Characterization of human GRK7 as a potential cone opsin kinase

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Purpose: Homozygous inactivation of the mouse gene for GRK1 (G protein-coupled receptor kinase 1, or rhodopsin kinase) causes severe defects in the recovery of cone phototransduction. However, electroretinographic (ERG) analyses of human *oguchi* patients with defective GRK1 alleles showed normal or slightly abnormal photopic responses. It remains unclear why the loss of GRK1 yields such different phenotypes in the recovery of mouse and human cones. We examined the localization and enzyme activity of GRK7, the human ortholog of the seventh member of the GRK family, in an attempt to understand its potential role in photopic vision.
Methods: Bioinformatic approaches were used to identify the human GRK7 gene. Human and bovine GRK7 cDNAs were isolated by RT-PCR. Recombinant GRK7, expressed in insect cells, was used to phosphorylate activated rhodopsin. Antibodies raised against GRK7 peptides were used to examine the retina specific expression of GRK7 by immunoblotting and its subcellular localization by immunocytochemistry.
Results: The human GRK7 gene is located on chromosome 3q21, spans at least 10 Kb and consists of 4 exons. In human, GRK7 is expressed exclusively in the retina and is found in all retinal neurons, and specifically, in cone outer segments. Recombinant human GRK7 catalyzes rhodopsin phosphorylation in a light dependent manner. We provide evidence that GRK1 and GRK7 are co-expressed in human cones. In contrast, mouse GRK7 is expressed in many tissues including retina where photoreceptors apparently do not express GRK7.
Conclusions: The presence of GRK7 in human, but not in mouse, cone outer segments suggests that GRK7 may function to provide the normal photopic vision reported by *oguchi* patients with a defective GRK1 gene. The absence of GRK7 expression in cone outer segments of mice is consistent with the notion that mouse cones rely solely on GRK1 to shutoff cone visual pigments.

Human *oguchi* disease is a recessive form of the congenital stationary night blindness (CSNB) that affects predominantly the scotopic vision of affected individuals (for review, see [1]). The molecular defects have been localized to two important genes involved in the shutoff and recovery of rod phototransduction, namely arrestin [2] and rhodopsin kinase (GRK1) [3,4]. GRK1 and arrestin act in sequence to quench photoexcited rhodopsin (R*) [5]. GRK1 recognizes and phosphorylates at least three C-terminal serine/threonine residues of R* [6]. Arrestin then forms a complex with the phosphorylated R* and prevents its interaction with the visual G-protein, transducin. Recessive mutations found in the GRK1 gene of the *oguchi* patients include the homozygous deletion of exon 5 [3,4] and a compound heterozygous missense mutation, V380D, in conjunction with a 4 bp deletion that causes a frame shift at position 523 [4]. These mutations render GRK1 catalytically inactive [7]. The most frequent mutation found in the arrestin gene of *oguchi* patients is a homozygous deletion of nucleotide 1147 (1147*del*A) that causes premature translation termination [2]. The resulting defect in R* deactivation manifests itself as night blindness in these patients because rods

remain saturated or desensitized, thus failing to register other photoisomerization events. These patients need prolonged dark adaptation time (>2 h as compared to minutes required for normal individuals) to fully recover their rod sensitivity. Since normal individuals rely on GRK1 and arrestin to deactivate R* the *oguchi* patients rely on complete regeneration of rhodopsin via the visual cycle [1].

The prolonged dark adaptation time required for *oguchi* patients to recover rod sensitivity is consistent with the phenotypes observed in mice lacking arrestin [8] or GRK1 [9]. The single photon responses recorded from mouse rods lacking arrestin (Arr-/-) revealed slowed recovery. In mouse rods null for GRK1 (GRK1-/-), the single photon response was abnormal and was characterized by prolonged activation, bigger response amplitude, and delayed recovery. These knockout mice are thus suitable models for night blindness. Further, ERG analyses determined that the cone derived retinal responses were greatly delayed in GRK1-/- mice, but not in Arr-/- mice [10], suggesting that GRK1 is essential for the recovery of both rod and cone photoreceptors. The arrestin gene is expressed specifically in rods while cone photoreceptors express a different type of arrestin, called X-arrestin [11,12]. Therefore, the cone derived retinal responses in Arr-/- mice are normal. The greatly delayed cone recovery in GRK1-/- mice is unexpected and contrasts sharply with the "normal to slightly abnormal" photopic vision reported by *oguchi* patients with

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defective GRK1. Since GRK1 is found in rod and cone photoreceptors of both human [13] and mouse [10], why would the loss of GRK1 affect the recovery of mouse cones but not human cones?

To address this question, we cloned and characterized the human ortholog of the seventh member of the G protein coupled receptor kinase (GRK) family, GRK7, and compared the localization of GRK7 in the retinas of human and mouse. GRK7 was initially identified as OlGRK-C in the retina of medaka fish and its message was localized to fish cone photoreceptors [14]. An ortholog of OlGRK-C, capable of phosphorylating rhodopsin in a light dependent manner when expressed in vitro, was then found in cone dominated ground squirrel retinas and because of its localization and abundance, GRK7 was suggested to be a potential cone opsin kinase [15]. Consistent with this notion, we found that GRK7 is expressed uniquely in human retina, present in all retinal neurons, including the cone outer segments. In mouse, GRK7 is widely distributed among tissues and is not localized in photoreceptor outer segments. In addition, recombinant human GRK7 catalyzes rhodopsin phosphorylation in a light dependent manner. Thus, our data support the hypothesis that GRK7 compensates for the loss of functional GRK1 in cones of the *oguchi* patients whose GRK1 alleles are defective.

METHODS

Materials: The human eyes were obtained from the Lions' Eye Bank and Dr. Nick Mamalis (University of Utah, Salt Lake City, UT). Bovine and pig eyes were obtained from a local slaughterhouse (Circle V Meat, Spanish Fork, UT). Chicken eyes were obtained from Dr. Sabine Fuhrmann (Department of Ophthalmology and Visual Sciences, University of Utah). Mouse and rat eyes were obtained from animals purchased from commercial vendors.

