Two types of arrestins expressed in medaka rod photoreceptors

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Abstract Similar to visual arrestins of other vertebrates, two subtypes of medaka visual arrestins, KfhArr-R1 and KfhArr-C, are selectively expressed in rods and cones, respectively [Hisatomi et al. (1997) FEBS Lett. 411, 12–18]. We isolated a cDNA encoding the third arrestin, KfhArr-R2, from a medaka retinal cDNA library. Phylogenetic analysis of arrestin sequences suggests that KfhArr-R2 is classified into the rod arrestin subtype. In situ hybridization indicated that KfhArr-R2 mRNA is localized in most of the rod photoreceptors, suggesting that both KfhArr-R1 and -R2 are co-expressed in rods. Antisera against KfhArr-R2 recognized outer segments, but anti-KfhArr-R1 antisera reacted with cell bodies and synaptic termini in light-adapted rods. It seems likely that KfhArr-R1 and -R2 play different roles in rod photoreceptors.

Key words: Arrestin; cDNA cloning; In situ hybridization; Immunocytochemistry; Medaka (Japanese killifish, *Oryzias latipes*)

1. Introduction

Light activates rhodopsin, which triggers the phototransduction cascade to induce the hyperpolarization of rod cells. The activated rhodopsin intermediate (metarhodopsin II) is phosphorylated by rhodopsin kinase, and then binds a regulatory protein, arrestin, to shut off further signaling [1]. In cone photoreceptors, cone arrestins are believed to specifically participate in deactivation of cone phototransduction [2–4].

It has been reported that light influences the intracellular localization of photoreceptor-specific proteins including arrestin. In the dark-adapted retina, arrestin is detected in all parts of the rod photoreceptor except for the outer segment [5]. This distribution is reversed in light-adapted retinas. Light-induced change in the subcellular topology of arrestin immunoreactivity was observed in rat [5], mouse [6], and toad [7]. Also, light-induced translocation has been reported for the transducin α subunit [8], but the mechanisms of such movements are poorly understood so far.

We have reported two kinds of cDNAs encoding medaka arrestins, KfhArr-R1 and KfhArr-C, selectively expressed in rods and cones, respectively [4]. In this paper, we report an additional cDNA encoding the second rod arrestin (KfhArr-R2) and subcellular localizations of these three medaka arrestins. Comparison of the three medaka arrestins may provide evidence for complex regulation of light-dependent localization and for unique desensitization mechanisms of teleost phototransduction.

2. Materials and methods

2.1. Isolation of cDNAs encoding KfhArr-R2

A cDNA fragment encoding a medaka (Japanese killifish, *Oryzias latipes*) arrestin, KfhArr-R2, was amplified by polymerase chain reaction with degenerate oligonucleotides as primers [4]. A medaka retinal cDNA library [9] was screened by plaque hybridizations using the amplified fragment as a probe, as described previously [10], and positive clones were sequenced by an ordinal deletion method [11]. Sequence data analysis was carried out as described by Hisatomi et al. [4].

2.2. In situ hybridization

The KfhArr-R2 cDNA fragment was cloned into a pGEM-3Zf(+) plasmid vector (Promega), and linearized with *Hin*dIII endonuclease. Antisense cRNA riboprobes (906 bases in length) were synthesized by run-off transcription from the T7 promoter with digoxigenin-UTP, as recommended in the manufacturer's protocol (Boehringer Mannheim).

Dark-adapted eyes were enucleated from medaka kept overnight in the dark, and light adaptation was carried out under room light for 4 h after dark adaptation. Preparation of medaka retinal cryosections and methods for in situ hybridization were as described previously [9,12,13]. Retinal cryosections of 3–5 μm were hybridized with 0.1–2.0 μg/ml (final concentration) cRNA probes, and the hybridization signal was visualized using the nucleic acid detection kit (Boehringer Mannheim).

