Evolution of the Genetic Machinery of the Visual Cycle: A Novelty of the Vertebrate Eye?

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Abstract

The discovery in invertebrates of ciliary photoreceptor cells and ciliary (c)-opsins established that at least two of the three elements that characterize the vertebrate photoreceptor system were already present before vertebrate evolution. However, the origin of the third element, a series of biochemical reactions known as the “retinoid cycle,” remained uncertain. To understand the evolution of the retinoid cycle, I have searched for the genetic machinery of the cycle in invertebrate genomes, with special emphasis on the cephalochordate amphioxus. Amphioxus is closely related to vertebrates, has a fairly prototypical genome, and possesses ciliary photoreceptor cells and c-opsins. Phylogenetic and structural analyses of the amphioxus sequences related with the vertebrate machinery do not support a function of amphioxus proteins in chromophore regeneration but suggest that the genetic machinery of the retinoid cycle arose in vertebrates due to duplications of ancestral nonvisual genes. These results favor the hypothesis that the retinoid cycle machinery was a functional innovation of the primitive vertebrate eye.

Key words: amphioxus, invertebrate ciliary photoreceptors, retinoid cycle, chromophore regeneration, photoisomerase.

Introduction

Light absorption causes isomerization of chromophore 11-cis-retinaldehyde to all-trans-retinaldehyde, which changes the conformation of the opsins and thereby leads to a signal transduction cascade. Following isomerization, all-trans-retinaldehyde is regenerated to the 11-cis-retinaldehyde to form a new functional visual pigment. In invertebrate rhabdomeric eyes, rhabdomeric (r)-opsins are able to regenerate 11-cis-retinaldehyde by photoisomerization, whereby the photoisomerase activity of the r-opsin itself regenerates the visual pigment. In contrast, vertebrate ciliary photoreceptors possess ciliary (c)-opsins that have lost the ability to photoreverse, and regeneration is accomplished through a complex series of light-independent enzymatic reactions known as the retinoid cycle (summarized in fig. 1). The discovery in invertebrates of ciliary photoreceptors with c-opsins (Arendt et al. 2004; Passamanek et al. 2011) raises the question of whether invertebrates possess a vertebrate-like retinoid cycle in their ciliary photoreceptors or whether they regenerate the chromophore by a different system. To answer this question, analysis of the existence of vertebrate orthologs of the vertebrate machinery for the retinoid cycle becomes an issue of interest.

The machinery for the retinoid cycle consists of several enzymes, including the retinal dehydrogenases Rdh8, Rdh12, and Rdh5, the lecithin retinol acyltransferase Lrat, and the isomerohydrolase Rpe65 as well as two eye-specific retinoid-binding proteins, the retinaldehyde-binding protein 1 (Rbp1) and the interstitial retinol-binding protein 3 (Rbp3) (fig. 1) (reviewed in Lamb and Pugh 2004). Several putative Rdh8 have been identified in the echinoderm Strongylocentrotus purpuratus and in the cnidarian Nematostella vectensis (Mindnich and Adamski 2009; Tarrant et al. 2009), 6 Rdh12-like and 1 ninaB (related to Rpe65) enzymes have been reported in Drosophila (vonLintig and Vogt 2000; Oberhauser et al. 2008; Belyaeva et al. 2009), 12 enzymes evolutionarily related to vertebrate Rdh5 have been found in amphioxus Branchiostoma floridae (Albalat et al. 2011), and some components of the cycle have been reported in ascidian Ciona intestinalis (Tsuda et al. 2003; Takimoto et al. 2006; Kusakabe et al. 2009), but a general picture of the retinoid cycle machinery in invertebrates has not been drawn. Moreover, a functional link of the invertebrate enzymes and the chromophore regeneration machinery had not yet been established, and complex orthologous and paralogous relationships between the invertebrate and the vertebrate components have blurred their evolutionary relationships, weakening any inference about the origin of the retinoid cycle in metazoans.

Under the assumption that an extensive study of the components of the machinery in the proper species might help to clarify the evolutionary origin of the cycle, I have surveyed genome databases of several invertebrates with special emphasis on the cephalochordate amphioxus Branchiostoma Floridae. Amphioxus was the most suitable choice because it is closely related to vertebrates, belonging to the same chordate phylum, and it is thought that, in many aspects, it resembles the vertebrate ancestor, with a fairly prototypical genome (Holland et al. 2008; Putnam et al. 2008). Importantly, amphioxus has both ciliary photoreceptors (Ruiz and Anadón 1991; Lacalli et al. 1994; Lacalli 1996) and c-opsins (Koyanagi et al. 2002; Holland et al. 2008). Phylogenetic and structural analyses of more than
200 invertebrate genes, including more than 90 amphioxus sequences, allow to reconstruct the evolution of the components involved in regeneration of the visual pigment and suggest that the genetic machinery of the retinoid cycle was a functional innovation of the vertebrate eye.

