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Phototransduction and Visual Cycle in the Ascidian Tadpole Larva

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Ascidians are lower chordates, and their tadpole-like larvae share a basic body plan with vertebrates. To study photoreceptive systems in ascidians, we have isolated and characterized cDNA clones for three opsins, five G protein α subunits (Gα), catalytic and regulatory subunits of cGMP phosphodiesterase (PDE), and arrestin from the ascidian *Ciona intestinalis* tadpole larva. Ci-opsin1 and Ci-opsin2 are vertebrate-type opsins, while Ci-opsin3 is a retinal photoisomerase similar to retinochrome and mammalian RGR. Both Ci-opsin1 and arrestin are specifically localized in the photoreceptor cells of the ocellus, whereas Ci-opsin2 is not expressed in the photoreceptors, but is co-localized in another population of neurons in the brain with PDE (Ci-PDE9 and Ci-PDEδ). Ci-opsin3 is present in the entire region of the brain. Though five different cDNAs encoding Gα have been cloned, no transducin-type G protein has been found yet. Interestingly, one of Gαi isoform is conspicuously expressed in the entire region of the brain. The *Ci-opsin3* gene expression was observed in a broad area of the brain vesicle as well as in the visceral ganglion. Genes encoding ascidian homologs of CRALBP and β–CD, whose function is required for the mammalian visual cycle, are co-expressed with Ci-opsin3 in the brain vesicle and visceral ganglion. Localization of Ci-opsin3, CRALBP, and β–CD in a broad area of the brain suggests that the brain of the ascidian larva has a visual cycle system similar to that of the vertebrate RPE. Based on these data, we discuss the evolution of vertebrate visual systems.

Key words : ascidian, ocellus, phototransduction, visual cycle, opsin, G protein, arrestin, cGMP phosphodiesterase

INTRODUCTION

The urochordate ascidian is a marine invertebrate and a close relative of the vertebrate in the animal phylogeny. The adult ascidian is a sessile organism with little resemblance to the vertebrate. In contrast, the ascidian larva is similar to a frog tadpole and the tadpole-like larva shares basic body structures with vertebrates. The ascdian larva has a remarkably simple central nervous system with about 100 neurons [1]. The brain contains an eyespot (ocellus), consisting of three lens cells, one pigment cup cell, and about 20 photoreceptor cells. The very small genome size and easiness of molecular genetic manipulation of embryos of ascidians allow us to study mechanisms that are common between ascidians and vertebrates. Thus the ascidian larva provides us a unique opportunity to study the evolution and basic mechanisms of visual systems of vertebrates.

There are a number of important differences between vertebrate and invertebrate phototransduction [2-4]. They use different types of rhodopsins and G proteins. Vertebrate rhodopsin couples with Gt (transducin), while invertebrate rhodopsin couples with Gq or Go. Morphology and electrophysiological response of photoreceptors are also very different between vertebrates and invertebrates. Vertebrate photoreceptors are ciliary photoreceptors and they hyperpolarize in response to light. On the other hand, most invertebrate photorecetpors are rhabdomeric and depolarize in response to light.

The absorption of light by rhodopsin leads to the *cis*-to-*trans* isomerization of the chromophore to generate all-*trans*-retinal. To regenerate rhodopsin and maintain visual sensitivity, the all-*trans*-retinal must be converted back into the 11-*cis*-retinal. This process is called "the visual cycle" [5,6]. In the mammalian eye, the retinal pigment epithelium (RPE) plays an essential role in the visual cycle. In contrast

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to the phototransduction, detailed mechanisms and regulation of the visual cycle in vertebrates remain to be elucidated.

In order to study the visual system in the ascidian, we have isolated and characterized cDNA clones encoding components of the phototransduction and visual cycle systems from the ascidian *Ciona intestinalis*. In this paper, we performed comparative analysis of strucutre and gene expression patterns of these molecules.

MATERIALS AND METHODS

Animals and embryos. Mature adults of *C. intestinalis* were collected from harbors in Murotsu and Aioi, Hyogo, Japan. The adults were maintained in indoor tanks of artificial seawater (Marine Art BR, Senju Seiyaku, Osaka, Japan) at 18°C. The embryos were prepared using gametes obtained from the gonoducts, as described previously [7].

