A novel subtype of G-protein-coupled receptor kinase, GRK7, in teleost cone photoreceptors

Osamu Hisatomi, Shinji Matsuda, Takunori Satoh, Shuichi Kotaka, Yoshikazu Imanishi, Fumio Tokunaga*

Department of Earth and Space Science, Graduate School of Science, Osaka University, Machikaneyama-chyo 1-1, Toyonaka, Osaka 560-0043, Japan

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Abstract Two kinds of retinal cDNA fragments (OlGRK-R and -C) encoding the putative G-protein-coupled receptor kinases (GRKs) were isolated from medaka, *Oryzias latipes*. OlGRK-R appears to be closely related to the rhodopsin kinase (RK) found in the outer segments of mammalian photoreceptors, but the deduced amino acid sequence of OlGRK-C shows less than 50% identity to those of GRKs known to date, suggesting that OlGRK-C is a novel GRK subtype (GRK7). The mRNA of OlGRK-R is detectable in rods, and that of OlGRK-C is found in all four types of cone photoreceptor. The C-terminal of OlGRK-R has a consensus sequence for farnesylation, whereas, surprisingly, OlGRK-C has a consensus sequence for geranylgeranylation. Our result are consistent with the concept that lower vertebrates have rod- and cone-specific opsin kinases.

Key words: G-protein-coupled receptor kinase (GRK); Opsin kinase; Medaka (*Oryzias latipes*); In situ hybridization; Geranylgeranylation

1. Introduction

G-protein-coupled receptors receive a wide variety of biological information, including for example photons, neurotransmitters and hormones, and trigger the enzymatic cascades which ultimately generate physiological responses. Activated G-protein-coupled receptors show rapid loss of responsiveness (desensitization) and phosphorylation of G-protein-coupled receptors by G-protein-coupled receptor kinases (GRKs) is one of the most representative mechanisms for desensitization [1,2]. Six subtypes of GRK have been reported so far, grouped into three subfamilies. Rhodopsin kinase (RK) alone forms the first rhodopsin kinase (RK or GRK1) subfamily, GRK2 (βARK1) and GRK3 (βARK2) form the second β-adrenergic receptor kinase (βARK) subfamily, and GRK4, GRK5, and GRK6 form the third subfamily (GRK4 subfamily) [3].

RK is one of the best studied GRKs. It phosphorylates the light-activated rhodopsin intermediate, metarhodopsin II [4,5], which increases arrestin (S-antigen) affinity to preclude further activation of transducin [6,7]. The C-terminal of RK is farnesylated and anchored to the membrane [8]. By immunohistochemical experiments, mammalian RKs have been suggested to be localized to the outer segments of both rod and cone photoreceptors [9], which are generally thought to be involved in twilight (scotopic) and daylight (photopic) vision, respectively.

*Corresponding author. Fax: +81 (6) 850-5480.
E-mail: tokunaga@ess.sci.osaka-u.ac.jp

Most studies of RKs carried out so far have been with mammalian RKs. Here, we report two kinds of cDNAs (OlGRK-R and -C) encoding the putative GRKs from medaka (Japanese killifish), *Oryzias latipes*. OlGRK-R shows a high amino acid similarity to mammalian RKs. In contrast, OlGRK-C shows less than 50% identity with GRKs, and appears to belong to a novel GRK subtype (GRK7). It was demonstrated that OlGRK-R is localized in rods and OlGRK-C is expressed in all four types of cone cells. It is suggested that medaka cone photoreceptors express a novel cone opsin kinase (CK).