2.3. Preparation of antisera against medaka arrestins

Expression and isolation of the fusion peptides containing KfhArr-R1, -R2, and -C were carried out as described by Hisatomi et al. [14]. The cDNA corresponding to the region (from D35 to S335) of KfhArr-R1 was inserted into the *Bam*HI site of pET-16b, and introduced into *Escherichia coli* cells (BL-21(DE3)). The cDNA corresponding to the region (from D35 to S335) of KfhArr-R2 were amplified using KfR2-F1 (5′-AGATCTGATCCAGTAGATGGAGTC-3′) and KfR2-R1 (5′-TTTGGATCCGATCCAGTAGATGGAGTC-3′) as primers, inserted between the *Bgl*II and *Bam*HI sites of a pQE-60 plasmid vector, and introduced into *E. coli* cells (SG13009, Qiagen). The cDNA containing the entire coding region of KfhArr-C was amplified with KfC-F1 (5′-CGCCATGGCAAAGATTTTC-3′) and KfC-R1 (5′-ATGGATCCCTCTGCGGGCTTTGG-3′) primers, inserted between the *Nco*I and *Bam*HI sites of pQE-60, and introduced into *E. coli* cells (SG13009, Qiagen). The fusion proteins, recombinant truncated KfhArr-R1 (or R2), and recombinant KfhArr-C with histidine hexamers were purified using Ni-NTA columns (QIA purification system, Qiagen) and used to immunize mice to produce antisera.

2.4. Western blotting

A medaka retina and other tissues were immersed in sodium dodecylsulfate (SDS) sample buffer and sonicated briefly. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the standard method using 8% acrylamide mini-gels. Proteins were transferred to PVDF membranes (Bio-Rad) using a semi-dry transfer cell (Transblot SD, Bio-Rad) in the presence of 100 mM Tris, 192 mM glycine and 20% methanol at 1.7 mA/cm^2 for 2 h. Membranes were blocked with 3% bovine albumin in PBS buffer, and were incubated with a 1000-fold dilution of the antiserum in PBS buffer containing 3% bovine albumin for 2 h. Horseradish peroxidase-conjugated anti-mouse IgG was reacted as recommended by the manufacturer (Vector

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Laboratories), and antibody binding was visualized with diaminobenzidine.

2.5. Immunocytochemistry

Immunocytochemical procedures were carried out as described by Hisatomi et al. [15]. Briefly, cryosections (3–5 μm) of the medaka eye cup were incubated with 100–1000-fold dilution of the antiserum, washed with PBS, and visualized with Histofine SAB-PO kit (Nichirei). Localization of arrestin was observed using Nomarski optics (Olympus-BX50). Immunofluorescent staining was carried out using TSA-indirect (NEN) and Cy2-labeled streptavidin (Amersham). Fluorescence was detected using a confocal microscope (Olympus-FLUOVIEW).

3. Results

3.1. Isolation of cDNA and the deduced amino acid sequence of KfhArr-R2

The cDNA encoding KfhArr-R2 consists of 1411 bases. High stringency screening yielded about 0.2% positive clones from 5×10^4 independent clones. This value is similar to the ratio of KfhArr-R1-positive clones (0.2%) in this library. Fig. 1 shows the deduced amino acid sequence of medaka arrestins aligned with bovine arrestin. The deduced amino acid sequence of KfhArr-R2 shows 63.6% identity with that of KfhArr-R1 and 54–59% identity with those of vertebrate rod arrestins. A phylogenetic tree calculated by the neighbor joining (NJ) method suggests that KfhArr-R2 is classified into the rod arrestin subtype (Fig. 2). It seems that the gene duplication event has occurred within the rod arrestin subtype after the divergence between teleosts and tetrapods.

3.2. In situ localization of KfhArr-R2 mRNA

The distribution of KfhArr-R2 was investigated by in situ hybridization. The digoxigenin-conjugated KfhArr-R2 cRNA probe only recognized the outer nuclear layer in radial sections of adult (Fig. 3a) and fry medaka retina (Fig. 3b), which closely resembles those of the medaka rhodopsin probe [9]. Signals were observed in the perinuclei and myoid region of most rods. Since KfhArr-R1 has been reported to be expressed in almost all rods [4], it was suggested that both KfhArr-R1 and -R2 co-exist in medaka rods.

