Review

Evolution and the origin of the visual retinoid cycle in vertebrates

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Absorption of a photon by visual pigments induces isomerization of 11-*cis*-retinaldehyde (RAL) chromophore to all-*trans*-RAL. Since the opsins lacking 11-*cis*-RAL lose light sensitivity, sustained vision requires continuous regeneration of 11-*cis*-RAL via the process called ‘visual cycle’. Protozoans and vertebrates use essentially different machinery of visual pigment regeneration, and the origin and early evolution of the vertebrate visual cycle is an unsolved mystery. Here we compare visual retinoid cycles between different photoreceptors of vertebrates, including rods, cones and non-visual photoreceptors, as well as between vertebrates and invertebrates. The visual cycle systems in ascidians, the closest living relatives of vertebrates, show an intermediate state between vertebrates and non-chordate invertebrates. The ascidian larva may use retinochrome-like opsin as the major isomerase. The entire process of the visual cycle can occur inside the photoreceptor cells with distinct subcellular compartmentalization, although the visual cycle components are also present in surrounding non-photoreceptor cells. The adult ascidian probably uses RPE65 isomerase, and *trans*-to-*cis* isomerization may occur in distinct cellular compartments, which is similar to the vertebrate situation. The complete transition to the sophisticated retinoid cycle of vertebrates may have required acquisition of new genes, such as interphotoreceptor retinoid-binding protein, and functional evolution of the visual cycle genes.

Keywords: visual cycle; opsin; chromophore regeneration; RPE65; photoisomerase; ascidian

1. INTRODUCTION

In the eyes of animals, including both vertebrates and invertebrates, the visual pigments responsible for sensing light in the photoreceptor cells consist of an 11-*cis*-retinaldehyde (11-*cis*-RAL) chromophore and a G-protein-coupled receptor, opsin. Absorption of a photon by the visual pigments induces isomerization of 11-*cis*-RAL to all-*trans*-RAL. This photoisomerization is the initial and only light-dependent reaction in the phototransduction cascade that converts light signal into electrical signal in the photoreceptors. Since the opsins lacking 11-*cis*-RAL chromophore lose light sensitivity, sustained vision requires continuous regeneration of 11-*cis*-RAL via the process called ‘visual cycle’ (Wald 1935). In this process, all-*trans* retinoids are isomerized to 11-*cis* retinoids and visual pigments are reconstituted.

In photoreceptor cells of the vertebrate retina, all-*trans*-RAL produced by the photoisomerization dissociates from opsin and enters into an enzymatic reaction pathway that regenerates 11-*cis*-RAL chromophore (Saari 2000; Rando 2001; Lamb & Pugh 2004; Travis *et al.* 2007). The retinal pigment epithelium (RPE) plays an essential role in the visual cycle. The isomerized chromophore (all-*trans*-RAL) released from opsin is reduced to all-*trans*-retinol (all-*trans*-ROL) and sent to adjacent RPE, where the *trans*-to-*cis* reisomerization reaction occurs. The regenerated chromophore re-enters the photoreceptor cells.

Protein components and their roles in a visual cycle pathway were first established in cephalopods by pioneer works by Hara and Hara. They found two RAL-bearing proteins, retinochrome and RAL-binding protein (RALBP) in addition to rhodopsin (Hara & Hara 1968, 1973, 1991). Retinochrome binds and allows light to photoisomerize all-*trans*-RAL to 11-*cis*-RAL. RALBP serves to send the 11-*cis*-RAL into the rhodopsin system and to carry away all-*trans*-RAL. Consequently, rhodopsin and retinochrome can interchange their chromophores through the mediation of

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RALBP to reform their original pigments, rhodopsin and retinochrome, in photoreceptor cells (Terakita *et al.* 1989). However, another chromophore regeneration system has also been evolved. In invertebrates, photoisomerized all-*trans*-RAL chromophore remains bound through its Schiff base to rhodopsin, forming a thermally stable metarhodopsin. Reisomerization of chromophore occurs through the absorption of a second photon by metarhodopsin, resulting in the regeneration of the original rhodopsin. This mode of chromophore regeneration can occur in rhodopsin in insects (Hardie 1986) and octopus (Tsuda 1987; Ashida *et al.* 2004). Thus, unlike the visual cycle in vertebrate eyes, the entire process of the visual cycle, including *trans*-to-*cis* reisomerization of chromophore, could occur in the photoreceptor cell of protostome eyes.

Protostomes and vertebrates use essentially different machinery of visual pigment regeneration, and the origin and early evolution of the vertebrate visual cycle is an unsolved mystery. Comparison of visual cycle systems between different vertebrate systems as well as between vertebrates and their close relatives would give us insights into the origin and evolution of the vertebrate visual cycle. Tunicates, including ascidians, are the closest living relatives of vertebrates (Delsuc *et al.* 2006; Putnam *et al.* 2008). Although the adult ascidian is a sessile organism with little resemblance to the vertebrate, the ascidian larva is similar to a frog tadpole and shares basic body structures with vertebrates. The photoreceptive system of the ascidian larva has recently been extensively investigated (Kusakabe & Tsuda 2007; Takimoto *et al.* 2007). In this article, we review our current understanding of the diversity of visual cycle systems in vertebrates and ascidians. We also present some original data on the visual cycle in the ascidians as well as phylogenetic analysis of protein families involved in the retinoid cycle with new sequence data obtained from the cephalochordate amphioxus genome. Taken together with the information obtained from these analyses, we discuss evolution and the origin of the retinoid cycle in vertebrates.