2. Materials and methods

2.1. Isolations of cDNAs encoding the putative medaka, goldfish, newt and lizard GRKs

Two kinds of oligonucleotide mixtures were prepared: RK-F2 (5′-CGTTAAGCTTGGN(AC)GNGGNGGNTT(CT)GGNGA-3′, N: A+G+C+T) for sense priming, and RK-R1 (5′-GGGAATTC(CT)(GT)NA(AGT)(AG)TCNCCNCC(AG)TTCAT-3′) for antisense priming. These correspond to the amino acid sequences GRGGFGE and MNGGDIR, conserved among bovine RK, human RK and β-adrenergic receptor kinases (βARKs). Retinal cDNAs of medaka (*O. latipes*), goldfish (*Carassius auratus*), a newt (*Triturus pyrrhogaster*) and a lizard (*Japalura polygonata*) were prepared, and cDNA fragments encoding GRKs were isolated as described previously [10,11]. A medaka retinal cDNA library [12] containing 10^6 independent clones was screened by plaque hybridizations using the amplified fragments as probes. Screening procedures were the same as described previously [13], except that formamide concentration in the hybridization solution was increased to 25% for low stringency screening. Positive clones were sequenced by an ordinal deletion method [14].

2.2. Sequence data sources

Sequence data used in the present analyses were taken from GenBank, EMBL and SWISS-PROT databases, with the following accession numbers: Human (*Homo sapiens*) RK (Q15835), GRK2 (P25098), GRK3 (P35626), GRK4 (U33054), GRK5 (P34947), and GRK6 (P43250); bovine (*Bos taurus*) RK (P28327), GRK2 (P21140), GRK3 (P26818), and GRK5 (P43249); Norway rat (*Rattus norvegicus*) RK (Q63651), GRK2 (P26817), GRK3 (P26819), GRK4 (X97568), GRK5 (Q62833), and GRK6 (Y09365); fruit fly (*Drosophila melanogaster*) GRK-A (P32865) and GRK-B (P32866); nematode (*Caenorhabditis elegans*) GRK-A (Q09639) and GRK-B (Q09537).

The deduced amino acid sequences of medaka RKs and 20 previously reported GRKs were aligned. Amino acid positions at which any GRK has deletions were excluded and the resulting 503 amino acids were used for the calculation of identities. The phylogenetic analysis was carried out for 350 amino acids, corresponding to positions 167 to 530 of bovine RK, except for the sites of deletions. Evolutionary distances of the sequences (k) were calculated using the proportion of different amino acids between the two sequences (p), with a correction for multiple substitutions of $k = -\ln(1-p-p^2/5)$ [15]. An unrooted tree was constructed by the neighbor-joining method [16] using a sequencing analysis program (ESAT) [17].

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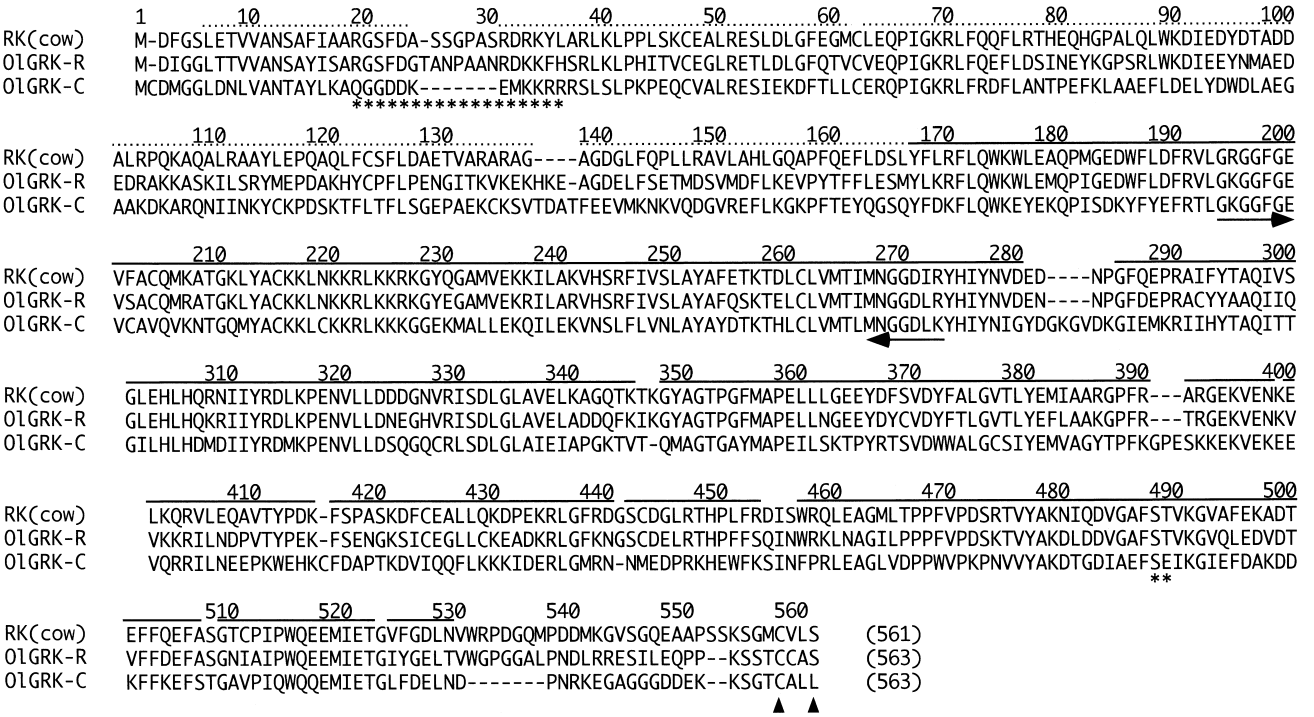

