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The Cloning of GRK7, a Candidate Cone Opsin Kinase, from Cone- and Rod-Dominant Mammalian Retinas

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Purpose: Desensitization in the rod cell of the mammalian retina is initiated when light-activated rhodopsin is phosphorylated by the G protein-coupled receptor kinase (GRK), GRK1, often referred to as rhodopsin kinase. A distinct kinase that specifically phosphorylates cone opsins in a similar manner has not been identified in mammals. To determine the existence of a cone opsin kinase, RNA from the retinas of cone- and rod-dominant mammals was analyzed by PCR.
Methods: RNA prepared from the retinas of two cone-dominant mammals, the thirteen-lined ground squirrel and the eastern chipmunk, and a rod-dominant mammal, the pig, was used to clone a new GRK family member by RT-PCR. The tissue distribution and localization of the kinase in retina were determined by Northern blot hybridization and in situ hybridization. The protein encoded by this cDNA was expressed in human embryonic kidney-293 (HEK-293) cells and compared with bovine GRK1 for its ability to phosphorylate bovine rhodopsin and to undergo autophosphorylation.
Results: The cDNA cloned from ground squirrel contains an open reading frame encoding a 548 amino-acid protein. Sequence analysis indicates that this protein is orthologous to GRK7 recently cloned from *O. latipes*, the medaka fish. Partial cDNA fragments of GRK7 were also cloned from RNA prepared from eastern chipmunk and pig retinas. In situ hybridization demonstrated widespread labeling in the photoreceptor layer of the ground squirrel retina, consistent with expression in cones. Recombinant ground squirrel GRK7 phosphorylates bovine rhodopsin in a light-dependent manner and can be autophosphorylated, similar to bovine GRK1.
Conclusions: These results indicate that cone- and rod-dominant mammals both express GRK7. The presence of this kinase in cones in the ground squirrel and its ability to phosphorylate rhodopsin suggests that it could function in cone cells as a cone opsin kinase.

In the rod cell of the mammalian retina, GRK1, a G protein-coupled receptor kinase (GRK) also referred to as rhodopsin kinase, phosphorylates light-activated rhodopsin and promotes the binding of arrestin to terminate visual signaling by transducin (G_t), the rod cell G protein [1]. Analogous events involved in cone visual signaling are less well-characterized, due to the fact that mammalian retinas generally have many more rods than cones. The cloning of a number of cone-specific proteins with significant homology to their rod cell counterparts [2-12] suggests that the signaling pathway is similar. However, dramatic physiological differences are observed between rods and cones in their responses to light. For example, cones are typically orders of magnitude less sensitive to similar light intensities, their response time is more rapid, and signal termination is faster than in rods [13]. Several mechanisms may account for the difference in the rate of signal termination. For example, the decay of the active forms (meta II) of the cone opsins is faster than the decay of rhodopsin [14,15]. It also seems likely that the lifetime of activated transducin is different in rods and cones, given the new evidence for regulation of GTP hydrolysis of the G protein αsubunits by members of the Regulators of G Protein Signaling (RGS) family. RGS9, which has been shown in vitro to accelerate the rate of GTP hydrolysis for α_{t1}, the rod cell transducin αsubunit, is actually present at higher levels in cones [16,17]. In addition, differences in the kinetics of opsin phosphorylation and arrestin binding could play a role in the different rates of signal termination observed for rods and cones.

Six GRKs have been cloned from mammals and biochemically characterized [18,19]. Recently, a novel member of the GRK family, GRK7, was cloned from the medaka fish (*Oryzias latipes*) and was localized specifically to cones, indicating that the cones of lower vertebrates have a distinct GRK [20]. Whether a unique GRK phosphorylates the cone opsins and plays a role in the termination of visual signaling in mammals is unknown. GRK1 has been localized to both rod and cone outer segments in mammals by immunohistochemistry [21], raising the possibility that GRK1 acts to desensitize both rod and cone opsins. However, in patients with Oguchi disease, which is caused by inactivating mutations in GRK1 [22,23], cone responses to light are relatively normal [24], suggesting that GRK1 does not play a major role in cone visual signaling.

The present study describes the cloning of a new mammalian member of the GRK family from the 13-lined ground squirrel and the eastern chipmunk, two cone-dominant mammals, as well as from the pig, a rod-dominant mammal. Sequence analysis suggests that this kinase is mammalian GRK7. The mRNA for this GRK is found exclusively in the retina and only in the photoreceptor cell layer of the ground squirrel

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retina. Like medaka fish GRK7, the 13-lined ground squirrel GRK7 contains a consensus sequence for geranylgeranylation of the C terminus. Functional studies demonstrate that this kinase phosphorylates bovine rhodopsin in a light-dependent manner and can be autophosphorylated, similar to GRK1. In addition to GRK7, we successfully cloned a partial cDNA fragment of GRK1 from both ground squirrel and pig retina RNA. These results support the existence of a new GRK family member (GRK7) in both cone- and rod-dominant mammalian retinas that is distinct from GRK1 and has the potential to regulate desensitization in cones.

