1. INTRODUCTION

Vertebrate cone visual pigments are classified into four groups according to their evolutionary lineages and spectral properties: short-wavelength-sensitive-1 (SWS1), short-wavelength-sensitive-2 (SWS2), middle-wavelength-sensitive (RH2) and long-wavelength-sensitive (LWS). In mammals, this complement is reduced to just two classes, LWS and SWS, with the latter either from the SWS1 class in marsupial and eutherian mammals, or from the SWS2 class in monotremes [1]. Most mammals are, therefore, dichromats, although monochromacy through the loss of sensitivity (UVS) and either Ser, Tyr or Val causing a shift to violet wavelengths. In primates, however, the tuning mechanism of violet-sensitive (VS) pigments would appear to differ. In this study, we examine the tuning mechanisms of prosimian SWS1 pigments. One species, the aye-aye, possesses a pigment with Phe86 but in vitro spectral analysis reveals a VS rather than a UVS pigment. Other residues (Cys, Ser and Val) at site 86 in prosimians also gave VS pigments. Substitution at site 86 is not, therefore, the primary mechanism for the tuning of VS pigments in primates, and phylogenetic analysis indicates that substitutions at site 86 have occurred at least five times in primate evolution. The sole potential tuning site that is conserved in all primate VS pigments is Pro93, which when substituted by Thr (as found in mammalian UVS pigments) in the aye-aye pigment shifted the peak absorbance into the UV region with a λmax value at 371 nm. We, therefore, conclude that the tuning of VS pigments in primates depends on Pro93, not Tyr86 as in other mammals. However, it remains uncertain whether the initial event that gave rise to the VS pigment in the ancestral primate was achieved by a Thr93Pro or a Phe86Tyr substitution.

Keywords: visual pigments; primates; colour vision; evolution
species, but an intact SWS1 gene is retained in two close relatives, the grey and Coquerel’s mouse lemurs. Members of the Tarsiiformes and the remaining species of Lemuriformes (Lemuridae, Lepilemuridae and Indriidae families) as well as the aye-aye, the sole extant member of the Daubentoniidae family of the Chiromyiformes, all appear to have an intact SWS1 gene [30], although functional information is limited. The residue present at site 86, however, varies substantially across prosimians [30]; Leu86 is found throughout the Haplorrhini but in the Strepsirrhini, either Cys, Ser, Val, Phe, Tyr or Asn is present, although Tyr and Asn are found only in SWS1 pseudogenes of the lesser, small-eared and greater galagos, and the slow lorises.

The objective of this study was to determine the underlying tuning mechanisms in the evolution of primate SWS1 pigments, by focusing on three prosimian species, each with a different residue at site 86.

2. MATERIAL AND METHODS

(a) DNA samples
Genomic DNA (gDNA) was extracted from brown lemur (Eulemur fulvus) liver tissue that was kindly donated by Prof. John Mollon (University of Cambridge, UK). Blood samples from Coquerel’s mouse lemur (Mirza coquereli) and the aye-aye (Daubentonia madagascariensis) were purchased from Duke University Primate Centre (USA) and gDNA was extracted using the Nucleon BACC Genomic DNA Extraction Kit (GE Healthcare, UK).

(b) Generation of expression vectors
The human SWS1 construct for in vitro expression was amplified from retinal cDNA using primers complementary to the 5’- and 3’-ends of the coding sequence. The coding sequences for the SWS1 opsin genes of all three prosimian species were generated using the SPICE technique for joining individual exons amplified from gDNA into a single transcription unit [31]. PCRs were performed using KOD Hot Start polymerase (Novagen, UK) and the primers listed in electronic supplementary material, table S1. The full-length coding sequences were cloned into the pMT4 mammalian expression vector as previously described [16]. Point mutations were introduced using the Quikchange site-directed mutagenesis kit (Stratagene, UK) and the oligonucleotides listed in electronic supplementary material, table S2.

(c) In vitro determination of the λmax of expressed pigments
Opsin expression and pigment production followed the methodology described in Cowing et al. [16]. Twelve 140 cm² tissue culture plates were used for the brown lemur, Coquerel’s mouse lemur and human SWS1 constructs, and 24 140 cm² tissue culture plates for the aye-aye SWS1 constructs. The absorbance spectra of the purified pigments (dark spectra) were measured at pH 7.2 by UV/visible spectrophotometry using a Spectronic Unicam UV500 dual-beam spectrophotometer, treated with either 1 M HCl to a pH < 2.0 or with hydroxylamine (56 mM) and measured again (treated spectra). For each pigment, the treated spectrum was subtracted from the dark spectrum to generate a difference spectrum. This eliminates protein absorbance scatter that may distort the dark spectrum. The λmax was then determined by fitting a standard visual pigment template [32] to the long-wavelength tail of the difference spectrum using a Solver add-in to Microsoft Excel.