**Materials and Methods**

The presence of invertebrate orthologs of the vertebrate components of the retinoid cycle machinery was investigated by exhaustive searches of invertebrate genomes, with special emphasis on the amphioxus (*B. floridae*) genome, version 1.0 (11.5-fold coverage). Human reference proteins for each component were used as starting queries for TBLASTN searches (Altschul et al. 1997) against genome databases. Automatically predicted genes were retrieved and revised manually to maximize the similarity with vertebrate proteins and adjusted with the Augustus gene prediction program (Stanke et al. 2004). The sequence identification numbers of all the sequences used in this work are indicated in the phylogenetic trees.

Orthology of the invertebrate proteins was inferred initially by reciprocal best-hit BLASTP searches against human protein databases and confirmed by phylogenetic reconstructions. For phylogenetic analyses, sequences identified by database searches were retrieved, and protein sequences alignments were generated with ClustalX (Thompson et al. 1997), including vertebrate (human and zebra fish), *B. floridiae*, and *N. vectensis* representatives. Other invertebrate sequences were optionally incorporated into the alignment depending on the analyzed group of proteins. The next closest related proteins to the analyzed group (i.e., the sister group), and other closely related sequences were also included in the alignments and being used as outgroups in phylogenetic reconstructions. Sequences from the cnidarian *N. vectensis* were used to root the phylogenetic trees when a proper outgroup was not available. Sequences alignments were manually verified before used for maximum likelihood (ML) and neighbor joining (NJ) phylogenetic analyses. The alignment files are available from the author. Robustness of the obtained tree topologies was assessed with 500 (ML) and 1,000 (NJ) bootstrap replicates. ML analyses were performed using the PHYML 2.4.5 program (Guindon and Gascuel 2003) from the MacGDE package, following the JTT model of amino acid substitution (Jones et al. 1992), estimating the amino acid frequencies from the data set and taking into account the among-site rate heterogeneity with four gamma-distributed categories. NJ trees were constructed excluding positions with gaps and visualized with the NJPlot program (Perrière and Gouy 1996).

Reconstructions of ancestral amino acid sequences were performed with the FASTML server (http://fastml.tau.ac.il/index.html), using a joint ML method, following the JTT model of amino acid substitution and a discrete gamma model with eight categories (Pupko et al. 2000, 2002).

**Results and Discussion**

**Retinoid Cycle Machinery in Invertebrates**

**Step 1: Retinaldehyde Reduction and Rdh8 and Rdh12 Enzymes**

The retinoid cycle begins with reduction of all-trans-retinaldehyde to retinol (fig. 1), mostly catalyzed by Rdh8 and Rdh12, two vertebrate enzymes that together account for >98% of all-trans-retinaldehyde reductase activity in the rodent retina (Maeda et al. 2007).

