

Arrestins expressed in killifish photoreceptor cells

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Abstract Two kinds of cDNA fragments (KfhArr-R and KfhArr-C) encoding the putative arrestins of killifish, *Oryzias latipes*, were isolated. The distributions of these transcripts were investigated by in situ hybridization, and it was demonstrated that KfhArr-R and KfhArr-C are expressed in, respectively, rod and all four types of cone cells. The deduced amino acid sequences of KfhArr-R and KfhArr-C are closely related to human S-antigen (rod arrestin) and X-arrestin (cone arrestin), respectively. Phylogenetic analysis of arrestin sequences suggests that vertebrate visual arrestins form a single cluster distinct from other arrestins and diverged to form rod and cone subtypes before the divergence between teleosts and tetrapods. It is speculated that the divergence pattern of vertebrate visual arrestins may prove to be reflected in the divergence of the proteins participating in the respective phototransduction cascades.

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Key words: Arrestin; S-antigen; cDNA cloning; Killifish (or medaka, *Oryzias latipes*); In situ hybridization

1. Introduction

Light activates rhodopsin, which triggers the phototransduction cascade to induce the hyperpolarization of rod cells [1]. The activated rhodopsin intermediate metarhodopsin II is phosphorylated by rhodopsin kinase [2,3]. Arrestin (S-antigen) binds to the phosphorylated rhodopsin, precluding further activation of transducin and so shutting down the phototransduction cascade [4,5]. The visual transduction cascade in cone cells are less well understood but cones have similar isozymes of rod phototransduction proteins [6–12], suggesting that the basic signal transduction pathway of cones and rods is similar.

Arrestin is a soluble protein with a molecular mass of about 48 kDa [13]. The primary structure of 13 visual arrestins in vertebrates (from representative mammals and amphibians) have been reported to date, but the molecular mechanism of arrestin function is not yet understood completely. Since the function of vertebrate visual arrestins do not seem to show any fundamental differences, the amino acid residues crucial for arrestin function(s) are expected to be conserved, and may be deduced by comparing the amino acid sequences of representatives from various animal groups. Comparison of the primary structure of various vertebrate arrestins can also provide insights into the diversity and molecular evolution of arrestins.

In this paper, we report the two kinds of cDNA fragments (KfhArr-R and KfhArr-C) encoding the putative arrestins of killifish (Japanese 'medaka'), *Oryzias latipes*, and describe the distributions of these transcripts as investigated by in situ

hybridization. It is suggested that KfhArr-R and KfhArr-C are expressed in, respectively, rods and all four types of cone cells. The phylogeny of vertebrate visual arrestin is discussed and compared with the divergence patterns of other proteins participating in the phototransduction cascade.

2. Materials and methods

2.1. Isolation of cDNAs encoding putative killifish arrestins

We prepared two kinds of oligonucleotide mixtures, Arr-F2 (GCGAATTCTA(CT)T(TA)(CT)CA(CT)GGNGA(GA)CC) for sense priming, and Arr-R1 (CTCAAGCTTGCNA(AG)(AG)TTNGT(AG)TC(CT)TC(AG)TG) for anti-sense priming. These correspond to the amino acid sequences Y(F/Y)HGEP and HEDTNLA, respectively, which are amino acid sequences conserved among bovine S-antigen, human X-arrestin and β -adrenergic receptor arrestins (β -arrestin). The cDNA fragments encoding killifish arrestins were amplified as described previously [14–16]. A killifish retinal cDNA library made with a Lambda ZAPII EcoRI/CIAF Kit [16] was screened by plaque hybridizations using the amplified fragments as probes. Screening procedures were the same as described previously [17], except that formamide concentration in the hybridization solution was increased to 25% for low stringency screening. Positive clones were transformed into plasmids by an EXASSIST-SOLR system (Stratagene), and sequenced by an ordinal deletion method [18].