2. DIVERSITY OF THE VISUAL RETINOID CYCLE IN VERTEBRATES

(a) Rod photoreceptor cells

The vertebrate retinas contain two types of photoreceptors: the rods and cones. Rods are highly sensitive to light and mediate black-and-white vision in dim light, whereas cones are less sensitive to light and provide high-resolution colour vision during daylight. Ever since the early discoveries by Wald (Wald 1935), the biochemistry of the rod visual cycle has been well explored.

The entire reaction of the rod visual cycle takes place in the photoreceptors and RPE (Zimmerman 1974). The first enzymatic reaction of the visual cycle is the reduction of all-*trans*-RAL to all-*trans*-ROL by an NADPH-dependent retinol dehydrogenase (RDH) (Rattner *et al.* 2000). This photoreceptor-specific RDH (prRDH) is localized to the outer segments of rods and cones via its C-terminal 16 amino acids sequence (Luo *et al.* 2004). Disruption

of prRDH in mouse did not affect the rate of 11-*cis*-RAL regeneration after short light exposure (Maeda *et al.* 2005). However, it caused significant accumulation of all-*trans*-RAL following exposure to bright lights and delayed recovery of rod function (Maeda *et al.* 2005).

All-*trans*-ROL is then released from photoreceptors to interphotoreceptor space, where it binds the interphotoreceptor retinoid-binding protein (IRBP), an abundant 140 kDa glycoprotein secreted by photoreceptors (Liou *et al.* 1982; Redmond *et al.* 1985). The endogenous retinoid ligands of IRBP include all-*trans*-ROL and 11-*cis*-RAL (Chen & Noy 1994). The binding of retinoids by IRBP protects them from oxidation and isomerization (Crouch *et al.* 1992). *In vitro* studies showed that IRBP promotes the release of all-*trans*-ROL from photoreceptors following the photobleach of the visual pigments (Qtaishat *et al.* 2005) and release of 11-*cis*-RAL from RPE cells (Carlson & Bok 1992), suggesting that IRBP functions as a transporter protein for all-*trans*-ROL and 11-*cis*-RAL during translocation between photoreceptors and RPE cells. However, the *irbp*^{-/-} mice exhibited a very mild visual cycle phenotype (Palczewski *et al.* 1999; Ripps *et al.* 2000).

In the RPE, lecithin:retinol acyltransferase (LRAT) is the dominant ester synthase responsible for esterification of all-*trans*-ROL (Ruiz *et al.* 1999). Ocular tissues of *lrat*^{-/-} mice contain a trace amount of all-*trans* retinyl esters (all-*trans*-REs) and 11-*cis*-RAL (Batten *et al.* 2004). A second ester synthase called acyl CoA:retinol acyltransferase may complement LRAT to provide additional RE synthase activity under high concentration of all-*trans*-ROL (Kaschula *et al.* 2006). This condition occurs in the RPE, following exposure to bright light.

All-*trans*-RE was shown to be the substrate for retinoid isomerase (or isomerohydrolase) in the RPE (Deigner *et al.* 1989). Phenotypes of *rpe65*^{-/-} mice suggested that RPE65 is involved in the all-*trans* to 11-*cis* isomerization. Photoreceptors in the *rpe65*^{-/-} retina contain only apo-opsin, but not 11-*cis*-RAL (Redmond *et al.* 1998). In contrast, all-*trans*-RE was accumulated in the RPE cells. These observations suggested that RPE65 may be the isomerase. Against this hypothesis are the reports that partially purified RPE65 possessed no isomerase activity (Znoiko *et al.* 2002). Moreover, depletion of RPE65 from RPE membranes has no effect on the isomerase activity in the membranes (Choo *et al.* 1998). Instead, it was suggested that RPE65 binds insoluble all-*trans*-REs and presents them to the isomerase (Mata *et al.* 2004). Recently, three groups (Jin *et al.* 2005; Moiseyev *et al.* 2005; Redmond *et al.* 2005) demonstrated that RPE65 itself is the retinoid isomerase in the visual cycle.

Oxidation of 11-*cis*-ROL to 11-*cis*-RAL in the RPE is catalysed by RDH5 (Driessen *et al.* 1995). RDH5 and prRDH (RDH8) are members of the RDH family, which belongs to the short-chain dehydrogenase/reductase superfamily. Disruption of the *rdh5* gene in mouse leads to the accumulation of *cis*-ROL and *cis*-REs (Driessen *et al.* 2000). However, the *rdh5*^{-/-} and the *rdh5-rdh11* double knockout mice

are still able to produce 11-*cis*-RAL efficiently, suggesting that the oxidation of 11-*cis*-ROL to 11-*cis*-RAL may involve additional, yet unidentified, RDH(s) (Kim *et al.* 2005).

RPE cells express two retinoid-binding proteins involved in the visual cycle. One is the cellular retinol-binding protein that binds all-*trans*-ROL, and the other is cellular retinaldehyde-binding protein (CRALBP) that binds 11-*cis*-ROL and 11-*cis*-RAL (Bunt-Milam & Saari 1983; Bok *et al.* 1984; Saari *et al.* 2001). Mutations in the human gene encoding CRALBP (*RLBP1*) cause autosomal-recessive retinitis pigmentosa: a condition characterized by progressive photoreceptor degeneration and night blindness (delayed dark adaptation) (Maw *et al.* 1997; Burstedt *et al.* 1999; Morimura *et al.* 1999). In CRALBP knock-out mice, isomerization of all-*trans*- to 11-*cis*-ROL in the visual cycle was substantially impaired and delayed dark adaptation was observed (Saari *et al.* 2001).

