Regeneration of ultraviolet pigments of vertebrates

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Abstract We report here the regeneration of the visual pigments of mouse, rat, goldfish and pigeon, which have wavelengths of maximal absorption at 359 nm, 358 nm, 359 nm, and 393 nm, respectively. The construction and functional assays of the ultraviolet or near-ultraviolet pigments from a wide range of vertebrate species will allow us to study the molecular bases of ultraviolet vision for the first time.

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Key words: Ultraviolet visual pigment; Opsin; Absorption spectrum; Molecular evolution; Vertebrate

1. Introduction

Ultraviolet vision has been used for social signaling [1], hunting [2], nectar localization [3], and mate-choice decisions [4]. Despite its important functions in many fish, bird, amphibian, reptilian, and mammalian species [5], the molecular mechanism for ultraviolet vision is not known. This is simply because it has been difficult to regenerate visual pigment from an ultraviolet opsin and 11-*cis* retinal [6], and consequently mutagenesis experiments, essential in understanding the structure-function relationships, could not be conducted.

The putative ultraviolet pigment genes from mouse (*Mus musculus*) [7], rat (*Rattus norvegicus*) [8], American chameleon (*Anolis carolinensis*) [9], and goldfish (*Carassius auratus*) [10] have been characterized. All of these pigments are known to belong to a specific evolutionary group, short wavelength-sensitive class 1 (SWS1) cluster [11]. Recently, the SWS1 pigment of American chameleon [9] was regenerated using cultured cells and was shown to be ultraviolet-sensitive, having a wavelength of maximal absorption (λmax) at 358 nm [12]. We have applied the procedure to the SWS1 pigments of mouse, rat, goldfish, and pigeon (*Columba livia*). Here we report the spectral sensitivities of these regenerated SWS1 pigments. The construction and functional assays of the ultraviolet or near-ultraviolet pigments from a wide range of vertebrate species will allow us to initiate the study of the molecular bases of ultraviolet vision for the first time.

2. Materials and methods

2.1. cDNA cloning by RT-PCR

The retinas of mouse and rat were obtained from Pel-Freeze (Rogers, AR), whereas those of pigeon and goldfish were isolated from animals purchased from a local pet store. Total RNAs were prepared from these retinas using the acid-guanidinium extraction method [13], from which the SWS1 opsin cDNAs were amplified using appropriate RT-PCR primers (Fig. 1). The primers for pigeon were based on the partial sequence data of a genomic SWS1 clone (S. Kawamura and S. Yokoyama, unpublished data), whereas others were based on the published sequence data [7,8,10]. The cDNA synthesis by RT-PCR amplification was performed as previously described [12]. Nucleotide sequences of the entire region of the cDNA clones were determined by standard dideoxynucleotide chain-termination method [14].

2.2. Regeneration of SWS1 pigments and spectral analysis

The large fragment of the *Eco*RI/*Sal*I-digested pMT expression vector contains the sequences necessary for expression in cultured COS1 cells and the last 15 amino acids of the bovine rhodopsin (Ser-Thr-Thr-Val-Ser-Lys-Thr-Glu-Thr-Ser-Gln-Val-Ser-Pro-Ala) that are necessary for immunoaffinity purification [15]. This *Eco*RI/*Sal*I fragment was ligated with the *Eco*RI/*Sal*I opsin cDNA fragments. The resulting plasmids were transiently expressed in COS1 cells and the transfected cells were incubated with 11-*cis* retinal (Storm Eye Inst., Medical University of South Carolina) in the dark. The pigments were purified by binding to the monoclonal antibody 1D4 Sepharose in buffer W1 (50 mM HEPES, pH 6.6, 140 mM NaCl, 3 mM $MgCl_2$, 20% (w/v) glycerol, and 0.1% dodecyl maltoside) [12].

Ultraviolet visible absorption spectra of visual pigments were recorded at 20°C, using a Hitachi U-3000 dual beam spectrophotometer. Visual pigments were bleached by a 366 nm ultraviolet light illuminator. The purified pigments were also denatured at pH 1.8 for 5 min at 20°C using 1 M sulfuric acid (H_2SO_4). Recorded spectra were analyzed using SigmaPlot software (Jandel).