Cloning: Human GRK7 (hGRK7) gene was identified by Blast search of the draft human genome using ground squirrel and teleost GRK7 cDNA sequences [14,15] as queries. Two genomic DNA entries, AC067952 and AC068693, were identified and analyzed. AC068693 contained 25 unordered contigs. The contigs that showed homology to ground squirrel GRK7 cDNA were further assembled and the hGRK7 gene structure was derived using the Blast algorithm. The hGRK7 cDNA was assembled by manually excising the introns. Full length hGRK7 cDNA was amplified by polymerase chain reaction (PCR) using the following primers: 5'-ATG GTG GAC ATG GGG GCC CTG (hGRK7N) and 5'-TTA CAA TAA CAA ACA CAC GCC (hGRK7C), from a commercial human retinal cDNA library (Clontech Laboratories, Palo Alto, CA) and from reverse transcribed human retinal mRNA. PCR conditions used were 2 min incubation at 94 °C followed by 35 cycles of 94 °C (1 min), 58 °C (1 min) and 72 °C (3 min). Partial bovine GRK7 cDNA fragment was amplified with specific primers 5'-CCC ATC ACC CAG AGG GCT GGA AC (bGRK7A3) and 5'-CTG CTT CCT AAG CGT TGC TCT GG (bGRK7B3) from bovine retinal yeast two hybrid cDNA library constructed in pGADT7 vector (Clontech Laboratories). The amplification was performed with a 2 min incubation at

94 °C, followed by 40 amplification cycles, each containing 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C. The resulting 290 bp PCR fragment was cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and sequenced using an ABI310 capillary automatic sequencer. The C-terminal/3'UTR was amplified with bovine GRK7 specific primer 5'-GGT TAT ATG GCT CCT GAA ATC CTA (bGRK7A) using GeneRacer kit on bovine retinal mRNA isolated using FastTrack™ 2.0 Kit (both kits from Invitrogen). The N-terminal sequence was obtained from bovine retinal yeast two hybrid cDNA library with primers 5'-GCC AAG AAG AGT CTG CAA ATA TCT (bGRK7B) and 5'-GA(T/C) ATG GGN G(C/G)N (T/C)TN GA(T/C) AA (bGRK7A5, corresponding to amino acids 3-9 of human GRK7 sequence). The 5'-UTR was obtained by 5'-TGG CGG AGC TGC TCG CAG CTC TG (bGRK7B2) and T7 primer. All the bovine GRK7's C-terminal/3'UTR, N-terminal and 5'UTR PCR fragments were amplified using Expand™ Long Template PCR System (Roche Molecular Biochemicals, Indianapolis, IN), and sequences confirmed from both ends with a LI-COR 4000L automatic DNA sequencer with infrared tagged universal primers.

RT-PCR: Human retinal total RNA was isolated using Trizol™reagent (GibcoBRL, Grand Island, NY) by following manufacturer's instructions. Other human total RNAs were obtained commercially (Human total RNA Panel I, Clontech Laboratories). The total RNAs (2 μg) were primed with poly(dT) and reverse transcribed using Superscript II™ reverse transcriptase (GibcoBRL) by following manufacturer's instructions. The resulting cDNAs were used as templates for subsequent PCR reactions. The presence of human β-actin transcript was verified by PCR using primers 5'-CTG CCC TGA GGC ACT CTT CCA GCC (hActinB-1) and 5'-AGG ATG GAG CCG CCG ATC CAC ACG (hActinB-2). The presence of hGRK7 transcript was checked by PCR using primers: 5'-AAT GGT TAC ATG GCT CCT GAG ATC CT (hGRK7-3) and 5'-AGG CCA CGT TCC AGG CGA GGA AAG TT (hGRK7-4). PCR conditions used were 94 °C for 2 min, followed by 30 cycles of 94 °C (1 min), 58 °C (1 min) and 72 °C (1 min). The resulting PCR products (330 bp for hGRK7, 280 bp for β-actin) were analyzed by 1.5% agarose gel electrophoresis.

Antibody production: Using the Imject™ Sulfhydryl Reactive Antibody Production and Purification kit (Pierce, Rockford, IL), 2 mg of the C-terminal peptide of hGRK7, NRPTGCEEGNSSKSGVC (GRK7-C), were synthesized and conjugated to bovine serum albumin (BSA). The BSA conjugated GRK7-C was used to immunize New Zealand White rabbits using the Freund adjuvant system (Cocalico Biologicals, Reamstown, PA). The resulting antiserum was purified over an immobilized GRK7-C column using the kit mentioned above following the manufacturer's instructions. Purified antibody (UU45APC) was stored at -20 °C. Pre-absorbed UU45APC was prepared by 30 min incubation of the antibody with immobilized GRK7-C and was used as controls for immunocytochemistry and immunoblotting. We also generated antibodies against the N-terminal peptides of hGRK7, GALDNLIANTAYLQARK (GRK7-N1) and

CDSKELQRRRRLAL (GRK7-N2). All three antibodies recognized recombinant hGRK7 expressed in H5 insect cells (data not shown) but only UU45APC recognized native hGRK7 in retinal extracts, presumably due to its higher affinity for endogenous hGRK7.

Immunoblotting: Retinal extracts of various animal species were prepared by homogenizing the retinas in phosphate buffered saline (PBS) in the presence of 250 µM phenylmethylsulfonyl fluoride (PMSF). Proteins (30 µg) were separated by 10% SDS-PAGE followed by electro-transfer to nitrocellulose membrane. The expression of GRK7 was examined by the binding of primary antibodies and horseradish peroxidase conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) to the membrane. Signals were detected using the Renaissance Chemiluminescence kit (NEN Life Science Products, Boston, MA). The dilutions of primary antibodies used were as followed: UU45APC (1:1,000), G8 (1:5,000), D11 (1:1,000). Extracts from other mouse tissues were prepared similarly. Human tissue extracts were purchased (Human Protein Medleys, Clontech).

Immunocytochemistry: Eyes were fixed in freshly pre-

Figure 1. Characterization of human GRK7 gene. **A**: Exon/Intron arrangement of the human GRK1 and GRK7 genes. Black boxes, exons; white boxes, UTRs. The genes are located on different chromosomes and their structures are unrelated. **B**: Dendrogram based on amino acid sequence alignment showing that GRK1 is ancestral to GRK7. The genes shown in the dendrogram are listed with their GenBank accession numbers: mouse GRK1 (AF085240), bovine GRK1 (M73836), human GRK1 (U63973), rat GRK1 (U63971), carp GRK1 (AB055657), chicken GRK1 (AF019766), ground squirrel GRK7 (AF063016), teleost GRK7 (AB009568), carp GRK7 (AB055658), human GRK7 (AF439409), and bovine GRK7 (AY049726).

pared 4% paraformaldehyde in 0.1 M phosphate buffer for 1-2 h or overnight at 4 °C, rinsed in buffer, and cryoprotected in 15% sucrose in buffer followed by 30% sucrose in buffer. After embedding in TBS (Triangle Biomedical Sciences, Durham, NC), sections 14 µm thick were cut and blocked with 0.3% hydrogen peroxide in 0.1% Triton X-100 in 0.1 M phosphate buffer (PBT) followed by 10% goat serum in PBT. For chromogenic detection, the sections were further blocked by the A-Block and the B-block (Vector Laboratories, Burlingame, CA) for 30 min. UU45APC and pre-absorbed UU45APC were used at 1:500 dilution in PBT at 4 °C overnight followed by three 10 min PBT washes. Biotin conjugated secondary antibodies were used at 1:200 dilution followed by three 5 min PBT washes. The GRK7 signals were amplified by the ABC (Avidin/Biotin/Complex) kit (Vector Laboratories) and visualized by 30 s 3,3'-diaminobenzidine (DAB) staining. For fluorescent detection, FITC and rhodamine conjugated secondary antibodies (1:300 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) were applied for 1 h at room temperature, and rinsed three times in PBT. Monoclonal antibody 7G6 (1:100 dilution) was used to identify human cones [16] whereas rhodamine labeled peanut agglutinin (PNA) was co-incubated with secondary antibody to demonstrate extracellular cone sheaths as described [17]. The fluorescent signals were imaged using a Zeiss LSM 510 confocal microscope.