METHODS

Preparation of RNA: Thirteen-lined ground squirrels were purchased from TLS Research (Bartlett, IL). Eastern chipmunks were obtained by trapping the animals in Jefferson County, Alabama. The animals were euthanized by carbon dioxide asphyxiation. The eyes were enucleated and the retinas frozen in liquid nitrogen. Animal care guidelines in accordance with those published by the Institute for Laboratory Animal research were followed. Total RNA from ground squirrel and eastern chipmunk retinas was prepared using the TRIzol Reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocols. Poly(A)$^+$ RNA was prepared using the polyATtract Kit (Promega, Madison, WI) following procedures described by the manufacturer. Rat poly(A)$^+$ RNA was purchased from Clontech (Palo Alto, CA).

Cloning of GRK7: Two degenerate primers used previously to clone members of the GRK family [25-29], were modified to recognize all GRKs and used to amplify sequence from rat, 13-lined ground squirrel, and eastern chipmunk retina RNA. Poly(A)$^+$ RNA or total RNA from rat, eastern chipmunk, and thirteen-lined ground squirrel were reverse-transcribed with MMLV reverse transcriptase using either a random hexamer or oligo dT primer (Advantage RT-for-PCR Kit, Clontech). Polymerase chain reaction (PCR) was performed with Pfu polymerase (Stratagene, La Jolla, CA) using two degenerate oligonucleotide primers, GT(ACGT)TA(CT)(AC)G(ACGT)GA(CT)(CT)T(ACGT)AA(AF)CC and A(ACG)(CT)TC(ACGT)GG(ACGT)GCCAT(AG)(AT)A(ACGT)CC, that correspond to amino acids 311-317 (I/VYRDLKP) and 354-360 (GF/YMAPEL/V) of bovine GRK1 within a conserved region of the catalytic domain. A 2-min incubation at 95 °C was followed by 30-35 amplification cycles, each containing 1 min at 95 °C, 1 min at 50 °C and 2 min at 72 °C. An approximately 150-bp fragment isolated on an agarose gel was ligated to pCR-script SK (Stratagene) and sequenced using the dideoxy sequencing method with the enzyme Sequenase (Amersham, Arlington Heights, IL). Alternatively, clones were sequenced at the UNC-CH Automated DNA Sequencing facility on a Model 377 DNA Sequencer (Perkin Elmer, Applied Biosystems Division, Foster City, CA) using the ABI PRISM™Dye Terminator cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin Elmer, Applied Biosystems Division).

The sequence of GRK7 from 13-lined ground squirrel RNA was extended using a Rapid Amplification of cDNA Ends (RACE) strategy [30]. For 3' extension, the 3' RACE System kit (Life Technologies) was used according to the manufacturer's protocols. The GRK7 cDNA was amplified by PCR using two primers, AUAP (Abridged Universal Amplification Primer) and the primer GAGAACGTG CTCCTGGATGACCTCGG, which corresponds to a site (nucleotides 1040-1065) within the 146-bp sequence of GRK7 originally cloned by PCR. A 2.1-kb fragment generated using this method was ligated to pCR-script SK (Stratagene) and sequenced as described above.

For 5' extension of GRK7 from 13-lined ground squirrel RNA, the Marathon cDNA Amplification Kit (Clontech) was used. Approximately 2 µg of total RNA was reverse-transcribed by MMLV reverse transcriptase using the primer GGAATAGCTCGCCTTGTCCATCAGGAT, which corresponds to a site (nucleotides 1163-1189) downstream from the original 146-bp sequence of GRK7. Second strand synthesis was followed by ligation of Marathon cDNA adapters. Amplification of the cDNA of the GRK7 was performed by PCR using the gene-specific primer (described above) and the Adapter-Primer 2. A 1.2-kb fragment was purified and ligated to pCR2.1 (Invitrogen, Carlsbad, CA) and sequenced.

To verify the accuracy of the cDNA sequence, a minimum of 3 clones from different PCR reactions were analyzed for each region. Searches against peptide and nucleotide sequence databanks were performed at the GSC using the BLAST network services. Other sequence analyses were performed using programs from the Wisconsin Package Version 9.0 (Genetics Computer Group, Madison, WI) as described in the legends to Figs. 1 and 2. The nucleotide sequence for 13-lined ground squirrel GRK7 has been deposited in the GenBank databse under the GenBank Accession Number AF063016. The accession numbers of sequences found to be related to GRK7 are: AB009568, *O.latipes* GRK7; AB009569, *O. latipes* GRK1; P28327, bovine GRK1; Q15835, human GRK1; P25098, human GRK2; P35626, human GRK3; P32298, human GRK4; P34947, human GRK5; P43250, human GRK6.

A partial fragment of the pig GRK7 cDNA was cloned by PCR using two degenerate oligonucleotide primers, GA(CT)TGGTT(CT)GC(ACGT)ATGGG(ACGT)TG and TC(AGT)AT(CT)TC(AG)TC(ACGT)AC(AG)TC(CT)TT, corresponding to amino acids 368-374 and 476-482 of the ground squirrel sequence, respectively. A partial fragment of GRK1 was isolated from ground squirrel and pig using two degenerate primers, GA(AG)AA(AG)GT(ACGT)GA(AG)AA(CT)AA(AG)GA and TC(CT)TG(AG)AA(AG)AA(CT)TC(ACGT)GT(AG)TC, corresponding to amino acids 394-400 and 499-505 of bovine GRK1, respectively.

