggesting that two proteins might be colocalized in the same population of pinealocytes. Confocal imaging of pineal sections double-labeled with antibodies to rhodopsin and GRK1 confirmed that cells containing both these proteins were present (Figure 4-5). Control sections in which primary antibodies were omitted or preabsorbed with purified antigen showed no labeling.

Very likely, rhodopsin/GRK1-positive pinealocytes also express hydroxyindole O-methyltransferase (HIOMT), an enzyme that is critical in melatonin synthesis, because most pinealocytes in mammalian pineal contain this enzyme (Kuwano et al., 1983). In bovine pineal, most pinealocytes were HIOMT positive and double-labeling experiments showed a subpopulation of these cells also express GRK1 (data not shown). However, the antibody against HIOMT (Kuwano et al., 1983) did not cross react with human HIOMT; thus, we could not determine if the rhodopsin/GRK1 positive cells are a subset of those expressing HIOMT in human pineal, although we believe that this is likely.

DISCUSSION

The high homology of rat kinase with other GRK1s indicates it is a rat homologue of human bovine and chicken GRK1. GRK1 localizes to the same pinealocytes that contain its putative substrate, opsin. The expression of these two phototransduction-related proteins may reflect the phylogenetic function of the pineal in photoreception

(Zimmerman and Tso, 1975). In this light, it is particularly surprising that not only rod opsin, but also putative blue cone opsin is found in the rat pineal, because blue cone cells are relatively rare in the rodent retina (Sz'el and Rohlich, 1992, Sz'el et al., 1993). The presence of cone opsin is consistent with the finding that both rod and cone arrestins are expressed in the rat pineal (Abe and Shinohara, 1990; Craft et al., 1994). The chicken retinal kinase has been shown to be in both rods and cones (Chapter 2). The expression of this kinase in chicken pineal gland which contains no rod opsin, is consistent with the existence of only one kinase for both rods and cones. However, the possibility of a pinopsin-specific photoreceptor kinase in chicken pineal is not excluded.

In addition to the possibility that the phototransduction proteins in pineal represent phylogenetic vestiges, it is also possible that these proteins are components of a functional photoreceptive system. It is known that light acutely suppress melatonin production in chicken pineal cells, and this effect persists in culture (Deguchi, 1981), suggesting that the pinealocytes contain a light-sensitive cascade coupled to melatonin production. Interestingly, neural retinas of golden hamster also exhibit circadian rhythms of melatonin synthesis for several days in culture (Tosini and Menaker, 1996). An argument was made that the mammalian pineal is a secretory organ and no longer responds to direct illumination, as measured by assaying enzyme activities related to melatonin production (Moore and Klein, 1974). This observation, however, does not exclude the possibility that a photoreceptive system is present in mammalian pineal that serves other functions, or is coupled to melatonin production via an indirect mechanism that involves other parts of the visual system.

In summary, these studies provide (1) the molecular identification of rat GRK1, (2) evidence that rod opsin and GRK1 are expressed in both mammalian retina and pineal

gland, (3) evidence that chicken retinal photoreceptor kinase is also expressed in opsin-less pineal gland, (4) colocalization of rod opsin and GRK1 in mammalian pinealocyte, and (5) identification of putative blue cone opsin in rat pineal gland.

Figure 3-1. Nucleotide and deduced amino acid sequence of rat GRK1.

Lower case letters indicate the DNA sequence. Upper case letters indicate the amino acid sequence. Asterisk (*) represents stop codon.

ctggccattcggtgggatcccaagcaccctggccacggccgtgtgcctccctttgtccc
acaagggcaggaacaggccataggccaagggcaccaggtcaagcctgctttggctgctgg
tgccttgagcttgacttcagcttatgggtcttaacacttgaagtggacacagggcatcag
caggatggatctcggtccttggaaaccgtggggccaactctgccttcattgcggcccg
M D F G S L E T V V A N S A F I A A R
aggcagctttgacggaagcagcaccctcttcccagagacaagaagtatctggccaagct
G S F D G S S T P S S R D K K Y L A K L
ccggttgcctccactgtccaagtgtgagggctctccgtgacagcatcagcctagagttga
R L P P L S K C E G L R D S I S L E F D
caacctgtgctcgagcagcccattggcaagaggctcttccagcagttcctgaagacaga
N L C S E Q P I G K R L F Q Q F L K T D
cgagaggcatgtgcccggctctggagctctggaaggacatcgaggactatgacactgccga
E R H V P A L E L W K D I E D Y D T A D
tgacgacctacggccacagaaggctcagggcatcctggctgagtacctggacccacaggg
D D L R P Q K A Q A I L A E Y L D P Q G
cacactcttctgcaacttctggaccagggcatggggcaagagtgaaggaagggccaac
T L F C N F L D Q G M V A R V K E G P T
tggggccaggatgggctcttccagcctctgctgcaggccacactggagcacttgagtca
G S Q D G L F Q P L L Q A T L E H L S Q
gggcccctccaggagtacctgggaagcctatatttctgcgggttcttccagtgggaagt
G P F Q E Y L G S L Y F L R F L Q W K W
gttggaaagcccagcccataaggtgaagactgggttttggacttcagggctcctggggaagg
L E A Q P I G E D W F L D F R V L G K G
gggcttggggaggtgtccgcctgccaatgaaggcagccggcaagatgtacgcttgcaa
G F G E V S A C Q M K A T G K M Y A C K
gaagctgaacaagaagcggtgaagaagaggaaggggtaccagggcgccatcgttgagaa
K L N K K R L K K R K G Y Q G A I V E K
gaggattctagctaaagtacacagcaggttcatcgtgtctctggcctatgcattcgaaac
R I L A K V H S R F I V S L A Y A F E T
caagaccgactctgtctggtgatgacctcatgaatggaggtgacgtaaggtaccacat
K T D L C L V M T I M N G G D V R Y H I
ctacaatgtgatgagagaacccccggcttccggagccacagagccatctactacacagc
Y N V D E E N P G F P E P R A I Y Y T A
acagatcatcagtgccctggagcactgcatcagagacgattgtctaccgtgacctcaa
Q I I S G L E H L H Q R R I V Y R D L K
gccagagaacgtgctgctggacaacgatggcaacatccgaatttctgacctggactggc
P E N V L L D N D G N I R I S D L G L A
cgtcgagttgaaggaagggcagaataagaccaagggctatgcagggacccaggttcat
V E L K E G Q N K T K G Y A G T P G F M
ggccccgagctcctgcgaggcgaagagtacgacttctctgtggactactttgcctggg
A P E L L R G E E Y D F S V D Y F A L G
ggtcacattgtacgagatgatcgcagccagaggacccttccgagcccaggagagaaggt
V T L Y E M I A A R G P F R A R G E K V
ggagaacaagagctttaaacagcgcacatctcagagcccgtgaagtaccagagaagtt
E N K E L K Q R I I S E P V K Y P E K F
cagccagggcagcaaggacttctgtgagcagctgttagagaaggaccgggagaagcgct
S Q A S K D F C E Q L L E K D P E K R L
gggttccgggatggaacctgtgacgccctaagggcgaatgtcctcttcaaagatatcag
G F R D G T C D A L R A N V L F K D I S
ctggagacagctcgaagccggaatgctcatccctccgttcatcccagactctaggactgt
W R Q L E A G M L I P P F I P D S R T V
ctatgccaaagaacattcaggatgtgggtgcctttccacagtcaaggggtgtgggttttga
Y A K N I Q D V G A F S T V K G V V D
caaagcagacacagaattcttccaagaattcgccctccggcaactgctccattccctggca
K A D T E F F Q E F A S G N C S I P W Q
ggaggagatgattgagactgggttcttccggggacctcaacgtgtggcgcccagacggctca
E E M I E T G F F G D L N V W R P D G Q
aatgcccgatgacatgaagggaatcaccgtggaggagcagcccctacagccaaatcagg
M P C D M K G I T V E E A A P T A K S G
gatgtgtctcatctcctagcctagtgttgggggaagcctggggctggctgtatagagag
M C L I S *
agggaaaggatcacagagctcaggggaggagagtgaccctgggtgtcagcctgtgccactcc
atccaggtcaccatgcaactgtgtcttcacaaggagccagcaagaatcccggtttagacc
ctagaagcatggcactccattttttgtcctgattcattcaaaaaagcaaaaaaaggc
cacagattgaacaataaa

Figure 3-2. Nucleotide and deduced amino acid sequence of rat blue cone opsin.

Lower case letters indicate the DNA sequence. Upper case letters indicate the amino acid sequence. Asterisk (*) represents stop codon.

gaattcggcagcagaccagaggtggtggtgagccatccaagcgcaagatgtcgggagag
M S G E
gacgagttttacctgtttcagaatatctcctcagtgggggccctgggatgggcctcagtac
D E F Y L F Q N I S S V G P W D G P Q Y
cacattgctcccgtctgggccttccacctccaggcagccttcatgggatttgtcttctt
H I A P V W A F H L Q A A F M G F V F F
gcagggacccccactcaatgccacagtggtggtggccacactgcactacaaaagttgagg
A G T P L N A T V L V A T L H Y K K L R
cagccccctcaactacattctgggtcaatgtatccctcggaggcttctctcttctgcattctc
Q P L N Y I L V N V S L G G F L F C I F
tctgtcttcacagtcttcatcgccagctgtcaggatacttctcttgggtcgccacggt
S V F T V F I A S C H G Y F L F G R H V
tgtgctctggaggccttcttgggctctgtagcaggtctagtacaggatgggtccttggct
C A L E A F L G S V A G L V T G W S L A
ttctggcctttgagcgtaccttgtcatctgtaaacctttggcaacatccgcttcaac
F L A F E R Y L V I C K P F G N I R F N
tccaagcagcactgacgggtggttctgattacttggaccattggcagcggggtgctccatc
S K H A L T V V L I T W T I G I G V S I
ccgcccccttcttgggtggagcaggttcatccctgagggcctgcagtgctcctgtggccccg
P P F F G W S R F I P E G L Q C S C G P
gactggtacaccgtgggcaccaagtatcgaagcgagcactatacctggtttctgttcatc
D W Y T V G T K Y R S E H Y T W F L F I
ttctgtttcatcttcttcttccctcatctgttctcctacttccagttgtctgaggact
F C F I I P L S L I C F S Y F Q L L R T
ctcagagctgtggcgcccagcagcaagagtcagctacaactcaaaaggctgaacggggag
L R A V A A Q Q Q E S A T T Q K A E R E
gtgagccataggtggtggtgatgggtgggatccttctgtctctgtctacgtgccctatgct
V S H M V V V M V G S F C L C Y V P Y A
gccctggccatgtacatggtcaacaatcgtaaccacgggctgtacttacggcttgtcacc
A L A M Y M V N N R N H G L Y L R L V T
atccccgccttctttccaagagttcatgtgtctacaaccccatcatctactgcttcatg
I P A F F S K S S C V Y N P I I Y C F M
aataagcagttccgggcctgcatcctggagatggtgtgcaggaagccatgacagacgaa
N K Q F R A C I L E M V C R K P M T D E
tctgacatgtctggctctcagaaaacagaagtttctactgtttcttccagcaaaagttggc
S D M S G S Q K T E V S T V S S S K V G
cctcactaaggaccctcagttggcctgctggcagcaacctggggccacattgaattatc
P H *
tacatctccattagaattcaaaccattaacaatgtagggaaacagacaggagcagtggtcat
ctggccgggtcagtttcattttctgttactctctccctgtcagcagggccaacagggcca
tttcaacagtcacagtttctaagtgatcccttcatctctgggagaactgtgagagaagggc
cgtgcctctggtgtatgggtaacacctgggtgttcttttattccgtgctgggatctgagt
tgatatactctgttagaataagcttggcatggggcagtggtgaagaaaaagcagcagacac
caaacacctgagtgagctgaagcttccctatgaggcctctccagttgctgcccgtctgg
gctatggctgccctgcccagaactgcccwccctcactcatctgacagttatttctaagctc
tctccccctcatttggctaaatgtatccatttaaagctgtgtgttgattaaagttagtg
cttaatctcttgacgacctcaaaaaaaaaaaaaaaaaaa

Figure 3-3. Sequence alignment of rat, mouse, human, and bovine blue opsins and chicken violet opsin.

The sequence of rat blue opsin was determined in the present study; the human, mouse, and bovine blue cone opsin sequences are as published (Nathans et al., 1986; Chiu, 1994). The sequence of the chicken violet opsin was taken from Okano et al. (1992). The shaded area indicates arbitrarily chosen transmembrane segments (I-VII); the blackened area indicates the unconserved substitutions (other than R=K, L=I=M=V, D=E, S=T=A, and W=Y); the shaded Lys (K) in the transmembrane segment VII is the site of chromophore attachment; the Cys residues marked near the C-terminus indicate putative palmitoylation sites; and the black arrows indicate potential phosphorylation sites.

R	MS--GEMFELFENSSGPGWDGPQYHIAPWAFLOAFMGVVFAGTPLNAT	52
M	MS--GEMFELFENSSGPGWDGPQYHLAPWAFLOAFMGVVFAGTPLNAT	52
H	MPEMDEEELFENSSGPGWDGPQYHIAPWAFLOAFMGVVFAGTPLNAT	54
B	MSKMEDEEELFENSSGPGWDGPQYHLAPWAFLOAFMGVVFAGTPLNAT	55
C	MS--GEMFELFENSSGPGWDGPQYHIAPWAFLOAFMGVVFAGTPLNAT	52

R	VLVATLRYKKLRQPLNYLLVNVSSGFLFCISVFEVFTIASRGGYFLFGRHVCAL	107
M	VLVATLRYKKLRQPLNYLLVNVSSGFLFCISVFEVFTIASRGGYFLFGRHVCAL	107
H	VLVATLRYKKLRQPLNYLLVNVSSGFLFCISVFEVFTIASRGGYFLFGRHVCAL	109
B	VLVATLRYKKLRQPLNYLLVNVSSGFLFCISVFEVFTIASRGGYFLFGRHVCAL	110
C	VLVATLRYKKLRQPLNYLLVNVSSGFLFCISVFEVFTIASRGGYFLFGRHVCAL	107

R	EAFLGVAAGLVTGWSLAFLAFERYLVICKPFGNERFSSKHALVVLITWTIGIGV	162
M	EAFLGVAAGLVTGWSLAFLAFERYVVICPFGNERFSSKHALVVLATWTIGIGV	162
H	EAFLGVAAGLVTGWSLAFLAFERYIVICKPFGNERFSSKHALVVLATWTIGIGV	164
B	EAFLGVAAGLVTGWSLAFLAFERYIIVICKPFGNERFSSKHALVVVATWTIGIGV	165
C	EAFVGTGAGLVTGWSLAFLAFERYIVICKPFGNERFSSKHALVVVATWTLIGVGV	162

R	SIPPPFGWSRFIPEGLQCSCGPDWYTVGTYRSEAYTWELFIFCFIIPLSLIQFS	217
M	SIPPPFGWSRFIPEGLQCSCGPDWYTVGTYRSEAYTWELFIFCFIIPLSLIQFS	217
H	SIPPPFGWSRFIPEGLQCSCGPDWYTVGTYRSEAYTWELFIFCFIIVPLSLIQFS	219
B	SIPPPFGWSRFVPEGLQCSCGPDWYTVGTYRSEAYTWELFIFCYIVPLSLIQFS	220
C	GLPPPPFGWSRYMPEGLQCSCGPDWYTVGTYRSEAYTWELFIFCFIIVPLSLIQFS	217

R	YFQLLRLRAVAAQQQESATTQKAEREVSERVVVMVGSFCLCYTPYAAEAMYMVN	272
M	YSQLLRLRAVAAQQQESATTQKAEREVSERVVVMVGSFCLCYTPYAAEAMYMVN	272
H	YTQLLRLRAVAAQQQESATTQKAEREVSERVVVMVGSFCLCYTPYAAEAMYMVN	274
B	YSQLLRLRAVAAQQQESATTQKAEREVSERVVVMVGSFCLCYTPYAAEAMYMVN	275
C	YSQLLRLRAVAAQQQESATTQKAEREVSERVVVMVGSFCLCYTPYAAEAMYMVN	272

R	NRN--HGLYLRLVLTIPAFFSSSSCVYNPIIYCFMKNQFRACILEWVCKRPMTDES	326
M	NRN--HGLDLRLVLTIPAFFSSSSCVYNPIIYCFMKNQFRACILEWVCKRPMADES	326
H	NRN--HGLDLRLVLTIPAFFSSSACIYNPIIYCFMKNQFRACIMWVCKRPMTDES	328
B	NRN--HGVDLRLVLTIPAFFSSSACVYNPIIYCFMKNQFRACIMWVCKRPMTDESE	330
C	NRD--HGLDLRLVLTIPAFFSSSACVYNPIIYCFMKNQFRACIMWVCKRPLTDDSD	326

R	MSGS--QKTEVSTVSSSKVGP	346
M	MSGS--QKTEVSTVSSSKVGP	346
H	TCSS--QKTEVSTVSSSTVGP	348
B	LSSS--QKTEVSTVSSSOVGP	350
C	ASTSA--QKTEVSSVSSSOVGP	347

▲ ▲ ▲ ▲

Figure 3-4 Distribution of rhodopsin and GRK1-positive cells in human pineal

Distribution of rhodopsin and GRK1 positive cells in the human pineal. At lower magnification, numerous rhodopsin positive (A) and GRK1 positive (B) cells are visible. These labeled cells have very similar morphology and patterns of distribution. Rhodopsin immunolabeling with a monoclonal antibody (A) or a polyclonal antibody (C) is indistinguishable. At higher magnification, the presence of rhodopsin (C) and GRK1 (D) can be detected in the somata and fine processes of these cells. Sections in which primary antibody have been omitted are devoid of label (E). The specificity of the GRK1 immune serum used in these studies was confirmed by preincubating the serum with purified heterologously expressed antigen, which abolished immunoreactivity (F). Scale bar=100 μm in A, B and E; scale bar =50 μm in C, D and F.