3.3. Western blot analysis and tissue distribution of medaka arrestins

We prepared antisera, anti-R1, anti-R2 and anti-C, raised

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Bovine Arr(rod)     1 MKANKPAPNHVIFKKISRDKSVTIYLGKRDYIDHVERVEPVDGVVLVDPELVKGKRVYVS    60
KfhArr-R1           1      MTPKQVIFKKVSRDKSVAVYLAKRDFVDHCDFVDPVDGVVVIDPTQLKGRKVYVM    55
KfhArr-R2           1      MSPKNVIFKKTCKDKSVGVFMGKRDFVDRVDSVDPVDGVILIDPEVLAGRKVFVT    55
KfhArr-C            1         MAKIFKKTSGNGGLTLYLGKRDYVDHVSSVEKLDGVVKVDTTDFGDRKAFVQ    52

Bovine Arr(rod)    61 LTCAFRYGQEDIDVMGLSFRRDLYFSQVQVFPPV--GASGATTRLQESLIKKLGANTYPF   118
KfhArr-R1          56 LSCTFRYGRQDMDVMGVAFRRDLFLVTRQVYPELQDKEKLTHTKIQQKLLRKLGDNAFPF   115
KfhArr-R2          56 LSCTFRYGRDDMDVMGIAFRRELYLVTRQVYPPMQDRDKTVHTRVQAKLLRKLGNNAYPF   115
KfhArr-C           53 MACAFRYGSDDLDVMGLCFRKDIWIQQIQIYPE---SSKPALSAMHDTLLKKAGDNSYPF   109

Bovine Arr(rod)   119 LLTFPDYLPCSVMLQPAPQDVGKSCGVDFEIKAFATHSTDVEEDKIPKKSSVRLLIRKVQ   178
KfhArr-R1         116 FFEFPDNLPCSVSLQPGPLDEGKKCAVEFEVKAF---CGEAQDEKIDKQSSVRLTIRKIQ   172
KfhArr-R2         116 FFEFPDNLPCSVALQPAPNDVGKQCAVEFEIKAF---SAESQDAKVRKRSTVKLMIRKVQ   172
KfhArr-C          110 SFEIPTNLPCSVSLQPGPDDKGKACGVDFEVKTYLAKEKNNPDEKIDKKDTARLVVRKIQ   169

Bovine Arr(rod)   179 HAPRDMGPQPRAEASWQFFMSDKPLRLAVSLSKEIYYHGEPIPVTVAVTNSTEKTVKKIK   238
KfhArr-R1         173 FSPENTELAPVAEMTFEFLMSEKPLQVKLSLPKETFYHGEVLKANVEITNSSSRNIKDIS   232
KfhArr-R2         173 YAPESQEVAPSVEITKDFVMSDKPLHVQATLDKELYYHGEPIKVHVNVTNNSNKNIKNII   232
KfhArr-C          170 YAPSQVGAGPKADVCKSFMMSDKPVHLEASMEKDLYFHGEDIPIKIKINNESNKTVKKIK   229

Bovine Arr(rod)   239 VLVEQVTNVVLYSSDYYIKTVAAEEAQEKVPPNSSLTKTLTLVPLLANNRERRGIALDGK   298
KfhArr-R1         233 LSVEQVTNVVLYSNDKYVKSVAKEETDDSVPSGTTLKKEYTLHPLLAYNKDRRGIALDGR   292
KfhArr-R2         233 VSVDQVATVVLFSNDSYTKCVDYEDNGDSVSAGATLKKVYTLLPLLANNRERRGIALDGK   292
KfhArr-C          230 ITVDQTTDIVLYSADKYTKTVLNQEFGETVDASSTADLCLTIKPALSENKEKRGLALDGR   289