2.3. Construction of a phylogenetic tree

At present, 17 SWS pigments have been characterized: blue (GenBank accession number M92037) and violet (M92039) pigments of chicken (*Gallus gallus*), blue pigment [16] of cavefish (*Astyanax fasciatus*), blue (L11864) and UV (D85863) pigments of goldfish (*Carassius auratus*), blue (AB001602) and violet (AB001605) pigments of killifish (*Oryzias latipes*), blue and UV [19] pigments of American chameleon (*Anolis carolinensis*), blue pigment (M13295–M13299) of human (*Homo sapiens*), blue pigment (U53875) of squirrel monkey (*Saimiri boliviensis*), blue pigment (L22218) of marmoset (*Callithrix jacchus*), blue pigment (L76226) of *Miopithecus talapoin*, blue pigment (U92557) of bovine (*Bos taurus*), UV pigment (U49720) of mouse (*Mus musculus*), UV pigment (U63972) of rat (*Rattus norvegicus*), and violet pigment of African water frog (*Xenopus laevis*).

The numbers (*K*) of amino acid replacements per site for pairwise comparisons were estimated by a Poisson correction. Topology and branch lengths of a phylogenetic tree are evaluated using the neighbor-joining (NJ) method [17] based on the *K* values. The NJ tree was tested by the bootstrap method [18] with 1000 replications. The rooted tree was constructed using the rhodopsins of cavefish (U12328) and human (U49742) as the outgroup.

3. Results and discussion

3.1. The phylogenetic position of UV opsins

The complete opsin cDNAs of the four species were isolated using the RT-PCR amplification method. The rooted phylogenetic tree for the currently known 17 SWS opsins and a newly acquired SWS opsin of pigeon has been obtained (Fig. 2). Fig. 2 shows that the vertebrate SWS opsins are distinguished into two (SWS1 and SWS2 [19]) groups. Previously, it was claimed that the UV gene of zebrafish (*Brachydanio rerio*) was cloned [20]. This UV opsin was shown to be

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Forward                 Eco RI

Mouse     5'-GCATCCGAATTCCACCATGTCAGGAGAGGATGACTT-3'
Rat       5'-GCATCCGAATTCCACCATGTCGGGAGAGGACGAGTT-3'
Pigeon    5'-CCGAGGGAATTCCACCATGTCGGGTGACGAGGAG-3'
Goldfish  5'-CACTAAGAATTCCACCATGGACGCGTGGACCTATC-3'

Reverse                 Sal I

Mouse     5'-GGTCCTGTCGACGGGCCAACTTTGCTAGAAGA-3'
Rat       5'-GGTCCTGTCGACGGGCCAACTTTGCTAGAAGA-3'
Pigeon    5'-GTCCCTGTCGACGGGCTGACCTGGCTGG-3'
Goldfish  5'-GGAATAGTCGACGCAGACACAGAGGAGGTTTCAG-3'
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Fig. 1. Oligonucleotide primers for RT-PCR amplification of SWS1 opsin mRNAs. The *Eco*RI and *Sal*I sites are for cloning into the expression vector pMT. A Kozak sequence (CCACC) was inserted between *Eco*RI and the start codon to promote translation.

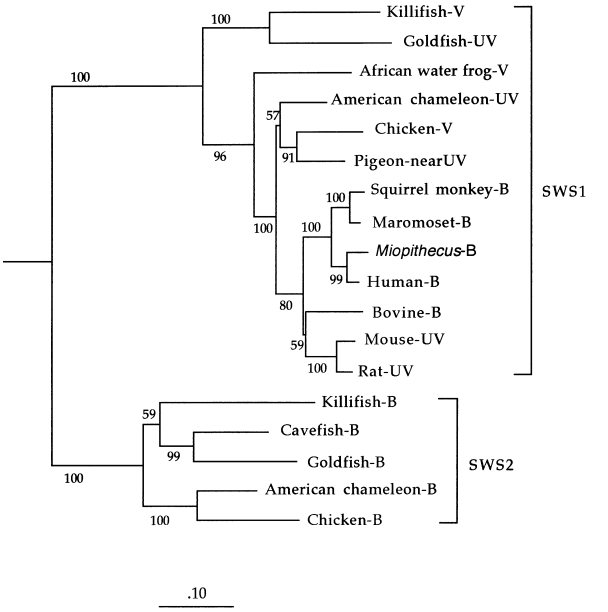

Fig. 2. Rooted phylogenetic tree for the SWS opsins. The numbers next to the different branches indicate clustering percent support generated by 1000 bootstrap analysis. B, V, and UV indicate blue, violet, and UV opsins, respectively.

