Assembly of the cnidarian camera-type eye from vertebrate-like components


*Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Videska 1083, 142 20 Prague 4, Czech Republic; ‡Graduate School of Frontier Sciences, University of Tokyo, S-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562, Japan; and ¶National Eye Institute, National Institutes of Health, Bethesda, MD 20892-3655

Edited by Eviatar Nevo, University of Haifa, Haifa, Israel, and approved April 11, 2008 (received for review January 14, 2008)

Animal eyes are morphologically diverse. Their assembly, however, always relies on the same basic principle, i.e., photoreceptors located in the vicinity of dark shielding pigment. Cnidaria as the likely sister group to the Bilateria are the earliest branching phylum with a well developed visual system. Here, we show that camera-type eyes of the cubozoan jellyfish, *Tripedalia cystophora*, use genetic building blocks typical of vertebrate eyes, namely, a ciliary phototransduction cascade and melanogenic pathway. Our findings indicative of parallelism provide an insight into eye evolution. Combined, the available data favor the possibility that vertebrate and cubozoan eyes arose by independent recruitment of orthologous genes during evolution.

The assembly of diverse animal eyes requires two fundamental building blocks, photoreceptors and dark shielding pigment. The function of photoreceptors is to convert light (stream of photons) into intracellular signaling. The photoreceptor cells (PRCs) are classified into two distinct types: rhodopsinic, characteristic of vision in invertebrate eyes; and ciliary, characteristic of vision in vertebrate eyes (1). In both ciliary and rhodopsinic PRCs, the seven-transmembrane receptor (opsin) associates with retinal to constitute a functional photosensitive pigment. Each photoreceptor type uses a separate phototransduction cascade. Rhodopsinic photoreceptors employ r-opsins and a phospholipase C cascade, whereas ciliary photoreceptors use c-opsins and a phosphodiesterase (PDE) cascade (2, 3). In general, the dark pigment reduces photon scatter and orients the direction optimally sensitive to light. The biochemical nature of the dark pigment appears more diverse than the phototransduction cascades used by the PRCs. Vertebrate eyes use melanin as their exclusive dark pigment. However, among invertebrates, pterins and ommochromes are accumulated in eyes of *Drosophila* (5), and melanin is found rare as such as the inverse cup-like eyes of the planarian, *Dugesia* (6).

Cnidaria, the likely sister group to the Bilateria, constitute the earliest branching phylum containing a well developed visual system. For example, Cubozoa (known as “box jellyfish”) have camera-type eyes with cornea, lens, and retina; unexpectedly, the cubozoan retina has ciliated PRCs that are typical for vertebrate eyes (7–9). Cubomedusae are active swimmers that are able to make directional changes in response to visual stimuli (10). The cubozoan jellyfish, *Tripedalia cystophora* (Fig. 1A), has four sensory structures called rhopalium that are equally spaced around the bell. In addition to two camera-type lens containing eyes at right angles to one another, each rhopalium has two pit-shaped and two slit-shaped pigment cup eyes (Fig. 1B). Thus, with six eyes located on each rhopalium, *Tripedalia* has 24 eyes altogether. Because the visual fields of individual eyes of the rhopalium partly overlap, *Tripedalia* (like other Cubomedusae) has an almost complete view of its surroundings. The lens-containing *Tripedalia* eyes have sophisticated visual optics as do advanced bilaterian phyla (11).

In the present work, we characterize genes required for the assembly of camera-type eyes in *Tripedalia*. We show that the genetic building blocks typical of vertebrate eyes, namely ciliary opsin and the melanogenic pathway, are used by the cubozoan eyes. Although our findings of unsuspected parallelism are consistent with either an independent origin or common ancestry of cubozoan and vertebrate eyes, we believe the present data favor the former alternative.