Bovine Arr(rod)   299 IKHEDTNLASSTIIKEGIDKTVMGILVSYQIKVKLTVS--GLLGELTSSEVATEVPFRLM   356
KfhArr-R1         293 LKHEDTNLASSSIVKQEVLKEIQGMLVSYKVVLKMIAS-----GMVGSSEVSLEVPFKLM   347
KfhArr-R2         293 LKHEDTNLASSSVVKEGVLKEVMGIMVSYRVMVKLIVG-----GMMGSSEVGLEVPFRLM   347
KfhArr-C          290 LKDEDTNLASTTMIRPGVEKEVLGILVSYKIKINLMVAGGGLLGGLTASDVTVELPLNLM   349

Bovine Arr(rod)   357 HPQPEDPDTAKESF-QDENFVFEEFARQNLKDAGEYKEEKTDQEAAMDE              404
KfhArr-R1         348 HSKPEA---AKESEPDDMVFEDFKRDYLKGVVYGDDDESPAEL                    387
KfhArr-R2         348 HPKPDA---VRESEMEDEMVFEEFKRSYLRGIIAGDDDDEEGNVSGGDDITPKEK        399
KfhArr-C          350 HPKPAE                                                         355
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Fig. 1. The deduced amino acid sequence of the medaka arrestin, KfhArr-R2, arranged with those of bovine visual arrestin and other medaka arrestins (KfhArr-R1 and KfhArr-C). The solid line above the sequences indicates one of the regions that may bind to rhodopsin [21]. Arrowheads indicate the residues that constitute the 'polar core' (see text). An arrow indicates the location of the termination codon of an alternatively spliced variant, p44. The sequence of KfhArr-R2 has been deposited in the EMBL nucleotide database with accession number AB029392.

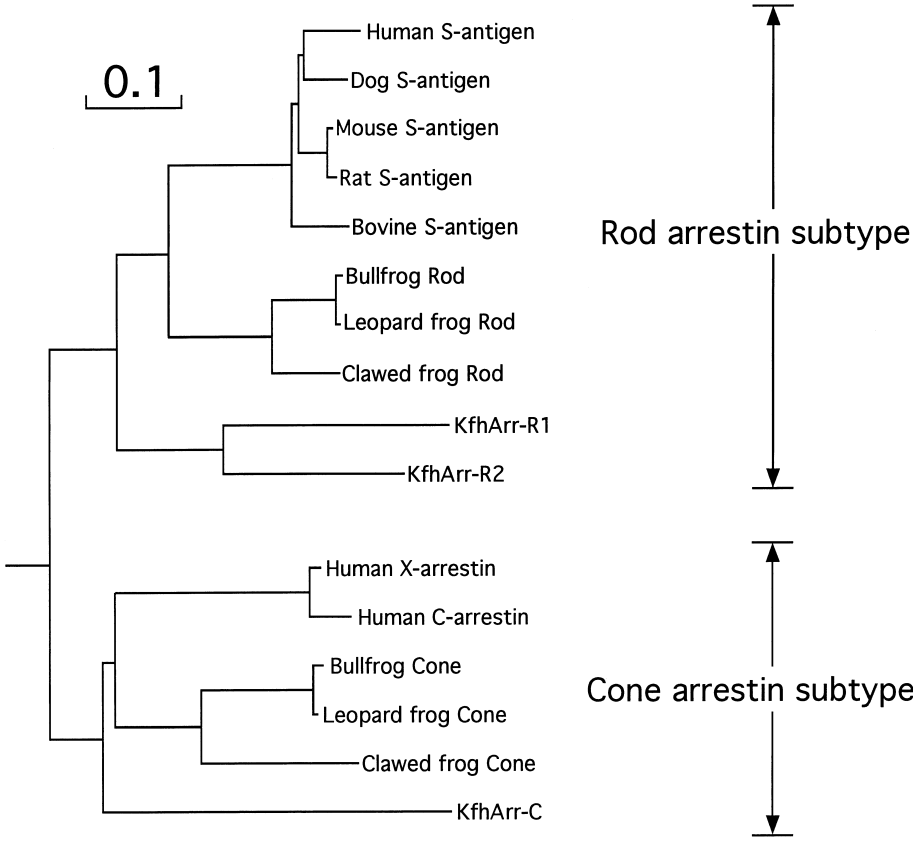
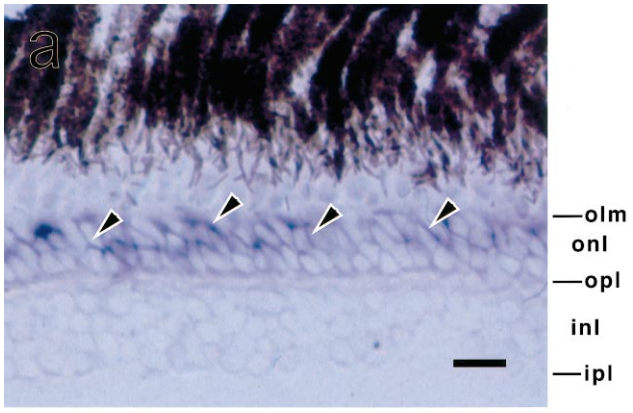