β -Carotene 15,15'-monooxygenase (BCO) in RPE supplies all-*trans*-RAL to the visual cycle via central cleavage of β -carotene (Redmond *et al.* 2001; Lampert *et al.* 2003; Chichili *et al.* 2005). Mammalian BCO is expressed in RPE (Hamel *et al.* 1993; Redmond *et al.* 2001; Yan *et al.* 2001). It is generally accepted that central cleavage of β -carotene by BCO gives rise to two molecules of all-*trans*-RAL (Redmond *et al.* 2001). Because animals cannot synthesize vitamin A (retinol) *de novo* from endogenous isoprenoid precursors, BCO is a crucial enzyme in development and metabolism that governs the *de novo* entry of vitamin A from plant-derivative precursors. BCO was formerly known as β -carotene 15,15'-dioxygenase. Recently, it was demonstrated that the reaction mechanism of enzymatic cleavage of the central carbon 15,15'-double bond in β -carotene involves a monooxygenase-type mechanism (Leuenberger *et al.* 2001; Lindqvist & Andersson 2002). In *Drosophila*, mutations in the *BCO* gene are responsible for the *ninaB* photoreceptor degeneration (von Lintig & Vogt 2000).

Retinal G-protein-coupled receptor (RGR) is a non-visual opsin expressed in RPE. RGR bound to all-*trans*-RAL is capable of operating as a photoisomerase that generates 11-*cis*-RAL in the light-dependent manner (Hao & Fong 1996; Chen *et al.* 2001a). This has a similar amino acid sequence to retinochrome that isomerizes all-*trans*-RAL to 11-*cis*-RAL in cephalopods. The photoisomerase activity of RGR was demonstrated only *in vitro* (Maeda *et al.* 2003; Wenzel *et al.* 2005). RGR has been shown to interact with proteins involved in the retinoid cycle (Chen *et al.* 2001b), and it has been suggested that RGR facilitates the mobilization of all-*trans*-REs from storage causing following enhancement of isomerizing activity (Wenzel *et al.* 2005). Thus, it is currently believed that RGR does not act as an isomerohydrolase, rather it behaves as a cofactor in the retinoid cycle that modulates isomerohydrolase activity (Wenzel *et al.* 2005).

(b) Cone photoreceptor cells

Although constituting only 5 per cent of all photoreceptors in the human retina, the cones are by far

Figure 1. Photoreceptive systems in the ascidian *C. intestinalis* larva. (a) Photoreceptor cells visualized by immunofluorescence staining using antibody against photoreceptor-specific protein, Ci-Arr. (b) Immunofluorescence staining of the visual pigment Ci-opsin1 visualizing outer segments of the photoreceptor cells. The *Ciona* brain contains three distinct groups of photoreceptor outer segments: groups I, II and III (Horie *et al.* 2008). (c) Immunofluorescence staining using antibody against the *C. intestinalis* orthologue of RPE65. Specific localization of Ci-RPE65 is not observed in the larva. oc, ocellus; ot, otolith.

the more important photoreceptors because they serve daylight vision. In order to investigate the visual cycle for cone visual pigment regeneration, Mata *et al.* (2002) analysed retinoid metabolism in the eyes of chickens and ground squirrels, in which cones constitute 60 and 96 per cent of total photoreceptors, respectively. Based on the biochemical data, the authors proposed a novel visual cycle for cone visual pigment regeneration. The cone visual cycle takes place in cones and Müller cells and involves four new enzymes: (i) an all-*trans*-ROL isomerase, (ii) an 11-*cis*-RE synthase, (iii) an 11-*cis*-RE hydrolase, and (iv) an 11-*cis*-ROL dehydrogenase (Mata *et al.* 2002).

Using cultured chicken Müller cells, Das *et al.* (1992) observed the formation of all-*trans*-retinyl palmitate (all-*trans*-RP), 11-*cis*-ROL and 11-*cis*-RP from all-*trans*-ROL, which was added into the culture medium after labelling with radioisotope. This observation suggests that Müller cells may express all-*trans*-ROL isomerase and 11-*cis*-RE synthase. Until now, there is no direct *in vivo* evidence that Müller cells provide visual retinoid for cone vision. The all-*trans*-ROL isomerase expressed in Müller cells is distinct from RPE65 isomerase, because the former converts all-*trans*-ROL to 11-*cis*-ROL (Mata *et al.* 2002, 2005; Muniz *et al.* 2007), whereas the latter uses all-*trans*-RP as its substrate. In another study, it was shown that only all-*trans*-RP is the direct precursor of 11-*cis*-ROL in chicken RPE/retina mixture membranes, suggesting that RPE65 may be involved in the synthesis of 11-*cis*-ROL in the chicken retina (Gollapalli & Rando 2003). Consistent with this suggestion, Znoiko *et al.* (2002) found that RPE65 is expressed in cone photoreceptors. Neural retina leucine zipper (NRL)-deficient mice, a mouse model

for cone-dominant retina (Mears *et al.* 2001), contained elevated levels of RPE65 (Wenzel *et al.* 2007). Similar to the phenotype of the *rpe65*^{-/-} mouse, ablation of RPE65 in *nrl*^{-/-} mice led to the absence of 11-*cis*-RAL, but increased all-*trans*-REs, suggesting that RPE65 is indispensable for the production of 11-*cis*-RAL in the cone-dominant mice (Wenzel *et al.* 2007; Feathers *et al.* 2008).

Although the 11-*cis*-RE synthase has not been identified, several early studies also suggested the existence of the enzyme in the cone-dominant retinas. Bridges *et al.* (1987a) found that 11-*cis*-RE was predominantly contained in the chicken retina. In contrast, rod-dominated frog and cow eyes had mainly all-*trans* RP in the RPE.