Northern blot hybridization analysis: Electrophoresis was performed following procedures described by Qiagen (Chatsworth, CA) in the RNeasy Mini Handbook. Approximately 8 µg of total RNA was electrophresed on a formaldehyde-agarose gel and electrophoretically transferred to Immobilon-S nylon membrane (Boehringer Mannheim Indiannapolis, IN). The RNA was immobilized by UV crosslinking. The membrane was prehybridized in 6X SSC, 5X Denhardt's solution, 0.5% SDS, 50% formamide and 100 µg/ml salmon sperm DNA at 42 °C for 2 h. A probe corresponding to the initial 146-bp fragment of GRK7 labeled with

[^{32}P]dCTP was heat-denatured and incubated with the nylon membrane overnight at 42 °C in the same buffer used for prehybridization. The membrane was washed twice in 2X SSC, 0.5% SDS at room temperature for 15 min and in 0.1X SSC, 0.5% SDS at 68 °C for 1 h and for 30 min. Radioactivity was detected using a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

In situ hybridization: Enucleated ground squirrel eyes were incubated overnight in PBS containing 4% paraformaldehyde, followed by incubation in PBS containing 30% sucrose. The eyes were frozen in liquid nitrogen, cryostat-sectioned at 10 µm and applied to Superfrost Plus microscope slides (VWR, Plainfield, NJ). In situ hybridization was performed essentially as described with minor modifications [31]. After prehybridization for 1 hour, the slides were hybridized in prehybridization buffer plus 0.2 mg/ml dextran sulfate containing an [^{35}S]UTP-labeled 146-bp fragment of GRK7 as a probe. Slides were hybridized overnight, then washed in 2X SSC, incubated for 30 min in 50% formamide, 1X SSC, 10 mM dithiothreitol (DTT) at 50 °C for 30 min, and for 30 min at room temperature in 0.5X SSC. After incubation in 0.1 mg/ml RNAse for 30 min at room temperature, the slides were washed in 0.1X SSC at 62 °C for 2 h and dehydrated through a series of ethanol concentrations in 300 mM NH_4 acetate. For visualization of probe hybridization, the slides were coated with emulsion (Kodak NTB-2), dried and developed according to the manufacturer's directions (Kodak, Rochester, NY). The sections were counterstained with toluidine blue.

Construction of GRK7 expression vectors: The full sequence of GRK7 was generated by PCR from N- and C-terminal clones. A histidine-tagged construct was created by PCR through the insertion of a 6x histidine tag sequence at the N terminus (MRGSHHHHHH) of the protein. *Hin*dIII and *Xho*I sites were created in both the wild-type and histidine-tagged constructs at the 5' and 3' ends, respectively. The constructs were ligated into pSP72 (Promega) for in vitro translation or into pcDNAI/Amp (Invitrogen) for transient expression in HEK-293 cells.

In vitro translation: The wild-type and histidine-tagged constructs of GRK7-pSP72 were linearized with *Xho*I and transcribed using T7 RNA polymerase. Approximately 1 µg of synthesized RNA was added to rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine and incubated at 30 °C for 1 h. The reaction mixture was analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [32] and radioactivity was detected by phosphorimage analysis of the dried gel.

Expression in HEK-293 cells: Transfection of HEK-293 cells was carried out using a DEAE-dextran transfection method essentially as described [33]. Cells were harvested approximately 70 h after transfection. Cell extracts were prepared by freezing and thawing the cells once at -80 °C and dounce homogenization in a buffer consisting of 20 mM HEPES, pH 7.2, 250 mM NaCl, 2 mM EDTA, 0.02% Triton X-100, 1 mM DTT, 0.5 mM PMSF, 20 µg/ml leupeptin, and 20 µg/ml benzamidine [34]. The cell lysates were centrifuged at 40,000 x g in a Ti70 rotor (Beckman, Palo Alto, CA) for 20 min at 4 °C and the supernatants containing the expressed GRK7 or GRK1 were used in experiments described below. Alternatively, the lysates were diluted to 50% with glycerol and stored at -20 °C.

Phosphorylation of rhodopsin by GRK7: An assay mixture containing 4 µg of urea-stripped rod outer segment (ROS) membranes [35] and 5 µg of the HEK-293 cell lysate in 20 mM Tris buffer pH 7.5, 2 mM EDTA, 5 mM $MgCl_2$ and 0.1 mM [γ-^{32}P]ATP (40 µCi/ml) was incubated in the presence or absence of light for 10 min at 30 °C. The reaction was stopped by addition of SDS-PAGE sample buffer [32] and electrophoresed on 10% SDS-polyacrylamide gels. The phosphorylation of rhodopsin was detected by phosphorimage analysis of the dried gels.