Fig. 2. A NJ tree calculated from the amino acid sequences of vertebrate visual arrestins, using β-arrestin and invertebrate arrestin sequences as outgroups. The bar indicates 10% replacement of amino acid per site. The arrestin subtype is indicated at the right of each sequence.

against recombinant truncated KfhArr-R1 and -R2 peptides, and recombinant full-length KfhArr-C, respectively. Anti-R1, anti-R2 and anti-C mainly recognized bands of about 46 kDa, 45 kDa and 41 kDa, respectively, in Western blot analysis of the retinal homogenate (Fig. 4a). It shows a good agreement of the molecular mass calculated from the deduced amino acid sequence of KfhArr-R1 (44 kDa), -R2 (45 kDa) and -C (39 kDa). In contrast to rod arrestins, the deduced amino acid sequence of KfhArr-C lacks a C-terminal region similar to p44, which is more efficient than arrestin in capping phosphorylated photoactivated bovine rhodopsin [16,17] (Fig. 1). The tissue distribution of these arrestins was examined for extracts from nine medaka tissues (Fig. 4b), and it was revealed that these arrestins are expressed specifically in retina.

3.4. Subcellular localization of medaka arrestins

In dark-adapted retina, strong anti-R1 immunoreactivity was found in inner segments, cell bodies and synapses, but not in outer segments of rods (Fig. 5a). Anti-R2 reacted with inner segments, and weakly with somas and synapses in dark-adapted rods (Fig. 5c). Neither anti-R1 nor -R2 immunoreac-



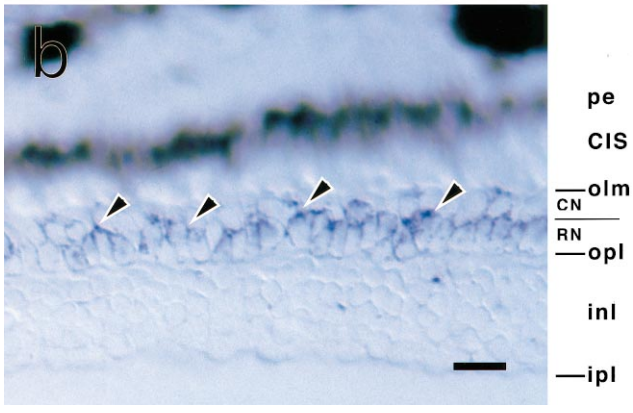


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Fig. 3. Localization of KfhArr-R2 mRNA in radial sections of (a) adult and (b) fry medaka retina. Arrows indicate the hybridization signals found at rod myoids and perinuclear regions in the outer nuclear layer (onl), but not at the cell bodies or myoids of cones. Abbreviations: pe, pigment epithelium; olm, outer limiting membrane; opl, outer plexiform layer; inl, inner nuclear layer; ipl, inner plexiform layer; RN, rod nuclei; CN, cone nuclei. Bar = 20 μm.