Rdh8 (also known as photoreceptor-associated retinol dehydrogenase or prRdh) accounts for ≈70% of the all-trans-retinaldehyde reductase activity in the rodent eye (Maeda et al. 2007). Rdh8 belongs to the SDR28C family of the short-chain dehydrogenase/reductase (SDR) superfamily (Kallberg et al. 2010), which also includes 17β-hydroxysteroid dehydrogenase type-1 (*Hsd17b1*) enzymes as the sister group of Rdh8. In the amphioxus database, neither Rdh8 nor *Hsd17b1* sequences were identified. The best-hit BLASTP amphioxus sequence retrieved by human RDH8, XP_002596816, recovered human DHRS7 in the reciprocal searches.
The absence of Sdr28c (Rdh8 and Hsd17b1) enzymes in amphioxus was, however, lineage specific because genomic surveys identified several invertebrate Sdr28c enzymes. Specifically, there were 14 sequences, named Sp_Sdr28c_1 to 14, in the echinoderm *S. purpuratus* (Mindnich and Adamski 2009), 5 sequences, Nv_Sdr28c_1 to 5, in the cnidarian *N. vectensis* (Tarrant et al. 2009), and 5 sequences, Ta_Sdr28c_1 to 5, in the placozoan *Trichoplax adhaerens* (this work; supplementary fig. S1, Supplementary Material online). Invertebrate sequences grouped outside the vertebrate Rdh8–Hsd17b1 cluster in the phylogenetic tree, indicating that Rdh8 (and Hsd17b1) arose in vertebrates from the duplication of an Sdr28c ancestor (fig. 2A and supplementary fig. S1, Supplementary Material online). To infer whether or not the Sdr28c ancestor might be active against retinoids, the sequences of the current enzymes were compared. It is known that retinoid or steroid discrimination relies mostly on one amino acid close to active site of the vertebrate enzymes (Haller et al. 2010): a bulky methionine (162.9 Å³) at position 144 for retinoid-active Rdh8 or a small glycine (volume 60.1 Å³) at position 145 for steroid-active Hsd17b (numbers refer to human enzymes). ML reconstruction of the protein sequence of the ancestral Sdr28c predicted small G or A (88.6 Å³) at this position in the vertebrate and metazoan ancestors (0.88 and 0.90 probability of marginal reconstruction, respectively). Moreover, 19 of the 24 invertebrate sequences contained a small- or medium-sized amino acid in the relative G145/M144 position. Small-sized amino acids G, A, or S (89.0 Å³) were present in 13 sequences, medium-
sized D (111.1 Å³), N (114.1 Å³), T (116.1 Å³), or V (140.0 Å³) in six sequences, and large M or L (166.7 Å³) in five sequences. It is noteworthy that none of the sequences of the basal placozoan *T. adhaerens* had an M or L at this position, pointing to steroid but not retinoid activity for placozoan enzymes.

The absence of Rdh8 in amphioxus, together with phylogenetic studies, reconstruction of the ancestral sequences and comparisons of the residues in the invertebrate Sdr28c did not support a role for this enzyme family in chromophore regeneration outside vertebrates. The Rdh8 enzyme probably arose from a duplication of an ancestor, biochemically close to the current Hsd17b1, that during the evolution of vertebrates modified the substrate specificity against retinoids and became highly expressed in the retina.

Rdh12 is a second enzyme involved in retinaldehyde reduction, accounting for ≈30% of the reductase activity in the eye (Maeda et al. 2007). Rdh12 belongs to the SDR7C family, which also includes vertebrate Rdh11, Rdh13, Rdh14, Dhrs13, and Dhrsx enzymes (Kallberg et al. 2010). In previous analyses (Belyaeva et al. 2009), six Drosophila, two Caenorhabditis elegans, and six *S. purpuratus* sequences had been related with the SDR7C family, but their phylogenetic relationship with vertebrate enzymes was unsolved.

The survey of genome databases retrieved 25 Sdr7c in the amphioxus *B. floridae*, 4 in the cnidarian *N. vectensis*, and the extended previous 6 *S. purpuratus* Sdr7c set to at least 13 (9 full-length and 4 partial) sequences. Phylogenetic analysis grouped three amphioxus sequences (*Bf_Dhrsx, Bf_Rdh13*, and *Bf_Rdh14*), three echinoderm sequences (*Sp_Dhrsx, Sp_Rdh13*, and *Sp_Rdh14*), and two cnidarian sequences (*Nv_Dhrsx* and *Nv_Rdh14*) with vertebrate Dhrs, Rdh13, and Rdh14, respectively. The remaining 22 amphioxus (*Bf_Sdr7c_1* to 22), 6 echinoderm (*Sp_Sdr7c_1* to 6; the partial sequences were not included in the phylogenetic analysis), 6 Drosophila (*Dm_CG2064, Dm_CG2065, Dm_CG2070, Dm_CG3842, Dm_CG30491*, and *Dm_CG30495*), and two cnidarian (*Nv_Sdr7c_1* and 2) sequences clearly grouped within the Sdr7c family but not strongly related to any of the vertebrate enzymes (fig. 2B and supplementary fig. S2, Supplementary Material online).

Phylogenetic tree topology indicated that Rdh12 (and Rdh11) enzymes arose from an Sdr7c precursor in invertebrates (fig. 2B and supplementary fig. S2, Supplementary Material online), probably during the evolution of mammals (Albalat R, unpublished data). The physiological role of this Sdr7c precursor is unknown, but it might be postulated based on the information from the invertebrate Sdr7c enzymes. Thus, *Drosophila* homologs are enzymes able to catalyze retinaldehyde reduction in many fly tissues and developmental stages (Belyaeva et al. 2009). The resemblance between the expression patterns of *Drosophila* Sdr7c homologs and the vertebrate Rdh11 in nonvisual tissues would favor the hypothesis that the Sdr7c precursor was active against retinoids, playing a general role in controlling retinoid homeostasis. Current Rdh12 enzymes would have derived, therefore, from a nonvisual enzyme of the retinoid metabolism that was duplicated and became specifically expressed in the retina during the evolution of vertebrates.