The 11-*cis* RDH involved in the cone visual cycle was also different from the 11-*cis*-specific RDH5 expressed in the RPE. In the cone-dominant retina, the oxidation of 11-*cis*-ROL to 11-*cis*-RAL is NADP dependent and takes place in cones (Mata *et al.* 2002), whereas in rod-dominant eyes, this oxidation reaction is NAD dependent. In an early study using isolated rods and cones from salamander, it was shown that 11-*cis*-ROL added to the medium could confer light sensitivity to photobleached cones, but not to rods (Jones *et al.* 1989), suggesting that cones contain RDH(s) that catalyses oxidation of 11-*cis*-ROL to 11-*cis*-RAL. Haeseleer *et al.* (2002) showed that RDH12 expressed in the photoreceptors could be involved in the production of 11-*cis*-RAL from 11-*cis*-ROL during regeneration of the cone visual pigments. Very recently, Miyazono *et al.* (2008) reported that the reduction of all-*trans*-RAL and the production of 11-*cis*-RAL are much more efficient in cones than in rods. The reducing activity in the outer segment of cones is 30 times higher than that of rods. The high activity of RDHs is attributed to the high content of RDH8 in cones (Miyazono *et al.* 2008). They further found a novel and effective pathway to convert 11-*cis*-ROL to 11-*cis*-RAL in cones: this oxidative conversion does not require NADP⁺ and instead is coupled with the reduction of all-*trans*-RAL to all-*trans*-ROL. The activity is 50 times more effective than the oxidizing activity of NADP-dependent RDHs. Miyazono *et al.* (2008) proposed that these highly efficient reactions of removal of all-*trans*-RAL by RDH8 and production of 11-*cis*-RAL by the coupling reaction are probably the underlying mechanisms that ensure effective visual pigment regeneration in cones that function under much brighter light conditions than rods.

Recently, Schonthaler *et al.* (2007) analysed the role of RPE65 in zebrafish, a species with a cone-dominated retina. The zebrafish genome contains two RPE65 genes: *RPE65a* and *RPE65b*. Only the *RPE65a* transcript was expressed in RPE cells. *RPE65b* mRNA was detectable in early larval stages, but faded out at later larval stages. Targeted knockdown of RPE65 by antisense morpholinos resulted in morphologically altered rod outer segments and overall reduced 11-*cis*-RAL levels. However, cone vision of RPE65-knockdown larvae remained functional as demonstrated by behavioural tests and by metabolite profiling for retinoids (Schonthaler *et al.* 2007). The zebrafish genome also contains two CRALBP

orthologues: *rlbp1a* and *rlbp1b* (Collery *et al.* 2008; Fleisch *et al.* 2008). CRALBP-a is exclusively expressed in the RPE, whereas CRALBP-b is localized to Müller cells. Knockdown of CRALBP in the RPE or Müller cells with antisense morpholinos results in the diminution of 11-*cis* RAL under both conditions. Light sensitivity in both CRALBP-a- and CRALBP-b-knockdown larvae was reduced, but it was more pronounced in CRALBP-b-knockdown larvae (Collery *et al.* 2008; Fleisch *et al.* 2008). Together, these studies suggest that zebrafish may possess an RPE65-independent pathway for the regeneration of 11-*cis*-RAL for cone vision.

(c) Retinoid cycles in non-visual photoreceptors

Vertebrates have several non-visual photoreceptive systems that use opsins as the photoreceptor molecule (Foster & Hankins 2002; Vigh *et al.* 2002; Mano & Fukada 2007). The most well-known non-visual photoreceptor organ is the pineal complex in non-mammalian vertebrates. In chick, the gene encoding RGR is expressed in the pineal organ (Bailey & Cassone 2004). The expression of *Rgr* mRNA was observed primarily in parafollicular cells broadly interspersed among pinealocytes. The distribution of *Rgr* mRNA is different from that of pinealocyte markers, arylalkylamine-*N*-acetyl transferase and pinopsin, suggesting that *Rgr* is not expressed in the photoreceptive pinealocytes (Bailey & Cassone 2004). The gene encoding another photoisomerase, peropsin, is also expressed in the chick pineal organ with expression patterns similar to those of *Rgr* (Bailey & Cassone 2004). The chick pineal organ expresses various visual cycle genes, including those encoding IRBP, RPE65, RGR, CRALBP and all-*trans*-ROL dehydrogenase (Bailey & Cassone 2004; Bailey *et al.* 2004). In zebrafish, the pineal organ expresses genes encoding orthologues of RPE65 and CRALBP (Schonthaler *et al.* 2007; Collery *et al.* 2008). Thus, the photoreceptive pineal organ in non-mammalian vertebrates may possess a visual cycle system similar to that of rod photoreceptors in the retina. Interestingly, however, IRBP and CRALBP are also present in the mammalian pineal gland, which is thought to be a non-photoreceptive organ (Rodrigues *et al.* 1986; van Veen *et al.* 1986; Bridges *et al.* 1987b). In bovine pineal, CRALBP-positive cells are glia-like and seem not to be pinealocytes (Saari *et al.* 1997).

Another mode of visual cycle in the pineal organ is the photoregeneration of parapinopsin in lamprey (Koyanagi *et al.* 2004). The lamprey parapinopsin is the bistable UV pigment that exhibits an absorption maximum at 370 nm. UV light causes *cis-trans* isomerization of its chromophore, forming a stable photoproduct having an absorption maximum at 515 nm, in the green region. The photoproduct reverts to the original pigment upon visible light absorption, showing photoregeneration of the pigment. The bistable nature of the parapinopsin can account for the photorecovery of the pineal UV sensitivity by background green light in the lamprey (Koyanagi *et al.* 2004).