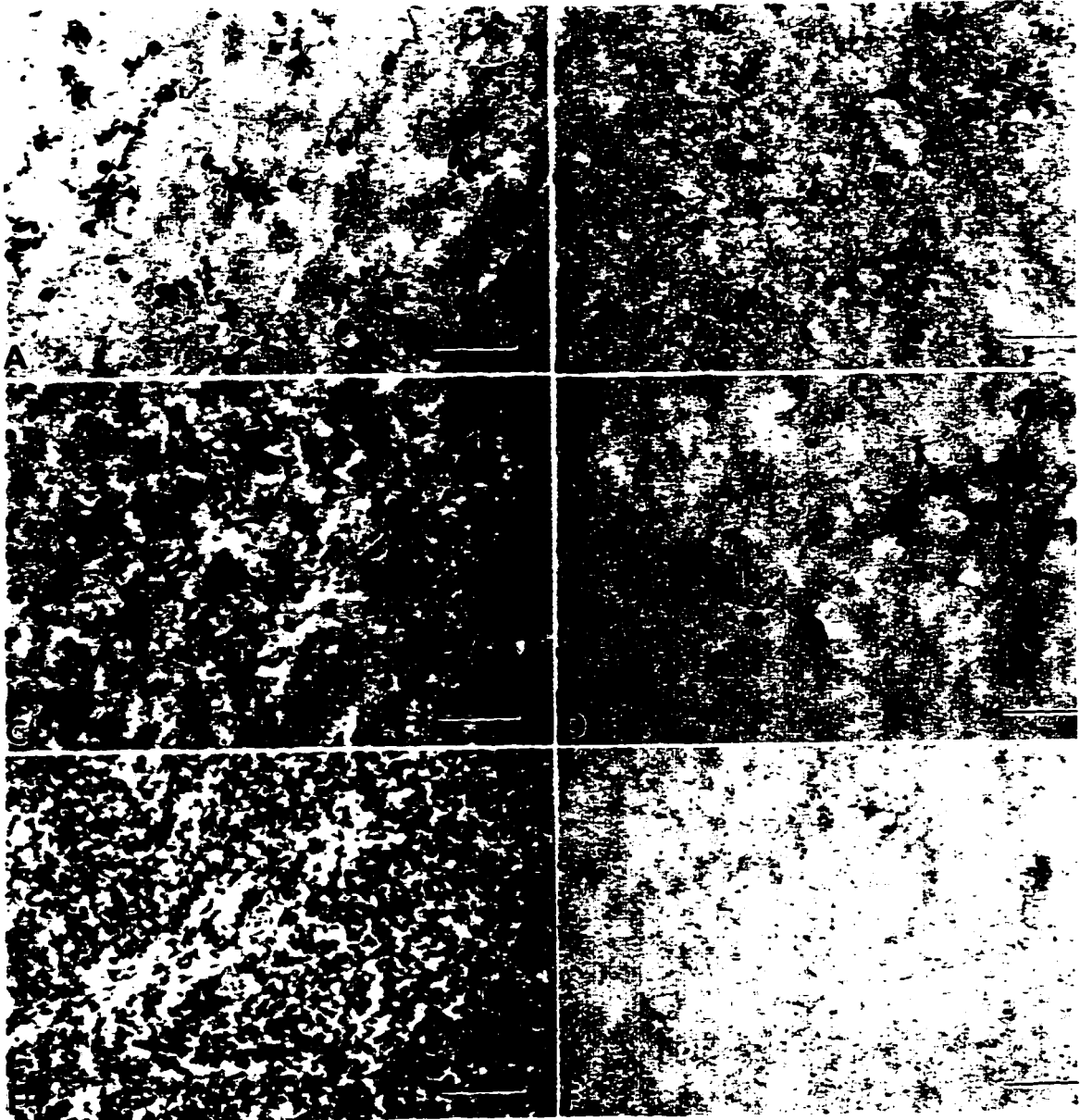
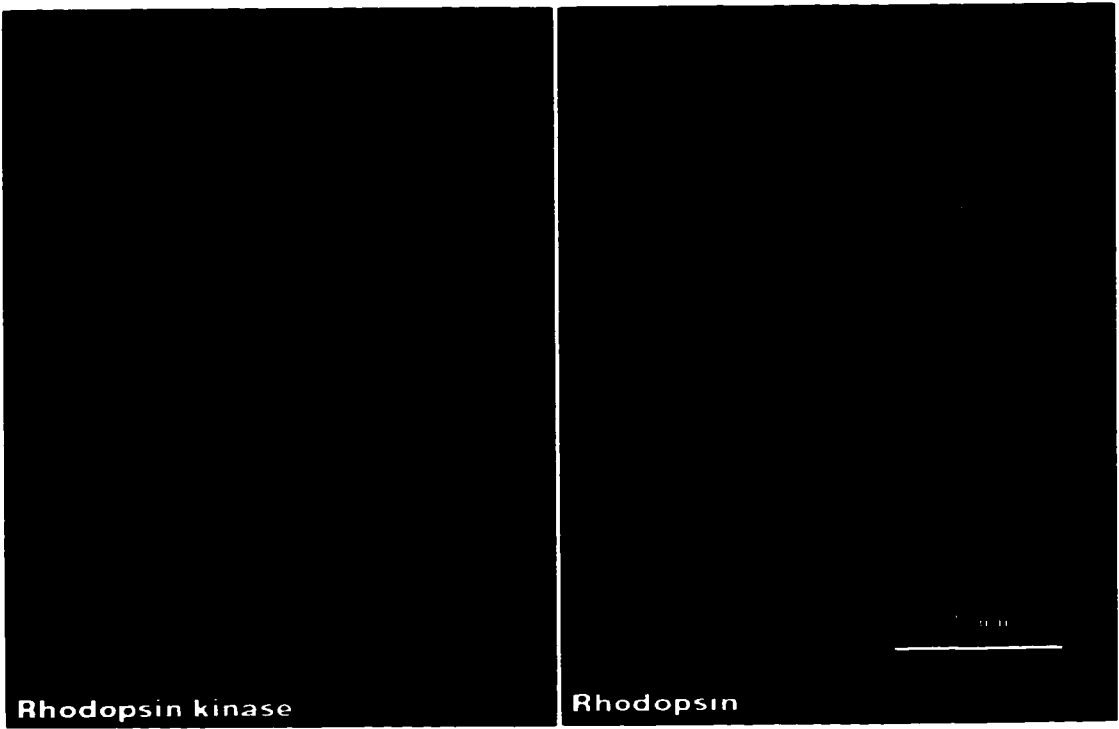


Figure 3-5. Colocalization of rhodopsin and GRK1 in human pineal.

The confocal images show immunolabeling of the soma and processes of a pinealocyte by antibodies to rhodopsin (red) and GRK1 (green), scale bar=25 μ m.



CHAPTER 4

CHARACTERIZATION OF MOLECULAR FORMS OF G PROTEIN-COUPLED RECEPTOR KINASE 1

INTRODUCTION

Desensitization of G protein-coupled receptors (GPCRs) is mediated, at least in part, by a family of Ser/Thr kinases called G protein-coupled receptor kinases (GRKs). Distinct properties set them apart from other protein kinases, including: (1) broad and overlapping substrate specificities that are, however, restricted to ligand-activated GPCRs; and (2) complex interaction with receptors that involves low affinity binding of GRKs to the region of the receptor that is phosphorylated and high affinity, multipoint interactions of GRKs with cytoplasmic loops of the receptor. To date, six members of the GRK family have been cloned from vertebrate species and *Drosophila*. Based on sequence homology, they are divided into three subgroups: group I contains GRK1 (rhodopsin kinase); group II contains GRK2 and 3 (β -adrenergic receptor kinase 1 and 2) and *Drosophila* GPRK1; and group III contains newly identified members GRK4, 5, and 6, and *Drosophila* GPRK2. The overall protein sequence similarity among these kinases is 53-93%, with the lowest sequence homology between group I and II (reviewed by Premont et al., 1995; Palczewski, 1997). In addition, four splice variants (α , β , γ , and δ) of GRK4, with different N- or C-terminal regions, are found primarily in the testis (Premont et al., 1996; Sallese et al., 1997).

Alternative splicing has been shown to be a common mechanism for producing diverse mRNA species. Splice variants can be generated by several mechanisms, including exon skipping, alternative selection of exons, differential usage of splicing sites, and intron retention (reviewed by Green, 1991). Many splice variant proteins have different tissue or cellular localizations, perform different physiological functions, and are differently regulated (Dirksen et al., 1995; Lai et al., 1995). Some of the variants have different sequences in the protein coding region, while others differ in their 5' or 3' untranslated regions. These untranslated regions frequently contain regulatory elements for transcription, translation and mRNA stability (Hérve et al., 1995).

Among GRKs, only GRK4 has been shown to be regulated by one of the splicing mechanisms. From four variants of GRK4, only the longest form, GRK4 α , phosphorylates rhodopsin in a light-dependent manner. This suggests that alternative splicing may be one of the mechanisms for generating GRK isoforms with different specificities. This diversification among the GRK family is important because only six members have been found so far, while hundreds of GPCRs are subject to receptor phosphorylation.

In rod photoreceptors, activated rhodopsin triggers a phototransduction cascade through the activation of G protein (G_t , also called transducin), leading to an increase in cGMP phosphodiesterase (PDE) activity. The hydrolysis of intracellular cGMP by PDE leads to the closure of cGMP-gated channels in the plasma membrane and the hyperpolarization of the photoreceptor cells. The quenching of activated rhodopsin is initiated by its phosphorylation, catalyzed by GRK1, and followed by the binding of the regulatory protein, arrestin, to the phosphorylated activated rhodopsin (reviewed by Polans et al., 1996). The importance of GRK1 in phototransduction, in addition to

biochemical and biophysical data, was further elucidated in its role in Oguchi disease, a special form of congenital night blindness (Fuchs et al., 1995, Yamamoto et al., 1997). A point mutation in the catalytic region, a deletion in the C-terminus, and a deletion of exon 5 (Chapter 5) were identified in the GRK1 gene obtained from Oguchi disease patients, and, as predicted from its domain structure, all of these mutations are likely to result in a nonfunctional kinase (Yamamoto et al., 1997).

In vivo, GRK1 phosphorylates photoactivated rhodopsin at Ser-338, Ser-343 and Ser-334 (Ohguro et al., 1995), near its C-terminus. Our lab proposed that phosphorylation at different sites results from the action of GRK1 (Palczewski et al., 1991), while others proposed additional involvement of protein kinase C (Greene et al., 1997). If GRK1 alone is responsible for phosphorylation at these sites, consistent with the Oguchi disease phenotype, phosphorylation at different sites could result from different conformations of the activated receptor, or alternatively two to three splice forms of GRK1 could each display different rhodopsin phosphorylation site patterns.

In this study, we have investigated the presence of GRK1 isoforms in the retina, using a combination of molecular, biochemical and immunocytochemical methods. We have shown that GRK1 has an alternative spliced form, GRK1b, which retains the last intron. The possible function of GRK1b will be discussed.

MATERIALS AND METHODS

Materials:

Human and bovine eyes were obtained from the same sources as in Chapter 2.

List of primers

Name	Sequence (5'—3')	Position
XZ-43(f)	AAGACCAAGGGCTACGCAGGGA	hRK: (e 4) bp1150-1169
XZ-37(f)	CCGAGAACGTGCTGCTGGAC	hRK: (e 6) bp1067-1086
XZ-49(f)	AGAAGGACCCGGAGAAGCGCCT	hRK: (e 6) bp1403-1424
XZ-44(r)	TCAAGCAAGTGCTGGTGGGTGGA	RK1b: (i 6) C-terminus
XZ-46B(r)	CTAGGAAACCAGACACATCCCTGA	hRK1a: (e 7) C-terminus
XZ-51(f)	GATGGATTCGGGTCTTTGGAGAC	hRK: N-terminus
XZ-54(r)	GCCTCCAGCTGCCTCCAGTTAAG	hRK: (e 6) bp 1480-1052
XZ-57(f)	GACTTCTCCGTGGACTACTTTGC	hRK: (e 5) bp 1216-1238
PA6(r)	AGGACCCAGAAGTCCAGGAA	hRK: bp 677-695
PA8(r)	TTCTCTCCACGGGCTCGGAA	hRK (e 5) bp 1282-1301
FH-13(r)	ATCGATGTCAATGTTGGAGAACA	CTGTATC bGCAP1: before EF4
FH-17(f)	AGCCTGGTCCTCAAGGGGAAG.	bGCAP1: bp 241-261

(f) and (r): forward (sense) or reverse (antisense) direction

e: exon; i: intron

hRK: human GRK1; bGCAP1: bovine guanylate cyclase activating protein.

All oligo primers were synthesized by Oligos Etc. Inc..

Determination of the size of human GRK1 introns 4, 5, and 6 by PCR.

The human GRK1 genomic clone containing exons 4 to 7 in pBluescript SK(-) was provided by Dr. T. Dryja (Khani et al., 1996). To obtain the size of introns 4, 5, and 6, the following primers were used in PCR: primer *b* (XZ-43: forward, from exon 4,); primer *c* (XZ-49: forward, from exon 6); XZ-57 (forward, from exon 5); primer PA8 (reverse,

from exon 5); primer XZ-54 (reverse, from exon 6); primer *d* (XZ-44: reverse, from intron 6); and primer *e* (XZ-46B: reverse, from exon 7). The conditions of PCR were similar to those previously described (Premont et al., 1996). Briefly, the reaction contained 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl₂, 0.2 mM dNTP, 1 μM primers, and 20 to 40 ng DNA. The samples were heated to 94°C for 5 min, followed by the addition of 2.5 U *Taq* DNA polymerase and 0.05 U *Tli* DNA polymerase. The reactions were cycled 40 times (94°C for 45 sec, 68°C for 1 min, and 72°C for 10 min). The identities of the products were verified by Southern blot analyses, using [α -³²P] dCTP-labeled probes corresponding to either the catalytic region, 3'-region, or intron 6 of human GRK1a.