**Results**

**Ciliary Opin Is Expressed in Camera-Type Eyes of *Tripedalia***. We screened an expressed sequence tag (EST) library derived from rhopalia of *Tripedalia* to identify the jellyfish genes that are involved in vision; orthologues of other invertebrates and vertebrates were identified by phylogenetic analysis. Of the four opsin types present at the base of the bilaterians [rhodopsinic (r-opsins), ciliary (c-opsin), G algae, and peropsin/RGR (12–14)], the *Tripedalia* opsin EST clustered with the c-opsins, an orthology consistent with the conservation of the characteristic stretch of deduced amino acids between the transmembrane domain VII and cytoplasmic tail [Supporting Information (SI) Fig. S1]. This region includes the c-opsin fingerprint tripeptide NR/KQ (NRS in *Tripedalia*) that is critical for coupling to the downstream phototransduction cascade through interaction with a GTP-binding subunit *Gα* (15). An antibody generated against *Tripedalia* c-opsin recognized a single electrophoretic band in protein extracts prepared from rhopalia and COS-7 cells transfected with c-opsin cDNA (Fig. 1C). Camera-type eyes of adult jellyfish (Fig. 1D) were immuno stained with an anti-c-opsin antibody. The c-opsin localized in the retinal ciliated PRCs of both complex eyes (Fig. 1E and 1F) in a pattern resembling that by staining with anti-acetylated tubulin antibody (Fig. 1G), which specifically labels stabilized microtubules in axons and cilia (13).

**Spectral Sensitivity of *Tripedalia* c-Opsin**. To address the question of whether the identified *Tripedalia* c-opsin can function as a true vision gene, we cloned and sequenced the c-opsin gene from *Tripedalia* (Fig. 1H). The deduced amino acid sequence of the *Tripedalia* c-opsin shows a high degree of similarity to the vertebrate c-opsins. The putative N-terminus of the *Tripedalia* c-opsin derived by translation of the reverse complement of the EST sequence is consistent with the c-opsin fingerprint tripeptide NR/KQ (14).


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

**Data deposition.** The sequences reported in this paper have been deposited in the GenBank database (accession nos. EU310498 (r-opsin), EU310502 (cox), EU310499 (mitf), EU310500 (catalytic polypeptide), EU310501 (inhibitory polypeptide) and EU310503 (guanylate cyclase)).

To whom correspondence may be addressed. E-mail: kozmik@img.cas.cz, joramp@nei.nih.gov, or vlcek@img.cas.cz.

J.R. and K.J. contributed equally to this work.

This article contains supporting information online at www.pnas.org/cgi/content/full/0800388105/DCSupplemental.

© 2008 by The National Academy of Sciences of the USA.

www.pnas.org/cgi/doi/10.1073/pnas.0800388105

PNAS | July 1, 2008 | vol. 105 | no. 26 | 8989–8993

© 2008 by The National Academy of Sciences of the USA.
visual opsin, we tested its photochemical properties. *Tripedalia* c-opsin was expressed in COS-1 cells and reconstituted as a functional photosensitive pigment with 11-cis-retinal. The reconstituted c-opsin was most sensitive to the blue–green region of the spectrum with a peak absorbance (A
\text{max}) at 465–470 nm (Fig. 1H), in agreement with the spectral sensitivity of the *Tripedalia* electroretinogram (16). We conclude that *Tripedalia* c-opsin is a functional, vertebrate-like photopigment expressed in the PRCs of the camera-type cubozoan eye.

**Tripedalia Orthologues of Vertebrate-Like Phototransduction Genes.** In vertebrates, activated heterotrimeric G proteins use cGMP PDE for signal transduction. In accordance with our identification of a vertebrate-like c-opsin in *Tripedalia*, we found that the catalytic subunit of pde expressed in the *Tripedalia* rhopaliun phylogenetically clusters with the group of GAF domain-containing PDEs including vertebrate rod- and cone-specific PDE6 (Fig. S2A). Furthermore, we identified other components of the ciliary-type cascade associated with deactivation or adaptation of phototransduction, such as the inhibitory subunit of phosphodiesterase (PDE6D), phosducin and guanylate cyclase (Fig. S2 B–D). Thus, the nature of the genes expressed in the rhopalia (detected by RT-PCR; Fig. S3) suggests that the camera-type eye of *Tripedalia* uses a ciliary-type phototransduction cascade similar to that of vertebrates.