2.2. Sequence data sources

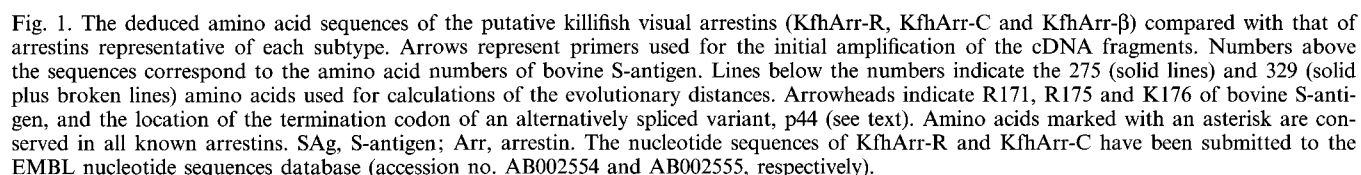
Sequence data used in the present analyses were taken from GenBank, EMBL, SWISS-PROT or NCBI databases, with the following accession numbers: Human (*Homo sapiens*) S-antigen (P10523), X-arrestin (P36575), arrestin-C (U03626), β -arrestin1 (P49407) and β -arrestin2 (P32121); bovine (*Bos taurus*) S-antigen (P08168), β -arrestin1 (P17870) and β -arrestin2 (P32120); mouse (*Mus musculus*) S-antigen (P20443); rat (*Rattus norvegicus*) S-antigen (P15887), β -arrestin1 (P29066) and β -arrestin2 (P29067); dog (*Canis familiaris*) S-antigen (NCBI seqID 1403297); bullfrog (*Rana catesbeiana*) rod (X92399) and cone (X92401) arrestins; leopard frog (*Rana pipiens*) rod (X92398) and cone (X92400) arrestins; clawed frog (*Xenopus laevis*) rod (U41623) and cone (L40463) arrestins; rainbow trout (*Oncorhynchus mykiss*) TRCarr (U48410); fruit fly (*Drosophila melanogaster*) arrestin A (P15372) and arrestin B (P19107); bluebottle fly (*Calliphora vicina*) arrestin1 (X79072) and arrestin2 (X79073); tobacco budworm (*Heliothis virescens*) arrestin (127926); migratory locust (*Locusta migratoria*) arrestin (S57174); horseshoe crab (*Limulus polyphemus*) arrestin (U08883); and nematode (*Caenorhabditis elegans*) arrestin (P51485).

The deduced amino acid sequences of killifish arrestins and 28 arrestins from previous reports were aligned. Amino acid positions in which the alignment have any ambiguity were excluded from the calculation, and analysis was carried out for the resulting 275 amino acids (see Fig. 1). 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 $k = -\ln(1-p-p^2/5)$ [19]. An unrooted tree was constructed by the neighbor-joining (NJ) method [20] using a self-made program (ESAT), and fluctuation of the bootstrap probabilities appeared to be $\pm 2\%$ with different random series ($n=3$).