Cloning of human GRK1b

Human retinas were dissected 2 to 15 hours *post mortem* from human eyes and stored at -80°C until needed. Total RNA was isolated using guanidinium isothiocyanate as previously described (Sambrook, 1989). cDNA used in PCR was prepared by reverse transcription with oligonucleotide (dT) primer (Gibco BRL) (Zhao et al., 1997). The 3' region of GRK1b was cloned by the Rapid Amplification of cDNA End (3'-RACE) method using a Marathon™ DNA Amplification Kit (Clontech Laboratories, Inc.) as described previously (Zhao et al., 1997). The 3'-RACE PCR contained 1X reaction buffer (Boehringer Mannheim), 1.5 mM MgCl₂, 0.2 mM dNTP, ~10 ng of double-stranded cDNA, 0.2 μM gene-specific primer (XZ-37), and the Marathon adapter primer 1 (AP1) (Clontech Laboratories, Inc.). The samples were heated to 94°C for 5 min, followed by the addition of 2.5 U *Taq* DNA polymerase (High Fidelity PCR system, Boehringer Mannheim). Reactions were cycled 40 times (94°C for 1 min and 68°C for 4 min). The secondary PCR was carried out using a nested gene-specific primer (primer *b*) and the

adapter primer 2 (AP2) (Clontech Laboratories, Inc.) under the same cycling conditions. The PCR products were cloned into pCRTM2.1 vector (Invitrogen) and sequenced. Both primers are derived from the catalytic region of human GRK1. To verify that the GRK1b transcript was not from genomic DNA contamination, genomic DNA and cDNA were amplified using primers derived from different exon sequences as shown in Figure 4-1. The PCR conditions and primers *b-e* were the same as for the genomic PCR experiments. Primer *a* is XZ-51.

Relative amounts of GRK1a and GRK1b mRNA in the human retina

To determine the relative amounts of GRK1a and GRK1b, quantitative PCR was performed as previously described (Jia and Gutierrez Ramos, 1995). Briefly, each PCR contained 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl₂, 0.2 mM dNTP, 0.5 µl cDNA, 0.5 µM primers, and 0.5 µCi [α -³²P] dCTP (300 cpm/pmol, DuPont NEN). The samples were heated to 94°C for 2 min, followed by the addition of 2.5 U of *Taq* DNA polymerase and 0.05 U of *Tli* DNA polymerase (Promega). The reactions were cycled 30 times (94°C for 45 sec, 65°C for 1 min, and 72°C for 1 min) to amplify human GCAP1 as an internal control or in separate experiments, the reactions were cycled 30 times (94°C for 45 sec, 68°C for 1 min, and 72°C for 1 min) to amplify GRK1a and GRK1b at the same time. The products were separated on a 1.5 % agarose gel, and bands corresponding to GRK1a, GRK1b, and GCAP1 were excised, dissolved in 6 M sodium perchlorate, and counted in a scintillation counter. The relative amount of GRK1a versus GRK1b mRNA was calculated as the ratio of the radioactivity associated with the GRK1a band to the radioactivity associated with the GRK1b band, taking the molecular weight differences of the PCR products into consideration. The primers for GRK1a were: primer *b* (XZ-43, as in genomic cloning), primer *e* (XZ-46B, as above).

The primers used for GRK1b were: primer *b* and primer *d* (XZ-44, as above). The primers used for human GCAP1 were: FH-13 and FH-17.

In vitro translation of GRK1a and GRK1b

Full length sequences of GRK1a (1,692 bp) and GRK1b (3.6 kb, containing intron 6) were cloned into the pGEM-T Easy vector (Promega). The plasmid DNA was purified through several steps under RNase-free conditions as described below. DNA was isolated using a spin miniprep kit (Qiagen), passed through a CentrifluxTM-AG column (Advanced Genetic Technologies Corp.), and precipitated by ethanol, then resuspended in diethyl pyrocarbonate-treated water. The *in vitro* transcription/translation reaction was carried out using a TnT-Coupled Transcription/Translation System (Promega), according to the manufacturer's protocol (TnT T7-coupled Reticulocyte Lysate System, L4611). Briefly, equal molar amounts of circular template DNA of GRK1a (1 µg) and GRK1b (1.8 µg) were added to the reaction mixture (total 50 µl) containing 25 µl of rabbit reticulocyte lysate, 1 µl provided amino acid mixture, 1 µl RNase inhibitor, and 1 µl T7 RNA polymerase (Promega). After two hours at 30°C, the samples were mixed with 1% SDS and 2µl β-mercaptoethanol, heated to 100°C for 5 min, and centrifuged at 86,000g for 30 min. The proteins were separated on a 10% acrylamide, 1.5 mm thick SDS-PAGE, and transferred to an Immobilon membrane (Millipore) at 90 V for 1.5 hours. The translational products were detected by immunoblotting using the D11 anti-GRK1 monoclonal antibody (1.5 mg/ml at dilution 1:10,000).

In situ hybridization

(In collaboration with Dr. Jing Huang). Human retinas were fixed for six hours and stored at -20°C in methanol until use (Gorczyca et al., 1995). The transcription template was a

cDNA fragment encompassing bases 1500 to 1890 of the human GRK1b sequence cloned into pBluescript. The digoxigenin-labeled probes were generated from linearized plasmid DNA using T3 RNA polymerase for the antisense probe and T7 RNA polymerase for the sense probe (Ambion). Both probes were hydrolyzed with 60 mM Na₂CO₃, 40 mM NaHCO₃, and 80 mM dithiothreitol at 60°C for 40 min to reduce the probe length to 150 to 250 bases. *In situ* hybridization was performed as previously described (Palczewski et al., 1994).

Protein expression and antibody generation

Full length or fragments of GRK1a and GRK1b cDNA were cloned into the pFR31 bacteria expression vector (modified from PQE40, Qiagen, Inc. See Chapter 2) to express His-tagged proteins. The constructions of expression vectors are as follows (Figure 4-7 upper panel): The full length GRK1a (1-563) and GRK1b (1-589) were generated by PCR using primer pairs XZ-51 and XZ-46B, and XZ-51 and XZ-44, respectively. The N-terminal region of GRK1, #3 (residues 1-126) and #4 (residues 1-228) were cloned by PCR using primer pairs XZ-51/PA10 and XZ-51/PA6, respectively. The above PCR products were cloned into pFR31 for corresponding expression vectors. The expression vector containing the catalytic region of GRK1 (#5-pFR31, residues 190-462) was generated by cloning the *NcoI/PvuII* fragment of GRK1 into pFR31. The expression vector containing the catalytic and C-terminal region of GRK1a (#6-pFR31, residues 190-563) was cloned by inserting C-terminal *SacI* fragment of GRK1a into #5-pFR. The expression vector containing partial catalytic region+GRK1a C-terminal region (#6-pFR, residues 363-563) was made by cloning C-terminal *SacI* fragment of GRK1a into pFR31. The expression vector containing the C-terminal region of GRK1a (#8-pQE40, residues 463-563) contains *PvuII* site to the XZ-46B primer site of GRK1a cDNA. Because the C-

terminal region of GRK1a is unstable when expressed alone in bacteria, this region was expressed as a fusion protein with dihydrofolate reductase (DHFR) fused at its N-terminus. The expression vector contains the C-terminal region of GRK1b (#9-pFR, residues 463-598) has from *PvuII* site to the XZ-44 primer site. The expression vector pQE40 expresses DHFR cDNA (#10). All above constructs were expressed in bacteria and purified by Ni-NTA resin (Qiagen) as described in Chapter 2.

The C-terminal fragment of GRK1b (#9, Figure 4-7) was used to immunize rabbits to generate a polyclonal antibody (Cocalico Biologicals, Inc.). The anti-human GRK1b polyclonal antibody (UW54) was purified using antigen coupled to CNBr-Sepharose (Pharmacia).

Kinase inhibition assay

Bacterially expressed human GRK1 fragments were purified in 8 M urea and dialyzed against decreasing concentration of Urea and finally to 10 mM BTP, 100 mM NaCl, pH 7.5. After centrifugation, the concentration of soluble proteins was determined by their absorbance at OD₂₈₀. Zero to 16 µg protein were used in the kinase inhibition assay. Bovine GRK1 was expressed and purified from High-FIVE insect cells as described by Ohguro et al. (1996). GRK1 activity was measured for 6 min using [α -³²P]ATP (100-500 cpm/pmol, DuPont NEN) and urea-washed bovine ROS membranes in 20 mM BTP buffer, pH 7.5, containing 1 mM MgCl₂ at 30°C (Palczewski, 1993).

RESULTS

Identification of a splice variant of human GRK1 containing intronic sequence

To identify novel forms of GRK1 from human retinal cDNA, RACE PCR and primers derived from the catalytic region were used to amplify the 3'- and 5'- regions of the kinase. The RACE products were cloned into the pCR2.1 vector and sequenced. The 5'-RACE PCR yielded clones identical to human GRK1 (Zhao et al., 1997). From 24 clones derived from the 3'-RACE PCR, 16 clones hybridized with the catalytic region but not with the C-terminal region of GRK1 probes on Southern blots. Since there is only one GRK1 gene in the genome (Khani et al., 1996), this latter product, named GRK1b (the original GRK1 is designated GRK1a) might be a splice variant of GRK1. To investigate the molecular structure of the GRK1b transcript, human GRK1 genomic DNA was analyzed using a genomic clone containing exons 4 to 7 (G2, Khani et al., 1996). The sizes of introns 4, 5, and 6 were identified using the long-accurate PCR method (Premont et al., 1996) (Figure 4-1, upper panel). Employing PCR primers residing at different exons and introns, it was determined that the GRK1b transcript was identical to GRK1a, except that it retained the last intron, intron 6 (Figure 4-1 middle panel). In addition, the sequence of intron 6 was identical to the 3'-end of GRK1b. Within the intron 6 sequence, there was a stop codon found ~300 bp from the catalytic region (Figure 4-2). GRK1b was not an amplification artifact of genomic DNA because the PCR primer pair *b* and *e* amplified an 11 kb fragment from the genomic DNA, but only 650 bp (corresponding to GRK1a) and 2.4 kb (corresponding to GRK1b) fragments from cDNA (Fig 4-1, lower panel). Using PCR pairs *a* and *e*, and *a* and *d*, we have amplified the full length coding sequence of both GRK1a and GRK1b (Figure 4-1, lower panel). All the PCR products from cDNA were sequenced and the identity of the PCR products from genomic DNA

was obtained by Southern blots. These results demonstrate human GRK1 has a splice variant, GRK1b, which retains the last intron in its mRNA.

Radioactive quantitative PCR was performed on the cDNA derived from four human retinas to investigate the abundance and prevalence of GRK1b. GRK1a and GRK1b (650 bp and 740 bp products, respectively) were amplified in the same PCR using primers *b* and *e*, and *b* and *d* (Figure 4-3, inset); GRK1a and GRK1b were amplified separately with an amplification of a fragment of GCAP1 (Gorczyca et al., 1995) as an internal control (generated a 200 bp product) (Figure 4-3, lower panel; see also Materials and Methods). Representative results from four individuals are shown in Figure 4-3. The relative abundance of GRK1b over GRK1a was calculated as the ratio of GRK1b counts to GRK1a counts, taking the molecular weight difference of the two PCR products into consideration. By both agarose gel electrophoresis and radioactivity measurements, the GRK1b level is between 20% to 80% of the GRK1a level in all cases. This result shows that the GRK1b transcript is abundant and prevalent in humans.

Localization of mRNA encoding GRK1b

In situ hybridization using human tissue and digoxigenin-labeled antisense and sense probes encoding the sequence of the 5'-terminal part of intron 6 was employed to determine if mRNA of GRK1b has nuclear or ribosomal localization. In the human retina, cells in the outer nuclear layer were specifically labeled with the antisense probe (Figure 4-4A), whereas no hybridization signal was produced by the sense probe (Figure 4-4B). The most intense staining was found in the cone and rod inner segments. Due to the size of the probes (~250 bp), however, some nuclear DNA was also non-specifically stained. This result shows that mRNA for GRK1b is exported from the nucleus.

Analysis of GRK1b protein by immunoblot and *in vitro* translation

To detect GRK1b form in the human retina, a specific antibody was raised against the unique C-terminal region. The antibody recognized bacterially expressed, full-length and C-terminal fragments of GRK1b, and detected small amounts of the splice form in the human retina (Figure 4-5A). Unfortunately, the antibody is weak and produces high background, making it unsuitable for immunolocalization of the splice form.

A monoclonal antibody specific for the N-terminal region of the kinase common to GRK1a and GRK1b failed to detect significant amounts of the splice form in partially purified preparations of GRK1 (Figure 4-5B), or in the retinal extract (data not shown). This low level of GRK1b did not result from abnormal structure of the transcript, since GRK1b can be translated in an *in vitro* translation system, as shown in Figure 4-5D. These results suggest that GRK1b protein is unstable in the human retina and that the splice form could be a result of transcriptional/translational regulation. The localization of GRK1b, for example, in the cell body or outer segments of photoreceptors, is at the present time uncertain. Due to *post mortem* effects, the human retina is not suitable for rod outer segment preparation.

Effects of GRK1a and GRK1b fragments on rhodopsin phosphorylation

The possible function of GRK1b protein was investigated by its effect on phosphorylation of photolyzed rhodopsin. As shown in Figure 4-6, fragments of GRK1a and GRK1b were expressed and purified from bacteria (Figure 4-7 lower panel). The protein, purified in 8 M urea, was renatured gradually by graded dialysis and used in *in vitro* GRK1 activity assay with urea-washed bovine ROS and insect cell-expressed bovine GRK1. The full length GRK1a and GRK1b were insoluble and were therefore not

used in the assay. The inhibitory effect of 4 μg of the fragment is shown in Figure 4-7 (upper panel). The N-terminal region of GRK1a had minimal inhibitory effect on rhodopsin phosphorylation by bovine GRK1. The catalytic region and the C-terminal regions of both GRK1a and GRK1b had little effect. This suggests that GRK1b protein, which contains the same N-terminal region as GRK1a, if expressed in outer segments, might compete with GRK1a for its substrate, photolyzed rhodopsin.

DISCUSSION

Gene expression is regulated at several levels, including mRNA processing. Alternative splicing occurs in at least in one out of 20 genes (Dirksen et al., 1995). Intron retention has also been shown to be responsible for several types of genetic diseases, such as the retention of intron 10 in the phosphofructokinase gene causes Tauri disease (Vasconcelos et al., 1995), and the retention of intron 9 in the CD44 gene causes certain bladder cancer (Matsumura et al., 1995). For the past several years, intron retention has also been shown as a mechanism in normal mRNA splicing, and intron-containing mRNA is associated with many normal cellular functions. For example, a fraction (0.1% to 20%) of bovine growth hormone cytosolic mRNA retains the last intron, intron D, in bovine anterior pituitary somatotrophs (Dirksen et al., 1995). Alternative mRNA of human nontransmembrane phosphotyrosine phosphatase (PTP-1B) retains the last intron and encodes a protein with different C-terminal regions. The amount of novel mRNA was increased upon growth factor stimulation (Shifrin and Neel, 1993). In some cases, intron retention serves as negative regulation for either functional mRNA production (the mouse tyrosinase encoding gene, Porter and Mintz, 1991) or functional protein synthesis (the kinase-deficient splice variant of Janus kinase 3, Lai et al., 1995).