Molecular phylogenetic analysis. The amino acid sequences were aligned and neighbor-joining trees were constructed using the Clustal W program.

In situ hybridization. Whole-mount *in situ* hybridization was carried out as described previously [8].

RESULTS AND DISCUSSION

Photoreceptors in ascidians

The action spectrum of photic behavior of ascidian larvae was similar to the absorption spectrum of human rhodopsin [7]. Photorecetor cells of the ascidian ocellus show morphological and electrophysiological properties that are similar to those of the vertebrate photoreceptor cells [9]. The photoreceptor cells hyperpolarize in response to light. Each photoreceptor cell extends a process through the pigment-cup cell towards the lens. The outer segment of the photoreceptor process is a modified cilium, consisting of many lamellae. The lamellae are thought to be homologous to photoreceptor disks of vertebrates.

Several photoreceptor organs have been proposed in the adult ascidian. The cerebral ganglion shows photoresponse [10]. In addition, there is a report of photoreceptor-like cells in the oral and atrial siphons of the adult [11].

Visual pigments

In vertebrate photoreceptors, the chromophore is released from opsin after photoactivation and it is regenerated in neighboring cells. On the other hand, the chromophore is retained and can be used repeatedly in invertebrate rhodopsins. These two types of rhodopsins can be clearly distinguished by the primary structure of opsins [2].

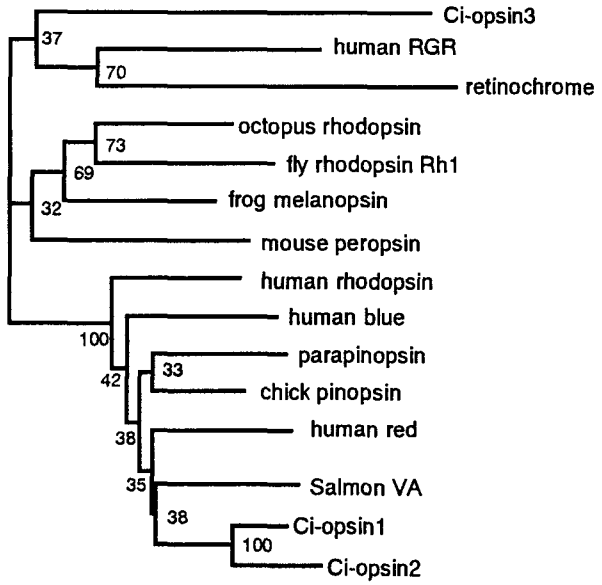

Figure 1. Phylogenetic tree of animal opsins. The phylogenetic tree was inferred from amino acid sequences by the neighbor-joining method. Numbers at nodes are bootstrap values based on 1000 replicates.

We have identified two genes, *Ci-opsin1* [8] and *Ci-opsin2* (T. Kusakabe et al., unpublished), encoding opsins from *C. intestinalis*. Figure 1 shows a molecular phylogenetic tree of animal opsins. The phylogenetic tree clearly shows that Ci-opsin1 is more closely-related to the vertebrate visual and pineal opsins than to the mollusk and arthropod opsins. The clustering of Ci-opsin1 and vertebrate opsins is supported by 100% of the bootstrap value.

Like most vertebrate opsins, Ci-opsin1 has a glutamate counter ion in the third transmembrane helix. The intron positions are also conserved between Ci-opsin1 and vertebrate visual pigments. Thus, both the amino acid sequence and the gene structure show a close relationship between Ci-opsin1 and vertebrate visual pigments.

The expression of *Ci-opsin1* in *C. intestinalis* larvae was detected by whole-mount *in situ* hybridization. The expression is observed specifically in photoreceptor cells of the ocellus (Fig. 2A). Thus the photoreceptor cells of the ascidian ocellus express a vertebrate-type opsins. Immunostaining of acidian larvae with a Ci-opsin1 antibody demonstrated that Ci-opsin1 localizes in outer segments of photoreceptor cells and no staining was observed in the cell bodies (T. Horie et al., unpublished). These results strongly suggest that Ci-opsin1 is responsible for light detection in the ascidian photoreceptor cells.