Expression of GRK7: The BAC-to-BAC baculovirus expression system (Invitrogen) was used to express human GRK7 according to manufacturer's instructions. Briefly, the full length human GRK7 cDNA was subcloned into pFastBac1 vector and transformed into DH10BAC strain in which recombination of the hGRK7 cDNA with the baculoviral genome was selected. The recombinant baculoviral genome carrying the hGRK7 cDNA under the control of the polyhedrin promoter was transfected into confluent H5 cells to produce the recombinant GRK7 virus. Recombinant GRK7 virus was amplified by repetitive rounds of infection/harvest. To express GRK7, confluent H5 cell cultures were infected with the GRK7 virus at m.o.i (multiplicity of infection) = 1 in Grace's insect medium (GibcoBRL) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 4 mM mevalonate lactone (Sigma, St. Louis, MO), 0.004% Yeastolate and 0.0035% Lactalbumin Hydrolysate (GibcoBRL). The infected H5 cells were harvested 48 h post infection and stored at -80 °C.

Light-dependent rhodopsin phosphorylation: All procedures were performed in a darkroom under a red safety light except otherwise noted. Bovine rod outer segments (ROS) were purified by sucrose floatation [18] and washed three times with 6 M urea in TM buffer (50 mM Tris.HCl, pH = 7.5, 5 mM MgCl$_2$). The urea washed ROS membranes (uROS) were washed three times with TM buffer, stored at -80 °C and used as substrates for GRK7. Rhodopsin content was determined by difference in absorption at 500 nm using the extinction coefficient of 40,000. Reaction mixtures (50 µl) containing 10 µM rhodopsin, 50 µg H5 cell extracts containing recombinant GRK7, 200 µM γ-^{32}P-ATP (specific activity = 2,000 cpm/pmole) and 5 mM MgCl$_2$ in 50 mM Tris/HCl (pH = 7.5) were prepared and exposed to room light for 30 min at 37 °C. The
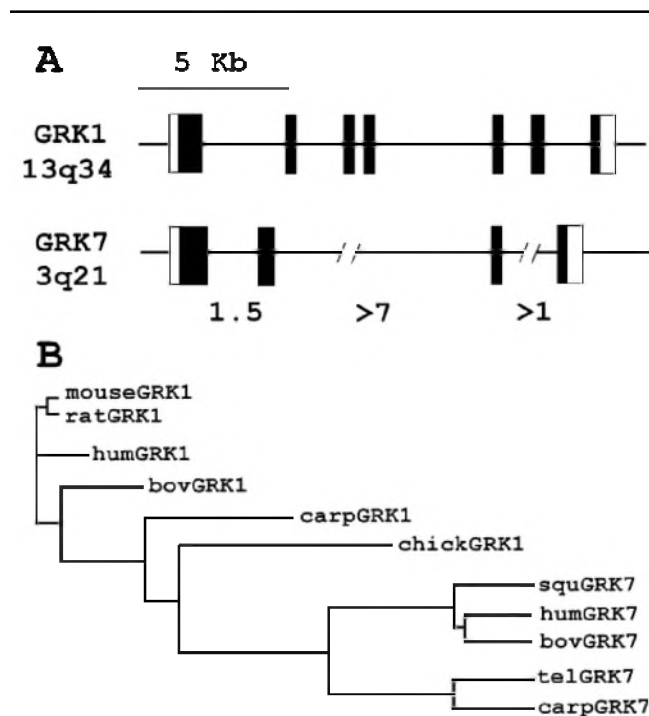
reaction was stopped with 50 µl 2X SDS-PAGE sample buffer. The unincorporated ATP was separated by 10% SDS-PAGE, and phosphorylated rhodopsin visualized by autoradiography.

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                 GRK7-N1                GRK7-N2
H7 MVDMGALDNLIANTAYLQARKPSDCDSKELQ----RRRRSLALPGLQGCAELRQKLSLNF
B7 MVDMGGLDNLIANTAYLQARKSSDADSKELQ----RRRRSLMLPGPQSCEQLRQALATDF
C7 MCDMGGLDNLVANTAYLKAQ---GGDDKEMK----KRRRSLSLPKPEQCAALRSTLDKDF
B1 -MDFGSLETVVANSAFIAARGSFDASSGPAS-RDRKYLARLKLPPLSKCEALRESLDLGF
C1 -MDIGGLTTVVANSAYISARGSFDGTANPAANRDKKYHAKLKLPHITVCEGLRETLDLQF
H1 -MDFGSLETVVANSAFIAARGSFDGSSSQPS-RDKKYLAKLKLPPLSKCESLRDSLSLEF
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H7 HSLCEQQPIGRRLFRDFLAT-VPTFRKAATFLEDVQNWELAEEGPTKDSALQGLVATCAS
B7 HSLCEQQPIGRRLFRDFLAT-VPAYQEARGFLEEVQSWELAEEGPAKGSALQGLVTTCAA
C7 ESLCEKQPIGKRFFRQYLDQGGPECNAAAEFLDDLNDWELS-EAAAKDKARTNIINKFCK
B1 EGMCLEQPIGKRLFQQFLRT-HEQHGPALQLWKDIEDYDTADDDALRPQKAQA--LRAAYL
C1 KSICVEQPIGKRLFQEFLET-TNEYKGPCRLWKDIETYDTAEDKDRAQKAAK--ILQRYH
H1 ESVCLEQPIGKKLFQQFLQS-AEKHLPALELWKDIEDYDTADNDLQPQKAQT--ILAQYL
   ..:* :****:::*::*      .   :   .::: :: : :      .*    :