Here, we showed that human GRK1 has a splice variant which retains the last intron, intron 6. To be translated into protein, the incompletely spliced mRNA has to be transported to the cytosol and reside on polysomes for protein synthesis. Sequences that are essential for the intron removal are limited to the intron/exon borders. Intron retention is believed to result from suppression of the utilization of both 5' and 3' splice sites on pre-mRNA. The 5' splice site in higher eukaryotes is the consensus sequence (C/A)AGIGU(A/G)AGU (the splice site is denoted by a vertical bar and invariant nucleotides are underlined). The 3' splice site is characterized by the sequence (T/C)AGIGU. These consensus sequences are well conserved within eukaryotic species (from yeast to human) (Mount, 1982).

The 5' splice site sequence of human GRK1 intron 6 (CUGIGUACUG) matches mammalian consensus sequences at only five out of nine positions. The 3' splice site of GRK1 (CAGIGG) matches four out of five positions (Figure 4-7). Studies have shown that unconserved or weak splicing sites, especially 5' sites, result in poor spliceosome binding, inefficient splicing and intron inclusion (Dirksen et al., 1995;), which were shown to be the mechanisms of generating several intron-containing mRNAs, including bovine and human growth hormone (Dirksen et al., 1995; MacLeod et al., 1991) and mouse β -tropomyosin (Wang and Rubenstein, 1992). It is likely that suboptimal splice sites surrounding GRK1 intron 6 lead to low efficiency of splicing and generation of GRK1b mRNA.

Using a combination of *in situ* hybridization, quantitative PCR, immunoblotting and immunocytochemistry, we have shown that GRK1b mRNA is abundant and prevalent among humans. The cytosolic localization of GRK1b message suggests that it is translated. Indeed, GRK1b mRNA was translated as efficiently as GRK1a mRNA in a

reticulocyte lysate. However, we were unable to detect the high level of GRK1b protein in retinal extracts. There are several explanations for this observation. First, the intron 6 sequence in GRK1b mRNA might form a secondary structure in the retinal environment, which suppresses its translation. Such a mechanism for translational attenuation has been observed previously (Zama, 1995; Lovett, 1996). Second, a photoreceptor cell-specific translational regulation system may inhibit GRK1b mRNA from being translated; third, the GRK1b protein maybe unstable. The deduced C-terminal amino acid sequence of GRK1b is rich in proline (P), glutamic acid (E), serine (S) and threonine (T) (Figure 4-2). "PEST" sequence-containing proteins are proposed to have a fast turn-over rate in eukaryotic cells (Rogers et al., 1986).

Biochemical data have shown that the C-terminal region of GRK1 performs important regulatory functions. Mutations in the autophosphorylation region have been shown to alter substrate binding, receptor specificity and catalytic activity (Buczylko et al., 1991; Palczewski et al., 1995). Deletion at the C-terminus of GRK1 was shown as part of the etiology of Oguchi disease (Yamamoto et al., 1997). Lack of this region in GRK1b suggests it is unlikely to be active. The possible function of this GRK1b protein was investigated by its effect on Rho* phosphorylation (Figure 4-6). The C-terminal region of GRK1b does not affect phosphorylation of photolyzed rhodopsin, while the protein encompassing the N-terminal region does. These results suggest that high levels of GRK1b protein could serve as an negative competitor of GRK1a for its substrate, Rho*.

The chicken GRK1 also has splice variants, which have the 3'-coding region replaced by a short untranslated region followed by poly(A) tails, suggesting a mechanism of exon deletion instead of intron retention was used. The alternative forms

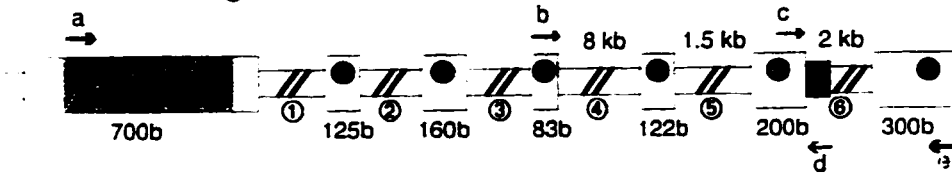
are also very abundant in chicken retina, because 70% of the clones obtained by RACE PCR and all three clones obtained by library screening belong to this group (Chapter 2, Figure 2-6). Analysis of the alternative splicing of GRK1 in both humans and chickens suggests that the loss of the functional C-terminal region is a common mechanism in GRK1 translational regulation. Attempts to clone a similar alternative GRK1 from bovine and rat retinal cDNA using primers residing on putative exons 6 and 7 have not been successful. It is possible that GRK1 in bovine and rat have similar alternative splicing mechanism as observed in the chicken--loss of the last exon.

In summary, the possible functions of GRK1b are: (1) to reduce the amount of GRK1a transcript by competing for the same set of transcriptional machinery, and thereby negatively regulating the amount of functional GRK1 in retina; and (2) the GRK1b protein, if expressed transiently in the retina, could serve as a negative competitor of GRK1a for photolyzed rhodopsin.

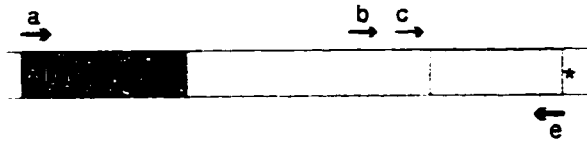
Figure 4-1. A splice variant of human GRK1.

Upper Panel. Diagram of the human GRK1 gene (Khani *et al.*, 1996) and its mRNAs produced by alternative splicing/intron retention. The size of the intron was determined by PCR from a genomic clone containing exons 4 to 7. The introns are not drawn to scale. Orange-colored boxes indicate the exons encoding the catalytic region; The blue boxes indicates the sequence encoding the N-terminus of GRK1. The red box indicates the intron sequence that encodes the C-terminus of GRK1b, the pink box within exon 7 indicates the C-terminal coding region of GRK1a, and the white boxes indicate the untranslated regions in DNA and mRNAs. Intron 6 is retained in GRK1b mRNA, which encodes a protein with a different C-terminus than GRK1a. The asterisks indicate stop codons. *Lower panel.* The presence of GRK1b transcript was verified by PCR. Closed arrowheads indicate the PCR products from human genomic DNA. Solid arrows and open arrows indicate PCR products from GRK1a and GRK1b cDNA, respectively. PCR primers used in the amplification (*a* to *e*) are indicated as in Panel A. Primers *b* and *e* amplified an 11 kb fragment from genomic DNA, but ~2.5 kb and 0.5 kb products from cDNA, respectively. This indicates that amplification of the GRK1b transcript does not result from genomic DNA contamination in the cDNA preparation.

Human GRK1 gene



GRK1a



GRK1b

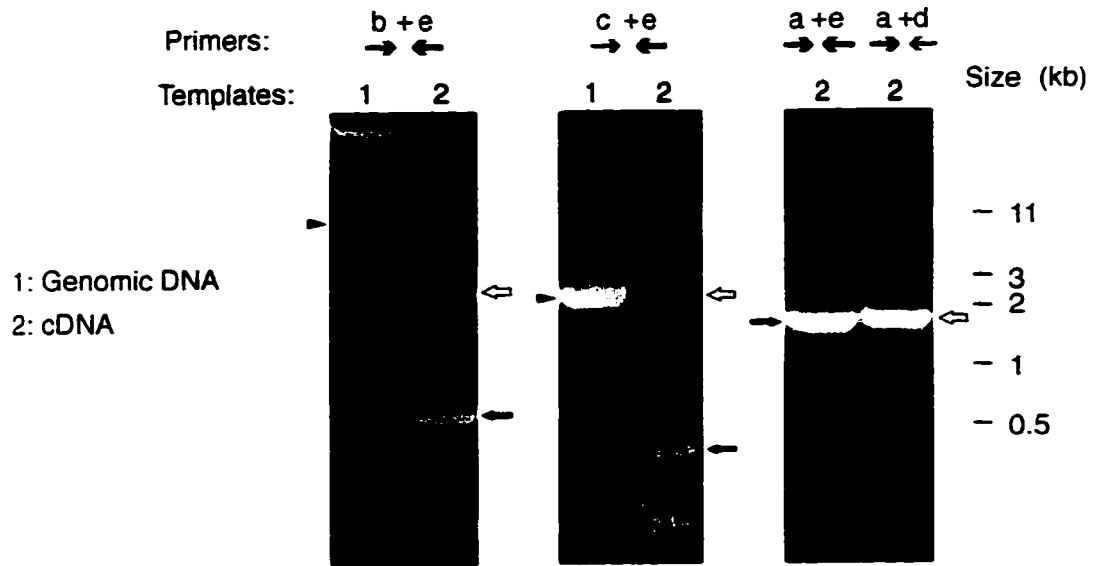
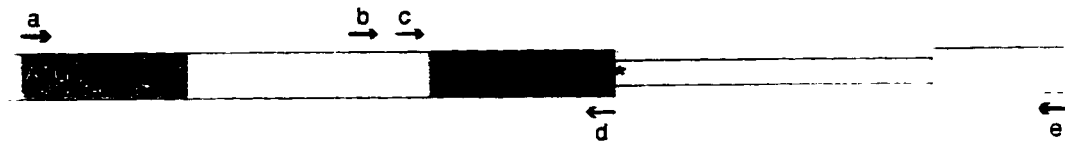


Figure 4-2. Intron 6 sequence of the human GRK1 gene.

The lower case letters represent the DNA sequence, and the upper case letters indicate the deduced amino acid sequence. The shaded boxes mark the boundary of exon 6 and exon 7 and their deduced amino acid sequences. The sequence between exon 6 and the in-frame stop codon within intron 6 consists of the 3'-coding region of GRK1b. Note that in the middle and at the 3' region of intron 6 there are repetitive segments of sequence, and the region is GT rich; therefore, the full length sequence of intron 6 was not elucidated.

exon 6

aaagctccctgccccccccctctctcagacccttactggaggcggcgggcccctgggtact
K L R L R E L E K D L N W R O E E A G T
gttggacgcctcagccccggagaggggtgggggttctgtgctgtgtggcccttgggtgtccg
V G R L S P G E G G V L C C V A L G C P
cccggtccagcctgtgagagtcggcagggaggagtgccctcagaccccccaaggctctcct
P G P A C E S R Q G G V P Q T P K A L P
ctgccccagcaaggccccagtcctccactcatcatcccagccccaggacaagccgatg
L P P A R P P V L H S S S Q P Q D K P M
gagccggcatcgggccagagggctctgggtgcaatgggaggcaggaaacacactggcccg
E P A S G Q R A L G A M G G R K H T G R
actggggcctcgagacccaaccttccaccacgtccccctgggtgctggaggggagcccaaga
T G A S R P K P S T T S P G A G G S P R
tcaaatggaggccagtggtcaggccgtctgccggggagaaagtcacccaccaccagca
S N G G Q W L R P S A G E K V I H P P A
cttgccttgacaagtggatgtggaagatactatgtgcgcacgtgtgtgtatgtgtgtgcac
L A *
gtgtgtgtgtatgtgtgcatgtgtgcgctgtgtgcgcacacgtgtgtgcatgtatgtgt
atgtgtgtgcatagctgtgtgtgacgtgtgcgcatgtgtatgtgtgtgtgcatagctgtg
tgcccatgttatgtgtgatacc.. (~100 bp) ...atgtgtgtgcatatgtctgtgca
tgtgtgtgcatgtgtgtatgtgtatgtgtgtgctgtgtgtgacacgtgctgctgcatgt
gtgtatgtgtgatacatgtgtgtgctgtgtgcatagctgtgtgctgtgtgtgctgtgt
gtatgtgtatacagtgctgctgtgtgcatgtgtgcatagctgtgtgctgtgtgtgata
cgtgtgtatgtgtgtgcatagctgtgtgtgctgtgtgcatagctgtgtgctgtgtgtg
cgcatgtgtgtgtgctgtgtgctgtgtgcatagctgtgtgctgtgtgtgtgtgtgtg
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acagcagtgactgccagacaggtgccctgggcaggccagcaaggcaaggatggctgtgt
tcagggaaaccaagggggctccaaggggtcacaggatgaggaggggaccccgcccgcct
gtaaggaagtctgagccacagaaggggttgagcccagaagtgttgaggtaaaagtgaggc
tgaggaggaaccacacggcggctgtgcatgtgtctgctggcactgggaggtggctcctgagg
ccgtcacagagggcgtctgggggcccagcatggggccagccgggtcagggctcgggtgcaccga
gaggagagtgatgtctgtgaccggctgtgacctggccatcggggggccgggtgctcagggag
ggacgctgggtggaaggaagctgccaagttcactggagcgtgtgcttgagtgctcgggggt
ctggggctctgcagagtgctgagggggaggccctgggtggggatggggccccaggggaaggcg
tgttcggggaggagaccgcttcatgacgagaccctaggggaggctcccaacaaggcagat
gtgagcggcaggtcctttcacaagaaggtccaaaatgagccctgggatctcaggttctt
ccggccccactcaagccccagctgtgtgtgtctcaggggaacccaggggcctctcgggaac
actgggctttctctctcagcctccacgacacttccctaaggaagagcggccccagggcctt
ngtgcactctgggagccatgggggagggggcttttggctaaacggcgtctcctcccacc
acgaggagcctggcgtctgtgttttctgtctcccacagggatgcccggcccccttccat
M L M P P P P T
cccagactccaaact
P D S A K T

exon 7

Figure 4-3. Relative abundance of GRK1a and GRK1b transcripts in the human retina.

Quantitative PCR was performed on four randomly selected donors. *Upper panel.* In each individual, the GRK1a level was set as 1.0. The length of the bars indicate the level of GRK1b message in comparison with GRK1a for each donor. Three independent results are shown as blue, purple, and green bars. The inset is one representative experiment using two pairs of primers that amplify both GRK1a (solid arrow) and GRK1b (open arrow) in the same reaction. *Lower panel.* Another representative experiment using GCAP1 as an internal control in each PCR reaction.

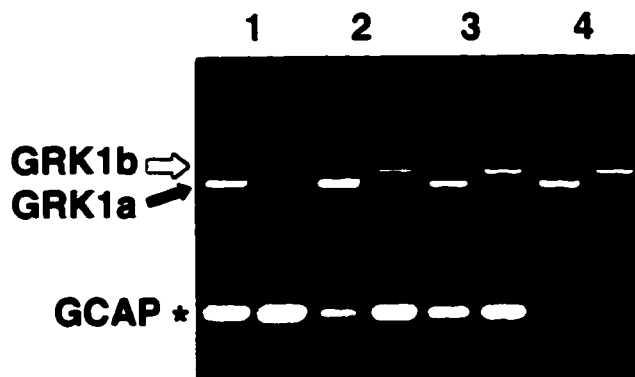
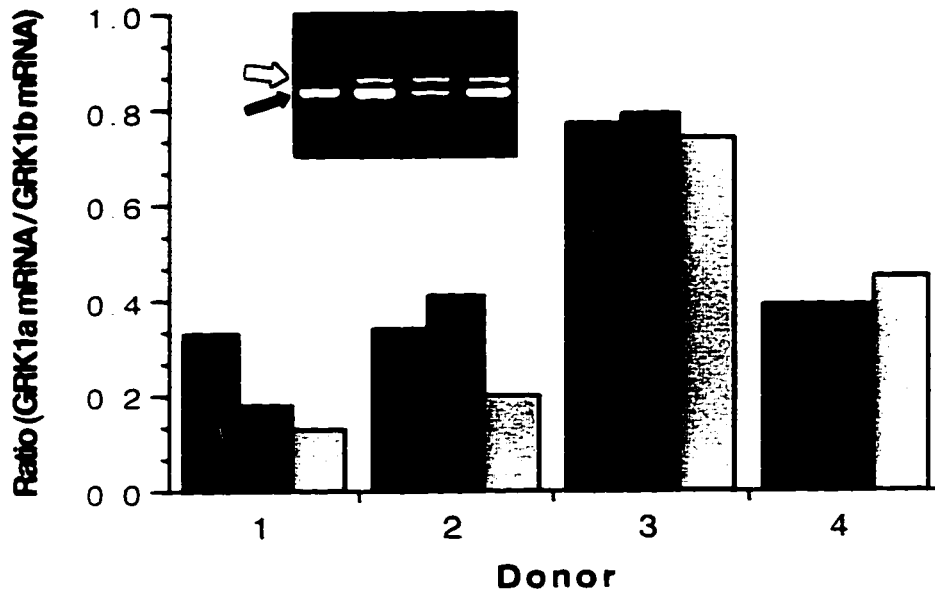


Figure 4-4. *In situ* hybridization of GRK1b mRNA to the human retina. Human retina was hybridized with digoxigenin-labeled antisense (*panel A*) and sense (*panel B*) human GRK1 intron 6 probes. The retina layers are indicated as follows: OS, outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; and NFL, nerve fiber layer. A specific hybridization of the inner segments of photoreceptors is observed with the antisense probe. (Scale bar = 50 μm .)