Fig. 1. The deduced amino acid sequences of the putative medaka GRKs, OlGRK-R and -C, arranged to correspond with those of bovine rhodopsin kinase (RK). Arrows represent the amino acid sequence corresponding to the primers used for the amplification of GRK cDNAs. Amino acids marked with asterisks are prospective sites for interaction with rhodopsin and major residues for autophosphorylation (see text). Arrowheads represent residues in CaaX motifs at the C-termini. Numbers correspond to the amino acid numbers of bovine RK. Solid lines above sequence represent amino acids used for construction of the phylogenetic tree, and solid plus dotted lines indicate the amino acids used to calculate identities. The nucleotide sequences of OlGRK-R and -C have been submitted to the EMBL nucleotide database with accession numbers AB009569 and AB009568, respectively.

2.3. In situ hybridization

cDNA fragments were cloned into a pGEM-3Zf(+) plasmid vector (Promega), and linearized with appropriate endonucleases. Antisense cRNA riboprobes (500–1000 bases in length) were synthesized by run-off transcription from the SP6 or T7 promoter with digoxigenin-UTP, as recommended in the manufacturer's protocol (Boehringer Mannheim). Preparation of medaka retinal cryosections and methods for in situ hybridization were as described previously [11,12,18]. Briefly, light-adapted eyes were fixed in 4% paraformaldehyde and embedded in 33% OCT compound diluted with 20% sucrose in phosphate buffer. 3–5 μm retinal cryosections were hybridized with 0.1–2.0 μg/ml (final concentration) cRNA probes. The hybridization signal was visualized using a nucleic acid detection kit (Boehringer Mannheim) and detected with Nomarski optics.

Table 1
Amino acid identities between medaka and other GRKs

	OIGRK-R	OIGRK-C
OIGRM-C	45.3	
RK (human)	68.8	46.7
RK (cow)	68.6	45.9
RK (rat)	67.6	47.3
GRK4 (human)	47.3	46.9
GRK5 (human)	48.3	48.1
GRK6 (human)	46.1	45.5
C. elegans GRK-B	45.9	42.1
GRK2 (human)	34.0	31.2
GRK3 (human)	34.8	32.0
Drosophyla GRK-A	35.2	31.8
C. elegans GRK-A	34.6	30.0

3. Results

3.1. Isolation of the cDNA fragments encoding putative medaka GRKs

Two kinds of cDNA fragments, OlGRK-R and -C, were amplified by polymerase chain reactions using medaka retinal cDNA as a template. Full length cDNA clones were isolated by screening a medaka retinal cDNA library [12] with the amplified cDNA fragments as probes. OlGRK-R and -C consist of 2449 and 2954 bases, respectively. The first ATGs of these GRKs were assigned as the translational initiation codons by comparison with those of other vertebrate GRKs. Fig. 1 shows the deduced amino acid sequences of medaka GRKs aligned with the bovine RK sequence [19]. The OlGRK-R and -C cDNA fragments each appear to encode proteins of about 560 amino acids with molecular masses of approximately 64 kD.