H7 APAPGNPQPFLSQAVATKCQAATTEEERVAAVTLAKAEAMAFLQEQPFKDFVTSAFYDKF
B7 APVPGRPHPFFSPALVTKCQAATTDDDRASLVELAKAEVMAFLQDQPFREFLASPFYDKF
C7 DGSK-SSLTFLTGDVATKCKAVT-DKDFEEVMGQVKEATKEFLEKGKPFTDYQTSEFFEKF
B1 EPQAQLFCSFLDAETVARARAG----AGDGLFQPLLRAVLAHLGCQAPFQEFLDSLYFLRF
C1 EPSAKHFCPFLPENDITKVKERH-EEAADDLFVQIQASVFDFLKEVPFTFYLETMYFKRF
H1 DPQAKLFCSFLDEGIVARFKEGP-VEIQDGLFQPLLQATLAHLGCQAPFQEYLGSLYFLRF
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H7 LQWKLFEMQPVSDKYFTEFPRVLGKGGFGEVCAVQVKNTGKMYACKKLDKKRLKKKGGEKM
B7 LQWKVFEMQPVSDKYFEEFPRVLGKGGFGEVCAVQVKNTGKMYACKKLDKKRLKKKNGEKM
C7 LQWKEYEKQPITEKYFYEFPRTLGKGGFGEVCAVQVKNTGQMYACKKLCKKRLKKKHGEKM
B1 LQWKWLEAQPHGEDWFLDFRVLGRGGFGEVFACQMKATGKLYACKKLNKKRLKKKRKGYQG
C1 LQWKWLEMQPVAEDWFLDFRVLGKGGFGEVSACQMKATGKLYACKKLNKKRLKKRKGYEG
H1 LQWKWLEAQPHGEDWFLDFRVLGKGGFGEVSACQMKATGKLYACKKLNKKRLKKKRKGYQG
   ****  *  :.: : :**.**:*:****** * *:.:**** ::*:******* *****:

H7 ALLEKEILEKVSSPFIVSLAYAFESKTHLCLVMSLMNGGDLKFHIYNVGT--RGLDMSRV
B7 ALLEKEILERVSSPFIVSLAYAFESKSHLCLVMSLMNGGDLKFHIYSVGE--PGLDMSRV
C7 ALLEKKILEKVNSLFIVSLAYAYDTKTHLCLVMSLMNGGDLKYHIYNIGE--KGIEMEPI
B1 AMVEKKILAKVHSRFIVSLAYAFETKTDLCLVMTIMNGGDIRYHIYNVDEDNPGFQEPRA
C1 AMVEKRILARVMSRFIVSLAYAFQTKTELCLVMTIMNGGDLRYHIYNVDENNPGFSEARA
H1 AMVEKKILMKVHSRFIVSLAYAFETKADLCLVMTIMNGGDIRYHIYNVNEENPGFPEPRA
   *::**.**  :* * ********:::*:.:.****::*****:.:*:.   :  *..:    *

H7 IFYSAQIACGMLHLHELGIVYRDMKPENVLLDDLGNCRLSDLGLAVEMKGGKPITQ-RAG
B7 IFYSAQITCGVLHLHSLGIVYRDMKPENVLLDDLGNCRLSDLGLAVQIQDGKPITQ-RAG
C7 IYYTAQITTGHLQLHNMDIVYRDMKPENVLLDSQGQCRLSDLGLAVEIPVGKTTTQ-KAG
B1 IFYTAQIVSGLEHLHQRNIIYRDLKPENLLDDDGNVRISDLGLAVELKAGQTKTKGYAG
C1 CYYAAQIIQGLEHLMQKRIIYRDLKPENVLDNEGHVRISDLGLAVELADDQLKTKGYAG
H1 LFYTAQIICGLEHLHQRRIVYRDLKPENVLLDNDGNVRISDLGLAVELLDGQSKTKGYAG
   :*::**  *::: :**.  *:***:*********.  *.:*********::  .:  *  **

H7 TNGYMAPEILMEKVSYSYPVDWFAMGCSIYEMVAGRTPFK---DYKEKVSKEDLKQRTLQ
B7 TNGYMAPEILHEKASYSYPVDWFAMGCSIYEMVAGRTPFR---DYKEKVSKEDLKQRTLK
C7 TGAYMAPEILTETP-YRTSVDWWALGCSIYEMVAGYTPFKGPEAKKEKVEKEEVQRIIN
B1 TPGFMAPELLLGEE-YDFSVDYFALGVTLYEMIAARGPFR---ARGEKVENKELKQRVLE
C1 TPGFMAPELLKGEE-YDYSVDYFTLGVTLYEFIAAKGPFR---TRGEKVENKEVKKRILN
H1 TPGFMAPELLQGEE-YDFSVDYFALGVTLYEMIAARGPFR---ARGEKVENKELKHRIIS
   *. *****:  *:  * .::*  :.*.::* :::*:  *:      .::**:::: *  :.

H7 DEVKFQHDNFTEEAKDICRLFLAKKPEQRLGSRE-KSDDPRKHHFFKTINFPRLEAGLIE
B7 EEVRFQHSNFTEEAKDICRLFLAKTPEQRLGSRE-KSDDPRKHHFFKTINFPRLEAGLVE
C7 EEPKFEHKWFNAPTIDIIKQFLKKKIDERLGCK---GDDPRKHEWFKSINFARLEAGLID
B1 QAVTFPDK-FSPASKDFCEALLQKDPEKRLGFRDGSCDGLRTHPLFRDISWRQLEAGMLT
C1 DPVTFPES-FSEKAKSVCEGLLAKEVDKRLGFKNGSCDELRTHPFFSEINWRKLDAGILP
H1 HPVKYPDK-FSQASKDFCEALLEKDPEKRLGFRDETCDKLRAHPLFKDLNWRQLEAGMLM
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H7 PPFVPDPSVVYAKDIAEIDDFSEVRGVEFDDKDKQFFKNFATGAVPIAWQEEIIETGLFE
B7 PPFVPDPSVVYAKDIEIDDFSEVRGVEFDDNDKQFFQRFATGAVPIAWQEEIIETGLFA
C7 PPWVPKPNVVYAKDTGDIAEFSEIKGIEFDAKDEKFFKEFSTGAVSIAWQKEMIDTGLFD
B1 PPFVPDSRTVYAKNIQDVGAFSTVKGVAFEKADTEFFQEFASGTCPIPWQEEMIETGVFG
C1 PPFVPDSKTVYAKNLDDVGAFSTVKGVCLEDDDKTFYDEFASGNISIPWQEEMIETGIYG
H1 PPFIPDSKTVYAKDIQDVGAFSTVKGVAFDKTDTEFFQEFATGNCPIPWQEEMIETGIFG
   **::*.*.:.****      :: :**   **   :  ::* :.  .*:.:**:*:*:**:  :

                         GRK7-C
H7 ELN---------DPNR-PTGCEEGNSSKSGVCLLL
B7 ELN---------DPNR-PAGCGEG-SSRSGVCLLL
C7 ELN---------DPNRKESSGGLDDDKKSGICTLL
B1 DLNVWRPDGQMPDDMKGVSGQEAAPSSKSGMCVLS
C1 ELNVWHGPNGTVPNDLR--RESILEQPPVSSTCSIS
H1 ELNVWRSDGQMPDDMKGISGG-SSSSKSGMCLVS
   :**                :  :      :*. * :
                               CaaX
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RESULTS