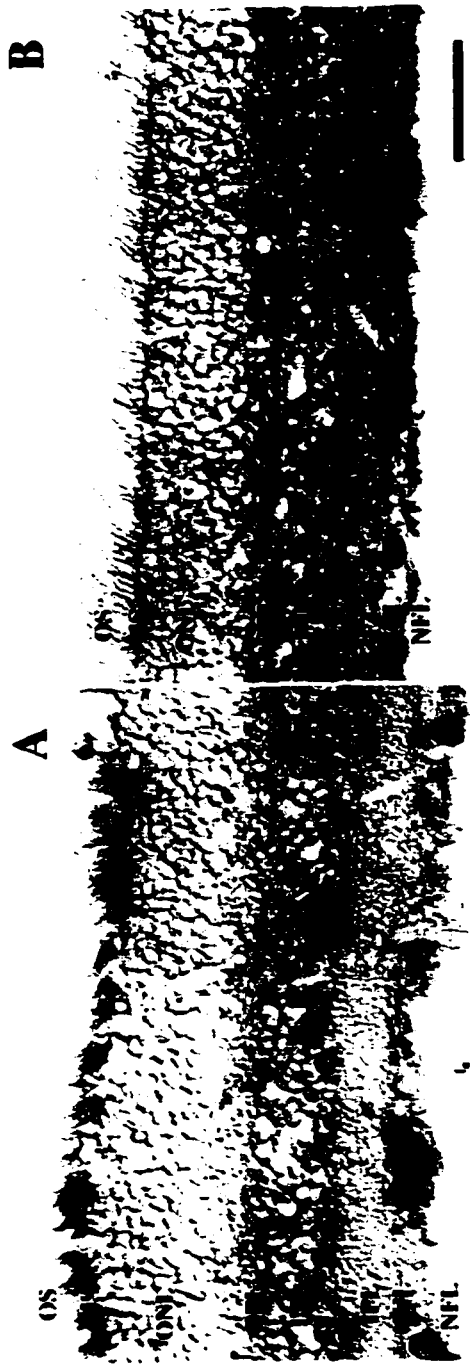


Figure 4-5. Analysis of GRK1b protein by immunoblot and *in vitro* translation.

Panel A: UW54 polyclonal antibody generated against the C-terminal region of GRK1b reacted with GRK1b (lane b) and C-terminal fragment of GRK1b (lane c), but reacted weakly with partially purified human GRK1 (lane a). *Panel B* GRK1a and GRK1b cDNA were employed in an *in vitro* transcription-translation system. D11 antibody recognized GRK1a (lane d) and a slightly higher molecular mass GRK1b (lane e).

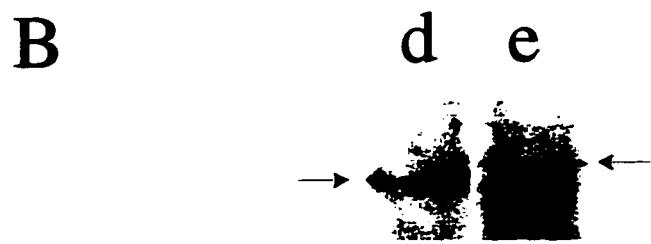
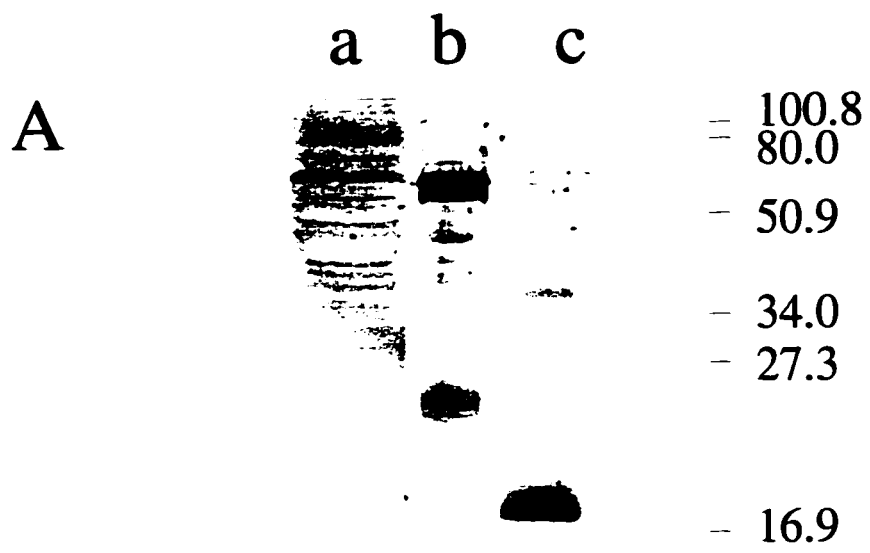











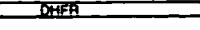


Figure 4-6. Effect of GRK1a and GRK1b fragments on rhodopsin phosphorylation.

Upper panel: Different regions of GRK1a and GRK1b were expressed in bacteria with an N-terminal His-tag and the soluble proteins were used in a kinase assay (See Materials and Methods). The percentage inhibition of GRK1 kinase activity is shown. In comparison with control (DHFR), 4 μ g of the N-terminal regions of GRK1 have an inhibitory effect on light-dependent rhodopsin phosphorylation. *Lower Panel:* The His-tagged recombinant proteins were purified by Ni-NTA resin. The purity of the protein was analyzed by SDS-PAGE.

Human RK Proteins Expressed in Bacteria (His-tagged)

			Residue	KDa	% Inhibition of RK activity	
1 (RKa)	N		 C	1-563	56	-
2 (RKb)	N		 C	1-589	59	-
3				1-126	12	30
4				1-228	20	22
5				190-462	28	Insoluble
6				190-563	38	Insoluble
7				363-563	20	0
8				463-563	32	Insoluble
9				463-589	13	13
10					22	12

MW (kDa) 1 2 3 4 5 6 7 8 9

94 —
67 —
43 —
30 —
20 —
14 —



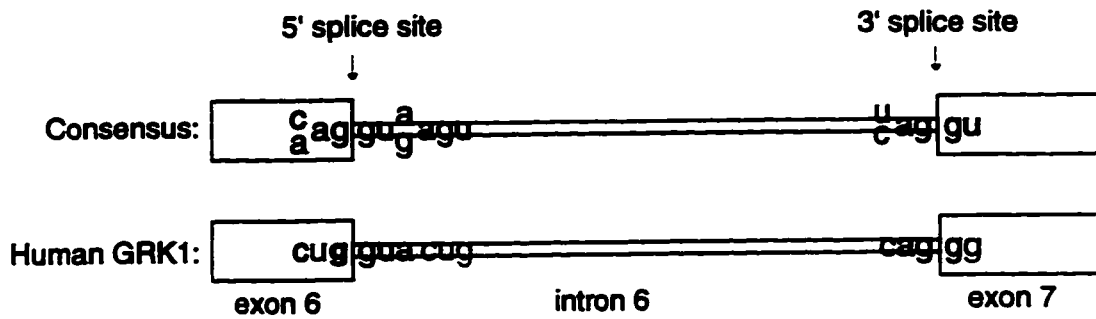


Figure 4-7. The 5'- and 3'-splicing sites of intron 6 of the human GRK1 gene.

The 5'- and 3'- splice sites of intron 6 are compared with the consensus sequences for mRNA splicing. The gray letters indicate variable nucleotides. The sequence of the 5'-splice site of intron 6 matches only five out of nine nucleotides when compared to the consensus sequence.

CHAPTER 5

NULL MUTATION OF GRK1 GENE IN A PATIENT WITH OGUCHI'S DISEASE

INTRODUCTION

Oguchi's disease is a form of congenital stationary night blindness, first reported by Oguchi early this century. This disease is characterized by a peculiar gray-white discoloration of the retina, which disappears after dark adaptation (Mizuo's phenomenon), a normal scotopic vision, and a prolonged dark adaptation of either rods only (Carr et al., 1967) or both rods and cones (Krill, 1972; Yamanaka, 1969). It was suggested that the disorder might be due to abnormality in rhodopsin kinetics; however, the rhodopsin concentration and regeneration was shown to be normal in these patients (Carr et al., 1967).

The breakthrough in understanding the pathology of Oguchi's disease comes from the finding that a frame shift mutation in the arrestin gene causes certain cases of this disease (Fuchs et al., 1995; Yamamoto et al., 1997). Recently, mutations within GRK1 genes were also shown to give rise to Oguchi's disease. A point mutation in the catalytic region, a deletion in the C-terminus, and a deletion of exon 5 were identified in the GRK1 gene. As predicted from its domain structure, all of these mutations result in a nonfunctional enzyme (Yamamoto et al., 1997). The Oguchi phenotype demonstrates the importance of GRK1 and arrestin in phototransduction. However, the precise roles of these proteins need to be defined in human vision.

In this study, we performed genetic analysis of a patient with Oguchi's disease, and demonstrated that he has a deletion of exon 5 in the GRK1 gene. To determine the

consequences of lacking GRK1 in photoreceptor function, the homozygote patient and his heterozygote parent were studied with psychophysical and electrophysiological tests. The results, in conjunction with previous biochemical data, suggest that GRK1 is a critical enzyme in the recovery phase of the rod phototransduction, but plays a minor role in light adaptation.

MATERIALS AND METHODS

Materials

A human genomic clone containing exons 4-7 was obtained from Dr. Dryja (Harvard University). Patient blood was obtained from Dr. Samuel G. Jacobson (University of Pennsylvania).

List of primers

Name	Sequence (5'→3')	Position
XZ-49 (f)	AGAAGGACCCGGAGAAGCGCCT	hRK: (e 6) bp1403-1424
XZ-46B (r)	CTAGGAAACCAGACACATCCCTGA	hRKa: (e 7) C-terminus
XZ-51 (f)	GATGGATTTCGGGTCTTTGGAGAC	hRK: N-terminus
XZ-54 (r)	GCCTCCAGCTGCCTCCAGTTAAG	hRK: (e 6) bp1480-1052
XZ-56 (r)	CTGTCCTGGCAGGACAGTCACATGT	hRK: (i 5) Deletion junction#
XZ-57 (f)	GACTTCTCCGTGGACTACTTTGC	hRK: (e 5) bp 1216-1238
XZ-59 (f)	CAGGGCCAGTGGTCGAAGGTCTCAA	hRK(i 4) Deletion junction#

(f) and (r): forward (sense) or reverse (antisense) direction.

e: exon; i: intron; #: Originally published in Yamamoto et al., 1997.

hRK: human GRK1; hRKa: human GRK1a.

All oligo primers were synthesized by Oligos Etc., Oregon.

Isolation of genomic DNA from blood

Genomic DNA was isolated from blood of the patient and his relatives using RapidPrep™ Genomic DNA Isolation Kit for Blood (Pharmacia). Typically, 1 ml of blood yielded 1 µg of DNA. The DNA was kept in an ice cold water bath before use.

PCR amplification of the GRK1 gene from patient DNA

PCR conditions were similar to those previously described (Premont et al., 1996). Briefly, each PCR reaction contains 1x reaction buffer (Promega), 1.5 mM MgCl₂, 0.2 mM dNTP, 1 mM each primers and 30 to 100 ng genomic DNA. The samples were first heated to 94°C for 5 min, followed by the addition of 2.5 U *Taq* polymerase and 0.05 U *Tli* polymerase (20:1=v:v) (Promega). The reactions were cycled 40 times (94°C for 45 seconds, 65°C or 68°C for 1 min, and 72°C for 4 min). The primers used were: exon 5-exon 6: XZ-57 and XZ-54; deletion junction: XZ-59: (Yamamoto et al., 1997, sense primer for deletion junction) and XZ-56 (Yamamoto et al., 1997, antisense primer for deletion junction); deletion junction-exon 6: XZ-59 and XZ-54; Exon 6-exon 7: XZ-49 and XZ-46B. Plasmid DNA containing exon 4 to 7 of human GRK1 (provided by Dr. T. Dryja) was used as a positive control template in PCR reactions. The PCR products containing deletion junctions from the patient were cloned into pCR™2.1 vector (Invitrogen, Carlsbad, CA) for automated sequencing using dye-deoxy terminator kit (ABI-prism, Perkin Elmer). The sequencing analysis was done at the University of Washington, Center for AIDS Research, DNA Sequencing Facility.

Psychophysical and electrophysiological studies

(In collaboration with Dr. Artur V. Cideciyan and Dr. Samuel G. Jacobson, Department of Ophthalmology, Scheie Eye Institute, University of Pennsylvania). Please see Appendix for details.

RESULTS

GRK1 gene mutation in one Oguchi patient

In our studies, a 13-year-old boy appears to have a deletion of exon 5. Our conclusion is based on PCR using genomic DNA and specific primers that amplified fragments of the GRK1 gene. All PCR-fragments obtained from the patient were cloned and sequenced, while those from other members of the patient's family were verified by restriction mapping and Southern blotting. Two primers within exon 5 and 6 (primers XZ-57 and XZ-54, respectively), were employed to obtain a 1.7 kb fragment (Figure 5-1, product I) encompassing a portion of exon 5 and 6 and the entire intron 5 from genomic DNA of the patient's mother (Figure 5-1, lower panel, Lane 2), father (Lane 3), sister (Lane 4), and from a control genomic clone of human GRK1 (Lane 5). No product was obtained from DNA of the Oguchi patient (Lane 1), suggesting a deletion of exon 5 or 6 (Figure 5-1). However, the 2.4 kb product II was obtained from all four members of the family based on primers encompassing a fragment of exon 6 and 7 (primers XZ-49 and XZ-46B), suggesting that exon 5, but not 6, is deleted from the gene encoding GRK1 from the patient. Furthermore, two primers from intron 4 and 5 (XZ-59 and XZ-56) yielded 1.2 kb fragments (product III) instead of 7.4 kb as detected using the genomic clone. The DNA sequence of the 1.2 kb fragment revealed a deletion of exon 5, for which the patient is homozygous, while other members of the family are heterozygotes.

Similarly, two primers located within intron 4 and exon 6 (XZ-59 and XZ-54), yielded 2.0 kb products (product IV) instead of the 8.2 kb as found using DNA of the genomic clone. The lack of the 7.4 and 8.2 kb products from heterozygotes likely resulted from the difficulty of PCR amplification of these long fragments from complex human genomic DNA. The sequence of the deletion junction and the genomic clone (Figure 5-2) showed that the patient has the same deletion of exon 5 in the GRK1 gene as previously published (Yamamoto et al., 1997). Based on biochemical data on and the domain structure of GRK1, the allele containing the deletion will be “null” and a functional GRK1 will not be produced.