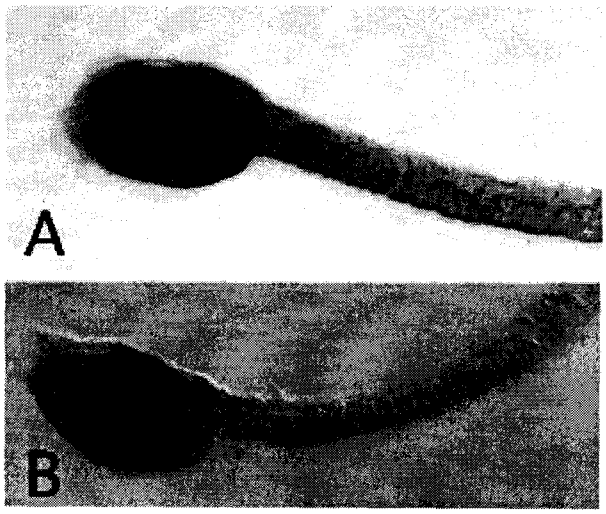

Figure.2. Spatial expression patterns of *Ci-opsin1* (A) and *Ci-opsin3* (B) visualized by whole-mount *in situ* hybridization using digoxigenin-labeled antisense probe.

The *Ci-opsin2* gene also encodes a vertebrate-type opsin (Fig. 1). Ci-opsin2 is most closely related to Ci-opsin1. Ci-opsin1 and Ci-opsin2 probably arose by gene duplication after the divergence of ascidians from the vertebrate lineage. In the genome database of *C. intestinalis*, we could not find any other vertebrate-type opsins. Therefore, the major opsin subtypes of vertebrates have probably diverged during vertebrate evolution after separation from the ascidian lineage.

The transcripts of *Ci-opsin2* were not found in the photoreceptor cells of the ocellus. *Ci-opsin2* is expressed in more ventral part of the brain as well as in small regions that is very close to the adult siphon rudiments (T. Kusakabe et al., unpublished). Therefore, Ci-opsin2 might be an opsin in the adult siphon photoreceptor cells.

Arrestin

Arrestin is an important protein for termination of active state of rhodopsin in vertebrate photoreceptor cells. Arrestin is also found in invertebrate photoreceptors, including those in *Drosophila* and planaria. Vertebrate arrestins can be divided into two distinct groups: visual arresin, which is expressed in photoreceptor cells, and β-arrestin, which is expressed in many different tissues. In the retina, cones and rods express different arrestin genes.

We have identifed Ci-Arr, arrestin of *C. intestinalis* (M. Nakagawa et al., submitted for publication). An extensive analysis of the genome sequence database and Southern blot analysis suggest that the ascidian has only one arrestin gene. Ci-Arr is more closely related to vertebrate arrestins than to invertebrate arrestins. However, β-arrestin and visual arrestin of vertebrates are closer to each other, suggesting that different isoforms of vertebrate arrestin appeared after the split between the vertebrate and ascidian lineages. Immunostainig using a Ci-Arr antibody revealed that, in the tadpole larva, Ci-Arr is only present in photoreceptor cells of the ocellus (T. Horie et al., unpublished).

Cyclic GMP phosphodiesterase

In the vertebrate retinal photoreceptor cells, transducin activates a specific isoform of phosphodiesterase, PDE6, which in tern rapidly hydrolyzes cGMP, causing the closure of cGMP-gated ion channels in the plasma membrane. So far, we have not found α, β, and γ subunits of PDE6 from ascidians. In stead, we have identifed a gene encoding a δ subunit of PDE6, which is thought to regulate membrane association of PDE6. In addition, we found a homologue of mammalian PDE9, which is a cGMP phosphodiesterase expressed in various tissues, including the brain, spleen, and small intestine. In the ascidian larva, these two genes are not expressed in photoreceptor cells. They are both expressed in the ventral part of the brain (T. Kusakabe et al., unpublished). These cells seem to very close to cells expressing *Ci-opsin2* in the brain vesicle.