Opsins and phototransduction cascade proteins have been demonstrated in neurons in the brain of various vertebrates. These photoreceptive systems, mainly comprising telencephalic and hypothalamic groups of cerebrospinal fluid-contacting neurons, are called 'deep brain photoreceptors' (Vigh *et al.* 2002). Although physiological roles and photoreceptive abilities of the encephalic photoreceptors have not been elucidated, visual cycle proteins have been reported in the brain of various vertebrates. For example, in the mammalian brain, CRALBP is expressed in oligodendrocytes (Saari *et al.* 1997). In chick, expression of *Rgr* and *peropsin* was observed throughout the brain (Bailey & Cassone 2004). In zebrafish, one of the two RPE65 orthologue genes, *RPE65b*, is expressed in the ventricular zone of the brain of early larvae (Schonthaler *et al.* 2007). At present, however, physiological and/or developmental relevance of visual cycle proteins in the vertebrate brain is totally unknown.

In the past decade, non-visual opsins have been identified and characterized in the inner retina (Provencio *et al.* 1998; Soni *et al.* 1998; Kojima *et al.* 2000). In zebrafish, one of the two duplicated IRBP genes is expressed in the inner nuclear layer and in the ganglion cell layer (Nickerson *et al.* 2006). Most fully characterized among inner retinal opsins is melanopsin. Vertebrate melanopsins are structurally similar to the cephalopod opsins (Provencio *et al.* 1998; Koyanagi *et al.* 2005); mouse melanopsin has been reported from knockout studies to be involved in the response of the pupil to light (Lucas *et al.* 2003) and in the entrainment of circadian rhythm by light (Hattar *et al.* 2003). Like invertebrate rhabdomeric visual pigments, melanopsin acts as a bistable photopigment and dark melanopsin can be regenerated by light (Koyanagi *et al.* 2005; Walker *et al.* 2008). However, the analysis of chromophore isoforms in melanopsin suggests that melanopsin must use light-independent mechanism to regenerate the photopigment (Walker *et al.* 2008). Thus the regeneration of melanopsin remains an important unresolved question.

3. PHOTORECEPTORS AND RETINOID CYCLES IN THE ASCIDIAN LARVA

The ascidian tadpole larva has a central nervous system derived from the dorsal neural tube, consisting of approximately 330 cells. The brain of the ascidian larva contains an eyespot (ocellus), which consists of three lens cells, one pigment cup cell and a group of photoreceptor cells (Kusakabe & Tsuda 2007; Horie *et al.* 2008) (figure 1). Larvae of the ascidian *Ciona intestinalis* start to show photo response behaviour at around 4 h after hatching (Nakagawa *et al.* 1999). The larvae start swimming when light intensity decreases and stop swimming when light intensity increases (Tsuda *et al.* 2003a).

Vertebrate retinal photoreceptors are ciliary photoreceptors and they hyperpolarize in response to light, whereas most photoreceptors of invertebrate eyes are rhabdomeric and depolarize in response to light. Ciliary photoreceptors and rhabdomeric photoreceptors use different types of opsins, which are clearly distinguished by their primary structures. The

ciliary photoreceptor cells of the ocellus of the *C. intestinalis* larva use a vertebrate-type ciliary opsin, Ci-opsin1, as the visual pigment (Kusakabe *et al.* 2001; Inada *et al.* 2003; Horie *et al.* 2008). Four genes encoding putative visual cycle proteins, orthologues of RGR (Ci-opsin3), CRALBP (Ci-CRALBP), RPE65 (Ci-RPE65) and BCO (Ci-BCO), have been identified in *C. intestinalis* (Nakashima *et al.* 2003; Tsuda *et al.* 2003b; Takimoto *et al.* 2006). Ci-opsin3 and Ci-CRALBP are localized in both ocellus photoreceptor cells and surrounding non-photoreceptor cells in the brain vesicle of the larva (Tsuda *et al.* 2003b). In contrast to Ci-BCO, which is predominantly localized in the ocellus photoreceptor cells of the larva, the *Ci-RPE65* gene is not significantly expressed in the ocellus and brain vesicle of the larva, as shown by *in situ* hybridization and expressed sequence tag (EST) profiles (Takimoto *et al.* 2006). We further examined localization of Ci-RPE65 in the larva by immunohistochemical analysis using anti-Ci-RPE65 antibody (see electronic supplementary material for experimental details). The result consistently suggests that Ci-RPE65 is not significantly present in the ocellus and brain vesicle of the larva (figure 1c). Ci-opsin3 binds all-*trans*-RAL, and irradiation with an orange light (greater than 560 nm) caused photoisomerization from the all-*trans*-RAL to the 11-*cis* form (Nakashima *et al.* 2003). Thus, Ci-opsin3, but not Ci-RPE65, most probably functions to supply 11-*cis*-RAL in the visual cycle of the ascidian larva. Co-localization of Ci-opsin3 and Ci-CRALBP suggests that *trans*-to-*cis* isomerization occurs in both photoreceptor cells and surrounding non-photoreceptor cells in the brain vesicle of the ascidian larva. It should be noted, however, that Ci-CRALBP, Ci-RPE65 and Ci-BCO were named based on their structural similarity to the vertebrate proteins, and their functional properties remain to be established.

To investigate compartmentalization of visual cycle within the photoreceptor cells, we examined subcellular localization of Ci-opsin3, Ci-CRALBP and Ci-BCO in the ocellus photoreceptor cells by immunofluorescence imaging using confocal microscopy (figure 2; see electronic supplementary material for experimental details). Ci-opsin3 is abundant in the basal region of the photoreceptor cell bodies, its amount is reduced towards the outer segments and the outer segments contained negligible amount of Ci-opsin3 (figure 2a–d). Ci-CRALBP shows a similar subcellular localization pattern, but it is more restricted to the basal peripheral region of the photoreceptor cell bodies (figure 2e–h). In contrast, Ci-BCO is abundant throughout the entire photoreceptor cells, including cell bodies and outer segments (figure 2i–l). The subcellular localization of Ci-opsin3 and Ci-CRALBP suggests compartmentalization of the visual cycle within the photoreceptor cells: *trans*-to-*cis* isomerization reaction may take place only in the basal region of photoreceptor cells.