Autophosphorylation of GRK7: Approximately 400 µg of cell lysate from HEK-293 cells transfected with the histidine-tagged construct was diluted into 2 ml of buffer containing 10 mM imidazole, pH 7.2, 100 mM NaCl, 2 µg/ml leupeptin, and 1 µg/ml aprotinin and incubated with 50 µl Ni-NTA resin (Qiagen) for 60 min at 4 °C. After washing the resin twice with buffer A containing 20 mM imidazole, 250 mM NaCl, 0.02% Triton X-100, 2 µg/ml leupeptin, and 1 µg/ml aprotinin to remove unbound protein, the GRK7-resin complexes were assayed for the ability of GRK7 to undergo autophosphorylation. The complexes were incubated for 10 min at 30 °C in a buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 10 mM NaF and 0.1 mM [γ^{32}P]ATP (20 µCi/ml) [36]. After washing with buffer

(A)

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GRK7 (GS)  315 ENVLLDDLGNCRLSDLGLAVEVQDDKPMTQRAGTN 349
               |||||||||||||||||||:||:|||.|||||||
GRK7 (CM)      ENVLLDDLGNCRLSDLGLAVQVQEDKPITQRAGTN
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(B)

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GRK7 (GS)  375 SIYEMVAGRTPFKDFKEKVSKEDLKERTMKDEVAFHHENFTEETKDICRLFLAKK 429
               |||||||||||||:|||:||||||:||:|:|| | |:.|||| ||||||||||
GRK7 (Pig)     SIYEMVAGRTPFKDYKEKISKEDLKQRTLKEEVRFQHQSFTEEAKDICRLFLAKT

GRK7 (GS)  430 PEQRLGSREKADDPRKHPFFQTVNFPRLEAGLVEPPFVPDPSVVYA 475
               ||||||||||.|||||| ||.|:||||||||||:||||||||||||
GRK7 (Pig)     PEQRLGSREKSDDPRKHHFFKTINFPRLEAGLVDPPFVPDPSVVYA
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(C)

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          401                                                 450
GRK1 (Bv)   LKQRVLSEPV AYSDKFGQAS RDFCEALLEK DPEKRLGWRD GSCDGLRAHA
GRK1 (GS)   LKQRVLEEAV TYSDKFSQAS KDFCEALLQK DPEQRLGFRD GTCDGLRASP
GRK1 (Pig)  LKQRVLEQAV TYPDKFSPAS KDFCEALLQK DPEKRLGFRD GSCDGLRTHP
Consensus   LKQRVL-~~V -Y-DKF-~AS -DFCEALL-K DPE-RLG-RD G-CDGLR-~-

          451                                                 498
GRK1 (Bv)   LFKDISWRQL EAGMLTPPFI PDSRTVYAKS IQDVGAFSTV KGVAFDNA
GRK1 (GS)   LFRDMNWRQL EAGMLAPPFI PDSRTVYAKN IQDVGVFSTV RGVVFDKG
GRK1 (Pig)  LFRDISWRQL EAGMLTPPFV PDSRTVYAKN IQDVGAFSTV KGVAFEKA

Consensus   LF-D-~WRQL EAGML-PPF- PDSRTVYAK- IQDVG-FSTV -GV-F-~~
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Figure 1. Comparison of partial fragments of GRK7 and GRK1 from ground squirrel with sequences from other mammalian species. **A.** Alignment of a partial fragment of GRK7 from ground squirrel (GS) with sequence from the eastern chipmunk (CM). Identical amino acids are aligned with vertical bars (|), highly conserved amino acids are aligned with colons (:), and weakly conserved amino acids are aligned with periods (.). Numbers correspond to the amino acid sequence of GS GRK7. **B.** Alignment of a partial fragment of GS GRK7 with the sequence from the pig. Symbols are as described in Figure 1A. **C.**Alignment of GRK1 sequences from bovine (Bv), ground squirrel (GS) and pig. Numbers correspond to the amino-acid sequence of bovine GRK1. Sequence comparisons were performed using the computer programs GAP, PILEUP and PRETTY from the Wisconsin Package Version 9.0.

A to remove unincorporated ATP, the protein was released from the resin by incubation with SDS-Laemmli buffer [32] and electrophoresed on 10% SDS-polyacrylamide gels. Phosphorylation of GRK7 was visualized by phosphorimage analysis of the dried gels.

RESULTS

Based on the hypothesis that a cone opsin kinase would be a member of the GRK family, a strategy was developed to clone new GRKs from the retinas of 2 cone-dominant mammals, the eastern chipmunk [37] and the 13-lined ground squirrel [38-39]. PCR primers corresponding to amino acids 311-317 and 354-360 of bovine GRK1 were designed to recognize the known GRK family members and used to screen retinal RNA from these animals. The cloned PCR products were classified according to their sequence and compared with the products from a PCR analysis of RNA from the rat, a rod-dominant animal [40]. As expected, the majority (85%) of GRKs cloned from rat retina RNA were GRK1 (data not shown). GRK1 was not detected in the eastern chipmunk or the 13-lined ground squirrel during this initial screening. However, a 146-bp fragment corresponding to a novel member of the GRK family was cloned from both the ground squirrel and eastern chipmunk. The sequences from these two animals were 91% identical at the amino acid level, excluding the primer hybridization sites, suggesting that they represent orthologous proteins (species homologues) (Figure 1A).