The psychophysical and electrophysiological analysis of the patient:

(Please see Appendix for details.) In summary, the results from these studies indicate: (1) After complete dark adaptation, the activation of both rod and cone phototransduction tested by an unsaturating light stimulus is normal for both the homozygote patient and his heterozygote parent. The sensitivity parameters for activation are normal. However, the recovery kinetics for rods are abnormally slow in both the homozygote and heterozygote, with the homozygote much slower. The recovery kinetics of cones are also delayed in the homozygote. (2) After intense bleaches (2% and 99% tested), the kinetics of rod dark adaptation are normal in the heterozygote, but about 10 times slower in the homozygote. Cone dark adaptation after 99% bleach is normal in both. (3) The light adaptation kinetics of rods and cones (measured as increment threshold at different background illumination) are normal in both.

The conclusion is that the abnormality of this patient is in the recovery of rod photoreceptor sensitivity from light exposure. The activation and light adaptation kinetics of rods appear normal in this patient.

DISCUSSION

To further understand the function of GRK1 in human vision, we have analyzed the GRK1 gene of one Oguchi patient. We have shown that he has a deletion of exon 5 encoding part of the catalytic region of GRK1. According to biochemical and physiological studies, this mutation will result in a nonfunctional kinase.

The electrophysiological studies performed on this patient show that the major abnormality of his vision is localized in the rod photoreceptors and specifically to the process of recovery of rods following light exposure. This result suggests that the recovery of photoreceptor sensitivity is achieved from at least two parallel processes: phosphorylation of photolyzed pigments and regeneration of the pigments. In the absence of GRK1, the recovery of sensitivity is likely achieved by the chromophore reduction/pigment regeneration mechanism, a much slower process than a combination of phosphorylation and regeneration.

I have shown that cone and rod photoreceptors contain the identical GRK1(Chapter 2). Why is cone recovery only mildly affected in Oguchi patients? This may be due to several reasons: (1) The regeneration rate of cone visual pigments (time constant ~100 sec) is much faster than that of rods (~ 400 sec following 99% bleach) (Smith et al., 1983); (2) The sensitive rods require nearly complete pigment regeneration to regain their sensitivity, while the less sensitive cones (at least 100 times less sensitive) do not; (3) Cones also do not saturate even under intense light (Chapter 1). Therefore,

after a 99% bleach, cones can regain their full sensitivity after 15 min, while rods need 60 min in a normal person, and 300 min in our Oguchi patient. It appears that pigment regeneration, not phosphorylation by GRK1, may play a major role in cone sensitivity recovery.

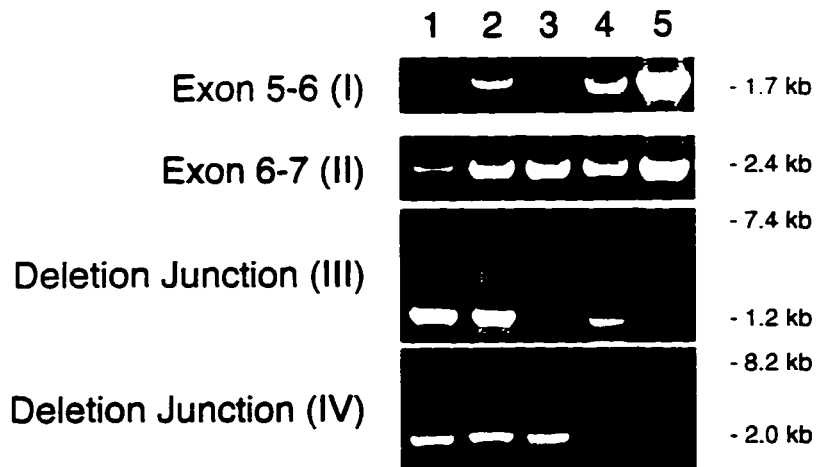
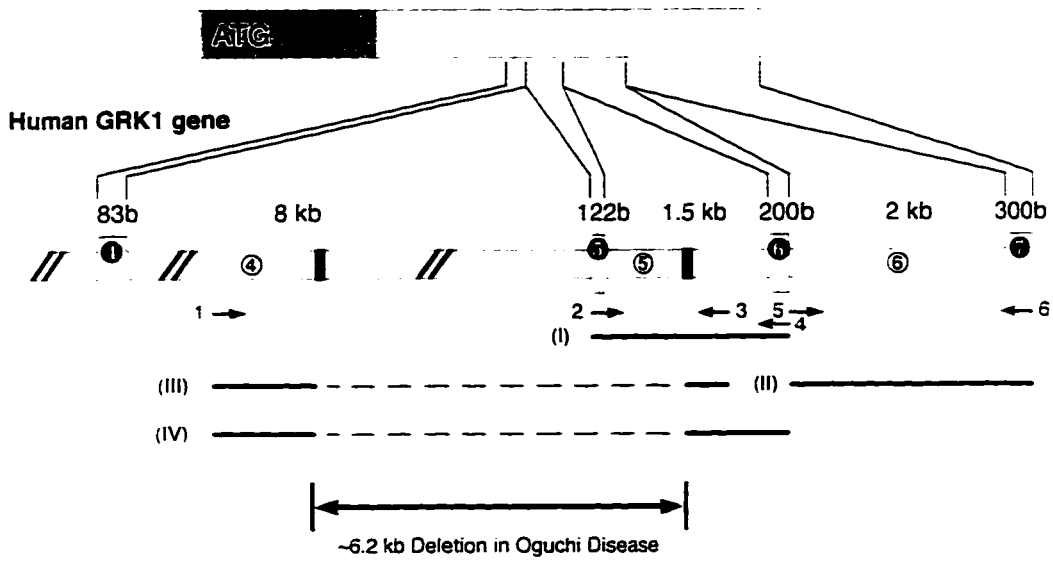
Oguchi patients clearly demonstrate the indispensable function of GRK1 *in vivo*. This disease also proved that phosphorylation of photolyzed rhodopsin by other kinases, such as protein kinase C (Greene et al., 1997) is not relevant to the quenching of activated rhodopsin. The present results also demonstrate that neither GRK1 nor recoverin has a role in photoreceptor cell light adaptation.

Unlike gene-knock out mice that suffer photoreceptor degeneration, Oguchi patients have nearly perfect vision during daytime, and no retinal degeneration has been observed until very late in the patients' lives. Physiological studies on Oguchi patients, in conjunction with the studies in reconstituted systems and in gene-knock out mice, should provide further understanding of GRK1 function in vision.

Figure 5-1. GRK1 mutation in Oguchi disease.

Upper panel: Structures of human GRK1 mRNA and genomic DNA containing exons 4 to 7. The arrows (numbered 1 to 6) indicate the PCR primers used to analyze the GRK1 gene. The black boxes in intron 4 and 5 indicate the deletion junction. The horizontal lines indicate the predicted PCR products from the patient (solid lines only) and from the positive control (both solid and dashed lines). *Lower panel:* Agarose gel electrophoresis showing the PCR products from amplifying the genomic DNA of the patient (lane 1), his relatives (lane 2: mother; lane 3: father; lane 4: sister), and from the genomic clone encoding normal human GRK1 gene (Lane 5). Variability in the intensity of the bands between samples resulted from differing amounts of non-specific hybridization of primers to genomic DNA

Human GRK1a mRNA



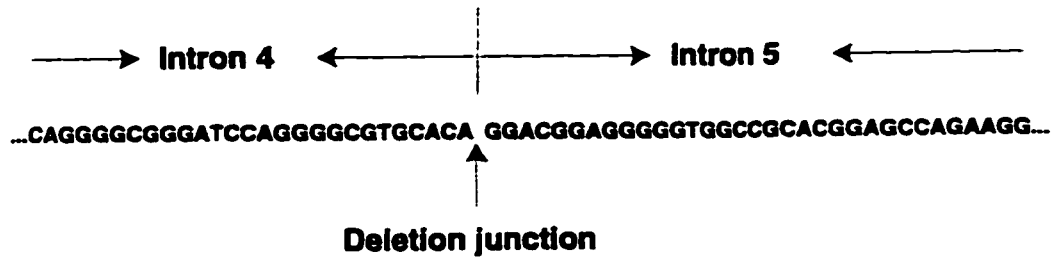


Figure 5-2. The deletion junction of the human GRK1 gene in Oguchi disease.

CHAPTER 6

CONCLUSION

Phosphorylation of rhodopsin by GRK1 serves as a model system for understanding G protein-coupled receptor desensitization. GRK1 plays an important role in photoreceptor quenching and recovery. This is best demonstrated by the phenotype of Oguchi's disease: the loss of GRK1 leads to delayed rod and possibly cone dark adaptation.

In this dissertation, I have investigated different molecular forms of GRK1 in both normal humans and in an Oguchi patient, in search for the molecular mechanisms underlying rhodopsin multiphosphorylation, cone pigment phosphorylation and the extraocular phototransduction system. The conclusions from my studies are:

(1) GRK1 is the only photoreceptor-specific GRK in retina. This is supported by biochemical (Palczewski et al., 1989), molecular biological (Chapters 2 and 3; Zhao et al., 1997), and immunocytochemical (Chapter 2; Zhao et al., 1997; Palczewski et al., 1993) data.

(2) Human and chicken GRK1 have splice variants. The mRNA of the human GRK1 splice variant, GRK1b is abundant and the GRK1b protein is present in the human retina at low levels. One possible function of GRK1b is to serve as a negative regulator in transcriptional/translational regulation of GRK1.

(3) Pineal glands, like the retina, also perform photoreceptive functions. We have demonstrated that mammalian pineal glands have three critical phototransduction

proteins: opsin, blue cone opsin and GRK1. The chicken pineal, in spite of its lack of opsin, also contains GRK1.

(4) Chicken GRK1 is likely to be modified by geranylgeranylation, and not farnesylation, in contrast to its mammalian homologues. This suggests both types of prenylation are compatible with GRK1 function in retina, and light-dependent translocation of GRK1 from cytosol to membrane is unlikely.

(5) Finally, analysis of GRK1 gene in an Oguchi patient indicates that this patient has a homozygous deletion of exon 5 of GRK1. Physiological results from analyzing the patient indicate that GRK1 is important in the fast recovery of rod photoreceptor sensitivity.

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APPENDIX

Classification: Biological Sciences - Neurobiology

Null mutation in the rhodopsin kinase gene slows recovery kinetics of rod and cone phototransduction in man

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ABSTRACT

Rhodopsin kinase (RK), a specialized G-protein-coupled receptor kinase expressed in retina, is involved in quenching of light-induced signal transduction in photoreceptors. The role of RK in recovery after photoactivation has not been specifically defined in man. We thus explored the effects on human vision of a mutation in the RK gene causing Oguchi's disease, a recessively-inherited form of night blindness. *In vitro* experiments revealed that the mutation, a deletion of exon 5, abolishes the enzymatic activity of RK and is likely to be a null mutant. Both a homozygote and heterozygote with this RK mutation had recovery phase abnormalities of rod-isolated photoresponses measured by electroretinography; photoactivation was normal. Kinetics of rod bleaching adaptation by psychophysics were dramatically slowed in the homozygote but normal final thresholds were attained. Light adaptation was normal at low backgrounds but became abnormal at higher backgrounds. A slight slowing of cone deactivation kinetics in the homozygote was detected by electroretinography. Cone bleaching and background adaptations were normal. In this *in vivo* condition without a functional RK and probable lack of phosphorylation and arrestin binding to activated rhodopsin (Rho*), reduction of photolyzed chromophore and regeneration processes with 11-*cis*-retinal are probably solely responsible for recovery of rod sensitivity. The role of RK in rods would thus be to accelerate inactivation of Rho* molecules which in concert with regeneration leads to the normal rate of recovery of sensitivity. Phosphorylation of Rho* appears not to be involved in the regulation of the catalytic properties of Rho*. Cones may rely mainly on regeneration for the inactivation of photolyzed visual pigment, but RK does also contribute to cone recovery.

INTRODUCTION

When light is absorbed in the retinal photoreceptors by visual pigments, these molecules are transformed from a quiescent form to a catalytically active form that is capable of activating hundreds of G-protein molecules. A cascade of biochemical reactions ensues and the result is an electrophysiological signal and vision (Polans et al., 1996). Recovery after photoactivation occurs rapidly in readiness for further light stimuli. The activated form of the rod visual pigment rhodopsin (Rho), for example, is inactivated by multiple steps including: phosphorylation by rhodopsin kinase (RK) and binding of a regulatory protein arrestin, removal and reduction of photolyzed chromophore all-*trans*-retinal by retinol dehydrogenase, and finally the complete inactivation which is accomplished by regeneration of rhodopsin with 11-*cis*-retinal. Parallel steps take place in the recovery of photolyzed species of cone visual pigments, but the kinetics are faster (Smith et al., 1983).

Much has been learned about the recovery mechanisms following photoactivation from biochemical reconstitution systems (Palczewski and Saari, 1997), but the role of these steps in human vision needs to be defined. Whereas it is usually basic science that increases our understanding of human retinal disease, there are occasional opportunities when the study of human retinopathy can help elucidate fundamental retinal processes as they relate to man. Such an opportunity presented recently in Oguchi's disease, an autosomal recessively inherited condition in which affected individuals experience night blindness due to a markedly prolonged insensitivity of their rod vision after light exposure (Carr, 1991). Two genes, both encoding photoreceptor proteins involved in recovery after photoactivation, have been found to be mutated in Oguchi's disease (Fuchs et al., 1995; Yamamoto et al., 1997). Mutations in the gene encoding arrestin were first discovered to be disease-causing in Oguchi patients of Japanese origin (Fuchs et al., 1995). More recently, the RK gene was incriminated in Oguchi patients of European ancestry (Yamamoto et al., 1997). RK is one of the most specialized of a family of Ser/Thr kinases involved in desensitization of G protein-coupled receptors (Palczewski, 1997). In the Oguchi patients, a point mutation in the catalytic region, a deletion in the C-terminus, and a deletion of exon 5 were identified in the RK gene (Yamamoto et al., 1997).

The present work seeks to understand the role of RK in human vision through study of Oguchi's disease caused by an exon 5 deletion in the RK gene. We first established with *in vitro* experiments that deletion of exon 5 abolishes the enzymatic activity of RK. The consequences on human photoreceptor function of absent functional RK were then explored in a homozygote for this null mutation of the RK gene and his heterozygous parent using psychophysical and electrophysiological tests. The results suggest that RK is critical to the recovery phase of rod phototransduction and bleaching adaptation, and RK contributes to rod background adaptation and

the recovery phase of cone phototransduction: RK plays no major role in cone bleaching or background adaptation.

MATERIALS AND METHODS

Isolation of Genomic DNA from Blood. Genomic DNA was isolated from blood of the patient and his relatives using RapidPrep™ Genomic DNA Isolation Kit for Blood (Pharmacia). Typically, 1 ml of blood yielded 1 µg of DNA. The DNA was kept in an ice cold water bath before use.