3. RESULTS AND DISCUSSION

(a) Absorbance peaks of in vitro expressed pigments
Inspection of the SWS1 opsin sequences of 26 primate species listed in GenBank shows that with one exception, Phe86 found in UVS pigments has been replaced by another residue as follows: Leu86 is consistently found in the Haplorrhini group, whereas the Strepsirrhini show much more variation with seven different amino acids at site 86 across the seven families (table 1). The exception is the retention of Phe86 in another member of the Strepsirrhini, the aye-aye [30], suggesting that this pigment may be UVS.

The prosimian SWS1 pigments selected for spectral analysis were derived from the brown lemur (GenBank AB111464), the aye-aye (GenBank DQ1918903-7) and Coquerel’s mouse lemur (GenBank DQ191898-902), as each SWS1 pigment possesses a different residue at site 86. The brown lemur pigment (with Cys86) gave a λmax at 413 nm (figure 1a), which is substantially shorter than the spectral peak values obtained by electroretinogram (ERG) flicker photometry for both the brown and ring-tailed lemurs at 437 nm [27]. However, such differences in peak absorbance values are not unexpected since ERG recordings arise from light filtered by the cornea and lens falling on the retina and frequently overestimate the spectral peak for SWS cones. The SWS1 pigment from Coquerel’s mouse lemur (with Ser86) gave a λmax of 409 nm (figure 1b). The only other reported pigment with Ser86 is in the elephant with a peak around 422 nm [20], suggesting that other sites may be important for the 13 nm difference between these two mammalian species. Finally, the aye-aye SWS1 pigment (with Phe86) gave a peak at 406 nm within the violet region of the spectrum (figure 1c), which contrasts with all other vertebrate SWS1 pigments with Phe86 that are consistently UVS with λmax values close to 360 nm [33,34].

The other residues encoded at site 86 in prosimians with a functional SWS1 gene are Val86 (e.g. as found in the woolly lemur) and Leu86 (e.g. as found in tarsiers and Coquerel’s sifaka). Since it has already been established in Old and New World primates that SWS1 pigments with Leu86 peak in the violet, the spectral tuning effect of this residue was not examined further. To determine the effect of Val86, the brown lemur SWS1 coding sequence was mutated to encode a Cys86Val substitution. The resulting mutant pigment gave a λmax at 401 nm (figure 1d). This is 12 nm shorter than the absorbance peak value for the brown lemur wild-type pigment but still spectrally tuned to violet wavelengths. The only other mammalian SWS1 pigment with Val86 is in the guinea pig [19] but this has a λmax some 19 nm more long wavelength-shifted; differences at other tuning sites identified from previous studies [29] may be responsible for this shift. Finally, the effect of Phe86 on the λmax of the aye-aye SWS1 pigment was assessed by substitution with Ser. As shown in figure 1e, the resulting pigment was long wavelength-shifted by 10 nm; Phe86 is able, therefore, to shift the short-wavelength λmax of the aye-aye pigment but not into the UV.

(b) Molecular basis for primate violet sensitivity
Previous studies have indicated that the simultaneous replacement of the residues present at seven sites (46, 49, 52, 86, 93, 114 and 118) in the human VS pigment with
Table 1. Residues present at seven sites in transmembrane helices II and III of the VS pigments of primates compared with the UVS pigments of two marsupials, the fat-tailed dunnart (GenBank accession number AY7724723) and honey possum (GenBank accession number AY772472), and two rodents, the mouse (GenBank accession number NM_007538) and rat (GenBank accession number NM_031015). The seven sites were identified by Shi et al. [29] from site-directed mutagenesis experiments. Species in italics possess a SWS1 pseudogene. Alignments of the amino acid sequences that form transmembrane helices 2–3 of the different opsins referred to in this table are presented in electronic supplementary material, figure S1, together with the pigments of the other species referred to in the text.

<table>
<thead>
<tr>
<th>Amino acid sites</th>
<th>dunnart/honey possum</th>
<th>mouse/rat</th>
<th>Old World monkeys</th>
<th>New World monkeys</th>
<th>Tarsier</th>
<th>Aye-aye</th>
<th>Brown lemur</th>
<th>Ring-tailed lemur</th>
<th>Sportive lemur</th>
<th>Coquerel’s silika</th>
<th>Woolly lemur</th>
<th>Grey mouse lemur</th>
<th>Fat-tailed dwarf lemur</th>
<th>Greater dwarf lemur</th>
<th>Lesser galago</th>
<th>Small-eared galago</th>
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*Ala is present in the honey possum, in three of four species of Old World and one of four species of New World monkeys.
those found in the mouse UVS pigment is required to shift the $\lambda_{\text{max}}$ of the human pigment into the UV [29]. However, an examination of these sites in the VS pigments of different primate species shows that only Pro93 is fully conserved across all primate groups (table 1). The other sites show variation in the amino acid present, and there are several instances where the residue present in the UVS pigments of certain rodents [35] and marsupials [14] is also found in primate VS pigments.