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,

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	280	290	300	310	320	330	340	350	360
Bovine SAg	NSSLTKTLTLVPLLANNRERRGIALDGKIKHEDTNLASSTIIKEGIDK	TVMGILVSYQIKVKLTVS---	GLLGELTSSEVATEVPFRLMHPQP						
KfhArr-R	GTTLKKEYTLHPLLAYNKDRRGIALDGRLLKHEDTNLASSIVKQEVLK	ETQGMVLVSYYVKVKMIAS----	GMVGSSEVSLEVPFKLMHSPK						
KfhArr-C	SSTADLCLTIKPALENKEKRGALDGRLLKDEDNLASTTMIRPGVEK	EVLGILVSYKIKTNLMVA-	GGGLLGGLTASDVTVELPLNLMHPKP						
Human SAg	NSTLTKTLTLLPLLANNRERRGIALDGKIKHEDTNLASSTIIKEGIDR	TVLGILVSYQIKVKLTVS---	GFLGELTSSEVATEVPFRLMHPQP						
Clawed frog (rod)	KASYNHTFSLPLLAYNREKREIALDGRLLKHEDTNLASSTLKEGTDR	TVMGILVDYKIKVTLTVS---	GLLGDMTSSEVSTELPFILMHPNP						
Human X-Arr	NSSFSQSFAVTPILAASCQKRGALDGRLLKHEDTNLASSTIIRPGMDK	ELLGILVSYKVRVNLNMS-	CGGILGDLTASDVGVLPVLVTHPKP						
Clawed frog (cone)	NANFSGSYSLTPLANNKEKRGALDGRLLKHEDTNLASSTLIRPGMDK	EVLGMLVSYKVRVSLVVA-	RGGILGDLTSSDVSVELPFTLMHPKP						
Drosophila-A	GSSLQKVMYLVPTLVANDRAGIAVEGDIKRKDTALASTTLIASQDAR	DAFGIIVSYAVKVKLFGLAGG-----	ELCAELPFILMHPKP						
Tobacco budworm	GSSLQKVLHLTPTLAHNRDKRGIALDGLKRSDDTLASTLLLDPDQR	DAFGIVVSYSKVKLYLGAISG-----	ELVAELPFILMHPKP						
Horseshoe crab	GATLSKVYTLPLASQNKDKRGIALDGLMKEGDNLASTL----	NSTGDAIGIVISYVIRVRLYMGATGG-----	ELVADVSKLANPKE						
Drosophila-B	GANLTKTFYLIPLAANNKDRHGIALDGHLLKDEDVNLASSTMVQEGKSTGDACGIVISYSVRILKNCGLTGG-----	EMQTDVPFKLLQPAP							
Migratory Locust	GASFTKVFYLVPCAASNDRYGIALDGYLLKDDVDNLASSTLVSEGKNTTDAIGIVISYSLRVKLNCGTLGG-----	ELQTDVPFKLLHPAP							
Human β Arr-1	SSTFCVKVYTLTPFLANNREKRGALDGRLLKHEDTNLASSTLREGANR	EILGIIVSYKVKVKLVVS-	RGGLLGDASSDVAVELPFTLMHPKP						
Human β Arr-2	SSTFCVKVYITPILLSNREKRGALDGRLLKHEDTNLASSTIKVGANK	EVLGILVSYRVKVKLVVS-RGG-----	DVSVELPFVLMHPKP						
Trout TRCarr1	SSTFCVKVYTLTPTLDKNREKRGALDGRLLKHEDTNLASSTIKVDVTNK	EVLGILVSYRVKVKLVIS-RGG-----	DVSVELPFVLMHPKP						
Nematode Arr	GGTLSKVFVAVCPILLSNNKDKRGALDGLLKHEDTNLASSTILDSKTSK	ESLGIVVQYRVKVRVAVLGPLNG-----	ELFAELPFTLTHSKP						
KfhArr- β	SSTFCVKVYTLTPTLDKNREKRGALDGRLLK	***			*	*		*	*
		*		*	*	*			
				Arr-R1					

	370	380	390	400
Bovine SAg	EDPDTAKESFQDENFVFEEFARQNLKDAGEYKEEKTQEAAMDE			
KfhArr-R	EAA---KESE-PDDMVFEFDRDYLLKGVVYGDDDESPAEL			
KfhArr-C	AE			
Human SAg	EDP---AKESIQDANLVFEFARHNLKDAGEAEEGKRDKNDA-DE			
Clawed frog (rod)	DGG---AKESEQEDDMVFEEFARDPLKGELQAEKEEEEDDEK			
Human X-Arr	SHE----AASESDIVIEEFTR----KGEESQKAVEAGDEGS			
Clawed frog (cone)	SPD----QTNIEDVVIEEFARQLQGAE-GEDDKDDA			
Drosophila-A	SRKAQLEAEGSIEA			
Tobacco budworm	GRVKMIHADSQADVEMFRQDTVHHQESVEVY			
Horseshoe crab	VPVVGSGSQAQEQKARMKKQLSREMSTDLIVEDFARRRQFSEDNE			
Drosophila-B	GTVIEKKRS-----NAMKKMKSIQHRNVKGYG-QDDDDNIVFEDFAKMRMNNVNMAD			
Migratory Locust	GTAEREKA-----QAIKMKMSIERTRYENSCYAADDDDNIVFEDFARLRLNEPE			
Human β Arr-1	KEEPPHRE-----VPENETPVDNTNIELDTN---	DDDIVFEDFARQLKGMEDDKEEEEEDGTGSPRLNDR		
Human β Arr-2	HDHPLPRPQSAAPET-----DVPVDNTNIEFDTN-YATDDDIVFEDFARLRLKGMKDDDYDDQLC			
Trout TRCarr1	TELPISR-----QSAPDSDPPIDNTNIEFETNSFSQDDDFVFEFARLRLKGMADDKDDC			
Nematode Arr	PESPERRDGLPSTFATNGSEFPVDIDLHLEELPRYDDDLIFEDFARMLHGNDSDESDSPSANLPPSL			