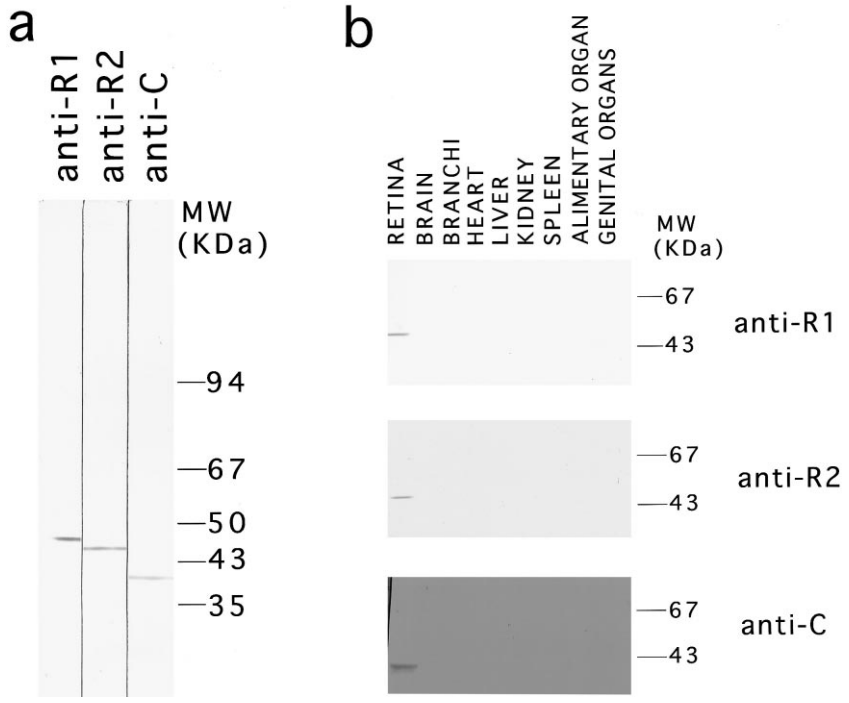

Fig. 4. a: Western blotting analysis of medaka retinal homogenate. Anti-R1, anti-R2, and anti-C recognized bands with molecular masses of 46 kDa, 45 kDa and 41 kDa, respectively. b: Tissue distribution of each arrestin analyzed by Western blots. Homogenates from nine tissues were examined.

tivity was observed in cone cells. In light-adapted retina, anti-R1 mainly reacted with somas and synapses (Fig. 5b). Immunoreactivity of anti-R1 was not detected in rod outer segments (ROS), even when signals were enhanced by immunofluorescent labeling (data not shown). This suggests that KfhArr-R1 does not localize in ROS. In contrast, anti-R2 recognized ROS, the part of phototransduction (Fig. 5d). To exclude the possibility of cross-reactions between KfhArr-R1 and -R2, we carried out immunocytochemical investigations using anti-R1 and -R2 pre-absorbed with recombinant truncated KfhArr-R2 and -R1, respectively. The results showed the same staining patterns with a slightly higher S/N (data not shown).

Anti-C reacted with cone photoreceptors, as expected from in situ hybridization experiments [4]. Medaka cones can be morphologically categorized into four types, principal and accessory members of double cones, and long and short single cones [9,18]. The reactivity was observed in cone inner segments, somas and synapses. Cones negative with anti-C were not detected in our study, suggesting that all cones express KfhArr-C. Note that anti-C immunoreactivity of short single cones was weaker than in other cones (Fig. 5f, arrows). Because of masking by pigment epithelium, it was difficult to judge whether cone outer segments were stained or not. Therefore, we enhanced the signal with immunofluorescent labeling and identified immunoreaction at cone outer segments (data not shown). It is suggested that KfhArr-C localizes to cone outer segments and possibly interacts with cone pigments.

4. Discussion

In this paper, we describe the isolation and characterization of a medaka cDNA encoding a rod arrestin, KfhArr-R2, similar to another rod arrestin (KfhArr-R1). High stringency screening with the KfhArr-R2 probe showed positive clones at a similar ratio to that with the KfhArr-R1 probe. Moreover, the signal intensities of in situ hybridization for KfhArr-R2 were almost comparable with that for KfhArr-R1 under the same condition. It is, therefore, speculated that the expression levels of KfhArr-R1 and -R2 are comparable.