A RACE strategy was used to obtain the 5' and 3' regions of the clone from ground squirrel; 2.1 kb and 1.2 kb fragments, respectively, were obtained that overlapped each other. The full-length cDNA is 3095 bases and contains a single open reading frame from nucleotides 98 to 1744 encoding a polypeptide of 548 amino acids. The calculated molecular mass of the protein is 61,993 Da. The C terminus contains a CAAX motif, coding for posttranslational attachment of a geranylgeranyl group to Cys-545. Alignment of this sequence with the recently cloned GRK7 from *O. latipes* (Figure 2) demonstrated an overall identity between these two proteins of 59%. In contrast, the ground squirrel GRK was found to be only 48% identical with bovine GRK1. The highly conserved catalytic domains of the GRK family members, consisting of approximately 280 amino acids, were also compared (Table 1). The catalytic domain of the ground squirrel kinase shows greatest identity with *O. latipes* GRK7 (67%), is more distantly related to GRK1, 4, 5 and 6 (approximately 55-57% identity), and shows the least sequence identity with GRK2 and GRK3 (approximately 40% identity). These results suggest that the ground squirrel GRK is a new mammalian GRK and orthologous to GRK7 cloned from *O. latipes*. Despite the similarity between ground squirrel GRK7 and GRK1, 4, 5 and 6, analysis of the evolutionary distance between these proteins

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GRK7 (GS)   M.DMGGLDNL IANTAYLQAR KT.DSDSREL QRRRRSLA.. .LPGPQGCAE   45
GRK7 (Ol)   -C-------- V------K-Q ...GG-DK-M KK-----S.. .--K-EQ-VA   44
GRK1 (Bv)   -.-F-S-ETV V--S-FIA-- GSF-AS-GPA S-D-KY--RL K--PLSK-EA   49
                                    *

GRK7 (GS)   LRQSLSPHFH SLCEQQPIGR RLFRDFLATV PKYSQAVAFL EDVQNWELAE   95
GRK7 (Ol)   --E-IEKD-T L---R----K --------NT -EFKL-AEF- DELYD-D---   94
GRK1 (Bv)   --E--DLG-E GM-LE----K ---QQ--R-H EQHGP-LQLW K-IEDYDT-D   99

GRK7 (GS)   EGPAKTSTLQ QLAATCARDP GP.QSFLSQD LATKCRAAST DEERKTLVEQ  144
GRK7 (Ol)   GAAKDKARQN IINKY-KP-S KTFLT---GE P-E--KSVTD ATFEEVMKNK  144
GRK1 (Bv)   DALRPQKAQA LR--YLEPQA QLFC---DAE TVARA--GAG -GLFQP-LR.  148

GRK7 (GS)   AKAETMSFLQ EQPFQDFLAS PFYDRFLQWK LFEMQPVSDK YFTEFRVLGK  194
GRK7 (Ol)   VQDGVRE--K GK--TEYQG- QYF-K----- EY-K--I--- --Y---T---  194
GRK1 (Bv)   ...AVLAH-G QA---E--D- LYFL------ WL-A--MGED W-LD--V--R  195

GRK7 (GS)   GGFGEVCAVQ VRNTGKMYAC KKLDKKRLKK KGGEKMALLE KEILEKVNSP  244
GRK7 (Ol)   ---------- -K---Q---- ---C------ ---------- -Q-------L  244
GRK1 (Bv)   ------F-C- MKA---L--- ---N------ RK-YQG-MV- -K--A--H-R  245

GRK7 (GS)   FIVSLAYAFE SKTHLCLVMS LMNGGDLKFH IYNVG..... .TRGLAMSRV  288
GRK7 (Ol)   -L-N----YD T--------T --------Y- ---I-YDGKG VDK-IE-K-I  294
GRK1 (Bv)   ---------- T--D-----T I-----IRY- ----DEDNP. ...-FQEP-A  291

GRK7 (GS)   IFYTAQMTCG VLHLHGLGIV YRDLKPENVL LDDLGNCRLS DLGLAVEVQD  338
GRK7 (Ol)   -H----I-T- I----DMD-I ---M------ --SQ-Q---- -----I-IAP  344
GRK1 (Bv)   ------IVS- LE---QRN-I ---------- ---D--V-I- -------LKA  341

GRK7 (GS)   DKPITQ.RAG TNGYMAPEIL MDKASYSYPV DWFAMGCSIY EMVAGRTPFK  387
GRK7 (Ol)   G-TV--.M-- -GA------- .S-TP-RTS- --W-L----- -----Y----  392
GRK1 (Bv)   GQTK-KGY-- -P-F----L- LGE.E-DFS- -Y--L-VTL- --I-A-G--R  390

GRK7 (GS)   ...DFKEKVS KEDLKERTMK DEVAFHHENF TEETKDICRL FLAKKPEQRL  434
GRK7 (Ol)   GPESK----E --EVQR-ILN E-PKWE-KC- DAP---VIQQ --K--IDE--  442
GRK1 (Bv)   ARG...---E NKE--Q-VLE QA-TYPDK.- SPAS--F-EA L-Q-D--K--  436

GRK7 (GS)   GSRE.KADDP RKHPFFQTVN FPRLEAGLVE PPFVPDPSVV YAKDVDEIDD  483
GRK7 (Ol)   -M-N.NME-- ---EW-KSI- ---------D --W--K-N-- ----TGD-AE  491
GRK1 (Bv)   -F-DGSC-GL -T--L-RDIS WRQ----MLT ------SRT- ---NIQDVGA  486