**Step 2: Retinal Esterification and Lrat Enzymes**

After all-trans-retinaldehyde reduction to retinol, lecithin retinol acyltransferase (Lrat) enzyme esterified all-trans-retinol to all-trans-retinyl esters (RE) in the retinal pig-ment epithelium (RPE) of the vertebrate eye (fig. 1). Lrat enzymes belong to the NlpC/P60 superfamily, specifically to the Lrat-like family, which also includes Hrasls (for Hras-like suppressor, also known as H-rev107-like proteins) and Fam84 (for family with sequence similarity 84) proteins (Anantharaman and Aravind 2003). One *C. elegans* sequence, named Egl26, has been associated with Lrat-like family (Anantharaman and Aravind 2003).

In *B. floridae*, 14 sequences were identified as members of the Lrat-like family by best-hit BLASTP surveys, although amphioxus sequences retrieved Hrasls and no Lrat proteins as best hit in the reciprocal searches. Genomic searches were extended to other invertebrate species, including *C. intestinalis, S. purpuratus, Drosophila melanogaster, Daphnia pulex, Capitella teleta, Lottia gigantea, Helobdella robusta,* and *N. vectensis*. Putative members of the Lrat-like family were only recognized in *D. pulex* (ID: 110055, 115643, 227171, and 307946), *L. gigantea* (ID: 162789, 162791, and 169625), and *N. vectensis* (XP_001626469) databases. As for amphioxus, protostome sequences retrieved Hrasls but no Lrat proteins in reciprocal best-hit BLASTP searches. The single cnidarian sequence, named *Nv_Fam84/Hrasls/Lrat*, represented a divergent member of Lrat-like family, and which was used as an outgroup in phylogenetic reconstructions.

In phylogenetic analyses, invertebrate sequences did not cluster strongly with any of the three vertebrate subfamilies. One amphioxus (*Bf_Fam84/Hrasls/Lrat_1*) and four *D. pulex* (*Dp_Fam84/Lras/Lrat_1 to 4*) sequences were weakly associated with vertebrate Hrasls; 13 amphioxus (*Bf_Fam84/Hrasls/Lrat_1 to 13*) and 2 *L. gigantea* sequences (*Lg_Fam84/Hrasls/Lrat_1 and 2*) appeared weakly related to vertebrate Fam84; and the *C. elegans* Egl26 sequence lay outside all other invertebrate and vertebrate enzymes (fig. 2C and supplementary fig. S3, Supplementary Material online). Structural information was used to evaluate functional conservation of vertebrate and invertebrate Lrat-like enzymes. Vertebrate Lrat have two conserved catalytic residues, histidine 60 and cysteine 161 (numbers refer to the human LRAT enzyme) (Mondal et al. 2000; Jahng et al. 2003), in strongly conserved contexts. C161 is the central cysteine of the strictly conserved asparagine-cysteine-glutamic acid box (NCE-box), which is replaced by serine in Fam84A members (asparagine-serine-glutamic acid box [NSE-box]). Invertebrate sequences showed conserved NCE-boxes—neither amphioxus nor any other invertebrate sequences showed an NSE-box—suggesting that the C to S replacement in the Fam84A subfamily took place during vertebrate evolution. For H60, the conserved context was HYGIY for Lrat enzymes, whereas the vertebrate Hrasls’ context was $H\gamma^C/\lambda^N/\iota^2/Y$. Interestingly, the H60 context present in
most invertebrate sequences was similar to that of the Hrasls enzymes, and ML reconstruction analysis predicted a Hrasls HWG/A to Hrasls enzymes, and ML reconstruction analysis predicted most invertebrate sequences was similar to that of the evolutionary of retinoid cycle.