as recommended in the manufacturer's protocol (Boehringer Mannheim). Preparation of killifish retinal cryosections and methods for *in situ* hybridization were as described previously [16,21,22]. 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 $\mu\text{g}/\text{ml}$ (final concentration) cRNA probes. The hybridization signal was visualized using a nucleic acid detection kit (Boehringer Mannheim), and detected with Nomarski optics.

3. Results

3.1. Isolation of the cDNA fragments encoding putative killifish arrestins

Two kinds of cDNA fragments, KfhArr-R and - β , were amplified by polymerase chain reactions (Fig. 1). A killifish retinal cDNA library [16] containing 5×10^4 independent clones was screened at high stringency using amplified cDNA fragments (KfhArr-R) as a probe. Another killifish cDNA fragment, KfhArr-C, was obtained by a screening of the library at low stringency using the KfhArr- β cDNA fragment as a probe.

3.2. The deduced amino acid sequences of killifish arrestins

KfhArr-R and -C consist of 2808 and 1676 bases, respec-

tively. The first ATGs of these arrestins were assigned as the translational initiation codons by comparison with those of other vertebrate arrestins. KfhArr-R and KfhArr-C cDNA fragments appeared to encode proteins of 387 amino acids (43.8 kDa) and 355 amino acids (39.0 kDa), respectively. Fig. 1 shows the deduced amino acid sequences of killifish arrestins aligned with the bovine S-antigen sequence [13]. In bovine S-antigen, the central basic region has been well characterized at the amino acid level [23–25]. Analysis of the site-directed mutants indicates that R171, R175 and K176 in bovine S-antigen play a primary role in phosphate interaction [25]. Positively charged amino acids corresponding to R171, R175 and K176 are found in the sequences of both KfhArr-R and -C, suggesting that these highly conserved amino acid residues may be among those crucial for arrestin function(s). A total of 35 amino acids are conserved in all arrestins from nematode to mammals (Fig. 1).

The deduced amino acid sequences of KfhArr-R and -C show 47% identity with each other and higher identities (54–59%) with those of vertebrate rod and cone arrestins, respectively. A phylogenetic tree calculated by the neighbor-joining method is shown in Fig. 2. This tree suggests that deuterocoele arrestins can be classified into three major types: vertebrate visual arrestins, vertebrate β -arrestins and arthropod