4. PHOTORECEPTORS AND RETINOID CYCLES IN THE ASCIDIAN ADULT

At least three types of light-responsive behaviours have been described in the adult ascidians: siphon

Figure 2. Localization of visual cycle proteins in the brain vesicle of *C. intestinalis* larvae. Localization of Ci-Arr (green) and visual cycle proteins (magenta) are visualized by double immunofluorescence staining. White colour indicates co-localization of Ci-Arr and a visual cycle protein in photoreceptor cells. Visual cycle proteins were visualized with antibodies against Ci-opsin3 (*a–d*), Ci-CRALBP (*e–h*) and Ci-BCO (*i–l*). Photographs shown in (*a*), (*e*) and (*i*) are confocal composite images generated from a series of optical sections. Photographs shown in (*b–d*), (*f–h*) and (*j–l*) are optical sections taken at different depths: (*b*), (*f*) and (*j*) position near the basal surface of the photoreceptor cell bodies; (*c*), (*g*) and (*k*) medial position of the cell bodies with respect to the apico-basal polarity of photoreceptor cells; (*d*), (*h*) and (*l*) inner (apical) position of the photoreceptor cells where outer segments are observed. OS, outer segments. Scale bar, 20 μ m.

contraction, phototropism and gamete release (Kusakabe & Tsuda 2007). The cerebral ganglion has been suggested to be a candidate photoreceptor organ underlying these behaviours (Kajiwarra *et al.* 1990; Ohkuma & Tsuda 2000; Ohkuma *et al.* 2000; Tsutsui & Oka 2000). In a previous study, expression of visual cycle protein genes was examined in adult tissues of *C. intestinalis* by RT-PCR (Takimoto *et al.* 2006). *Ci-RPE65* is expressed in the neural complex, a photoreceptor organ of the adult ascidian, at a level comparable to that of *Ci-opsin3* and *Ci-CRALBP*. These results suggest that the visual cycle of the adult photoreceptors is RPE65 dependent. *Ci-RPE65* is also expressed in various adult tissues, including the gill, body wall and intestine, suggesting that Ci-RPE65 plays an unrecognized role in addition to that in the visual cycle (Takimoto *et al.* 2006).

The neural complex is roughly explained to consist of three components: neural gland, ciliated funnel and cerebral ganglion. The cerebral ganglion and neural gland do not have direct connection. Ciliated duct connects neural gland and ciliated funnel, which opens into the pharynx, constituting the neural gland complex (Deyts *et al.* 2006). To elucidate localization of the visual cycle system in the neural complex, spatial expression patterns of *Ci-opsin3*, *Ci-CRALBP*, *Ci-BCO* and *Ci-RPE65* were analysed by *in situ* hybridization (figure 3; see electronic supplementary material for experimental details). All of these genes are expressed in the neural complex. *Ci-opsin3* expression was detected specifically in the ciliated duct (figure 3*a–c*). Similarly, Ci-CRALBP is specifically expressed in the ciliated duct (figure 3*d–f*). *Ci-BCO* was expressed widely in the neural gland and

duct-like structure circumscribing the ciliated funnel (figure 3*g–i*), and *Ci-RPE65* was broadly expressed in the neural gland, including the ciliated duct (figure 3*j–l*). Thus, in contrast to the larval brain, where *Ci-RPE65* is not expressed, the adult neural complex expresses *Ci-RPE65* in a specific manner. These results suggest that *Ci-RPE65* may be involved in the visual cycle of the adult ascidians. The results also suggest that the *trans*-to-*cis* isomerization reaction occurs in the neural complex and ciliated duct in adult ascidians. Since the existence of photoreceptor cells has been suggested in the cerebral ganglion (Kajiwarra *et al.* 1990; Ohkuma & Tsuda 2000; Ohkuma *et al.* 2000; Tsutsui & Oka 2000), the visual cycle of the adult ascidian may occur in two distinct cellular compartments: the 11-*cis* to all-*trans* isomerization of the visual pigment chromophore in the cerebral ganglion and *trans*-to-*cis* regeneration in the neural gland/ciliated duct. This presumed compartmentalization of the adult ascidian, together with the possible involvement of RPE65, is reminiscent of the visual cycle in the vertebrate retina. Thus, the visual cycle in the adult ascidian might be more similar to the visual cycle in the vertebrate retina than the larval visual cycle.

5. MOLECULAR AND FUNCTIONAL EVOLUTION OF RETINOID CYCLE PROTEINS

(a) Molecular phylogeny of retinoid cycle protein families

(i) RPE65/BCO/BCO2 family

RPE65 and BCO are similar in amino acid sequences, evolved from a common ancestor (Wyss 2004). Both

Figure 3. Spatial localization of mRNAs for visual cycle proteins in the neural complex of the adult *C. intestinalis*. Spatial localizations of transcripts of Ci-opsin3 (*a–c*), Ci-CRALBP (*d–f*), Ci-BCO (*g–i*) and Ci-RPE65 (*j–l*) were visualized by whole-mount *in situ* hybridization (ISH). In (*a*), (*d*), (*g*) and (*j*), anterior is up and dorsal is to the left (right lateral view). In (*b*), (*e*), (*h*) and (*k*), anterior is up (dorsal view). After ISH, the neural complexes were embedded in paraffin and sectioned for further observation (*c*), (*f*), (*i*) and (*l*). In these sagittal sections, anterior is up and dorsal is to the left. Arrowheads indicate specific expression of Ci-opsin3 and Ci-CRALBP in the ciliated duct. cg, cerebral ganglion; ng, neural gland. Scale bar, 200 μ m.