Step 3: Retinoid Isomerization and Rpe65 Enzymes

RPE-specific protein 65 kDa (Rpe65) is a key enzyme in the vertebrate retinoid cycle because it acts as a light-independent isomerase, converting all-trans-RE to 11-cis-retinol for chromophore regeneration. Rpe65 enzymes belong to a protein family that also includes β,β-carotene-15,15′-monooxygenase (Bcmo1) and β,β-carotene-9′,10′-dioxygenase (Bco2) (Giuliano et al. 2003). In cephalechordates, five sequences, Bf_Bcmo1/Bco2/Rpe65_1 to 5, showed sequence similarity with vertebrate Bcmo1/Bco2/Rpe65 enzymes (fig. 2D and supplementary fig. S4, Supplementary Material online). In addition, ascidian Ci_Bco and Ci_Rpe65 (Takimoto et al. 2006), sea urchin Sp_Bcmo1/Bco2/Rpe65_1 and 2, insect Dm_NinaB and Gm_NinaB (von Lintig and Vogt 2000; Oberhauser et al. 2008), annelid Ct_Bcmo1/Bco2/Rpe65_1 and 2, and cnidarion Nv_Bcmo1/Bco2/Rpe65_1 to 4 were also clearly similar to vertebrate Bcmo1/Bco2/Rpe65 enzymes. Unfortunately, except for Ci_Bco, which grouped with vertebrate Bcmo1 enzymes, phylogenetic analysis did not classify the invertebrate enzymes among the three vertebrate clusters (fig. 2D and supplementary fig. S4, Supplementary Material online). Neither gene structure nor syntenic analyses provided insights into the appearance and evolution of Rpe65 enzymes in the animal kingdom.

At the functional level, a number of mutations have been shown to be detrimental for Rpe65 isomerization activity or have been found in patients with Rpe65 malfunction, which causes Leber congenital amaurosis or a milder retinitis pigmentosa. Although most of these mutations involve residues highly conserved among Rpe65, Bcmo1, and Bco2 enzymes, 13 of them affect positions highly conserved in Rpe65 but that differ in Bcom1 and Bco2 enzymes (supplementary table S1, Supplementary Material online). These positions are highly conserved within vertebrate Rpe65, from 77% to 100% identical (supplementary table S1, Supplementary Material online), and they might be considered Rpe65-specific residues that, based on mutation analyses, are relevant for Rpe65 function. To assess their functional conservation in invertebrate enzymes, these Rpe65-specific residues were identified in 17 invertebrate Bcmo1/Bco2/Rpe65 sequences (supplementary table S1, Supplementary Material online). Interestingly, in most cases, invertebrate residues did not concur with the typical Rpe65 sequences, questioning the function of invertebrate enzymes in chromophore regeneration. In fact, there is no experimental evidence that NinaB, an insect β,β-carotene-15,15′-oxygenase responsible for the centric cleavage of β-carotenes, could convert all-trans-RE to retinol for photoreceptor regeneration (Gu et al. 2004; Oberhauser et al. 2008). NinaB is not expressed in the retina, and it has been implicated in production of the visual pigments and photoreceptor development but not in chromophore regeneration. Moreover, ascidian enzymes, Ci-Rpe65 and Ci-Bco, would not be involved in retinoid isomerization either. Ci-Rpe65 and Ci-Bco are not able to synthesize 11-cis-retinol from all-trans-RE (Kusakabe et al. 2009), Ci-Rpe65 is not significantly expressed in the ocellus and brain vesicle of the ascidian larva, and Ci-Bco knockdown does not show any morphological defect or abnormality in photic behavior (Takimoto et al. 2006). In summary, there is no phylogenetic, structural, or functional data connecting invertebrate Bcmo1/Bco2/Rpe65 enzymes with the processing of all-trans-RE for chromophore regeneration in the retinoid cycle.

Step 4: Retinol Oxidation and Rdh5

In the final step of the retinoid cycle, 11-cis-retinol is transformed to 11-cis-retinaldehyde by Rdh5 (also known as 11-cis-Rdh), the major retinol dehydrogenase in the vertebrate RPE. Rdh5 is a member of the vertebrate Rdh cluster (also known as Rdh1-7/9 [Dalfó et al. 2007] or RODH-like group [Belyaeva and Kedishvili 2006]) that belongs to the SDR9C family (Kallberg et al. 2010), together with 3-hydroxybutyrate dehydrogenase type-1 (Bdh1) and Hsd type-2 enzymes. SDR9C family includes six human RDH genes (RDH5, RDH16, similar to RDH16, HSD17B6, SDR9C7, and DHR64), one BDH1, and two HSD2 genes (HSD11B2 and HSD17B2). Evolutionary analyses have led to the suggestion that vertebrate Rdh multiplicity, derived from a combination of gene-tandem and genomic duplication events, occurred at the base of the vertebrate lineage (Dalfó et al. 2007), and therefore, that Rdh5 enzymes arose after the vertebrate-cephalochordate split.