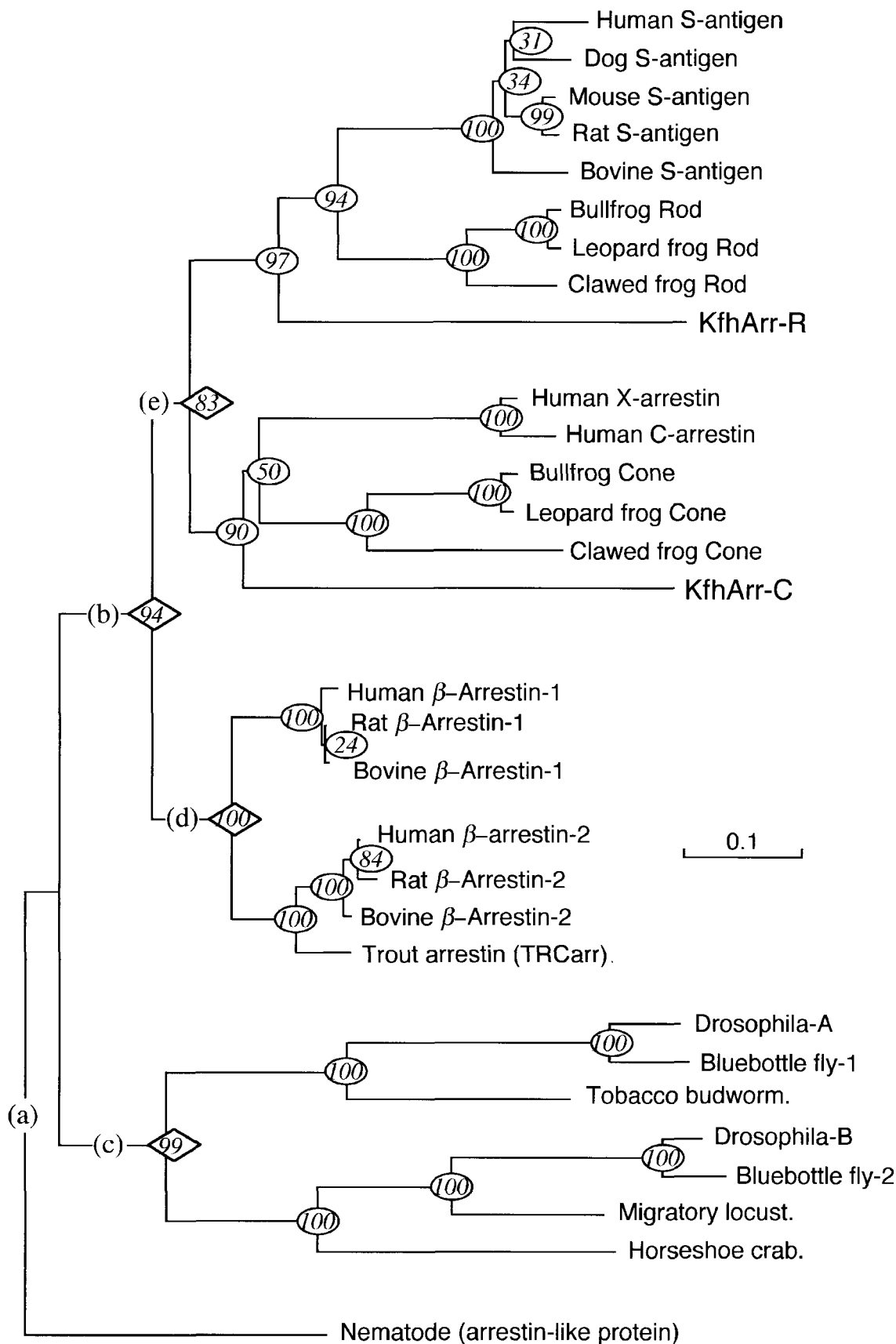


Fig. 2. A phylogenetic tree calculated from the amino acid sequences of arrestins. (a)–(e) represent the five possible locations of the root. Circles and diamonds represent the putative speciation and gene duplication nodes, respectively. Numbers indicate clustering percentage obtained from 1000 bootstrap resamplings. Bar indicates 10% replacement of an amino acid per site ($k=0.1$; see Section 2).