Recent studies using site-directed mutants, and biochemical and crystallographic techniques support a model for the conversion of arrestin from an 'inactive' conformation to one that can bind to and inhibit the light-activated form of rhodopsin [19]. The polar core of arrestin is responsible for maintaining the 'inactive' conformation and disruption of this polar core permits structural adjustment that allows arrestin to bind to the activated rhodopsin. Residues in bovine arrestin (Asp-30, Arg-175, Asp-296, Asp-303, and Arg-382) are essential residues of the polar core [20]. In KfhArr-R1 and KfhArr-R2, these residues are well conserved (Fig. 1). In addition, residues 109–130 were recently implicated in rhodopsin binding [21]. This region of bovine arrestin is highly conserved among bovine arrestin KfhArr-R1 (15 residues per 22) and -R2 (15 residues per 22). We could not point out any difference in functional features between KfhArr-R1 and -R2 from their primary structures.

Interestingly, *Drosophila* has two visual arrestins both of which are expressed in all photoreceptors (R1–R8) in the eye. In vivo, these arrestins have at least partially redundant roles [22]. Transgenic mouse rods lacking arrestin (−/−) display a rapid partial recovery followed by a prolonged final phase, but when arrestin expression was halved (−/+), photoresponses are not affected at all [23]. The result indicates that arrestin binding does not constitute a rate-limiting step in recovery of the mouse rod flash response, while it does in *Drosophila* [24].