GRK7 (GS)   FSEVRGVEFD DKDKQFFQRF STGAVPVAWQ EEIIETGLFE ELN.......  526
GRK7 (Ol)   ---IK-I--- A--DK--KE- ------IQ-- Q-M----L-D ---.......  534
GRK1 (Bv)   --T-K--A-E KA-TE---E- AS-TC-IP-- --M----V-G D--VWRPDGQ  536
             **

GRK7 (GS)   ..DPNRPSGD GKG.DSSKSG VCLLL  548
GRK7 (Ol)   ..-----KE-A -G-D-EK--- T-A--  557
GRK1 (Bv)   MP-DMKGVSG QEAAP----- M-V-S  561
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Figure 2. Comparison of the amino-acid sequence of ground squirrel GRK7 to GRK7 from Oryzias latipes and bovine GRK1. The hyphens (-) represent sequence in GRK7 from Oryzias latipes (Ol) and bovine (Bv) GRK1 that is identical with the sequence of ground squirrel (GS) GRK7. Gaps in the sequence alignment are represented by periods (.) The blue lettered region represents the predicted catalytic domain (amino acids 185-464 of the ground squirrel sequence). The asterisks (*) mark serine and threonine residues that serve as known or potential autophosphorylation sites. The sequence with the double underline represents the CAAX motif, which codes for isoprenylation at the cysteine residue. Sequence comparisons were performed using the computer programs PILEUP and PRETTY from the Wisconsin Package Version 9.0. The nucleotide sequence for 13-lined ground squirrel GRK7 has been deposited in the GenBank database under the GenBank Accession Number AF063016.

Table 1. Homology between the catalytic domains of GRK7 and other members of the GRK family.

	GRK7 (GS)	
	Identity (%)	Similarity (%)
GRK7 (GS)	100.0	100.0
GRK7 (O. latipes)	67.4	77.1
GRK1 (bovine)	55.4	66.2
GRK1 (human)	56.5	65.8
GRK2 (human)	40.4	52.4
GRK3 (human)	39.6	50.9
GRK4 (human)	56.8	67.3
GRK5 (human)	56.5	68.3
GRK6 (human)	56.1	67.3

Protein sequence data was analyzed using the computer program GAP from the Wisconsin Package version 9.0

indicates that this GRK7 is most closely related to GRK1 (not shown), similar to the observations of Hisatomi et al. for *O. latipes* GRK7 [20].

RT-PCR was used to amplify GRK7 sequence from RNA isolated from the pig retina. The pig retina is rod-dominant, composed of 89% rods and 11% cones [41,42]. Two degenerate olignucleotide primers, based on the sequence of ground squirrel GRK7, were used to clone a 303-bp fragment from pig RNA corresponding to amino acids 375-475 of the ground squirrel protein (Figure 1B). The identity of this region between ground squirrel and pig GRK7 is 84%. In addition, partial fragments of GRK1 were also cloned from both ground squirrel and pig (Figure 1C). This region of ground squirrel GRK1 is 79% and 83% identical to pig and bovine GRK1, respectively. An overlapping region of the GRK7 and GRK1 partial cDNAs, consisting of 78 amino acids, demonstrates only 43% identity to each other in ground squirrel and 47% identity in the pig. Therefore, GRK7 and GRK1 are distinct kinases present in both rod- and cone-dominant mammals.

The distribution of GRK7 in 8 ground squirrel tissue RNA preparations was determined using Northern blot hybridization (Figure 3). A probe corresponding to the original 146-bp sequence of GRK7 hybridized only to retinal RNA. The molecular size of the major band is approximately 3 kb, which corresponds closely to the length of the complete cDNA sequence (3095 bp) of GRK7. Several minor bands are visible above and below the major band. Some of these may be processed forms, or splice variants, of GRK7, such as those reported for GRK1 and GRK4 [21,43,44]. These results suggest that ground squirrel GRK7 is highly tissue-specific in its expression and is found predominantly in the retina. The same probe was used to localize GRK7 messenger RNA by in situ hybridization in histological preparations of ground squirrel retina (Figure 4). The antisense-strand RNA probe hybridized to the photoreceptor cell layer, which is composed of approximately 94% cones and 6% rods [38]. In contrast, the sense-strand RNA probe showed no specific hybridization. These results indicate that GRK7 is distributed exclusively in photoreceptor cells of the 13-lined ground squirrel retina, consistent with a function in cone visual signaling.

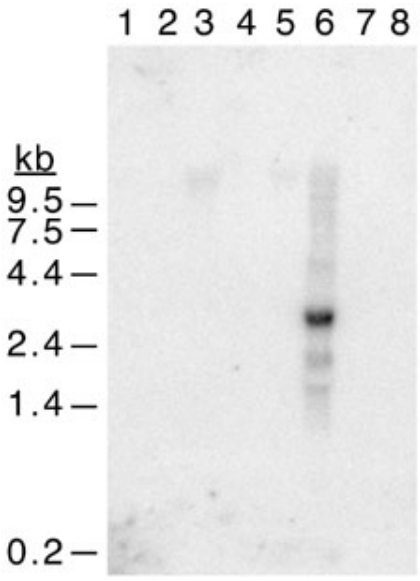

Figure 3. Northern blot analysis of GRK7. A ^{32}P-labeled probe of GRK7 was hybridized to a blot containing ground squirrel tissue RNA as described in the "Methods". Lane 1: brain, Lane 2: heart, Lane 3: kidney, Lane 4: liver, Lane 5: lung, Lane 6: retina, Lane 7: skeletal muscle, Lane 8: spleen. Numbers at left represent molecular size markers for RNA (Life Technologies).