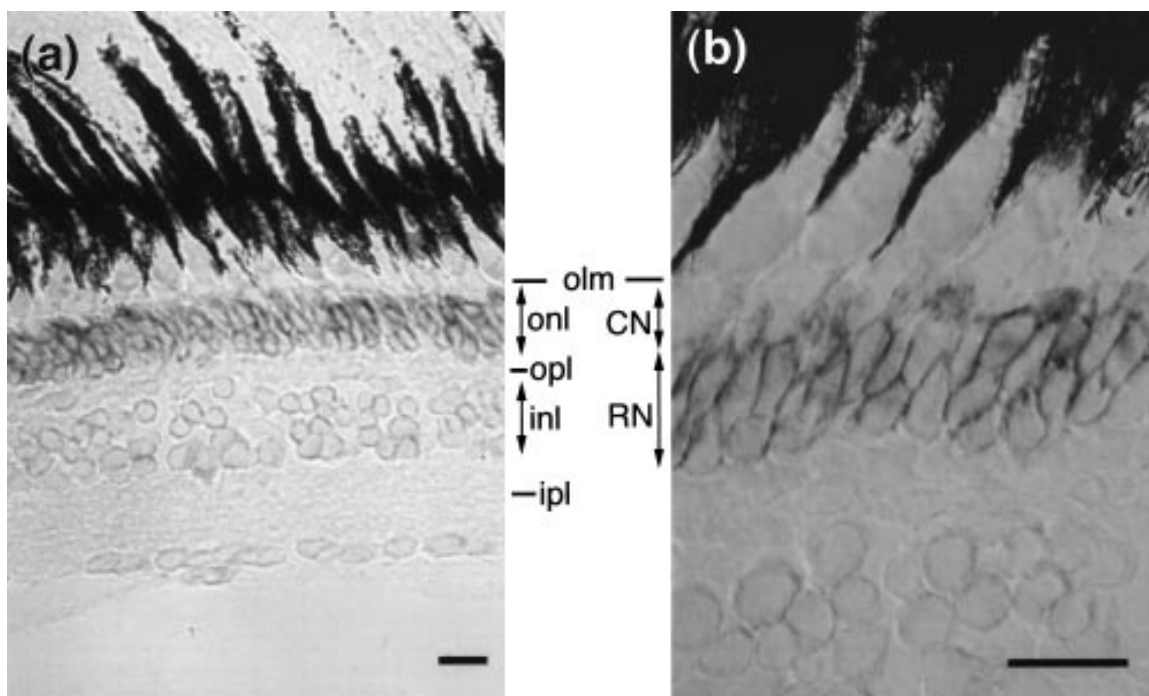


Fig. 3. (a) Localization of KfhArr-R mRNAs in radial sections of light-adapted killifish retina, and (b) a higher magnification image. Hybridization signals are found in the outer nuclear layer (onl), but not in the cell bodies or myoids of cones. Abbreviations: olm, outer limiting membrane; opl, outer plexiform layer; ipl, inner plexiform layer; inl, inner nuclear layer; RN, rod nuclei; CN, cone nuclei. Bar = 10 μ m.

arrestins. Vertebrate visual arrestins are distinguished as rod or cone subtypes, and KfhArr-R and -C can be classified into rod and cone arrestin subtypes, respectively.

3.3. *In situ* localization of killifish arrestin mRNAs

Killifish photoreceptor cells can be categorized morphologically into five types: rods, principle and accessory members of double cones, long single cones, and short single cones. The cone cells are reported to be arranged in a square mosaic pattern [16,26]. The distribution of the killifish arrestin mRNAs was investigated by *in situ* hybridization. The digoxigenin conjugated KfhArr-R cRNA probe only recognized the outer nuclear layer in radial sections of adult killifish 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 killifish rhodopsin cRNA as a probe [16], so we conclude that KfhArr-R is expressed exclusively in rods.

Fig. 4 shows the localization of KfhArr-C mRNA. Hybridization signals were only found around the outer limiting membrane (Fig. 4a), and localized in the myoid regions of principle and accessory members of double cones (Fig. 4b), and of long (Fig. 4c) and short (Fig. 4d) single cones. The localization of the signals in the myoid regions of the cone inner segments is as shown previously for killifish and goldfish cones [16,22,27], suggesting that KfhArr-C is expressed in all four types of cone photoreceptors, but not in rods. Cone cells negative with the KfhArr-C cRNA probe were not identified in our *in situ* hybridization experiments.

4. Discussion

4.1. C-terminal structure of killifish rod and cone arrestins

It has been reported that the acidic C-terminal region (res-

idues from 365 to the C terminus) may interact with the N-terminal basic region and play a role in controlling conformational changes in arrestins [23]. An alternatively spliced variant of bovine S-antigen lacking the C-terminal region (Fig. 1), p44, is a more potent inhibitor of transducin activation by rhodopsin [28]. KfhArr-C lacks this C-terminal region and may have similar physicochemical properties to p44. It was suspected that KfhArr-C is itself a spliced variant similar to bovine p44. However, spliced variants of KfhArr-C were not obtained in our screening. Moreover, Northern blot analyses of human and *Xenopus* retinal mRNAs show only a single band with the cone arrestin cDNA probe [11,29]. An alternatively spliced variant similar to p44 may not exist in killifish cone arrestins.