evolutionarily most closely related to the goldfish rhodopsin in the RH1 group [21] and had an intriguing characteristic, i.e. only one amino acid change from Trp to Lys at site 126 appeared to be responsible for the development of UV vision of this species [20]. However, these claims turned out to be incorrect [22,23]. Thus, all currently known putative UV opsins of goldfish, mouse, rat, and pigeon and the UV opsin of American chameleon belong to the SWS1 group (Fig. 2).

It should be noted that the spectral sensitivities of the two groups of SWS pigments differ. That is, the SWS1 pigments have λmax values of 360–420 nm, while the SWS2 pigments have more red-shifted λmax values, ranging from 437 nm to 455 nm [19].

3.2. Construction of the SWS1 opsins

The amino acid sequences deduced from the PCR products differ only slightly from the corresponding published sequences. Amino acid at site 279 is Asp rather than Tyr in the rat pigment and those at sites 176 and 193 in the goldfish pigment are Leu and Ser rather than Ile and Thr, respectively (Fig. 3). We cannot eliminate the possibility of protein polymorphism. However, since amino acids at these three sites are almost completely conserved among the SWS1 pigments (see also Fig. 2), the present PCR products may present more accurate sequences. Since the amino acid at site 5 in rat has not been determined [5], it is assumed to be Asp like in mouse pigment. In both mouse and rat pigments, the last amino acid, His, at site 346 is deleted. As mentioned earlier, the expression vector pMT contains an extra 15 amino acids of the bovine rhodopsin next to the last amino acid site and these additional amino acids do not affect the spectral property of pigments [24]. Thus, the deletion of the last amino acid in the murine opsins is not expected to modify their spectral sensitivities.

3.3. Absorption spectra of the regenerated pigments

The SWS1 pigments were regenerated by expressing these opsins in cultured cells and reconstituting with 11-*cis* retinal. Absorption spectra of the purified visual pigments were directly measured from the dark spectra (Fig. 4a–d), where we can see that all pigments show spectra with prominent absorption of a λmax value together with a protein absorbance at 280 nm. The λmax values of the SWS1 pigments of mouse,

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Mouse      MSGEDDFYLFQNISSVGPWDGPQYHLAPVWAFRLQAAFMGFVFFVGTPLNAIVLVATLHYKKLRQPLNYILVNVSLGGFLFCIFSVFTVFIASCH 95
Rat        MSGEEEFYLFQNISSVGPWDGPQYHIAPVWAFHLQAAFMGFVFFAGTPLNATVLVATLHYKKLRQPLNYILVNVSLGGFLFCIFSVFTVFIASCH 95
Goldfish     MDAWTYQFGNLSKISPFEGPQYHLAPKWAFYLQAAFMGFVFFVGTPLNAIVLFVTMKYKKLRQPLNYILVNISLGGFIFDTFSVSQVFFSALR 93
Pigeon     MSGDEEFYLFKNGSSVGPWDGPQYHIAPPWAFYLQTAFMGFVFLVGTPFNAIVLVVTIKYKKLRQPLNYILVNISFSGFISCIFSVFTVFVSSSQ 95

Mouse      GYFLFGRHVCALEAFLGSVAGLVTGWSLAFLAFERYVVICKPFGSIRFNSKHALMVVLATWIIGIGVSIPPFFGWSRFIPEGLQCSCGPDWYTVG 190
Rat        GYFLFGRHVCALEAFLGSVAGLVTGWSLAFLAFERYLVICKPFGNIRFNSKHALTVVLITWTIGIGVSIPPFFGWSRFIPEGLQCSCGPDWYTVG 190
Goldfish   GYYFFGYTLCAMEAAMGSIAGLVTGWSLAVLAFERYVVICKPFGSFKFGQSQALGAVALTWIIGIGCATPPFWGWSRYIPEGIGTACGPDWYTKN 188
Pigeon     GYFIFGKDMCALEAFVGATGGLVTGWSLAFLAFERYIVICKPFGNFRFNSKHALMAVVATWVIGLGVALPPWFGWSRYVPEGLQCSCGPDWYTVG 190