To determine whether the cDNA for ground squirrel GRK7 encodes a protein of the correct molecular size, the cDNA was expressed by in vitro translation. The [^{35}S]methionine-labeled, in vitro-translated product of the GRK7 cDNA migrates at approximately 62 kDa, consistent with the molecular size of the protein predicted from the length of the open reading frame (data not shown). Ground squirrel GRK7 and bovine GRK1 were also transiently expressed in HEK-293 cells to compare their ability to phosphorylate rhodopsin. Cytosolic extracts prepared from these cells were analyzed for their ability to phosphorylate bovine rhodopsin in ROS membranes in the light and in the dark (Figure 5). GRK7 and GRK1 were similarly effective at phosphorylating rhodopsin in a light-dependent manner. Extracts prepared from nontransfected cells showed no detectable phosphorylation of rhodopsin. These results indicate that GRK7 can phosphorylate rhodopsin in a light-dependent manner similar to GRK1, consistent with its classification as a GRK family member and with a potential role as a cone opsin kinase.

Autophosphorylation sites have been identified for GRK1, 5, and 6 downstream from the catalytic domain [28,45-48]. Autophosphorylation of GRK1 may regulate the binding of ATP to the catalytic domain and the selectivity for different substrate sites on rhodopsin [46]. Ground squirrel GRK7 has a threonine at position 21, equivalent to a minor autophosphorylation site at Ser-21 in bovine GRK1 (Figure 2). A serine is also present at position 485, which is equivalent to one of two major autophosphorylation sites at Ser-488 in bovine GRK1 [46], suggesting that GRK7 may also be autophosphorylated. A histidine-tagged GRK7 construct expressed in HEK-293 cells was examined for its ability to un-

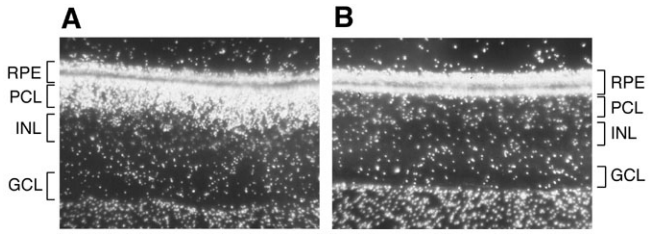

Figure 4. Localization of GRK7 mRNA in ground squirrel retinas using in situ hybridization.. ^{35}S-labeled antisense and sense RNA probes were produced from the 146-bp fragment corresponding to nucleotide sequence 1019-1164 by in vitro transcription and hybridized to cryostat sections of ground squirrel retina as described in the "Methods". (**A**) Dark field image of ground squirrel retina hybridized with an antisense probe for GRK7. Retinal pigment epithelium (RPE), photoreceptor cell layer (PCL), inner segment layer (INL), ganglion cell layer (GCL). (**B**) Dark field image of ground squirrel retina hybridized with a sense probe for GRK7. Abbreviations are as described in (**A**). Since the retinal pigment epithelium is black, it appears white in dark field images.

dergo autophosphorylation. Extracts from transfected and nontransfected cells were partially purified on Ni-NTA resin and incubated with [γ^{32}P]ATP (Figure 6). The results using extracts from transfected cells show the presence of a radioactive protein bound to the resin with a molecular size similar to that of GRK7 identified by in vitro translation. In contrast, this band was not detected in ^{32}P-labeled extracts from nontransfected cells or in the flowthrough from either transfected or nontransfected cell extracts. These results suggest that GRK7, like GRK1, GRK5 and GRK6, is capable of autophosphorylation.

DISCUSSION

The present report describes the cloning of a new member of the mammalian GRK family from the retinas of the 13-lined ground squirrel, the eastern chipmunk and the pig. Comparison of this sequence with known GRK family members and the sequence of the newly cloned GRK7 from *O. latipes*, the medaka fish, suggests that these proteins are mammalian GRK7. Although its sequence is closely related to GRK1, partial fragments of a different cDNA cloned from the 13-lined ground squirrel and pig show greater homology to GRK1. Therefore cone- and rod-dominant mammals both express GRK1 and GRK7. Northern analysis and in situ hybridization demonstrate that GRK7 is expressed exclusively in the photoreceptor cell layer of the ground squirrel. Approximately 94% of the photoreceptors of the ground squirrel retina are cones [38], making GRK7 an excellent candidate for a cone opsin kinase. Furthermore, in vitro phosphorylation studies show that rhodopsin can serve as a substrate for this kinase, strengthening the likelihood that it can phosphorylate the homologous cone opsins.