4.2. Distribution of arrestin mRNAs in killifish photoreceptor cells

The photoreceptor distribution of mammalian arrestins has been investigated by immunohistochemical experiments. It was reported that blue cones of human, baboon and ground squirrel had epitopically similar arrestins to rods, which were distinguishable from red/green cones [30–32]. On the other hands, it has been suggested that human X-arrestin is expressed in red-, green- and blue-sensitive cones in the human retina [33]. Our *in situ* hybridization in killifish demonstrate that two arrestins are expressed separately in rods and in all four types of cones, respectively. Although so-called rod and cone arrestins usually play a role in their respective cell type, it may not be detrimental if they are expressed in a different cell type.

4.3. Amino acid substitution rates among arrestins

The phylogenetic analysis suggests that amino acid substitution rates (k) are lower in β -arrestins than those of other

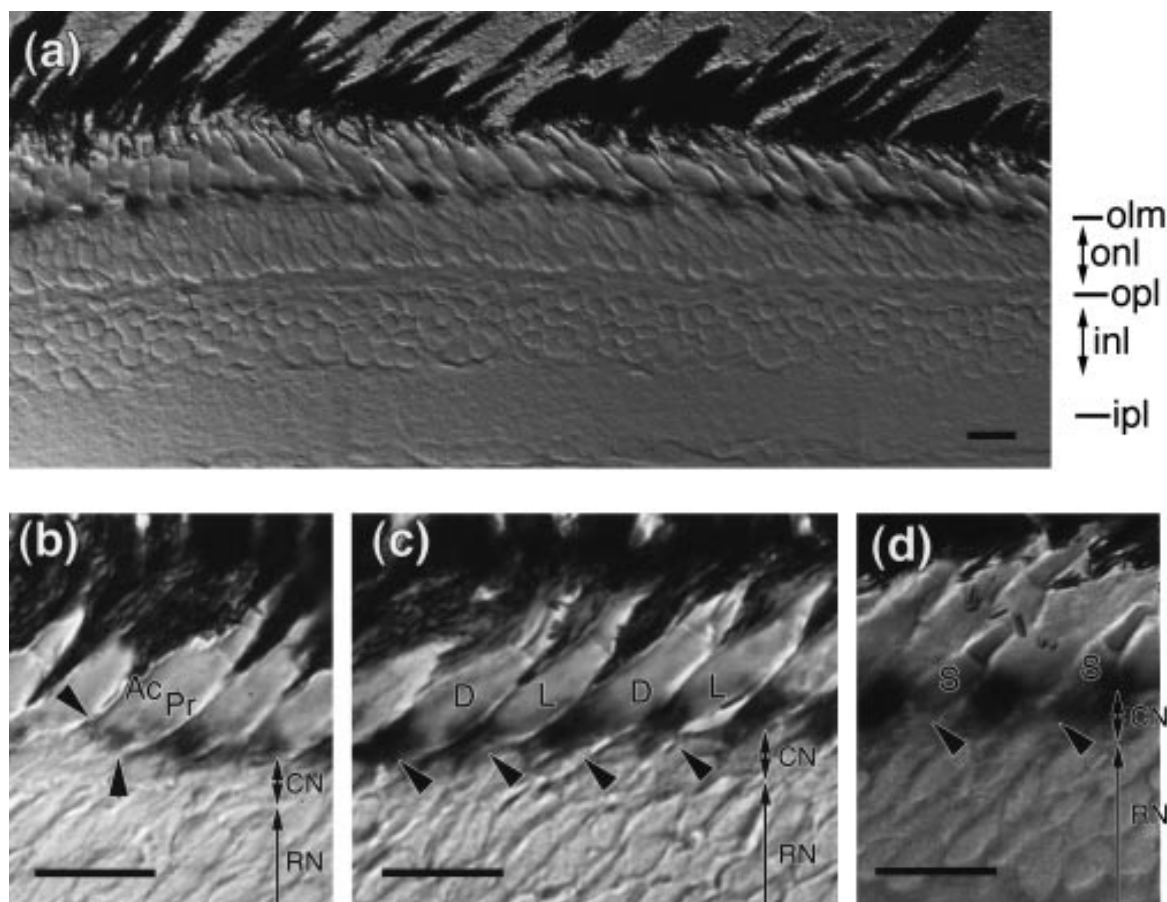


Fig. 4. (a) Localization of KfhArr-C mRNAs in radial sections of light-adapted killifish retina, and (b–d) higher magnification images. Signals are detected in the myoid regions of all four types of cone cell. Arrowheads indicate examples of hybridization signals. D, double cone (Pr, principle member; Ac, accessory member); L, long single cone; S, short single cone. Other abbreviations as in Fig. 3.