3.2. Phylogenetic analysis of OlGRK-R and -C

The deduced amino acid sequence of nearly full length OlGRK-R shares 64–69% identity with those of mammalian RKs (Table 1). Fig. 2 shows a NJ-tree calculated for 350 amino acids which could be aligned in all GRKs. Judging from the high clustering probability, OlGRK-R belongs to the RK (GRK1) subtype, and the amino acid difference between these medaka GRKs and mammalian RKs is likely due to the teleost mammalian divergence.

In contrast, OlGRK-C shows only 45% amino acid identity with OlGRK-R, and is more similar (45–49% identities) to

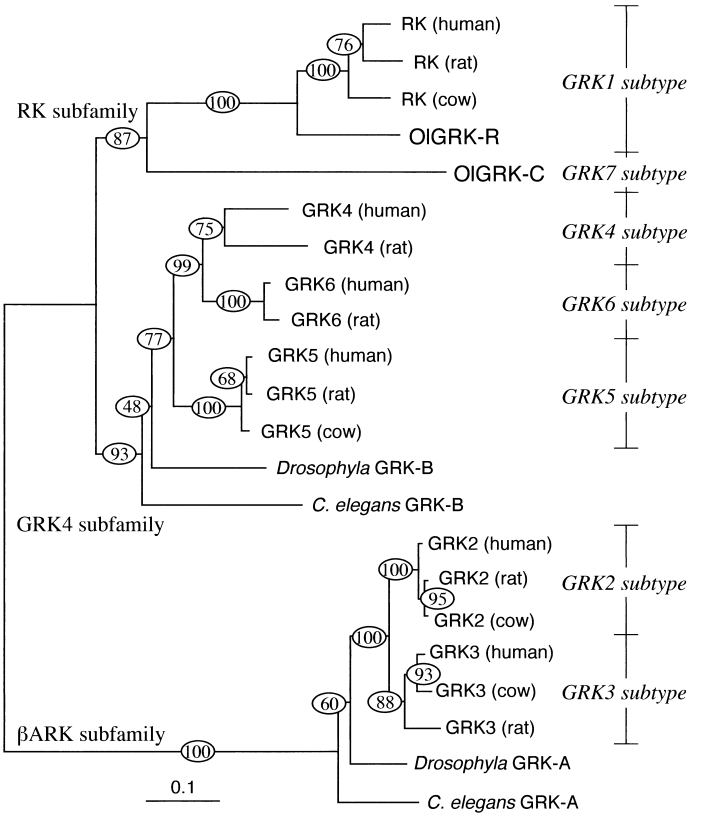

Fig. 2. A NJ-tree calculated from the amino acid sequences of GRKs. Circled numbers indicate mean clustering percentage obtained from 1000 bootstrap resamplings ($n=5$). The fluctuations of these percentages are less than 3% with different random series. Bar indicates 10% replacement of an amino acid per site ($k=0.1$; see Section 2). GRK subtype is indicated at the right of each sequence. Phylogenetic analysis in the kinase domain locates the putative root on the branch between βARK and other subfamilies.

GRKs in the RK and GRK4 subfamilies than in the βARK subfamily (Table 1). The NJ-tree suggests that OlGRK-C is phylogenetically related to RKs but does not belong to any of the GRK1-6 subtypes (Fig. 2). It is therefore concluded that OlGRK-C is a member of a novel GRK subtype, designated GRK7. The clustering probability of OlGRK-C and GRK1 is 86.6% (±0.9% standard deviation, $n=5$) but that of OlGRK-C with the GRK4 subfamily is only 13.1% (±0.9%). A NJ-tree calculated from the overall coding region (503 amino acids), gives a clustering probability of 95% (±0.7%) for RKs and OlGRK-C, so it is likely that OlGRK-C forms a single cluster with RKs within a GRK1 subfamily and plays a similar role in medaka retina to mammalian RKs.