Surveys of amphioxus and cnidarian databases retrieved 12 B. floridab, Bf_Rdh_1 to 12, and 14 N. vectensis, Nv_Rdh_1 to 14 (2 very short sequences were not included in the phylogenetic tree), sequences similar to vertebrate Rdh sequences. In phylogenetic analyses, amphioxus and cnidarian Rdh clustered with vertebrate Rdh enzymes, but in separated groups (fig. 2A and supplementary fig. S1, Supplementary Material online), further supporting the vertebrate origin of Rdh5 enzymes. Tree topology indicated that vertebrate, amphioxus, and cnidarian Rdh enzymes expanded by lineage-specific duplication events from an ancestral Rdh form. Scant information is available about function of invertebrate Rdh enzymes that can give clues about
the physiological role of the ancestral Rdh enzyme. Only 2 Bf_Rdh (AAC48487 and AAC48488) of 12 amphioxus enzymes have been biochemically characterized, and kinetic studies revealed that these amphioxus enzymes catalyze the reduction of retinaldehyde to retinol rather than act as retinol dehydrogenases for retinaldehyde production (Dalfó et al. 2007). Considering the amphioxus biochemical data, the diversity in substrate specificity of vertebrate Rdh enzymes and their expression patterns (reviewed in Parés et al. 2008) as well as the evolutionary history of the vertebrate cluster (Dalfó et al. 2007), the most likely scenario is that the Rdh ancestor was a nonvisual enzyme. A duplicate of the ancestral Rdh was recruited to catalyze retinol oxidation in the eye during early vertebrate evolution. This duplicate, the current Rdh5 enzymes, became isomer specific for cis-retinoids and highly expressed in the RPE, thereby providing the essential 11-cis-retinol dehydrogenase activity for the retinoid cycle of vertebrates.

**Evolution of Eye-Specific–Binding Proteins**

Two eye-specific retinoid–binding proteins, such as retinaldehyde-binding protein 1 (Rlbp1, also known as cellular retinol-binding–protein, Crlbp) and interstitial retinol–binding protein 3 (Rbp3, also known as interphotoreceptor retinoid–binding protein, Irbp) are required for transport and protection of retinoids during the retinoid cycle.

Rlbp1 is a vertebrate protein abundantly expressed in the RPE and Müller cells that may function carrying 11-cis-retinol and 11-cis-retinaldehyde and favoring the oxidation of 11-cis-retinol. Vertebrate Rlbp1 belongs to the CRAL-TRIO family and shows sequence similarity with C. elegans 1 and 2 proteins (Clvs1 and Clvs2, formerly known as Rlbp1-like1 and Rlbp1-like2), two neuron-specific proteins involved in the regulation of lysosome morphology, and with tocopherol (alpha) transfer protein (Ttpa), a critical mediator of vitamin E function.

Surveys of the amphioxus genome identified five putative Rlbp1/Clvs/Ttpa sequences, which, however, did not retrieve Rlbp1 as best hit in the reciprocal searches. In fact, except for *C. elegans*, all the genomes surveyed contained numerous Rlbp1/Clvs/Ttpa-related sequences, but only two ascidian *C. intestinalis* and one cnidarian *Hydra magnipapillata* sequences retrieved Rlbp1 in the reciprocal searches. For phylogenetic analysis, the Rlbp1/Clvs/Ttpa-related sequences from eight selected species were used: human (five sequences), zebra fish (five sequences), amphioxus (five sequences), ascidians (six sequences), *Capitella* (five sequences), *Lottia* (two sequences), *Nematostella* (one sequence), and *Hydra* (three sequences). Phylogenetic reconstructions confirmed the reciprocal best-hit results showing that only *C. intestinalis* and *H. magnipapillata* sequences clustered with vertebrate Rlbp1 proteins, whereas amphioxus, *Capitella*, *Lottia*, and *Nematostella* sequences grouped with the Clvs and Ttpa proteins (fig. 2E and supplementary fig. S5, Supplementary Material online). Analysis of additional invertebrate genomes, including *Drosophila*, *Anopheles*, *Aedes*, and *C. elegans*, did not reveal any convincing Rlbp1 ortholog in these species. The topology of the phylogenetic tree and the presence of an Rlbp1 in *Hydra* indicated an ancient origin of Rlbp1 proteins in metazoans, but the absence of Rlbp1 in nearly all the species analyzed suggested that Rlbp1 would have been lost in many animal lineages during evolution.