arrestins found in vertebrate photoreceptors and arthropod sensory systems. For example, the k values of rod and cone arrestins estimated from the amino acid identities between human and killifish arrestins are 0.59 (59% identity) for rod and 0.57 (60% identity) for cone arrestins. These values are about 6 times higher than the 0.10 that estimated from the identity (91%) between human β -arrestin2 and TRCarr, a rainbow trout arrestin found in red blood cells [34]. Recently, it was suggested that β -arrestins (but not visual arrestin) interact with clathrin and play a role in the internalization of β_2 -adrenergic receptors [35–37]. The low k value of β -arrestins may be due to the difference of functional constraints between β -arrestins and visual arrestins [38]. Also, the k value between *Drosophila* (Diptera) arrestin-A and tobacco budworm (Lepidoptera) arrestin is estimated to be 0.42 (68% identity), which has occurred during the approximately 250 million years since the last common ancestor of these insect groups. This value is comparable to those of vertebrate visual arrestins, assuming the teleost-tetrapod divergence occurred 400 million years ago [39,40].

4.4. Phylogenetic relationships among arrestins

It seems likely that the root of the phylogenetic tree is located at one of the five positions (a) to (e) in Fig. 2. Considering that nematodes obviously diverged from vertebrates and arthropods before the vertebrate-arthropod divergence

[40,41], the tree which gives the least gene duplication events are obtained when the root is located at (a). This tree leads us to speculate that vertebrate arrestins diverged after the vertebrate-arthropod divergence, to form visual arrestin and β -arrestin types, each of which has further branched out into two subtypes. In contrast to the arrestin phylogeny, it has been suggested that visual pigments diverged from neuro-transmitter receptors before the specific divergence between vertebrates and arthropods [42,43]. However, the large difference of amino acid substitution rates between β -arrestins and other sensory arrestins possibly distorts the structure of the tree presented here, so it may be necessary to reconstruct the arrestin tree more precisely by obtaining the arrestin sequences from many other representative organisms.

The existence of rod and cone arrestin subtypes has already been pointed out by an investigation of the clawed frog arrestin similar to human C-arrestin [29]. Unlike their tree, the present analysis suggests that vertebrate rod and cone arrestins form their own cluster phylogenetically distinct from other arrestins despite the uncertainty of root location. The clustering patterns of our tree are not fundamentally perturbed by the alignment of the sequences. For example, a tree calculated from 329 amino acids (gaps were excluded, see Fig. 1) showed the same clustering patterns for rod arrestins, cone arrestins and visual (rod and cone) arrestins with high bootstrap probabilities (95%, 94% and 97%, respectively).

It seems likely that the divergence between rod and cone arrestin subtypes occurred in the period between the vertebrate-arthropod divergence (700 million years ago) and the teleost-tetrapod divergence (400 million years ago). Divergence, by gene duplication, among vertebrate visual pigments appears to have occurred during the same period [42,43], except that visual pigments have branched out into five subtypes [14]. The divergence of visual arrestins into photoreceptor type-specific (rod and cone) subtypes may be more similar to that of S-modulins (recoverins) [12], which modulate photoreceptor sensitivity by controlling opsin kinase activity [44]. S-modulin homologues in vertebrate photoreceptor cells have been classified into rod and cone subtypes, which have branched from an ancestral S-modulin before the amphibians diverged from the rest of the higher vertebrates [12]. Both arrestins and S-modulins participate in the shut-off mechanism of the phototransduction cascade, so these proteins have possibly co-evolved with specific photoreceptor types. It may be that many of the proteins participating in the phototransduction cascade have a similar phylogeny to these proteins.

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