An interesting feature of this kinase is the presence of a C terminal CAAX motif coding for the addition of a geranylgeranyl group to Cys-545. In contrast, GRK1 is farnesylated in every species examined, except chicken, where it is geranylgeranylated [21]. The functional consequence of this difference in isoprenylation may be predicted from studies of Inglese et al. [49], who reported that mutation of the C-terminal CAAX box of GRK1 from CVLS to CVLL, to promote geranylgeranylation instead of farnesylation, resulted in constitutive association with the plasma membrane. Therefore GRK7 may be more tightly associated with the membrane than GRK1. It is intriguing to speculate that this difference in posttranslational modification could contribute to the differences in rates of signal termination between rods and cones through differences in rates of phosphorylation. Further analysis of the biochemical properties of this kinase will be necessary to verify such a hypothesis.

Another feature shared by GRK7 with several members of the GRK family is its ability to be autophosphorylated. Thr-21 in GRK7 from the 13-lined ground squirrel is conserved with Ser-21 in bovine GRK1, but this site is absent in *O. latipes* GRK7, suggesting that it may not be critical for function. Both the 13-lined ground squirrel and *O. latipes* GRK7 possess a serine at a position corresponding to Ser-488 in bovine GRK1, but Thr-489 in GRK1, which is also autophosphorylated, is a glutamic acid in both GRK7 proteins. It has been suggested that phosphorylation of these residues in GRK1 enhances its release from phosphorylated rhodopsin, thereby serving to limit the stoichiometry of phosphorylation [50,51]. These residues may also play a role in ATP binding [46].

The existence of GRK7 in the retinas of the 13-lined ground squirrel, the eastern chipmunk and the pig, and the

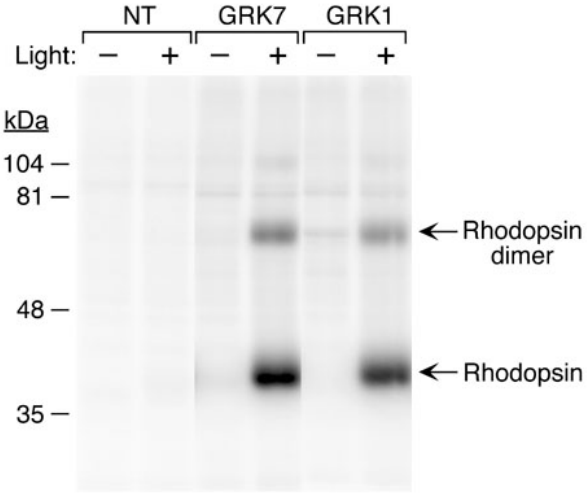

Figure 5. Phosphorylation of bovine rhodopsin by GRK7 and GRK1. Extracts from nontransfected (NT) HEK-293 cells or cells expressing bovine GRK1 or ground squirrel GRK7 were used to phosphorylate bovine ROS membranes as described in the "Methods". ROS membranes containing bovine rhodopsin were incubated with [γ^{32}P]ATP in the presence (+) or absence (-) of light, then electrophoresed on a 10% SDS-polyacrylamide gel. Phosphorylation was visualized by phosphorimage analysis. The numbers at left represent protein molecular size markers (Biorad, Hercules, CA).

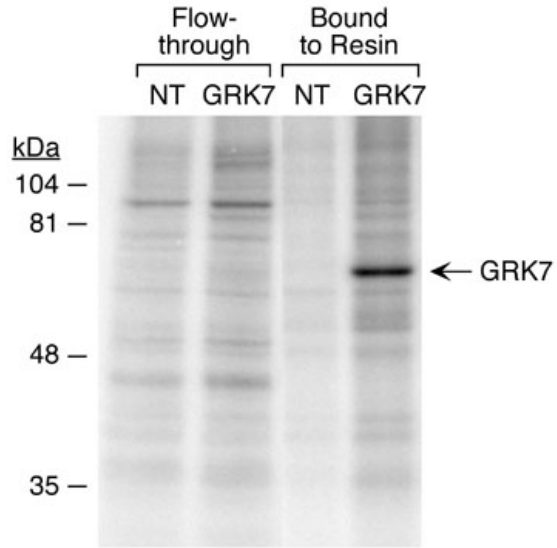

Figure 6. Autophosphorylation of GRK7. Extracts from nontransfected HEK-293 cells (NT) and cells transfected with histidine-tagged GRK7 were incubated with Ni-NTA resin and washed to remove unbound protein (Flowthrough). Both the flowthrough and the protein bound to the resin were incubated with [γ^{32}P]ATP as described in the "Methods". The numbers at left represent protein molecular size markers (BioRad).

ability of rhodopsin to serve as a substrate for this kinase, raise several interesting questions: (1) Does this novel kinase plays a role in mammalian cone visual signaling pathways, similar to the role played by GRK1 in rods, by phosphorylating the cone opsins? (2) If GRK7 participates in cone signaling, do differences exist in the kinetics of cone opsin phosphorylation that may account for the faster signal termination in cones? In the medaka fish, GRK7 was expressed only in cones and GRK1 only in rods [20]. However in mammals, it has been suggested that GRK1 is also in cones [21]. If GRK7 is also found in both rods and cones, it raises the interesting possibility that these two closely-related members of the GRK family cooperate in visual signaling.

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The typographical corrections below were made to the article on the date noted. These changes have been incorporated in the article and the details are documented here.

9 December 1998: In paragraph 6 of Results, "Figure 1" was changed to "Figure 2".
16 December 1998: In paragraphs 7, 12 and 13 of Methods and the Figure 5 caption,"chromatographed" was changed to "electrophoresed."