The cloning of human and bovine GRK7. To explore whether GRK7 is expressed in human tissues, we identified orthologous human GRK7 genomic contigs and derived the complete cDNA sequence and gene structure. The hGRK7 gene maps to chromosome 3, spans at least 10 Kb (Figure 1A), consists of 4 exons and encodes a protein of 553 amino acids. By screening the Stanford TNG3 radiation hybrid panel, the hGRK7 gene was fine mapped to chromosome 3q21, closely linked to the marker D3S1309 with a LOD score of 8.9. One unidentified retinal disease locus, GLC1C (adult onset primary open angle Glaucoma 1 C) [19] was mapped to the vicinity of hGRK7. The full length cDNA of hGRK7 was subsequently cloned (GenBank accession number AF439409). A bovine GRK7 cDNA fragment was identified by degenerate RT-PCR from bovine retinal RNA and full length cDNA was obtained by RACE (GenBank accession number AY049726). Among the six known members of the GRK family, members of the GRK1 subfamily show the greatest sequence homology to GRK7 (Figure 1B). According to this dendrogram, GRK1 is ancestral to GRK7. Similarity between the two GRKs places GRK7 within the GRK1 subgroup of the GRK family. Figure 2 shows the alignments of GRK1 and GRK7 in human, carp and bovine. A hallmark of this subgroup is the presence of the carboxyl terminal CaaX motif (Figure 2), a common feature of isoprenylated proteins [20]. The CLLL motif of GRK7 specifies protein geranylgeranylation, while the CLVS motif of GRK1 specifies protein farnesylation [21]. Mutagenesis analysis on GRK1 showed that these prenyl modifications are essential for the interaction with rod outer segment membranes [22].

Localization of GRK7 in human. Human GRK7 appears to be retina specific as shown by RT-PCR using RNA from various human tissues (Figure 3A). A polyclonal antibody (UU45APC) produced against the peptide GRK7-C, recognized a 64 KDa band in the retina but not in other human tissues (Figure 3B), consistent with the results obtained by RT-PCR. The sequence of the GRK7-C peptide is not contained in GRK2-6, and is sufficiently different from the C-terminus of GRK1 such that the UU45APC antibody is specific to GRK7. This 64 KDa signal was blocked by absorbing
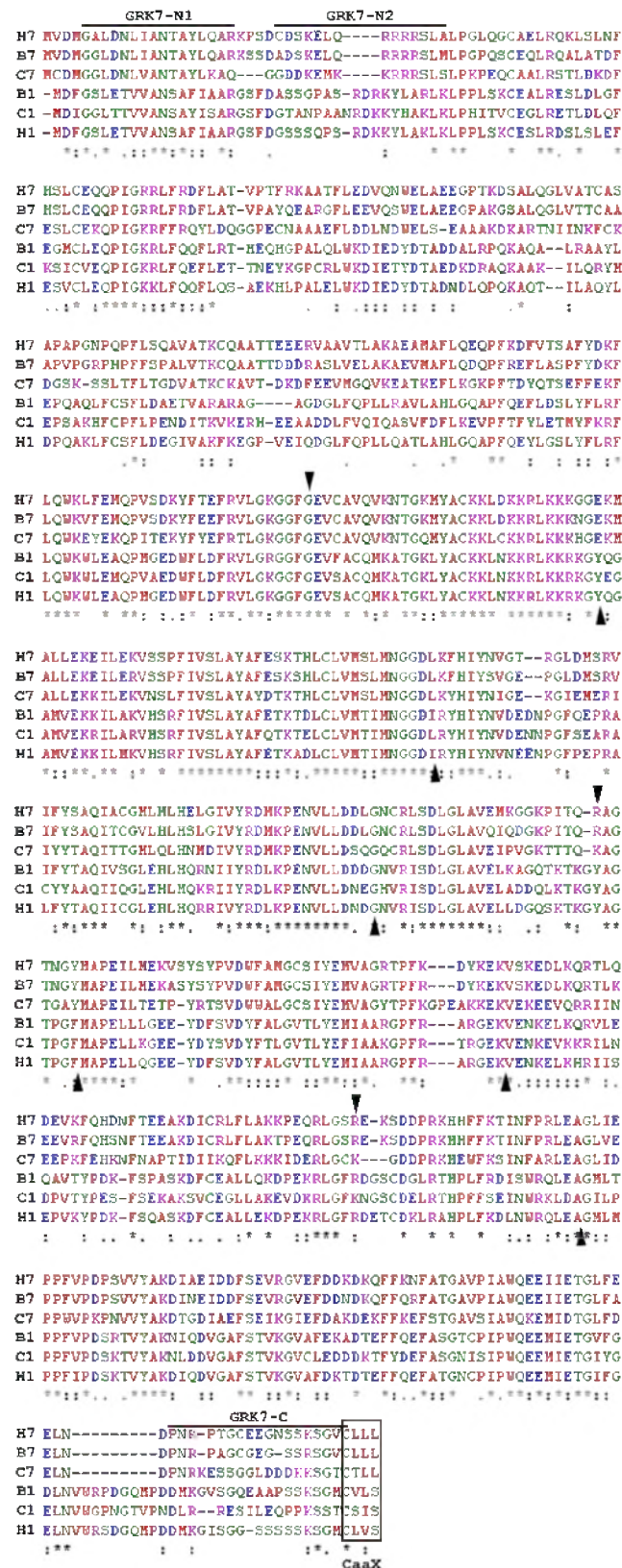
Figure 2. Amino acid sequence alignment of vertebrate GRK1 and GRK7 polypeptides. The alignment was produced using ClustalW 1.8 method. H7: human GRK7, B7: bovine GRK7, C7: carp GRK7, B1: bovine GRK1, C1: carp GRK1, H1: human GRK1. Acidic residues are presented in blue, basic residues in magenta, small and hydrophobic residues in red, other residues in green. In the consensus line immediately below each alignment, identical or conserved residue is marked by an asterisk, conserved substitution by a colon and semi-conserved substitution by a period. The carboxyl terminal Caax motif is indicated by a rectangle. The CLVS motif of human GRK1 specifies protein farnesylation, while the CLLL motif of human GRK7 specifies protein geranylgeranylation. Sequences of peptides used for antibody production, GRK7-N1, GRK7-N2 and GRK7-C, are as indicated. The exon boundaries are marked by downward arrowhead for human GRK7 and by upward arrowhead for human GRK1.

UU45APC with immobilized GRK7-C peptide (data not shown). Using chromogenic immunocytochemistry we found that the nuclear layers, the inner and outer plexiform layers as well as inner segment layer of the human retina stained positive for GRK7. In particular, UU45APC stained the cone outer segments (COS) but not the rod outer segments (Figure 4A). All the signals were blocked by pre-absorbing UU45APC with immobilized GRK7-C, strongly suggesting that the labeling was specific (Figure 4B). In adult retina, the GRK7 staining appears uniformly throughout the cone outer segments (Figure 4D,E). In retina from a 4 month old donor, GRK7 was specifically localized to the proximal portion of the cone outer segments by fluorescent double labeling (Figure 4F-H) using UU45APC and a monoclonal antibody 7G6 that is specific for primate cones [16].