3.3. *In situ localizations of medaka GRK mRNAs*

The photoreceptor cells of medaka can be categorized morphologically into five types: rods, principle (Pr) and accessory (Ac) members of double cones, long single cones (LS), and short single cones (SS). The cone cells are arranged in a square mosaic pattern [12,20]. The distribution of medaka GRK mRNAs was investigated by in situ hybridization. The digoxigenin-conjugated OlGRK-R cRNA probe only recognized the outer nuclear layer in radial sections of adult medaka retina (Fig. 3a). Signals were localized in the cell bodies and myoids of rods, but were not found in cone cells (Fig. 3b). A similar staining pattern was observed with in situ hybridization using medaka rhodopsin and rod arrestin cRNAs as probes [12,17], so it is therefore concluded that OlGRK-R is expressed exclusively in rods.

Fig. 4 shows the localization of OlGRK-C mRNA. Hybridization signals were found only around the outer limiting membrane (Fig. 4a) and localized in the myoid regions of

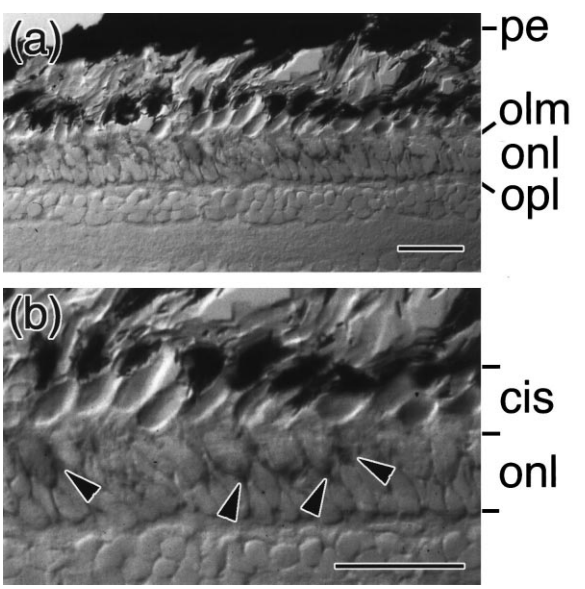

Fig. 3. Localization of OlGRK-R mRNAs in radial sections of medaka retina. Arrowheads indicate signals that hybridize with OlGRK-R cRNA probes. pe, pigment epitherium; olm, outer limiting membrane; onl, outer nuclear layer; opl, outer plexiform layer; and cis, cone inner segment. Scale bar, 20 μm.

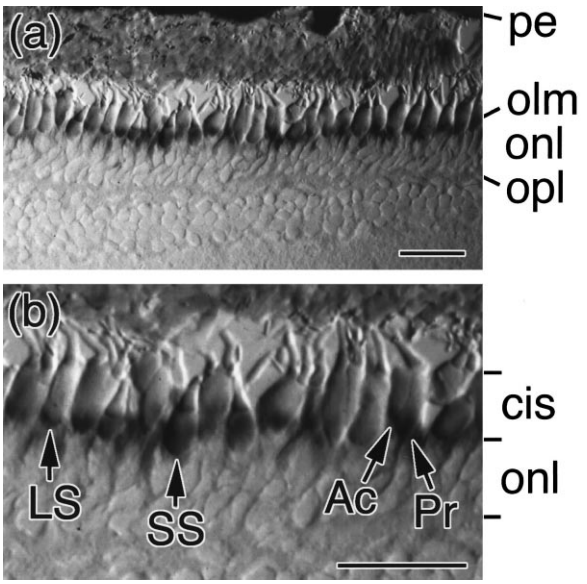

Fig. 4. Localization of OlGRK-C mRNAs in radial sections of medaka retina. Arrows indicate signals that hybridize with OlGRK-C cRNA probe in the myoid regions of principle (Pr) and accessory (Ac) members of double cones, long single cones (LS), and short single cones (SS). Other abbreviations as in Fig. 3. Scale bar, 20 μm.

Pr, Ac, LS, and SS (Fig. 4b). The localization of the signals in the myoid regions of the cone inner segments is as shown previously for medaka cone pigments and a cone arrestin [12,17], suggesting that OlGRK-C is expressed in all four types of cone photoreceptors.