mammalian RPE65 and BCO are expressed in RPE (Hamel *et al.* 1993; Redmond *et al.* 2001; Yan *et al.* 2001). RPE65 and BCO constitute a protein family with β -carotene 9',15'-dioxygenase (BCO2), another enzyme involved in carotenoid metabolism (Kiefer

et al. 2001; Wyss 2004). The molecular phylogenetic analysis performed using amino acid sequences of vertebrate and ascidian RPE65/BCO/BCO2 family proteins suggested that the ascidian *C. intestinalis* has distinct orthologues of RPE65 (Ci-RPE65) and BCO

(Ci-BCO) (Takimoto *et al.* 2006). Thus, the functional divergence of RPE65 and BCO seemed to have occurred before the emergence of the last common ancestor of chordates. The analysis also suggested that there is no BCO2 orthologue in the *Ciona* genome (Takimoto *et al.* 2006). Therefore, BCO2 might have arisen by gene duplication of either ancestral BCO or RPE65 after the divergence from the common ancestor of ascidians and vertebrates. Alternatively, ascidians might have lost BCO2 during evolution.

Since the cephalochordate amphioxus occupies a key position to understand early chordate evolution (Putnam *et al.* 2008), we analysed RPE65/BCO/BCO2 family proteins in the amphioxus genome. The amphioxus genome contains four RPE65/BCO/BCO2 family genes. All the four genes show strong similarity (*E*-values less than 10^{-30}) to human RPE65, BCO and BCO2, whereas they show only moderate similarity (*E*-values greater than 10^{-20}) to *Drosophila* BCO, the only member of this family found in the *Drosophila* genome, suggesting that all RPE65/BCO/BCO2 family proteins in chordates diverged from a single ancestral gene after the protostome/deuterostome divergence. To further resolve evolutionary history of the RPE65/BCO/BCO2 family in chordates, molecular phylogenetic analysis (figure 4a) was conducted with newly identified amphioxus sequences. The phylogenetic tree clearly indicates that the *Ciona* orthologues of RPE65 and BCO are closer to the vertebrate cognates than the amphioxus proteins, respectively (figure 4a). This is consistent with the recent consensus of the phylogenetic relationships among Vertebrata, Tunicata and Cephalochordata (Delsuc *et al.* 2006; Putnam *et al.* 2008). The four amphioxus proteins are classified into two groups—RPE65-like and BCO/BCO2-like—each consisting of two members (RPE65-like1/RPE65-like2 and BCO-like1/BCO-like2, respectively) that probably arose by gene duplication in the amphioxus lineage.

(ii) CRALBP family

A previous phylogenetic analysis of the CRALBP family revealed that the *Ciona* CRALBP (Ci-CRALBP) is more similar to the vertebrate cognates than that of *Drosophila* (Nakashima *et al.* 2003). As we did for the RPE65/BCO/BCO2 family, we analysed the CRALBP family in the amphioxus genome. The amphioxus genome contains two CRALBP paralogues, CRALBP1 and CRALBP2, derived from a lineage-specific gene duplication (figure 4b). Molecular phylogenetic analysis revealed that Ci-CRALBP is closer to mammalian CRALBPs than to amphioxus CRALBPs. Thus, all of the three visual cycle proteins, RPE65, CRALBP and BCO, show the closer relationships between ascidian and vertebrates, suggesting that a basic system of the vertebrate visual cycle had already established in the last common ancestor of vertebrates and tunicates.

(iii) Photoisomerase opsins

In the opsin family, RGR, retinochrome and Ci-opsin3 constitute a photoisomerase opsin subfamily, although

they show only moderate amino acid sequence similarity to each other (Nakashima *et al.* 2003). Peropsins have been identified in various vertebrates and amphioxus and form another subfamily of photoisomerase opsin that can photoisomerize between 11-*cis*-RAL and all-*trans*-RAL (Sun *et al.* 1997; Koyanagi *et al.* 2002). The widespread distribution of photoisomerase opsins among different animal groups suggest an ancient origin of the light-dependent *trans*-to-*cis* isomerization system in metazoans.

(iv) IRBP family

IRBP has been reported only in vertebrates. It is not found in the *Drosophila* genome (Gonzalez-Fernandez 2002). We have not been able to identify IRBP homologues in the amphioxus and *Ciona* genomes. IRBP consists of four consecutive modules, each approximately 300 amino acid residues in size (Gonzalez-Fernandez 2002). The gene appears to have arisen from the quadruplication of an ancestral gene present in an early common ancestor of vertebrates (Nickerson *et al.* 2006). In teleosts, the IRBP gene has been further duplicated into two genes, each of which shows distinct structural features and expression patterns (Nickerson *et al.* 2006). The three-dimensional structure of an individual module of IRBP suggested a probable homology between IRBP and two diverse protein families, the C-terminal transferase (CPTase) and the crotonase families (Gonzalez-Fernandez 2002). The early vertebrate ancestor might have recruited a CPTase/crotonase family protein for a new purpose, retinoid transport between photoreceptor cells and their neighbour cells, having facilitated the establishment of the vertebrate-type visual cycle.