Light dependent phosphorylation of rhodopsin by recombinant GRK7. The BAC-to-BAC system was used to produce recombinant baculovirus expressing hGRK7 in High-Five (H5) insect cells. Recombinant GRK7 has an apparent molecular weight of 64 KDa (Figure 5A). When incubated with urea washed rod outer segment (uROS) membranes, recombinant GRK7 catalyzes rhodopsin phosphorylation in a light dependent manner (Figure 5B). In human, the presence of GRK7 in cone outer segments and the light dependent rhodopsin phosphorylation catalyzed by recombinant GRK7 suggest that it may function as a cone opsin kinase.

Co-localization of GRK7 and GRK1 in human cone outer segments. During characterization of recombinant GRK7 we found that monoclonal antibody G8 (Affinity BioReagents,

Golden, CO), raised against GRK1 and used to localize GRK1 to human rod and cone outer segments [13], reacts with recombinant hGRK7. This initially cast doubt as to the presence of GRK1 in human cones. Subsequently we observed that a monoclonal antibody D11 (Affinity BioReagents), also raised against GRK1, does not cross react with GRK7 (Figure 6A). Monoclonal antibody D11 and polyclonal antibody UU45APC were then used to determine whether human cones express GRK1 and GRK7, respectively. As shown in Figure

Figure 4. Localization of GRK7 in human retina. **A** and **B**: Chromogenic detection of hGRK7 by UU45APC (1:1,000 dilution, **A**) and pre-absorbed UU45APC (1:500 dilution, **B**) on sections of a 2 h fixed retina from an adult donor 3 h postmortem. Bar represents 20 μm. **C** to **E**: Fluorescent detection of GRK7 in sections of an overnight fixed adult retina >6 h postmortem. **C**: Labeling of cone sheath by rhodamine conjugated peanut agglutinin. **D**: Labeling of the photoreceptor layer using UU45APC (1:1,000 dilution) visualized by FITC conjugated secondary antibody. **E**: Co-labeling of UU45APC and peanut agglutinin. **F** to **H**: Fluorescent staining of a 1 h fixed retina from a 4 month old female whose eye was enucleated due to retinoblastoma. The eye was received 2 h post enucleation. **F**: Labeling of cones using monoclonal antibody 7G6 (1:100 dilution) visualized by rhodamine conjugated secondary antibody. **G**: Labeling of the photoreceptor layer using UU45APC (1:500 dilution) visualized by FITC conjugated secondary antibody. **H**: Co-labeling of UU45APC and 7G6. Bar represents 10 μm. GRK7 is present in most retinal layers, but is absent in rod outer segments (**A**). The presence of GRK7 in the cone outer segments is obvious in **D**, **E**, **G**, and H. However, the proximal part of cone outer segments is more intensively labeled by UU45APC in the 4 month old retina fixed for 1 h. OS: outer segments, IS: inner segments, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, GCL: ganglion cell layer.

Figure 3. Retina specific expression of human GRK7. **A**: RT-PCR analyses of human total RNAs using GRK7 specific primers (left panel) and β-actin specific primers (right panel). Shown here are reverse images of ethidium bromide stained agarose gels. The leftmost lanes of each panel are 1 Kb DNA markers (GibcoBRL). **B**: Western blot analysis of various human tissue extracts (30 μg/lane from Clontech human protein medley) using UU45APC (1:1,000 dilution). B: brain, H: heart, K: kidney, Li: liver, Lu: lung, Ln: lymph node, R: retina, S: spleen, T: testis, Th: thyroid gland, Tr: trachea. Human GRK7 transcript and polypeptide can only be detected in the retina.
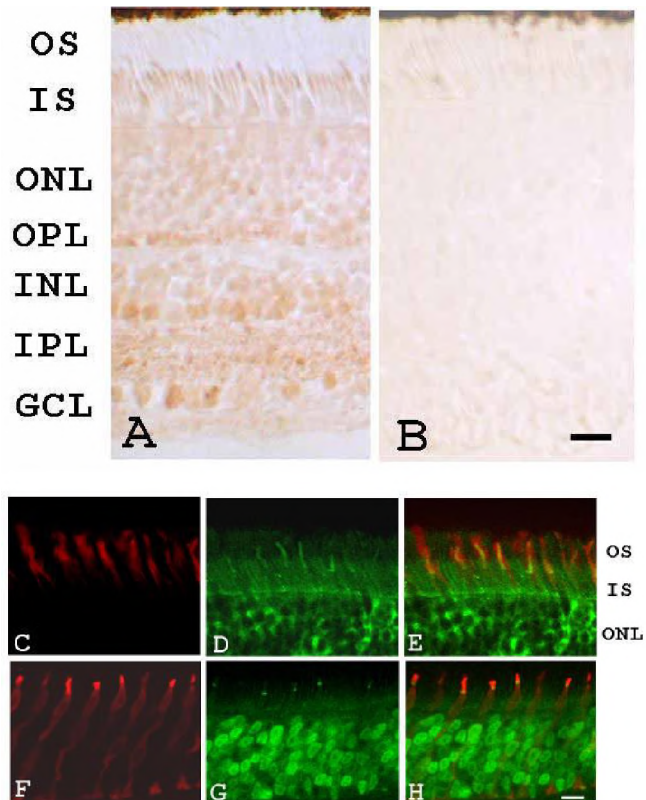
6B, both GRK1 and GRK7 were expressed in human cones. Interestingly for all human retina specimens examined, GRK1 is present throughout the cone outer segments regardless of the age of the retinas. Similar localizations were found for GRK7 in all adult retinas examined. However, GRK7 is localized to the proximal portion of the cone outer segments in the retina of a 4 month old donor (Figure 6B, right panels), indicating that GRK7 may be contained in specific cellular compartment during development.

Expression of GRK7 in many mouse tissues. Approaches used successfully to clone bovine and human GRK7 (degenerate RT/PCR and library screening) thus far failed to isolate sequences resembling GRK7 from mouse retina. In addition, we failed to identify mouse and human expressed sequence tags (ESTs) that contain GRK7-like sequences in GenBank. A genomic Zoo blot demonstrated the presence of GRK7-like sequences in human, rat, mouse, dog, bovine and pig (results not shown). Western blot analyses showed that the UU45APC antibody recognized a specific band in the retinal extracts of mouse, rat, bovine and pig, demonstrating the presence of GRK7 gene in mouse (Figure 7A). In contrast to human, mouse GRK7 is not retina specific (Figure 7B), it can also be found in brain, olfactory bulb, lung and pancreas among the tissues examined. In the mouse retina, UU45APC stained the inner and outer plexiform layers and the nucleus of the inner nuclear and ganglion cell layers. The outer nuclear layer could be stained weakly if the retina were fixed for less than 2 h. Overnight fixation significantly reduced the signals of all nuclear layers (data not shown). Regardless of the fixation time, the outer segment layers were negative for GRK7. The lack of outer segment layer staining by UU45APC in mouse retina was confirmed by fluorescent double labeling using rhodamine labeled peanut agglutinin (Figure 8). Similar immunocy-

tochemical results were obtained in rat (data not shown). GRK7 is therefore unlikely to be a cone opsin kinase in mouse. Unlike human cones where both GRK1 and GRK7 are present, mouse cones apparently express only GRK1, providing a molecular explanation for the dramatic difference in phenotype in *oguchi* patients and GRK1-/- mice.