Melanin Granules in *Tripedalia* PRCs. A conspicuous ring of dark shielding pigment surrounds the area of c-opsin expression (compare Fig. 1D and E). Most if not all PRCs in the *Tripedalia* retina contain pigment granules (Fig. 2A). These PRCs thus resemble what one might imagine a prototypical ancestral cell combining photoreceptor and pigment functions to look like (17). The biochemical nature of the *Tripedalia* pigment was suggested by the identification of an orthologue of the vertebrate ocular and cutaneous albinism-2 (*Oca2*) gene in our EST library (Fig. S4A). *Oca2* (also known as pink-eyed dilution) is an essential gene for melanin biosynthesis. It is the most commonly mutated gene in cases of human albinism (18). In addition, mutations in *Oca2* are responsible for pigmentation defects in mouse (19), medaka (20), and independently arisen populations of the cave fish, *Astyanax* (21). In situ hybridization analysis revealed that *Tripedalia oca* is conspicuously expressed near the pigmented retina layer of PRCs (Fig. 2C). Control staining with an *oca* sense probe (Fig. 2D) did not yield a signal. The results of a direct chemical assay (Fontana–Masson) were consistent with melanin being the pigment in the *Tripedalia* retina (Fig. 2E–G). In vertebrates, development of melanin-producing cells and specification of retinal pigment cells require the conserved
microphthalmia-associated transcription factor, Mitf (22–24). Mitf regulates expression of tyrosinase and tyrosinase-related protein-1 and -2, which are necessary for melanin biosynthesis (for review, see ref. 25). Here, we have cloned a *mitf* orthologue from *Tripedalia* (Fig. S4A), expressed in a ring-like pattern just outside of the melanin deposits (Fig. 3A and B); this area contains the PRC nuclei (Figs. 2A and 3D). Thus, *mitf* is a conserved transcription factor with shared expression patterns in the complex eyes of *Tripedalia* and vertebrates.

**Mitf Expression in Lens and Crystallin Expression in Pigmented PRCs of Nonlens Eyes of *Tripedalia***. In addition to expression in the pigmented PRCs of camera-type eyes, *mitf* mRNA was detected in the outermost cells of the *Tripedalia* lens (Fig. 3C). Consequently, to investigate a possible relationship between the pigmented PRCs and the cellular lens we examined whether J1-crystallin, the major protein of the *Tripedalia* lens (26), is expressed in PRCs. The J1-crystallin antibody immunostained the slit and pit eyes as well as the cellular lens (Fig. 3 D and E). The presence of J1-crystallin in the slit and pit eyes of *Tripedalia* was unexpected because these cup-like eyes lack cellular lenses. They do, however, have pigmented PRCs, suggesting a relationship between the PRCs and cellular lens that warrants further study.

**Discussion**

The present work reveals surprising similarities in the genetic components used for visual system development in vertebrates and cnidarian jellyfish. If Cubozoa and vertebrates express orthologous c-opsins in their PRCs and make use of the same pigmentation pathway including the key transcription factor Mitf, does this represent a parallel evolution or conservation of an ancestral “eye” program between those evolutionarily distant animal phyla (Fig. 4)? Although our data are formally consistent with both evolutionary scenarios, we believe that they favor the former.

Even though ciliary and rhododermic photoreceptive systems coexist throughout the animal taxa (1), the present evidence suggests that their evolutionary histories differ. For photode-
after the separation of the cnidarian and bilaterian lineages (Fig. 4) (27).

All eyes have shielding pigment typically found in cells adjacent to the PRCs. Melanin, the dark pigment of Tripedalia eyes, presumably performs the same function in vertebrate eyes as in the simple cup-like eyes of a basal lophotrochozoan, Dugesia (6). Interestingly, Dugesia uses another pigment, an ommochrome, as the body pigment (2, 6). Pterins constitute the dark eye pigment of the polychaete P. dumerilii (4), and pterins and ommochromes are the pigments in eyes of Drosophila (5). Thus, as with the opsin, Tripedalia shares the same dark pigment in the eye with vertebrates.

Unlike in the Dugesia eye, the camera-type Tripedalia eye combines the photoreceptor and pigment functions in the same cell consistent with an ancestral (basal) condition (Fig. 4A). However, the medusae stage of cubozoans may well be a derived rather than ancestral condition for Cnidaria, complicating discussions about the basal state of the cubozoan visual system (Anthozoa, for example, do not have eyes). Nevertheless, it remains possible that the pigmented PRCs in Tripedalia are descendants of one of the postulated ancient prototypical photosensitive cells diversified by natural selection (17, 28). However, this does not require a common origin for the eyes. It was estimated through computer-based modeling (29) that fewer than a half-million generations would be required under selective pressure to proceed from a cluster of light-sensitive cells to a sophisticated camera-type eye. In theory, this relatively short time interval would allow sophisticated eyes to have originated de novo several times during evolution (polyphyletic eye origin).

For the common-ancestry model to be true, the cnidian-bilaterian ancestor (CBA) must have had the same genetic determinants as its descendants. The common-ancestry scenario for cubozoan and vertebrate eyes requires, however, that animals in many bilaterian phyla lost their eyes that were initially assembled by using the same building blocks as in present-day vertebrates and Cuboza (c-opsins, melatonin) to explain the exclusive occurrence of rhodameric PRCs in invertebrate eyes. There is no obvious explanation for such a specific selection against ciliary PRCs to be used for visual purposes. Eyes in general provide a freely moving animal with a tremendous advantage, and as such there should be a constant selection for eye maintenance, except in, for example, cave or underground animals.