3.4. Distribution of GRK7 in teleost, amphibia and reptile retinas

To investigate the distribution of GRK7 within vertebrate retinas, we carried out amplifications using, as templates, retinal cDNAs of goldfish (*C. auratus*), a newt (*T. pyrrhogaster*), and a diurnal lizard (*J. polygonata*). The lizard, *J. polygonata*, has a pure-cone retina (our unpublished observation), as with all diurnal lizards [21]. The deduced amino acid sequences of the isolated cDNA fragments are shown in Fig. 5a, and a NJ-tree was calculated from these sequences (Fig. 5c). Though amino acids used for the calculation are limited (65 amino acids), OlGRK-C, CaGRK-2, TpGRK, and JpGRK apparently constitute a cluster, suggesting that GRK7 is present in the retinas of many lower vertebrates.

4. Discussion

The amino acid residues crucial for RK functions have been investigated by several groups. The N-terminal region (Ala-17-Lys-34) of bovine RK plays a role in the recognition of rhodopsin [9], Ser-488 and Thr-489 are the major sites of autophosphorylation [22], and the C-terminal Cys-558 is post-translationally farnesylated [23]. OlGRK-C has a rather different sequence in the N-terminal region, with a glutamic acid residue located at the autophosphorylation site corresponding to Thr-489 of bovine RK. A site-directed mutant at residue Thr-489 to Ala (T489A) of bovine RK showed a difference in the initial sites of phosphorylation of activated rhodopsin from those of the wild-type [24]. It is therefore likely that this residue affects the properties of OlGRK-C also.

The amino acid at the C-terminal of OlGRK-R is a serine

(a)

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                          210       220       230       240       250       260
OlGRK-R          SACQMRATGKLYACKKLNKKRLKKRKGYEGAMVEKRILARV---HSRFIVSLAYAFQSKTELCLVMTI
OlGRK-C          CAVQVKNTGQMYACKKLCKKRLKKKGGEKMALLEKQILEKV---NSLFLVNLAYAYDTKTHLCLVMTL
CaGRK-1 (goldfish) SACQMKATGKLYACKKLNKKRLRKRKGYEGAMVEKRILARV---HSRFIVSLAYAFQTKTELCLVMTI
CaGRK-2 (goldfish) CAVQVKNTGQMYACKKLCKKRLKKKHGEKMALLEKKILEKV---NSLFIVSLAYAYDTKTHLCLTMSL
TpGRK (newt)     CAIQVKDTGKMYACKKLDKKRLKKKNGEKMALLEKEILEKV---HSPFIVKLAYAYESKRHLCLVMSL
JpGRK (lizard)   CAIQVRNTGKMYACKKLDKKRLKKKGGEKMALLEKVILEKV---NSRFIVTLAYAYQSKTALCLVMSL
RK (bovine)      FACQMKATGKLYACKKLNKKRLKKRKGYQGAMVEKKILAKV---HSRFIVSLAYAFETKTDLCLVMTI
RK (human)       SACQMKATGKLYACKKLNKKRLKKRKGYQGAMVEKKILMKV---HSRFIVSLAYAFETKADLCLVMTI
GRK2 (human)     YGCRKRDTGKMYAMKCLDKKRIKMKQGETLALNERIMLSLVSTGDCPFIVCMSYAFHTPDKLSFILDL
GRK3 (human)     YGCRKADTGKMYAMKCLDKKRIKMKQGETLALNERIMLSLVSTGDCPFIVCMTYAFHTPDKLCFILDL
GRK4 (human)     CACQVRATGKMYACKKLQKKRIKKRKGEAMALNEKRILEKV---QSRFVVSLAYAYETKDALCLVLTI
GRK5 (human)     CACQVRATGKMYACKRLEKKRIKKRKGESMALNEKQILEKV---NSQFVVNLAYAYETKDALCLVLTI
GRK6 (human)     CACQVRATGKMYACKKLEKKRIKKRKGEAMALNEKQILEKV---NSRFVVSLAYAYETKDALCLVLTL
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(b)

	Identitiy(%)	
	OlGRK-R	
		OlGRK-C
OlGRK-C	58	
CaGRK-1 (goldfish)	95	60
CaGRK-2 (goldfish)	58	91
TpGRK (newt)	62	78
JpGRK (lizard)	66	82
RK (bovine)	88	63
RK (human)	86	62
GRK2 (human)	38	40
GRK3 (human)	38	42
GRK4 (human)	69	69
GRK5 (human)	63	72
GRK6 (human)	66	74