Substantial efforts have been made to characterize ligand interactions and the structure of the retinoid-binding pocket of Rlbp1. Ten residues have been implicated as potentially interacting with retinoids, including W166, Y180, F198, C199, M209, Q211, M223, V224, M226, and W244 (Liu et al. 2005; Saari and Crabb 2005). Biochemical studies have shown that mutations at these positions affect the ligand-binding capacity of Rlbp1. The amino acids at these relevant positions were analyzed in *Ciona* and *Hydra* proteins to estimate their capability to bind retinoids (supplementary table S2, Supplementary Material online). It is noteworthy that many of these positions differed in nonvertebrate proteins: 50% in *C. intestinalis* Rlbp1 and 70% in *H. magnipapillata* Rlbp1 when compared with the human sequence, whereas they are highly conserved within vertebrates, from 80% to 100% identical (supplementary table S2, Supplementary Material online).

Although functional information for these invertebrate Rlbp1 was not available, the data question the capacity of *Ciona* and *Hydra* proteins to bind retinoids, which would imply that the retinoid-binding capacity of Rlbp1 might have arisen during the evolution of the vertebrate eye.

Rbp3 is a second eye-specific retinoid–binding protein involved in retinoid transport between different eye compartments in vertebrates (reviewed in Gonzalez-Fernandez 2003). No Rbp3 homologs were found in any of the invertebrate genomes analyzed. This result supported the previous statements about the absence of Rbp3 homologs outside vertebrates (Nickerson et al. 2006; Kusakabe et al. 2009), and on the recruitment of a CPTase/crotenase family protein for a new purpose, retinoid transport between photoreceptor cells and their neighbor cells. Actually, it is though that the evolution of Rbp3 proteins was probably linked to the evolution of the optic vesicle in vertebrates (Gonzalez-Fernandez 2003).

In summary, genome surveys, phylogenetic reconstructions and structural analyses of invertebrate components similar to those of the vertebrate retinoid cycle—that is, Rdh8, Rdh12, Lrat, Rpe65, Rdh5, Rlbp1, and Rbp3—did not provide any evolutionary or functional support for the existence of the genetic machinery of the retinoid cycle outside vertebrates.

**Chromophore Regeneration in the Ancestral Ciliary Photoreceptor Cell**

The vertebrate specificity of the genetic machinery for retinoid cycle raised new questions. How is chromophore regeneration accomplished in invertebrate ciliary photoreceptor cells? What might we speculate about the regeneration system in ancestral ciliary photoreceptor cells? To address these questions, two possibilities have to be considered. The first possibility would be that invertebrate
visual ciliary opsins are bistable photopigments that can readily regenerate their chromophores by photoconversion, whereby the photoisomerase activity of the opsin itself, without any other additional element, regenerates the visual pigment. Supporting this possibility, it has been reported that lamprey parapinopsin, a nonvisual ciliary opsin involved in ultraviolet light reception, has photoisomerizing two stable states that can account for chromophore regeneration (Koyanagi et al. 2004). The second possibility would be that invertebrate ciliary photoreceptors rely on an enzymatic machinery different from that of the retinoid cycle for chromophore regeneration. In this regard, the hypothetical existence of an alternative “visual cycle” in rhabdomeric Drosophila eyes has been recently proposed (Wang et al. 2010) based on the characterization of a pigment cell–enriched dehydrogenase (Pdh) enzyme in the ommatidia of flies. Unfortunately, other enzymatic components of the Drosophila cycle have not been yet identified, and therefore, the evolutionary conservation of this new cycle cannot be evaluated. Moreover, to establish that this rhabdomeric cycle has a role in ciliary photoreceptors requires further investigation.