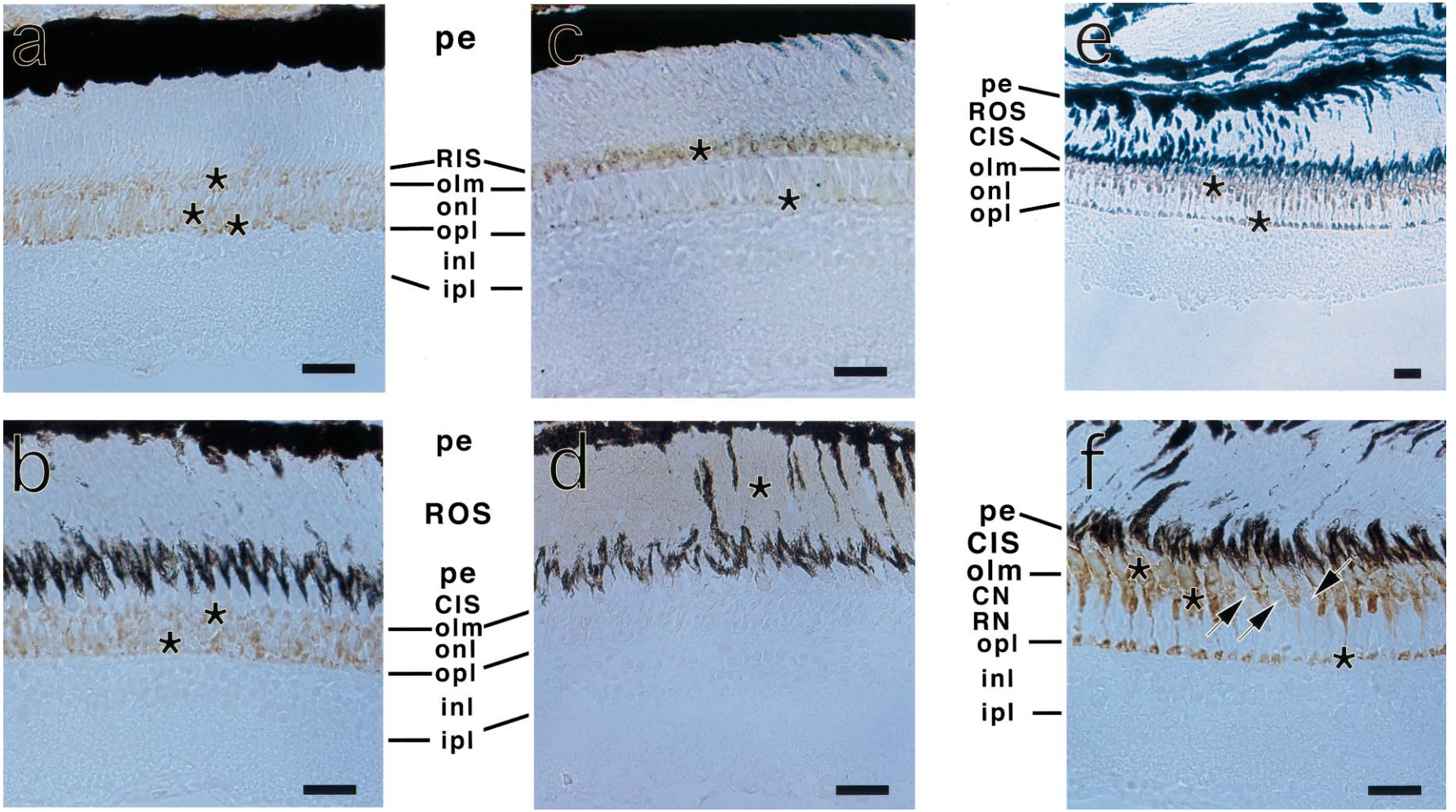

Fig. 5. Anti-R1 immunoreactivity in (a) dark- and (b) light-adapted medaka retina. Anti-R1 recognizes the synaptic terminus more heavily in dark-adapted retina. Anti-R2 immunoreactivity in (c) dark- and (d) light-adapted medaka retina. Anti-C immunoreactivity in light-adapted retina (e), and a higher magnification image (f). Shifts of pigment granules are observed (to cover cone outer segments) in light-adapted retina. Asterisks indicate signals. Arrows indicate short single cones labeled more weakly than other cones. Scale bar = 20 μm. Abbreviations as in Fig. 3.

Medaka rod arrestins are more closely related to mouse visual arrestin than to *Drosophila* arrestin, and so it is intriguing that two types of visual arrestins are expressed in medaka rods, although a single allele of arrestin seems to be sufficient for mouse rod phototransduction. A recent study indicated that medaka possesses two types of guanylate cyclases [25] and two types of GRK1 orthologues (our unpublished results) expressed in rods. These proteins and arrestin participate in the shut-off mechanism of phototransduction. One explanation for the existence of two arrestins in rods is that teleosts may have evolved unique desensitization mechanisms adapted to their photic environments.

It has been reported that light influences the intracellular localization of visual arrestin [5–8]. From our results, KfhArr-R2 is able to function in deactivation of medaka rhodopsin, because it is localized at outer segments in light-adapted rods (Fig. 5d) like other vertebrate arrestins.

Anti-R1 did not recognize the rod outer segments even in light-adapted rods. It is possible that the observed absence of anti-R1 immunoreactivity in rod outer segments results from epitope masking due to binding to rhodopsin or other protein-protein interaction (e.g. arrestin-arrestin interaction which may regulate visual arrestin activity [19,26]). However, the antibodies used in our study were polyclonal, which does not favor this hypothesis. The most readily acceptable explanation is that our observations reflect the change of arrestin localization.

KfhArr-R1 is probably localized at inner segments, cell bodies and synapses, but not at outer segments of rods. Anti-R1 reactivity in the synaptic terminus is heavier in the dark-adapted retina. It was recently reported that immunocytochemical experiments in rodent ocular tissues indicated that β-arrestin-related protein is located at inner segments and synaptic spherules in rods [27]. β-Arrestin is implicated not only in receptor desensitization but also in endocytosis of corresponding receptors [28]. The function of arrestin-related proteins in synaptic termini may be implicated in this manner.

Consequently, these results led us to speculate on the significance of two arrestins in rods, that is, KfhArr-R1 may play a role for an unknown function in the synaptic region of dark-adapted rods, and KfhArr-R2 may take part in recovery of photoactivation as known among other visual arrestins.

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