Mouse      TKYRSEYYTWFLFIFCFIIPLSLICFSYSQLLRTLRAVAAQQQESATTQKAEREVSHMVVVMVGSFCLCYVPYAALAMYMVNNRNHGLDLRLVTI 285
Rat        TKYRSEHYTWFLFIFCFIIPLSLICFSYFQLLRTLRAVAAQQQESATTQKAEREVSHMVVVMVGSFCLCYVPYAALAMYMVNNRNHGLDLRLVTI 285
Goldfish   EEYNSESYTYFLLVSCFMMPIMIITFSYSQLLGALRAVAAQQAESASTQKAEKEVSRMVVVMVGSFVVCYGPYAITALYFSYAEDSNKDYRLVAI 283
Pigeon     TKYKSEYYTWFLFIFCFIVPLSLIIFSYSQLLSALRAVAAQQQESATTQKAEREVSRMVVVMVGSFCLCYVPYAALAMYMVNNRNHGLDLRLVTI 285

Mouse      PAFFSKSSCVYNPIIYCFMNKQFRACILEMVCRKPMADESDVSGSQKTEVSTVSSSKVGP*  346
Rat        PAFFSKSSCVYNPIIYCFMNKQFRACILEMVCRKPMTDESDMSGSQKTEVSTVSSSKVGP*  346
Goldfish   PSLFSKSSCVYNPLIYAFMNKQFNACIMETVFGKKIDESSEVSSKTETSSVSA          336
Pigeon     PAFFSKSSCVYNPIIYCFMNKQFRACILELVCGRPMTDDSDVSSSAQRTEVSSVSSSQVSPS 347
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Fig. 3. The deduced amino acid sequences of SWS1 opsins. Amino acids boxed indicate those differing from the published sequences. Asterisks (*) in the mouse and rat opsins show the site of a deleted amino acid, His, in the regenerated pigments.

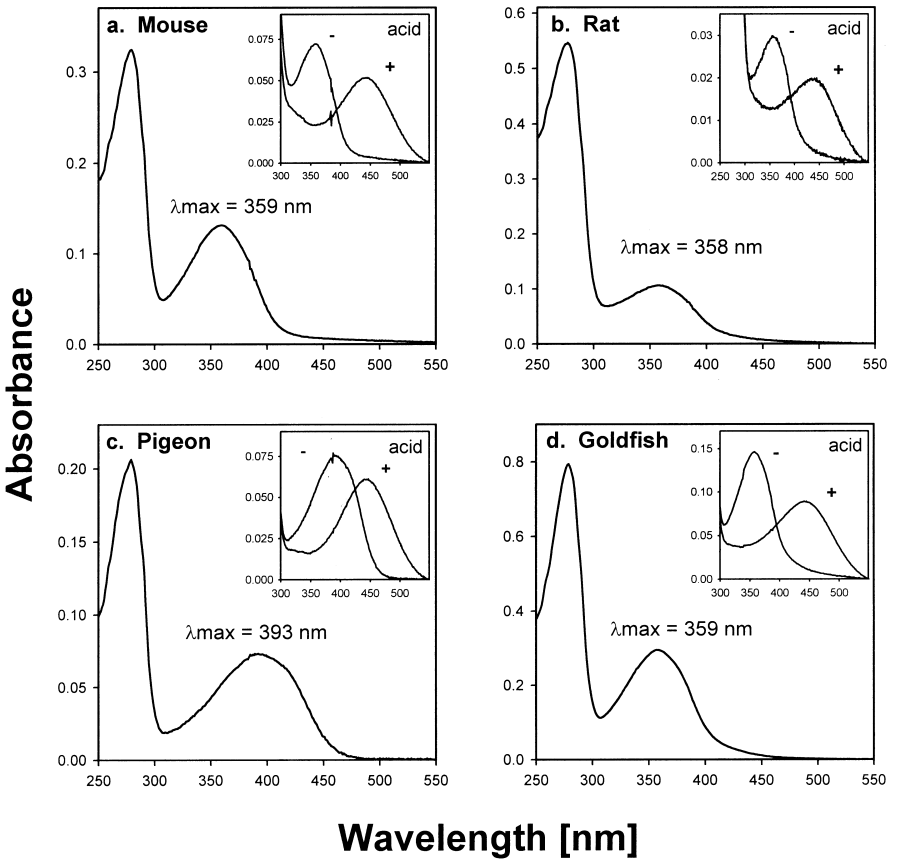

Fig. 4. Absorption spectra of the SWS1 pigments of mouse (a), rat (b), pigeon (c), and goldfish (d). Insets: Acid denaturation of the expressed SWS1 pigments. Absorption spectra were measured before (−: no acid added) and after (+: with acid) treatment with H_2SO_4 to decrease the pH to 1.8. Both spectra were normalized to 0 at 550 nm.