An appealing alternative regeneration system to the Drosophila cycle is based on the photoisomerase activity of the RPE-retinal G protein–coupled receptor (Rgr). Rgr belongs to the Rgr/Rrh/Opn5/G0-opsin group of opsins (Suga et al. 2008), and in vertebrate RPE and Müller cells, Rgr is able to regenerate the visual chromophore in a light-dependent reaction (Chen et al. 2001) (fig. 1). Photons of light directly convert all-trans-retinaldehyde to 11-cis-retinaldehyde within Rgr, without any further enzymatic machinery (Chen et al. 2001). The role of Rgr on chromophore regeneration is still controversial (Wenzel et al. 2005) but because photoisomerase activity has been also shown for nonvertebrate Rgr enzymes, such as Todarodes pacificus retinochrome (Hara and Hara 1982; Seki et al. 1982) and C. intestinalis opsin3 (Nakashima et al. 2003), and for closely related Rrh (for RPE-derived rhodopsin homolog, also known as peropsin) proteins, namely B. belcheri Amphiop3 (Koyanagi et al. 2002) and Hasarius adansoni Rrh (Nagata et al. 2010), Rgr/Rrh photoisomerization is a good candidate for mediating chromophore regeneration in invertebrate ciliary photoreceptors and deserves further analyses. Thus, I searched for Rgr and Rrh orthologs in the invertebrate databases. Reciprocal best-hit BLASTP searches of Rgr/Rrh/Opn5/G0-opsins identified seven sequences in the B. floridana genome (Bf_Rrh, Bf_G0-opsin1 to 3 and Bf_Opn5_1 to 3), four in the ascidian C. intestinalis (Ci_Nut, Ci_LOC100186963, Ci_LOC100182476, and Ci_Opsin3), one in the hemichordate Saccoglossus kowalevskii (Sk_Opsin-like), and four in the echinoderm C. teleta (Ct_similar to Opn5_1 to 5) but none from the leech H. robusta. Finally, although there were many opsins in cnidarians, previous analyses had revealed that there are no Rgr/Rrh/Opn5/G0-opsins in this phylum (Suga et al. 2008).

Phylogenetic analysis ascribed all the new invertebrate sequences to the Rgr/Rrh/Opn5/G0-opsin group (fig. 2F and supplementary fig. S6, Supplementary Material online). This classification was further supported by the analysis of the gene structure (supplementary fig. S7, Supplementary Material online). Three intron positions (introns 1, 3, and 5 referred to human RGR) were strictly conserved in vertebrate and invertebrate genes, distinguishing the Rgr/Rrh/Opn5/G0-opsin genes from c-opsins genes, which are thought to be the closest sister group (Suga et al. 2008). Unfortunately, neither the phylogenetic analysis nor the gene structure resolved the orthology relationship of vertebrate and invertebrate Rgr/Rrh/Opn5/G0-opsin sequences but suggested the existence of Rgr/Rrh in deuterostome (Ci_Opsin3, Ci_Nut, Ci_LOC100186963, Ci_LOC100182476, Bf_Rrh, Bb_Amphiop3, Sk_Opsin-like, and Sp_Rrh) and protostome (retinochrome, Ha_Rrh, and Lg_Rrh_1 and 2) genomes. Photoisomerase activity has been previously experimentally demonstrated for Rgr/Rrh members in three different phyla (Chordata, Arthropoda, and Mollusca) (Hara and Hara 1982; Seki et al. 1982; Chen et al. 2001; Koyanagi et al. 2002; Nakashima et al. 2003; Nagata et al. 2010) and, from an evolutionary perspective, the phylogenetic analysis extends the genetic machinery to two other phyla, Hemichordata and Echinodermata. Although the taxonomic sampling is still too limited, and biochemical data of the new opsins are necessary to state general conclusions, it is tempting to speculate that photoisomerization mediated by Rgr/Rrh enzymes might represent an ancient and general system for regeneration of the visual pigment in ciliary photoreceptor cells of bilaterians.

Retinoid Cycle and the Evolution of the Vertebrate Eye

It has been suggested that when vertebrates began to colonize habitats where light intensities were low (e.g., deep waters), the evolution of eyes with a high sensitivity to light became advantageous. The enhancement in light sensitivity was achieved by a number of structural and functional changes of vertebrate eyes (reviewed in Lamb et al. 2007), including the use of ciliary opsins that were more sensitive to light. In these new light conditions, a photoisomerase system for chromophore regeneration based on Rgr/Rrh enzymes or in the photoreversal capacity of ciliary opsins might be insufficient, and the capability to synthesize...
11-cis-retinaldehyde in darkness could become advantageous. The data reported here describe how this capability might be achieved by recruiting duplicates of nonvisual elements in a new functional machinery of the vertebrate eye. This machinery includes an isomerase, Rpe65, which functions in the dark; several specialized enzymes, Rdh5, Rdh8, Rdh12, and Lrat, for retinoid oxidation, reduction, and esterification in the eye; and two eye-specific–binding proteins, Rlbp1 and Rbp3, for retinoid protection and transport. This evolutionary process yielded a light-dependent system for chromophore regeneration known as the retinoid cycle, which might have been vital for primitive vertebrates colonizing dim environments in Cambrian seas.

Supplementary Material

Supplementary figures S1–S7 and tables S1 and S2 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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References