PCR Amplification of the RK Gene from Patient DNA. PCR conditions were similar to those previously described (Premont et al., 1996). Briefly, each PCR reaction contains reaction buffer (Promega), 1.5 mM MgCl₂, 0.2 mM dNTP, 1 mM each primers and 30 to 100 ng genomic DNA. The samples were first heated to 94°C for 5 min, followed by the addition of 2.5 U *Taq* polymerase and 0.05 U *Tli* polymerase (20:1=v:v) (Promega). The reactions were cycled 40 times (94 °C for 45 seconds, 65°C or 68°C for 1 min, and 72°C for 4 min). The primers used are: exon 5-exon 6: XZ-57 (5'-GACTTCTCCGTGGACTACTTTGC-3'), and XZ-54 (5'-GCCTCCAGCTGCCTCCAGTTAAG-3'); deletion junction: XZ-59: (Yamamoto et al., 1997, sense primer for deletion junction: 5'-CAGGGCCAGTGGTTCGAAGGTCTCAA-3'), and XZ-56 (Yamamoto et al., 1997, antisense primer for deletion junction: CTGTCCCTGGCAGGACAGTCACATGT-3'); deletion junction-exon 6: XZ-59 and XZ-54; Exon 6-exon 7: XZ-49 and XZ-46B. Plasmid DNA containing exon 4 to 7 of human RK (provided by Dr. T. Dryja) was used as a positive control template in PCR reactions. The PCR products containing deletion junctions from the patient were cloned into PCR™ 2.1 vector (Invitrogen, Carlsbad, CA) for automated sequencing using dyedeoxy-terminator kit (ABI-prism, Perkin Elmer, Ramsay, NJ). The sequencing analysis was done at the University of Washington, Center for AIDS Research, DNA Sequencing Facility.

Heterologous expression of wild-type and the deletion mutant of RK in COS7 cells. The eukaryotic expression plasmid pCMV-HRK encoding wild-type human RK was constructed by ligating *Hind III*-*Xba I* digested pCMV5 vector (Andersson et al., 1989) to a *Hind III*-*Nhe I* cDNA cassette containing the coding wild-type RK cDNA sequence flanked by 33 bases of 5'- and 85 bases of 3'- untranslated sequences. The plasmid pCMV-HRK(X5 del) encoding the mutant form was constructed by ligating digested pCMV5 with an analogous mutant *Hind III*-*Nhe I* cDNA cassette containing RK sequence lacking in exon 5. The mutant *Hind III*-*Nhe I* cDNA cassette was generated from the wild-type RK cDNA in Bluescribe by a combination of standard techniques including site-directed mutagenesis on double-stranded template using QuickChange kit (Stratagene). The wild-type sequences immediately surrounding the proximal and distal junction of

exon 5 were converted to unique *Stu I* and *Pml I* sites respectively on the cDNA in two sequential mutagenesis steps using the mutagenic oligonucleotides,

5'-GGGACCCCAGGCCTCATGGCCCCGAGCTCC-3' (codon 354-363), and

5'-CCGTGGAGAGCACGTGGAGAACAAGGAGCTG-3' (codon 395-404) as primers in Pfu polymerase reaction. The resultant DNA was then digested with *Pml I* and *Stu I* and religated to excise the *Pml I-Stu I* fragment corresponding nearly to the entire sequence of exon 5 from the cDNA. Finally, oligonucleotide 5'-CTACGCAGGGACCCCAGGTGGAGAACAAGG-3' was used in a site-directed mutagenesis reaction to delete a single remaining junctional nucleotide from the religated cDNA to generate the mutant cassette for construction of pCMV-HRK(X5 del).

Alterations in the sequences were verified by dideoxy sequence analysis in both directions as well as restriction mapping. The expression plasmids were transfected into subconfluent monolayers of COS7 cells in the presence of DEAE dextran by standard techniques (Andersson et al., 1989). The cells were harvested 48 hours after transfection, lysed, and the supernatants were assayed for the presence of immunoreactive protein and light dependent phosphorylating activity.

Clinical Examination. The patient and his mother underwent a clinical evaluation, including refraction, visual acuity, visual fields and ophthalmoscopy. The Mizuo phenomenon noted on the patient's examination was documented with fundus photography by taking photos in the light-adapted state and then, on another occasion, having the patient wear an occluder patch on this eye overnight and photographing while "dark-adapted".

Psychophysics. Dark- and light-adapted static threshold perimetry, bleaching and background adaptometry were performed with a modified automated perimeter previously described (Jacobson et al., 1986). All stimuli were 1.7° in diameter and 200 ms in duration; pupils were fully dilated. For the static threshold perimetry, thresholds were measured at 75 loci (12° grid) across the visual field in the dark-adapted state with 500 and 650 nm stimuli; midspectral cone thresholds were measured in the light-adapted state (2.7 log phot.td white background) with a 600 nm stimulus. For bleaching adaptometry, monochromatic stimuli of 500 and 650 nm were used at a test locus 12° in the inferior field (Cideciyan et al, 1997). Pre-bleach baseline dark-adapted (>10 hrs for the homozygote and >3 hrs for the heterozygote) thresholds were established. Full (99%) bleaches were delivered with Maxwellian optics to a 20° diameter region of retina, centered on the test locus, under visualization of the fundus with a Zeiss fundus camera. The retinal illuminance of the yellow (>510 nm) bleaching light was 6 log scot.td and lasted 60 seconds. Partial (2%) bleaches were delivered with a ganzfeld sphere coated internally with white reflective paint and illuminated by a white xenon flash (5.3 log scot.td.s). Thresholds were determined until pre-bleach levels (within 0.1 log unit) were attained. Details of the testing techniques, methods of analysis, and normal data have been published (Jacobson et al., 1994, 1996a; Cideciyan et al., 1997). Increment thresholds

for background adaptometry were measured at 12° inferior field on an achromatic background which varied over -3.3 to +2.2 log scot.td range. Thresholds for two wavelengths (500 and 650 nm) were determined alternately at each background. Evaluation of the difference in thresholds allowed identification of the rod or cone mechanism mediating detection (Jacobson et al, 1986; Cideciyan et al, 1997).

Electroretinography: Standard Protocol. Full field ERGs were performed using bipolar Burian-Allen contact lens electrodes, fully dilated pupils and a computer-based system. Details of the methods and normal data have been published (Jacobson et al, 1996a). A standard protocol consisted of a rod ERG (-0.1 log scot.td.s blue flash, dark adapted > 30 min); a mixed cone and rod ERG (2.4 log phot.td.s white flash, dark adapted); a cone ERG at 1 Hz (2.4 log phot.td.s white flashes on a 3.2 log.phot.td white background); and cone ERGs at 29 Hz (2.4 log phot.td.s white flashes on a 2.5 log.phot.td white background).

Electroretinography: Photoresponse Activation. The leading edge of ERGs in response to high energy chromatic stimuli were used to quantify the activation phase of rod and cone phototransduction. Instrumentation, techniques and normal data have been published (Cideciyan & Jacobson, 1993, 1996; Jacobson et al, 1994, 1996a, 1996b). A protocol to isolate the rod photoreceptor response in the homozygote was specifically designed with consideration of the published reports of prolonged recovery of the suprathreshold ERG after a single light flash in Oguchi's disease (Carr & Gouras, 1965; Gouras, 1970; Sharp et al, 1990). After >8 hours of dark adaptation, a single blue (Wratten 47A) stimulus (4.6 log scot.td.s) was delivered. This stimulus was followed by a single red (Wratten 26) stimulus (3.7 log phot.td.s) which was photopically matched to the blue stimulus. In the homozygote, this stimulus produced a dark-adapted cone ERG (see photoresponse recovery results) which was digitally subtracted from the first blue response to determine the rod-isolated photoresponse. In the heterozygote and normal subjects, the protocol and rod-isolation were accomplished as previously described (Cideciyan & Jacobson, 1996). Red (Wratten 26) stimuli (2.2 to 4.1 log phot.td.s) on a rod-desensitizing background (3.2 log phot.td) were used to isolated cone photoresponses in all subjects. A physiology-based model that has been shown to describe rod and cone phototransduction in normal subjects (Cideciyan & Jacobson, 1996) and patients (Jacobson et al, 1994, 1996a,b) was fitted to the leading edges of rod- and cone-isolated photoresponses to quantify the activation kinetics.

Electroretinography: Rod Bipolar Cell Activation. The rod-isolated ERG b-wave component to a 4.6 log scot.td.s blue flash was estimated by digitally subtracting the cone component as well as subtracting the rod photoresponse model fitted to the leading edge of the a-wave (Hood & Birch, 1992; Cideciyan & Jacobson, 1993; Hood & Birch, 1996). In the homozygote, the cone component was the photopically matched red response. In the heterozygote

and normal subjects, the red response was a mixture of rods and cones, the rod component of which was estimated by a dimmer blue response scotopically matched to the red response. The derived rod-isolated b-wave was normalized by the maximum amplitude of the rod photoresponse: this allowed comparison of the relative rod bipolar cell activity independent of rod receptor activity.

Electroretinography: Photoresponse Recovery. The kinetics of the recovery of maximal photoresponse amplitude following a high energy stimulus can be non-invasively quantified using a double-flash paradigm in rods (Birch et al, 1995; Pepperberg et al, 1996, 1997) and in cones (Hood et al., 1996a). To estimate the recovery of the rod photoresponse, we used an abbreviated protocol with a conditioning flash followed by a probe flash (both were blue, Wratten 47A, 4.6 log scot.td.s) separated by an inter-stimulus interval (ISI) ranging from 7 to 300 sec. Two ISIs were used with the homozygote and one ISI was used with the heterozygote. The cone component of the probe response was estimated and subtracted as described above. The probe response was fitted with the model of rod phototransduction and the fraction of maximum amplitude recovered was plotted against ISI. The recovery of the cone photoresponse was performed in the light-adapted state (3.2 log phot.td) with an achromatic conditioning stimulus (4.1 log phot.td.s) and a red (Wratten 26) probe stimulus (4.1 log phot.td.s). The intervals between the two stimuli were varied over a range of 100 to 200 ms. The probe response was fitted with the model of cone phototransduction and the model amplitude at 10 ms following the probe was plotted against ISI.

RESULTS

The patient, at age 4 years, was noted by his parents to be severely visually disabled in the dark but seemed fully sighted under well lit conditions. No other family members had similar visual symptoms and there was no known parental consanguinity; both parents had Ashkenazi Jewish origins. On an examination at age 6 years, the patient had normal visual acuity and kinetic visual fields. A standard ERG revealed no detectable rod b-wave after 45 minutes of dark adaptation. A mixed cone-rod ERG to a bright flash of white light, dark-adapted, had an a-wave of normal amplitude but a reduced b-wave. Cone ERGs to 1 Hz and 30 Hz stimuli were normal. On ophthalmoscopic examination, there was a metallic sheen to the retina ; the coloration became normal in appearance following 12 hours of dark adaptation. The diagnosis was Oguchi's disease. The eye examinations of the patient's mother and sister were normal. On re-evaluation at age 13 years, the homozygote patient had normal thresholds on light-adapted static perimetry. Dark-adapted (>12 hrs) perimetry showed rod threshold elevation (> 2 s.d. from mean at each locus) at 30.5% of the loci tested (normal subjects average 1.7% of loci, range 0-6.9%). The threshold elevations at the patient's abnormal loci were between 0.5 and 1 log unit.

PCR using genomic DNA and specific primers that amplified fragments of the RK gene in

the patient indicated a deletion of exon 5 (Fig. 5-1). All PCR fragments obtained from the patient were cloned and sequenced, while those from his parents and an older female sibling were verified by restriction mapping and Southern blotting. Two primers within exon 5 and 6 (primers XZ-57 and XZ-54, respectively), were employed to obtain a 1.7 kb fragment (product I) encompassing portion of exon 5 and 6 and entire intron 5 from genomic DNA of the patient's mother (lane 2), father (lane 3), sister (lane 4), and from a control genomic clone of human RK (lane 5), respectively. No product was obtained from DNA of the patient (lane 1), suggesting a deletion of exon 5 or 6 (Fig. 5-1). However, the 2.4 kb product II was obtained from all four members of the family based on primers encompassing a fragment of exon 6 and 7 (primers XZ-49 and XZ-46B), suggesting that exon 5, but not 6, is deleted from the gene encoding RK from the patient. Furthermore, two primers from intron 4 and 5 (XZ-59 and XZ-56) yielded 1.2 kb fragments (product III) instead of 7.4 kb as detected using the genomic clone. The DNA sequence of the 1.2 kb fragment revealed a deletion of exon 5, for which the patient is homozygous, while other members of the family are heterozygotes. Similarly, two primers located within intron 4 and exon 6 (XZ-59 and XZ-54), yielded 2.0 kb products (product IV) instead of the 8.2 kb as found using DNA of the genomic clone. The lack of the 7.4 and 8.2 kb products from heterozygotes likely resulted from the difficulty of PCR amplification of these long fragments from complex human genomic DNA. The sequence of the deletion junction and the genomic clone showed that the patient has the same deletion of exon 5 in the RK gene as previously published (Yamamoto et al., 1997). Based on the domain structure of RK, the allele containing the deletion will be null and a functional RK is not produced (Zhao et al., 1995). An analysis of exon 11 (Fuchs et al., 1995) of the arrestin gene in the patient showed no mutation (data not shown).

Based on the domain structure of RK, the allele containing the deletion will be "null" and a functional RK is not produced. To further verify this prediction, RK and RK lacking fragment encompassing exon 5 was expressed in COS-7 cells. RK lacking the sequence encompassing exon 5 was inactive (data not shown).

Rod photoreceptor responses (Fig. 3A), representing the activation phase of phototransduction, had a maximum amplitude (354 μV) that was just within 2 s.d. from mean normal ($456 \pm 54 \mu\text{V}$) for the homozygote and normal for the heterozygote (523 μV); the sensitivity parameter was normal in both patients (1.49 and 1.56 $\log \text{scot.td}^{-1} \cdot \text{s}^{-3}$ for the homozygote and heterozygote respectively; normal = $1.52 \pm 0.15 \log \text{scot.td}^{-1} \cdot \text{s}^{-3}$). The derived rod b-wave (data not shown) was normal in the heterozygote (peak normalized amplitude = 1.xx; normal = $1.xx \pm xxx$) and the homozygote (1.xx). Cone photoresponse maximum amplitude (Fig. 3B) was normal ($85 \pm 9 \mu\text{V}$) in the homozygote (110 μV) and the heterozygote (96 μV).

Recovery kinetics for rods (Fig. 3 C and E) were abnormal in both homozygote and

heterozygote. For the homozygote, the rod response probed 30 sec after the conditioning flash recovered to 5% of baseline maximum amplitude whereas normal subjects recover to 100% in this interval. At 300 sec following the conditioning flash, the homozygote had recovered to 14% of baseline. The rod photoresponses in the heterozygote recovered to 81% of baseline in 30 seconds. Cone deactivation kinetics (Fig. 3 D and F) were normal in the heterozygote. The homozygote, however, showed a subtle but definite delay at short (100 and 120 msec) ISIs.

The kinetics of rod dark adaptation following a 2% partial bleach or a 99% full bleach had a dramatically slowed time course in the homozygote (Fig. 4A) but were normal in the heterozygote (Fig. 4C). The major component of rod recovery (Cideciyan et al, 1997) in the homozygote has a slope of $-0.03 \text{ log units} \cdot \text{minute}^{-1}$ (corresponding to a time constant of 870 sec), which is approximately 9 times slower than the normal second component (normal range=80-113 sec), but only slightly slower than the normal 3rd component of recovery (range=580-745 sec). The rod-cone break is prolonged to 120 minutes following a 99% bleach in the homozygote (normal mean=12.5±0.9 minutes). Cone dark adaptation following a 99% bleach is normal in both patients (Fig. 4 A and C, insets). The borderline thresholds of the heterozygote measured with the 650 nm stimulus could be explained by her carrier state of an X-linked color defect.

In the homozygote, rod mediated increment thresholds performed dark-adapted and on a range of achromatic backgrounds were normal at low backgrounds (Fig. 4B). Starting at backgrounds of approximately -2.0 log scot.td the rod system adaptation became abnormal; the amount of abnormality increased with higher backgrounds. Cone mediated increment thresholds (Fig. 4B, inset) were normal in the homozygote. The rod (Fig. 4D) and cone (Fig. 4D, inset) increment thresholds of the heterozygote were normal.