rat, pigeon, and goldfish are given by 359 ± 1 nm, 358 ± 1 nm, 393 ± 2 nm, and 359 ± 1 nm, respectively (Fig. 4a–d). The ratios of the protein absorbance to the pigment absorbance were 2.3, 5.1, 2.8, and 2.7 for the SWS1 pigments of mouse, rat, pigeon, and goldfish, respectively. Most of these values are more comparable to the ratio of 1.5–1.9 for the bovine rhodopsin than 8.8 for the ultraviolet pigment of American chameleon [12].

There exists a possibility that these pigment peaks are either from residual free 11-*cis* retinal in solution or from residual 11-*cis* retinal that formed random Schiff base adducts with other proteins. However, the following three observations strongly suggest that the regenerated pigments using cultured cells are in fact photosensitive molecules. First, when these regenerated pigments were exposed to ultraviolet light, new absorbance peaks of 380 nm were achieved (data not shown), showing that 11-*cis* retinal in the pigments was isomerized by light and all-*trans* retinal was released. Second, when the regenerated visual pigments were denatured by sulfuric acid (H_2SO_4) in the dark, the resulting dark spectra had peak absorbances of 440 nm (Fig. 4a–d, insets), which is identical to that of a protonated Schiff base 11-*cis* retinal free in solution [25]. Since acid has no effect on the absorbance of free 11-*cis* retinal, this results strongly suggests that the observed pigment peaks were generated by opsin covalently linked to 11-*cis* retinal in a Schiff base linkage [12]. Third, the UV and near-UV peaks appear only in the transfection experiments involving selected SWS1 pigments [12].

Using the electroretinogram flicker photometric procedure, both mouse and rat are shown to have ultraviolet pigments with λmax values of about 360 nm [26]. Goldfish is shown to have ultraviolet pigments with λmax values of 355–360 nm [27]. Thus, the observed λmax values of the SWS1 pigments of mouse, rat, and goldfish are consistent with previous estimates of spectral sensitivities of photoreceptors obtained using different methods.

Pigeon is suspected to have ultraviolet sensitivity from both behavioral and electrophysiological analyses ([28]; references therein). However, applying microspectrophotometry (MSP) analysis to pigeon, four types of cone pigments with λmax values of 567 ± 3 nm, 507 ± 2 nm, 453 ± 5 nm, and 409 ± 7 nm have been identified [28], which correspond very closely to the red (571 nm), green (508 nm), blue (455 nm), and violet (415 nm) pigments of chicken [29], respectively. The screening of the pigeon genomic DNA library also strongly suggests that there exist only four types of cone pigment genes (S. Kawamura and S. Yokoyama, unpublished data). Since the MSP estimate of the violet pigment has a large standard deviation [28], the pigeon SWS1 pigment may be considered to be maximally sensitive in the violet-ultraviolet range and the existence of the 'true' ultraviolet pigment in pigeon remains to be resolved. The more definite answer will come from the molecular analyses of the cone pigment genes isolated from the pigeon genome.

Comparison of the ultraviolet pigments and other pigments reveals no amino acid changes common to all ultraviolet pig-

ments that are uniquely distinguished from those of the non-ultraviolet pigments (data not shown). This demonstrates that no single molecular mechanism is responsible for ultraviolet vision in vertebrates. One effective way to study the mechanisms of ultraviolet vision is to first identify amino acid changes that may be responsible for the λmax shifts toward 360 nm and then to test such hypotheses by performing mutagenesis experiments [19]. Successful regeneration of the ultraviolet pigments of mouse, rat, American chameleon, and goldfish and ultraviolet (or near-violet) pigment of pigeon clearly demonstrates that such analyses are now well within our reach.

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