(b) Functional evolution of the RPE65/BCO1/BCO2 family

RPE65 was first described as an abundant protein of the RPE (Bavik *et al.* 1992; Hamel *et al.* 1993). In *Sf9* cells and bovine RPE, RPE65 is expressed in two forms (Ma *et al.* 2001): one is a cytosolic form (sRPE65), and the other is a membrane-associated form (mRPE65). The sRPE65 has a molecular mass of 61 161, which is close to the calculated value (60 944), whereas the molecular mass of mRPE65 was 61 961, suggesting that RPE65 contained post-translational modification. Jahng *et al.* (2003) showed that RPE65 binds all-*trans*-RP. Later, it was shown that RPE65 is S-palmitoylated by LRAT on three cysteine (Cys) residues (Cys231, Cys329 and Cys330) and that this palmitoylation is required for isomerase activity and the association of RPE65 with membranes (Xue *et al.* 2004). The authors proposed a novel 'palmitoylation switch model': the palmitoylation of RPE65 serves to switch off the visual cycle in darkness and to switch it on in the daylight (Xue *et al.* 2004). However, substitution of Cys231, Cys329 and Cys330 with Ala or Ser in all combinations did not significantly change enzyme activity and/or association of RPE65 with membrane (Redmond *et al.* 2005; Takahashi *et al.* 2006; Jin *et al.* 2007).

Figure 4. Molecular phylogenetic trees of the RPE65/BCO/BCO2 family (a) and the CRALBP family (b). Phylogenetic trees were inferred from amino acid sequences using the neighbour-joining method using CLUSTALX. Numbers at nodes are bootstrap values based on 1000 replicates. Scale bar, 0.1 amino acid replacements per site. See electronic supplementary material for amino acid sequences used to reconstruct the phylogenetic trees.

RPE65 belongs to the evolutionarily diverse carotenoid oxygenase family. This family also contains BCO and BCO2, as mentioned earlier. The entire *Drosophila* genome contains only one *bco* gene of the family. In vertebrates, however, three genes encode RPE65, BCO and BCO2 proteins, respectively. Mammalian genomes contain a single copy of the RPE65 gene, whereas the zebrafish genome has *RPE65a* and *RPE65b* genes, a consequence of the genome duplication in the teleost ancestry. RPE65, BCO and BCO2 share approximately 40 per cent overall sequence identity (Kiefer *et al.* 2001). The basic function of the family is to catalyse the oxidative cleavage of carbon–carbon double bonds in the polyene backbone of RE and carotenoid. BCO cleaves β -carotene into all-*trans*-RAL, the first step of vitamin A metabolism in animals.

X-ray diffraction analysis of the crystallized apo-carotenoid oxygenase, a member of the family, suggests that the proteins share a common seven-bladed β -propeller structure containing a non-haeme

ferrous iron coordinated by four histidine residues (Kloer *et al.* 2005). These four histidines are strictly conserved among the members of the family. On binding of iron, the apo-carotenoid oxygenase converted all-*trans* carotenoid to *cis-trans-cis* conformation. This observation suggests that the apo-carotenoid oxygenase of cyanobacterial *Synechocystis* may have potential isomerase activity. However, we observed that Ci-RPE65 and Ci-BCO could not synthesize 11-*cis*-ROL from all-*trans*-RP, whereas bovine RPE65 converted all-*trans*-RP to 11-*cis*-ROL in the same condition (M. Jin, N. Takimoto, T. G. Kusakabe & M. Tusada 2007, unpublished data). The individual family members may differ in their substrate preference and the position of the cleaved double bond. Interestingly, the bovine and mouse RPE65 expressed in a β -carotene accumulating *Escherichia coli* strain did not synthesize apo-carotenal cleavage products, suggesting that vertebrate RPE65 does not have β -carotene cleaving activity (von Lintig & Vogt 2000).

Figure 5. Possible scenarios for the chordate visual cycle evolution. Schematic of cellular and subcellular localizations of visual cycle proteins in vertebrates, tunicates and protostomes. The dendrogram represents proposed evolutionary routes from the ancestral state in a bilaterian ancestor to the extant systems. Dashed branches indicate two possible origins of the visual cycle in the neural complex of the adult ascidian. Events occurred during evolution are indicated by purple arrows. Arrows with a question mark indicate alternative possibilities for the events. OS, outer segments of photoreceptor cells.

6. EVOLUTIONARY PERSPECTIVES

The genome-wide survey and molecular phylogeny of gene families involved in the visual cycle across the chordate subphyla suggested that basic components used in the visual cycle of the vertebrate eye had been already established in the last common ancestor of tunicates and vertebrates. The visual cycle systems in tunicates, namely ascidians, show a kind of an intermediate state between vertebrates and non-chordate animals such as cephalopods. Similar to the cephalopod visual cycle, the ascidian larva uses retinochrome-like opsin (Ci-opsin3), but not RPE65, as the major isomerase; the entire process of the visual cycle can occur inside the photoreceptor cells with distinct subcellular compartmentalization. On the other hand, the ascidian larva also shows a vertebrate-like character: the visual cycle components are also present in non-photoreceptor cells surrounding the ocellus. The visual cycle system of the adult ascidian may be more similar to that of the vertebrates: it probably uses RPE65, and *trans-to-cis* isomerization may occur in distinct cellular compartments. The current data together with previous findings enabled us to deduce possible scenarios for the chordate visual cycle evolution (figure 5). A visual cycle system using photoisomerase opsin was probably present in photoreceptor cells of the last common ancestor of bilaterian animals. The vertebrate/tunicate common ancestor had acquired visual cycle in adjacent non-photoreceptor cells. RPE65 was also recruited to the system as

another isomerase in a chordate ancestor. In the lineage towards vertebrates, photoreceptor cells lost *trans-to-cis* isomerization machinery. The complete transition to the vertebrate-type system may have required the acquisition of some genes with new function, such as IRBP, which is absent in the genomes of ascidians and amphioxus. Thus, recruitment of new genes and functional evolution within the gene families might have facilitated the evolution of the sophisticated retinoid cycle system for the vertebrate photoreceptors.

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