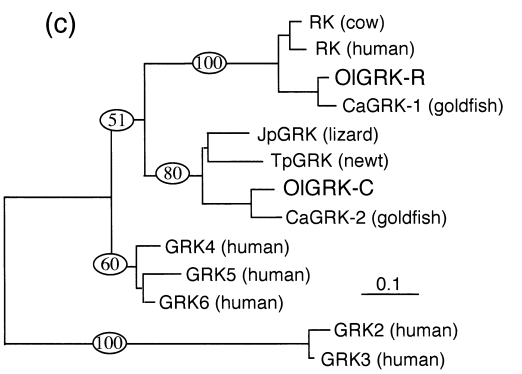

Fig. 5. Amino acid sequences (a), identities (b), and a NJ-tree (c) deduced from amplified cDNA fragments. Numbers above these sequences correspond to amino acid numbers of bovine RK. Identities are calculated for the 65 amino acid sites without gaps. Fluctuations of the clustering percentages obtained are less than 3% with 1000 bootstrap resamplings (n=5). Abbreviations as in Fig. 2. The nucleotide sequences in this figure have been submitted to the EMBL nucleotide sequences database with accession numbers AB009570 (CaGRK-1), AB009571 (CaGRK-2), AB009572 (TpGRK) and AB009573 (JpGRK).

residue but, surprisingly, that of OlGRK-C is leucine. It has been reported that a protein farnesyltransferase and a protein geranylgeranyltransferase type I modify cysteine residues in carboxy-terminal CaaX motifs in which 'a' is an aliphatic amino acid, and 'X' is one of several possible residues. When 'X' is Ala, Met, Ser, Cys, or Gln, the protein substrate is farnesylated, and when 'X' is Leu or Phe, the protein is geranylgeranylated [25–29]. A site-directed mutant of bovine RK, in which the serine residue at the C-terminal was replaced with leucine, was geranylgeranylated in COS-7 cells [30]. These post-translational modifications by thioether linkage to isoprenylated groups have been found with a variety of proteins in eukaryotic cells (see [31,32]). It is therefore suggested that OlGRK-R is farnesylated and that OlGRK-C is geranylgeranylated.

Farnesylated wild-type RK was associated with rod outer segment membrane only in the light-adapted state, but the geranylgeranylated mutant was found mainly in the membrane fraction even in the dark [30]. The non-isoprenylated mutant could not be associated with membrane, and showed much low activity of rhodopsin phosphorylation even in the light-adapted state [30]. Bovine S-modulin (recoverin), which interacts directly with RK, is heterogeneously acylated with four kinds of fatty acids at its N-terminal [33]. Since differences in the acylation of recoverin affect the efficiency of rhodopsin phosphorylation [34], the efficiency may also be tuned by acylation of RKs with different isoprenoids [30].

In the present paper, both kinds of medaka GRKs (OlGRK-R and -C) are shown to be resemble mammalian RKs. OlGRK-R and OlGRK-C are selectively expressed in rods and cones, respectively, and rods and cones are known to have similar isozymes of various phototransduction proteins including opsins [35], transducins [36], phosphodiesterases [37], cGMP-gated channels [38], arrestins [39] and S-modulin (recoverin) [40]. It is believed that the molecular properties of these isozymes influence the physiological responses of rods and cones [41], such as lower sensitivity [42], faster time course and more pronounced adaptation [43] in cones than in rods. Cone subtypes of S-modulin, visinin and s26, have been found only in non-mammalian species [40,44]. Similarly, cone subtypes of RK (cone opsin kinase, CK) have not been identified in mammals but are expected to correspond to those of lower vertebrates. The divergence of rod and cone subtypes of S-modulin is similar to that of GRK1 and GRK7, and the molecular diversities of these proteins may have some relationship [17]. Our data, presented here, are consistent with the concept that lower vertebrates have rod- and cone-specific enzymes participating in the phototransduction cascades, which tune the physiologically different responses of rods and cones.

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