Figure 6. Coexistence of GRK1 and GRK7 in human cone outer segments. **A**: Western Blot analyses of two fold serial dilutions of H5 cell extracts (10, 5, 2.5, 1.25 µg from left to right) containing recombinant hGRK7 using various antibodies as indicated. The monoclonal anti-GRK1 antibody G8 (1:5,000 dilution) recognized GRK7 while GRK1 specific antibody D11 (at 1:1,000 dilution) did not. UU45APC was used at 1:3,000 dilution. **B**: fluorescent double labeling of human retinas using D11 (1:500 dilution) and UU45APC (1:1,000 dilution) on sections of an adult retina described in Figure 4C-E (left panels) and of a 4 month old retina described in Figure 4F-H (right panels). Rhodamine conjugated anti-mouse antibody was used to localize D11 and FITC conjugated anti-rabbit antibody was used to localize UU45APC. Upper panels: GRK1 signal (red), middle panels: GRK7 signal (green) and lower panels: co-localization of GRK1 and GRK7 in the cone outer segments (yellow). Images were taken and analyzed using a Zeiss LSM510 confocal microscope.

Figure 5. Light dependent phosphorylation of rhodopsin by recombinant hGRK7. **A**: Western blot analysis of 50 µg protein extracts of H5 cells infected with wild type AcNPV virus (lane 1) and recombinant GRK7 virus (lane 2) using UU45APC (1:1,000 dilution). **B**: Phosphorylation of rhodopsin by recombinant GRK7 under various conditions. See Methods for experimental details. The phosphorylated rhodopsin monomer and dimer are indicated by arrows.
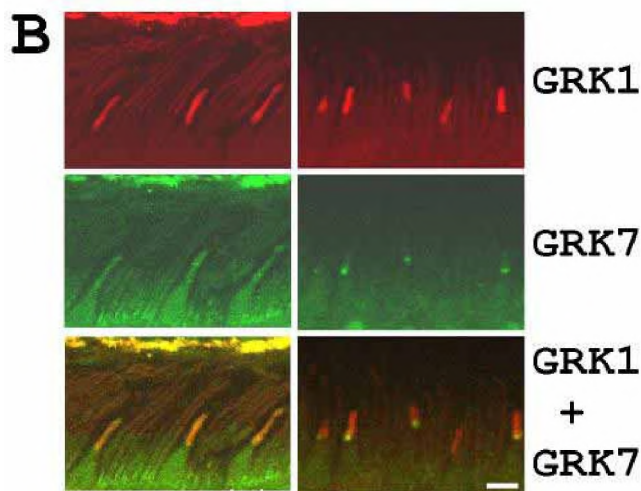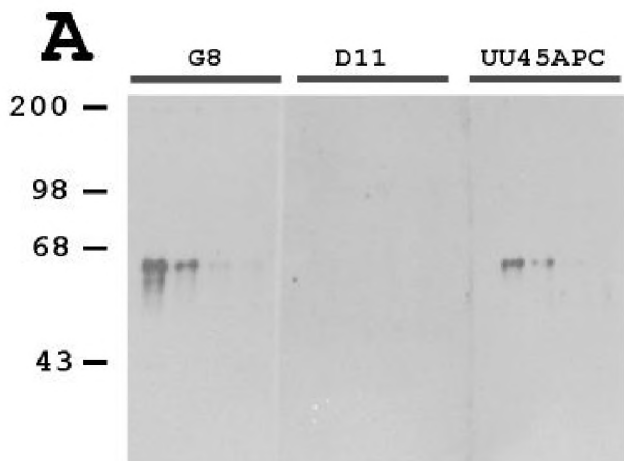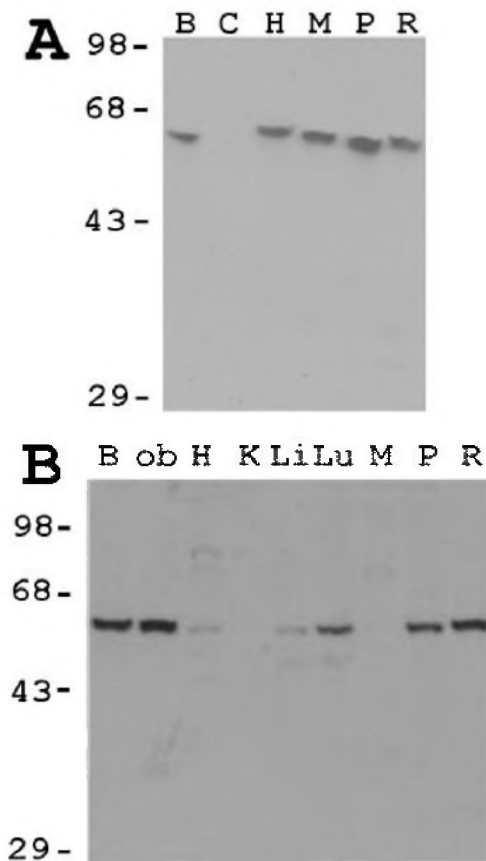
Figure 7. GRK7 from other species. **A**: 50 μg of retinal extracts from bovine (B), chicken (C), human (H), mouse (M), rat (R) and pig (P) retinas were analyzed by Western blot using UU45APC (1:1,000 dilution). The presence of GRK7 in all species examined except in chicken is demonstrated. **B**: Western Blot analysis of 30 μg protein extracts from various mouse tissues using UU45APC (1:1,000 dilution). GRK7 protein is found in several mouse tissues and is especially prevalent in brain, olfactory bulb, pancreas and retina. B: brain, Ob: olfactory bulb, H: heart, K: kidney, Li: liver, Lu: lung, M: skeletal muscle, P: pancreas, R: retina.