DISCUSSION

Analysis of Phenotype in a Known Oguchi Genotype. Oguchi's disease, considered for decades to be caused by a post-receptor retinal defect like other forms of congenital stationary night blindness (Berson, 1994), has now been found to result from mutations in genes encoding proteins involved in photoreceptor function (Fuchs et al., 1995; Yamamoto et al., 1997). The current prospective analysis of the phenotype in Oguchi's disease caused by a homozygous null mutation in the RK gene shows that the major abnormalities in disease expression could be localized to rod and cone photoreceptors and, specifically, to the process of recovery following light exposure.

Rod-isolated ERG photoresponses in the homozygote, although normal in phototransduction activation, showed a pronounced disturbance in recovery phase kinetics. Dark adaptation experiments showed that after partial and full bleaches rod-mediated thresholds did return to normal levels, but the dynamics of the recovery process were markedly abnormal. A less

dramatic but interesting result was that a relatively large number of loci in the visual field had abnormally elevated rod thresholds, even after dark adaptation for 12 hours. This finding, taken together with the borderline maximal amplitude for the rod photoreponse, could result from a small degree of persistent desensitization from incomplete dark adaptation. An alternative hypothesis is that there may be some rod cell damage (reduced outer segments or cell loss) detectable by these methods but insufficient to produce clinical hallmarks of retinal degeneration. Normal rod bipolar cell maximum activity would not be inconsistent with subtle and diffuse rod damage (Shady, Hood, Birch, 1995). In the heterozygote, a small but significant rod photoreceptor recovery phase abnormality was also detected, but other rod function measurements were normal. In the homozygote, light adaptation of the rod system was normal at low backgrounds but became increasingly abnormal at higher backgrounds. Cone-isolated ERG photoresponses of the homozygote were normal in phototransduction activation but showed a subtle but definite disturbance in recovery phase kinetics. Cone-mediated recovery after a full bleach as well as light adaptation of the cone system were within normal limits for both homozygote and heterozygote.

What is the relationship of the present results to previous descriptions of phenotype in Oguchi's disease of known or unknown genotype? Of the three unrelated cases of Oguchi's disease recently reported to have RK gene mutations (Yamamoto et al., 1997), two patients were studied in detail with visual function tests about 30 years ago (Carr and Gouras, 1965; Carr and Ripps, 1967). Both a homozygote with an exon 5 deletion and a compound heterozygote with Val380Asp and Ser536 (4-bp del) mutations had prolonged dark adaptation; the former patient had a slightly elevated dark-adapted threshold, even after 24 hours (Carr and Gouras, 1965). Standard ERGs in the patient with an exon 5 deletion had a normal a-wave amplitude after many hours of dark adaptation but a reduced amplitude after only 10 minutes in the dark. Retinal densitometry, performed in the compound heterozygote, indicated that rhodopsin kinetics were normal. Cone function measured with adaptometry and ERGs was mainly normal. In general, these results are consonant with those in the present study. Rod- and cone-isolated photoresponses and recovery phase kinetics, background adaptation and dark adaptation following a partial bleach have not previously been described in these patients or in heterozygous relatives.

In Japanese Oguchi patients with an arrestin gene mutation (1bp deletion in codon 309 causing a frameshift and premature termination, Fuchs et al., 1995), there is a reduced standard ERG a-wave after 30 minutes of dark adaptation. There were cone ERG, visual field, and visual acuity abnormalities in some patients (Nakazawa et al., 1997). Activation or recovery phase kinetics for rod- and cone-isolated photoresponses, the rod and cone background adaptation and the exact time course of bleaching adaptation in these patients or heterozygotes with arrestin mutations were not reported.

Among the many descriptions of Oguchi patients of unknown genotype, common features include prolonged and unmeasurable rod dark adaptation (Doesschate et al., 1966; Krill, 1977) and rod ERG b-wave losses probably attributable to insufficient dark adaptation or rod desensitizing stimuli (Gouras, 1970; Krill, 1977; Carr, 1991; Miyake et al., 1996). Some studies of Oguchi patients show reduced rod a-waves (Kubota, 1966; Doesschate et al., 1966; Miyake et al., 1996). Persistent rod threshold elevations of about 0.5 log units, even after 24 hours of dark adaptation, have been reported (Krill, 1977; Sharp et al., 1990). Cone system abnormalities such as delayed cone dark adaptation, elevated cone thresholds, and cone ERG abnormalities, have also been documented (Doesschate et al., 1966; Sharp et al., 1990). The basis of the potentially interesting differences in the Oguchi phenotype should be explored, with detailed characterization of rod and cone function in patients with known genotypes, now that the diagnosis is recognized to represent a genetically heterogeneous disease category.

What is the current understanding of inactivation steps in the rod visual cycle? A series of conformational changes of Rho starting with light-dependent isomerization of the chromophore, 11-*cis*-retinal, result in an active form of the receptor, Rho*, that can continuously activate the phototransduction cascade with high efficiency. Some studies suggest that other intermediates of the visual cycle like non-covalent complexes of opsin and all-*trans*-retinal (Jaeger et al, 1996; Buczylko et al, 1996; Crouch et al, 1996) or free opsin (Fain, Matthews & Cornwall, 1996) can also activate the cascade albeit with lower efficiencies. For rods to regain full sensitivity, all of these (and other) activating species need to be turned off. In reconstituted systems and *in vivo*, RK phosphorylates Rho* and thus attenuates its activity (for example Ohguro et al., 1995). RK may also phosphorylate opsin/all-*trans*-retinal complexes. Subsequently, the phosphorylated receptor binds a regulatory protein, arrestin, completely quenching the activation of transducin (Wilden et al., 1986). Inhibition of RK leads to prolonged photoresponses in functionally intact gecko rod outer segments (Palczewski et al., 1992). These and many other observations have led to the hypothesis that RK is important for inactivation of Rho*.

Concurrent with these inactivation steps, the photolyzed chromophore, all-*trans*-retinal, is removed (or just simply dissociates) from the binding site of Rho and is reduced to all-*trans*-retinol by retinol dehydrogenase (reviewed in Palczewski and Saari, 1997). The reduced chromophore, through a series of enzymatic steps in retinal pigment epithelium cells, is converted to 11-*cis*-retinal, which combines with opsin to regenerate Rho. How both Rho* phosphorylation/arrestin binding and reduction of the chromophore contribute *in vivo* to the inactivation is uncertain. It is possible that the two processes are decoupled from each other, independently reducing the catalytic power of Rho* and other activating intermediates.

How do our *in vivo* results relate to the role of RK in rods? From the rod-

isolated photoresponse recovery experiments and the rod bleaching adaptation data in the Oguchi patient of this study, it is evident that RK is an indispensable element in the 'fast' recovery of sensitivity in rods. However, it is equally apparent that in the dark following light exposure rods do recover without RK, albeit much slower. Thirty seconds following a bright conditioning flash (0.4% rhodopsin bleach) that saturates the rods, no more than 5% of the dark-current is re-established in the patient whereas 100% of the dark-current recovers in normal subjects. Upon more intense bleaching (2% or 99%), rod cells are desensitized for relatively long periods of time in normals, with full recovery of pre-bleach sensitivity occurring in 15 minutes and 60 minutes, respectively. Lack of RK increases the times to 60 minutes and >300 minutes. Recovery of sensitivity in the absence of RK can be speculated to occur by the reduction/regeneration cycle of the visual pigment, which would be a much slower process than the normal combination of phosphorylation and regeneration.

The exact cellular and molecular mechanisms underlying psychophysically-determined bleaching adaptation remain speculative (Lamb, 1981; Stabell & Stabell, 1996). In normal subjects, following a range of bleach levels most of the psychophysically measurable dynamic range of rod-mediated vision recovers with an invariant time constant of approximately 100 seconds (Lamb, 1981; Jacobson et al, 1996a; Cideciyan et al, 1997). Without functional RK, the time constant of the major component of recovery increases to approximately 900 seconds both after 2% and 99% bleaches. Of further interest, the initial desensitization (extrapolated to time zero, see Lamb, 1981; Cideciyan et al, 1997) caused by this portion of recovery is linearly related to bleach level suggesting first order removal of one of the earlier bleach products. Based on these data, we speculate that major component (the so-called second component, Lamb 1981) of recovery in normal bleaching adaptation represents the rate of phosphorylation of Rho* and opsin/all-*trans*-retinal complex. The approximate rate of phosphorylation determined *in vivo* during the first 2.5 minutes after a 45% bleach is consistent with this speculation (Ohguro et al, 1995). The results of the patient, on the other hand, suggest that the rate of reduction of all-*trans*-retinal and dissociation of opsin may be approximately a first order reaction with a time constant of 900 secs. It is of interest that the third component in normal bleaching adaptation which has been speculated to represent Rho regeneration (Lamb, 1981; Kemp et al., 1994), has a similar time constant (Lamb, 1981; Jacobson et al., 1996a). It is possible that intracellular calcium-mediated feedback (Kawamura, 1994, Chen et al., 1995) turns RK activity off during the final log unit of recovery in the normal thus slowing the recovery rate to approximate that of the Oguchi patient. This hypothesis is also consistent with the background adaptation abnormalities seen in the homozygote. At low intensity backgrounds the thresholds of the patient are normal, but at higher backgrounds the thresholds become increasingly abnormal possibly because at these background levels

phosphorylation is turned on in normal subjects.

The phosphorylation of rhodopsin may not be involved in the regulation of the catalytic properties of Rho*, because our results show that the activation steps of rod phototransduction are indistinguishable in the presence or absence of RK. This is not concordant with single cell recordings in transgenic mice carrying S334ter rhodopsin mutation where rhodopsin shut-off was shown to start before the peak photoresponse after a dim flash (Chen et al, 1995). However it is important to point out that the activation of the phototransduction in our patient was measured for 10 msec following relatively bright flashes. The slower time course of channel closures following single photon responses were not being measured.

Does RK play a role in cone deactivation? Light activated species of cone pigments are believed to inactivate with processes similar to rods, but the kinetics are more rapid. The differences between rods and cones could result in part from a different subset of gene products involved in the inactivation of cone pigments (for example cone-specific arrestin has been identified Craft and Whitmore, 1995). RK may represent an exception; a cone-specific kinase has not been identified. Molecular cloning and immunolocalization studies have shown that both rod and cone photoreceptors express the same photoreceptor kinase and only RK has been cloned from the cone-dominated chicken retina (Zhao et al., 1997, unpublished). The finding of an abnormality in cone deactivation kinetics in the Oguchi patient suggests that RK plays a subtle role in cone recovery from light. The principle mechanism of cone inactivation, however, may be reduction of the chromophore and regeneration of cone pigments which occur faster in cones than in rods (Smith et al., 1983). This hypothesis is consistent with the normal bleaching adaptation found following a 99% cone bleach in the Oguchi patient. If true, this hypothesis suggests that isolated cone photoreceptor cell studies may be lacking the important and fast contribution of pigment regeneration to the recovery phase following light stimulation.

In summary, the lack of RK, resulting from a recessive human disease caused by a null mutation in the RK gene, causes a profound abnormality in recovery of rod photoreceptor function after light activation; and, for the first time, physiological evidence is provided that RK may also be involved in cone deactivation kinetics. This observation should be confirmed and extended by studying cone deactivation kinetics in other Oguchi patients with RK mutations and the results compared to those in patients with Oguchi's disease caused by mutations in the arrestin gene which is believed to be rod-receptor specific. It will also be important to compare these human data with results from isolated rods and cones in animals with targeted deletion of the RK gene to gain detailed insights into the role RK plays in the subtle balance between inactivation and regeneration of visual pigment as the quiescent state of the photoreceptor is restored.

FIG. 3. Electrophysiological activation and recovery kinetics in rods and cones. Rod- (A) and cone-isolated (B) photoresponses representing activation in the homozygote (filled circles) and the heterozygote (filled squares) evoked by 4.6 log scot.td.s blue (A) or 4.1 log phot.td.s. red stimuli (B). Lines show the fitted model of phototransduction. Arrows denote the lower limit (mean - 2 s.d.) of photoresponse maximum amplitude. Recovery kinetics of the rod- (C) and cone-isolated (D) photoresponses at a fixed inter-stimulus interval (ISI=30 sec in C, 120 msec in D) are shown for the patients and a representative normal subject. Baseline responses are solid lines and probe responses are dashed lines. The fraction of baseline rod (E) and cone (F) photoresponse amplitudes that recovered for different ISIs in the homozygote (filled circles), and the heterozygote (filled squares); gray lines represent the normal range.

FIG. 4. Psychophysical bleaching and background adaptation. Dark adaptation after 2% (black triangles) or 99% (black circles) bleaches in the homozygote (A) and the heterozygote (C). Cone dark adaptation following 99% bleach shown in insets (gray circles). Rod-mediated (black squares) background adaptation in the homozygote (B) and the heterozygote (D). Cone mediated background adaptation shown in insets (gray squares). All main panels show thresholds to 500 nm stimuli (black symbols) and all insets to 650 nm stimuli (gray symbols). Gray lines represent the normal range. Pre-bleach thresholds are shown preceding time zero in panels A and C. The horizontal axes of insets in panels B and D are in log phot.td. units.

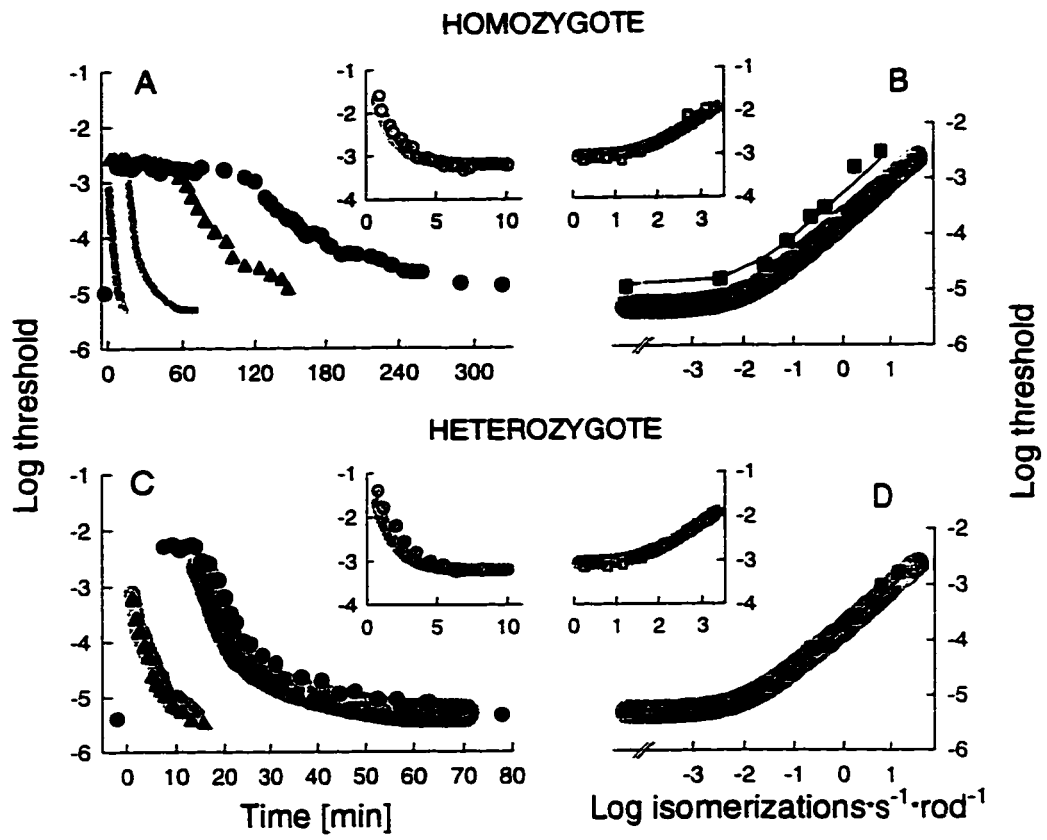


Fig. 4

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ABSTRACTS:

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