Although not definitive, there are at least two additional complications to the common-ancestry model that arise if one invokes the developmental argument that similar transcription factor cascades may direct development of vertebrate and cubozaan eyes. The first is that PaxB, a Pax2/6/8-related transcription factor, is used in Tripedalia (30) instead of Pax6 as in vertebrates (31) as well as flies (32, 33) and other species (34). The second is the apparent evolutionary "promiscuity" of developmental cascades in general; entire regulatory circuits can be co-opted for specific elements and independent recruitments of similar genes during evolution of the diverse eyes.

Materials and Methods

Jellyfish Collection and Culture. T. cystophora was collected and cultured as described in ref. 43.

Isolation of Rhopaliun-Expressed Genes and Phylogenetic Analysis. An EST library was generated from rhopalia mRNA, and 2,433 individual clones from the library were sequenced by using an ABI capillary sequencer. The accession numbers for the clones are as follows: c-opsin (EU310498), oca (EU310502), mitf (EU310499), catalytic pde (EU310500), inhibitory pde (EU310501) and guanylate cyclase (EU310503). Details on phylogenetic analysis including the accession numbers of individual sequences are described in SI Materials and Methods.

RNA in Situ Hybridization. Jellyfish were fixed in 4% paraformaldehyde (PFA), cryoprotected in 30% sucrose overnight at 4°C, and embedded and frozen in OCT (Tissue Tek). RNA in situ hybridization was performed as described in ref. 43.

Immunohistochemistry. The cryosections were fixed in 4% PFA for 10 min, washed three times with PBS, permeabilized with PBT (PBS + 0.1% Tween 20) for 15 min, and blocked in 10% BSA in PBT for 30 min. The primary antibodies were diluted in 1% BSA in PBT, incubated overnight at room temperature, washed three times with PBT, and incubated with secondary antibodies in 1% BSA in PBT. The sections were counterstained with DAPI and mounted. Primary antibodies used were: anti-Tripedalia c-opsin, anti-Tripedalia J1-crystallin, and anti-acetylated tubulin (Sigma). The following secondary anti-
Rhopalia excised from juvenile medusae
Transmission Electron Microscopy.
room temperature. The sections were treated with 5% oxalic acid for 5 min,
distilled water and exposed to 0.25% potassium permanganate for 30 min at
were treated with Karnovsky fixative (2.5% glutaraldehyde, 2.5% parafor-
formation of multimeric opsin complexes, protein extracts from transfected
VADAATQPVHK was attached to KLH via the C-terminal lysine and used for
Bleaching was performed either after Fontana–
immunization. Monkey kidney COS-7 cells were transfected with CMV-c-opsin
Expression, Reconstitution, and Spectroscopic Analysis of Tripedalia c-opsin.
Tripedalia c-opsin cDNA was expressed in transfected COS-1 cells. Transfected
cells were resuspended with 5 μM 11-cis-retinal, solubilized with 1% dodecyl
maltoside, and the resulting c-opsin photopigment was purified by using immobilized
ID1 (Cell Culture Center, Minneapolis, MN). The UV-visible
sorption spectrum was recorded for the c-opsin photopigment from 250 to
560 nm at 0.5-nm intervals by using the Hitachi U3010 dual-beam spectrome-
ter at 20°C. Five replicates were performed in the dark and five more after 3
min of light exposure (with a <440-nm cut-off filter). The Amax value was taken from the dark-light difference spectrum.
For additional details, see SI Materials and Methods.

ACKNOWLEDGMENTS. We thank Drs. Ales Cvekl and Stanislav Tomarev for comments on the manuscript and Mrs. Veronica Noskova for excellent technical assistance. We are grateful to Prof. Tom Tosteson for kind support during our stay at the Boxe Institute. This work was supported in part by Project AVGZ5025014 awarded by the Academy of Sciences of the Czech Republic and by Center for Applied Genomics Grant 1M6837805002 awarded by the Ministry of Education, Youth, and Sports of the Czech Republic and by the intramural research program of the National Eye Institute, National Institutes of Health.

4. Viscontini M, Hummel W, Fischer A (1970) Isolation of pterin dimers from the eyes of
Flatoyneres dumerilii (German). Helv Chim Acta 53:1207–1209.