G protein α subunits

We have identfied five isoforms (CiGαi1a, CiGαi1b, CiGαi2, CiGαq, and CiGαx) of G protein alpha subunits in *Ciona intestinalis* by cDNA cloning (Yoshida et al., submitted for publication). Sequence analysis of cDNA and genomic DNA demonstrated that CiGαi1a and CiGαi1b are generated by alternative splicing of one gene *CiGαi1*. An exhaustive search failed to identify transducin-type G protein from ascidians.

Spatial expression patterns of five Gα isoforms were investigated by whole-mount *in situ* hybridization. None of these isoforms are photoreceptor-specific. The *CiGαi1* gene is specifically expressed in the brain vesicle and in the visceral ganglion. Therefore, CiGαi1 may couple with Ci-opsin1 or Ci-opsin2.

The visual cycle

Various proteins are involved in the visual cycle [5,6]. For example, RGR isomerizes all-*trans*-retinal to 11-*cis*-retinal in a light-dependent manner. Cellular retinaldehyde binding protein (CRALBP) selectively binds to 11-*cis*-retinoids. An RPE-specific protein RPE65 is also necessary for isomerization. RPE65 is related to β-carotene dioxygenase (β-CD) that generates two molecules of all-*trans*-retinal by central cleavage of β-carotene. All these proteins are localized in RPE in mammals. We have identified cDNAs encoding these proteins from *C. intestinalis*. (T. Kusakabe et al., unpublished).

Ci-opsin3 is a divergent member of the rhodopsin family in *C. intestinalis*. Although it is divergent, Ci-opsin3 is most similar to retinal photoisomerases, squid retinochrome and mammalian RGR. Experiments using recombinant Ci-opsin3 demonstrated that Ci-opsin3 exhibits characteristics of photoreaction similar to those of retinal phoisomerases, retinochrome and RGR (A. Terakita and Y. Shichida, unpublished). Whole-mount *in situ* hybridizations revealed that Ci-opsin3 is expressed in a broad area of the brain vesicle as well as in the visceral ganglion, which contains motor neurons (Fig. 2B).

Genes encoding ascidian homologues of β-CD and CRALBP show basically the same expression pattern as that of *Ci-opsin3* (T. Kusakabe et al., unpublished). They are expressed in the brain and the visceral ganglion. Distribution of retinal proteins visualized by the retinal imaging method is also consistent with the expression pattern of *Ci-opsin3* (M. Tsuda et al., unpublished). Therefore, these cells in the brain and the visceral ganglion of the ascidian larva may play physiological roles similar to those of RPE in the vertebrate retina. It is very interesting to know the role of visual cycle-like system in the visceral ganglion, which locates far from the ocellus photoreceptors.

CONCLUSIONS

We have found a number of molecules involved in the visual systems of ascidians and investigated their expression patterns. To further understand mechanisms of the phototransduction and visual cycle, however, we need to study function of these molecules. We have just started such direction of studies. Arrestin was depleted in *Ciona intestinalis* larvae developed from eggs injected with antisense morpholino oligos for arrrestin (K. Inada et al., unpublished). Antisense morpholino oligos effectively knock down gene function in ascidian larvae. We expect that this kind of studies will elucidate the mechanisms of phototransduction in ascidians.

Photoreceptor cells of ascidian larvae exhibit morphological, physiological, and molecular characteristics similar to those of the vertebrate photoreceptors. Photoreceptor cells of the ocellus of the ascidian larva have a vertebrate-type opsin, Ci-opsin1. In the ascidian genome, we could only find one arrestin gene, and two vertebrate-type opsin genes. The two opsin genes seem to originate by gene duplication in the ascidian lineage. Therefore, different subtypes of opsin and arrestin in vertebrates probably appeared after the divergence between ascidians and vertebrates. The brain of the ascidian larva has a visual cycle system similar to that of the vertebrate RPE. In addition to these similarities, there are substantial differences between ascidians and vertebrates. The differences may be also important to understand the evolution and diversity of visual systems.

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