DISCUSSION

Three hypotheses have been raised to account for the discordance regarding the recovery of cone function in GRK1-/- mice and human *oguchi* disease patients with defective GRK1 gene [10]. The first hypothesis downplays the necessity of phosphorylation of activated cone pigments for its deactivation and suggests that regeneration of cone pigments plays a more important role [3]. Support for this hypothesis comes from the fact that dark adaptation of cone photoreceptors was normal in *oguchi* patients after 99% bleach of the cone pigments [3] and a recent finding that the non-covalent binding of 11-cis-retinal to opsin activates the phototransduction cascade in rods but deactivates phototransduction in cones [23]. However, the defect in the recovery of GRK1-/- cones indicates that regeneration of cone pigments is not sufficient to account for the normal recovery [10], providing that regeneration of cone pigments proceeds normally in GRK1-/- mice. A second hypothesis calls for a different action of GRK1 in terminating the activities of R* and activated cone visual pigments. This hypothesis speculated that the binding of GRK1 to activated cone pigment is enough to quench its activity, even in the absence of kinase activity. Support for this hypothesis came from a finding that GRK1 binding to metarhodopsin II could block the activation of transducin [24]. However, a recent report by Mendez et al. negates the physiological relevance of this effect of GRK1 on rod phototransduction [6]. The single photon responses of transgenic rods with all phosphorylation sites in the C-terminal tail of rhodopsin removed was found to be indistinguishable from those of the GRK1-/- rods, implying that the binding of GRK1 to rhodopsin is not sufficient to quench rod phototransduction [6], let alone quenching cone phototransduction. The third hypothesis calls for the presence of another protein kinase in human cones to compensate for the loss of functional GRK1 in *oguchi* patients. Support for this hypothesis came from the cloning of GRK7, a potential cone opsin kinase showing high homology to GRK1, from several vertebrate species including medaka fish [14], ground
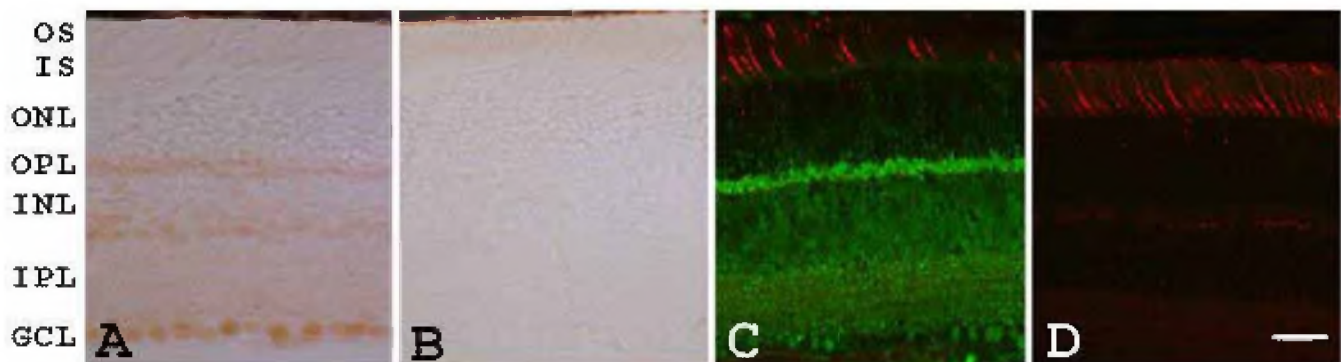

Figure 8. GRK7 is not present in mouse cone outer segments. Localization of GRK7 using UU45APC (**A** and **C**, used at 1:1,000 dilution) and pre-absorbed UU45APC (**B** and **D**, used at 1:500 dilution) in overnight fixed mouse retina by chromogenic detection (**A** and **B**) and by fluorescent detection (**C** and **D**). GRK7 is present in the OPL (outer plexiform layer), INL (inner nuclear layer), IPL (inner plexiform layer) and GCL (ganglion cell layer), but absent in the ONL (outer nuclear layer), the IS (inner segments) and the OS (outer segments). Fluorescent double labeling using rhodamine conjugated peanut agglutinin to bind to mouse cone sheaths reveals the absence of GRK7 signal in mouse cone outer segments. Bar represents 30 μm.

squirrel and pig [15]. Our data provide additional support for the third hypothesis by showing the localization and the activity of human GRK7. Phosphorylation of cone visual pigments by GRK7 has yet to be demonstrated as a final proof. Nonetheless, our study indicates that human cone photoreceptors have a G-protein coupled receptor kinase, GRK7, not found in the cone outer segments of mice. It is possible that in human cones GRK1 plays only a minor role deactivating photolyzed cone visual pigments while GRK7 plays a much more prominent role. Alternatively, the expression or the activity of GRK7 maybe up regulated in those patients to compensate for the loss of functional GRK1. In mouse cones GRK1 is the only kinase known to deactivate cone pigments [10]. Human and mouse apparently rely on different GRKs to turn off cone pigments, a phenomenon which may be linked to their daily activities. Humans are diurnal while mice are nocturnal. There is no selective pressure for the mouse to rely on the expression of GRK7 in the photoreceptors. On the other hand, expressing two GRKs in human cones may provide an additional mechanism of phototransduction regulation for human cones to cope with the much more dynamic light input they are facing.

GRK7 has been cloned from a variety of species including medaka fish, carp, ground squirrel, pig, cattle and human, but not from mouse, rat or chicken. The presence of GRK7 in mouse is established in this study at the protein level. The peptide antibody UU45APC recognized a specific band in the retinas of mouse, rat, pig, and bovine (Figure 7A). The sequence of the peptide used to produce UU45APC is 70-90% identical among ground squirrel, bovine and human. In addition, the immunoblot and immunocytochemical signals recognized by UU45APC from mouse retinas could be blocked by GRK7-C peptide absorption (Figure 8), strongly suggesting that they are authentic mouse GRK7 signals. However, we have not yet isolated the mouse GRK7 cDNA. When screening a mouse retinal cDNA library using pieces of hGRK7 cDNA as probes, we found only GRK1 and GRK6 (data not shown). By degenerate cloning techniques we have successfully isolated the bovine GRK7 cDNA from a bovine retinal cDNA library. A similar approach so far failed to isolate the mouse GRK7 cDNA. Prior to this report and completion of the draft of the human genome, the existence of a human "cone opsin kinase" was once doubtful [13]. Therefore the failure of cloning does not contradict our conclusion that GRK7 exists in mouse. In addition, our data show that mouse GRK7 is not a cone opsin kinase because it is not present in the cone outer segments.

The localization of GRK7 in human cone outer segments can be observed in many human retina specimens but the signal strength varies among specimens, presumably due to various postmortem and fixation times [25]. Notably in the retina of a 4 month old donor whose eye was enucleated due to retinoblastoma and fixed within 120 min after surgery, GRK7 was localized to the proximal end of the cone outer segments, indicating that it may be involved in other cellular activities of cone photoreceptors. In addition we also found GRK7 in other neurons of all human retinas examined, strongly sug-

gesting that it has functions in addition to being a cone opsin kinase. GRK7 may be involved in other G-protein signaling pathways presumably by phosphorylating other GPCRs. We are currently using the antibody UU45APC to screen various mouse cDNA expression libraries in order to obtain the mouse GRK7 cDNA clone for further studies.

Note added in proof:
During revision of this manuscript, a paper entitled "Species-specific differences in expression of G-protein-coupled receptor kinase (GRK) 7 and GRK1 in mammalian cone photoreceptor cells: implications for cone cell phototransduction." by Weiss et al. appeared in the December 2001 issue of the Journal of Neuroscience [26]. While the major conclusions of that paper are similar to those drawn here, we found, as a major difference, expression of GRK7 polypeptide in mouse retina (but not in photoreceptor outer segments) and in other tissues.

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