If, as seems likely, a single tuning mechanism is responsible for the VS of all primate SWS1 pigments, then the only consistent feature to account for this is Pro93. Substitution at this site may be responsible, therefore, for the long-wavelength shift, regardless of the residue present at site 86. This hypothesis is supported by the observation that in other mammalian pigments, Thr93 is consistently combined with Phe86 in UVS pigments, but it is always replaced in mammalian VS visual pigments by either Val93 as found in the tree shrew, Tupaia belangeri (GenBank accession number: EU487780), and grey squirrel [3], Ile in the cow [16] and elephant [20], Ala in the guinea pig [19] or Ser in the pig [16]. The retention of Thr93 may therefore preclude the generation of a VS pigment.

To investigate this hypothesis, a Pro93Thr mutant was generated in the human pigment. However, although the wild-type pigment gave an in vitro spectral peak at 414 nm as previously reported [29], the Pro93Thr mutant failed to generate a pigment in several repeat experiments (data not shown). As the human SWS1 VS pigment contains Leu86 instead of Phe86, the failure of this mutant pigment to regenerate in vitro is consistent with our hypothesis that UVS pigments require both Phe86 and Thr93. Therefore, we generated a Pro93Thr substitution in the aye-aye SWS1 pigment that naturally exhibits five out of the seven substitutions (Phe46, Phe49, Thr52,
Phe86 and Ser118) used previously [29] to shift the human VS pigment into the UV (table 1). Furthermore, the wild-type aye-aye pigment and the UVS pigment of the dunnart possesses common residues at six out of the seven sites (table 1); the aye-aye Pro93Thr mutant will therefore be identical over these seven tuning sites to that of the dunnart. As shown in figure 1, the absorbance of the aye-aye Pro93Thr mutant pigment was significantly shifted by 35 nm from the wild-type VS pigment (λmax value of 406 nm) into the UV region with a peak at 371 nm. Thus, we conclude that a Pro93 is responsible for the VS of primate SWS1 pigments.

(c) Evolution of violet-sensitive pigments

From the above discussion, it would appear that the residue present at site 86 is not responsible for the VS of primate SWS1 pigments. Rather, substitutions at this site may serve to ‘fine-tune’ the pigments in the violet region of the spectrum. However, it does not necessarily follow that substitution at site 86 was not important in the evolution of VS pigments in primates; an alternative explanation is that a Phe86Tyr substitution occurred at the base of the primate lineage, thereby releasing codon 86 from selective constraint and allowing further changes to occur. To examine this further, the eight different residues found at site 86 in primate SWS1 opsins, together with the corresponding codon sequences, were mapped onto the primate phylogeny reported recently by Perelman et al. [36], together with the tree shrew as the species most closely related to primates for which spectral [37] and sequence data (GenBank accession number EU487780) are available (figure 2). The ancestral Phe residue, as found in non-primate mammalian UVS pigments [14,15,35,38], is encoded by TTC, which is also found in the aye-aye. This codon differs, however, in the SWS1 gene of all other primates. If Phe86 was retained in the ancestral primate and passed unchanged onto the aye-aye, then each residue change at site 86 in the other branches of the tree can be explained by single-step nucleotide changes. The alternative explanation, that a Phe86Tyr substitution occurred at the base of the primate lineage, requires two nucleotide changes in the Tarsiiformes/Simian branch to achieve the Tyr86Leu (TAC to CTC) substitution, and an additional change (TAC to TTC) in the branch to Coquerel’s sifaka and the woolly lemur, which makes this the less parsimonious interpretation of the sequence data. It would also mean that Phe86 in the aye-aye is a back mutation. Within the Lemuriformes, Phe (TTC) is replaced by Ser (TCC) in the Coquerel’s sifaka, the gray mouse lemur, the fat-tailed dwarf lemur, greater dwarf lemur, red-tailed sportive lemur, and Coquerel’s sifaka; Leu (GTC) in the woolly lemur. In the Lorisiformes with non-functional SWS1 genes, Phe is replaced initially by Tyr (TAC), followed by Asn (AAC) in the small-eared and thick-tailed galagos. Finally, there is a synonymous transition (CTC to CTT) in New World monkeys. Overall, therefore, if it is assumed that the substitution of Leu...
and Val in the Lemuriformes were separate events, then there have been either ten or nine amino acid substitutions in the primate lineages depending, respectively, on the presence of either Phe86 or Tyr86 in the ancestral primate. The Phe86Tyr substitution in the tree shrew may also be ancestral to the primate lineage and, as found in many other non-primate species [3,16–20], was almost certainly responsible for the generation of a VS pigment [37,39]. These alternative interpretations are shown in figure 2. Depending, therefore, on the sequence of substitutions at sites 86 and 93, either a Phe86Tyr substitution or a Thr93Pro substitution was the primary change in SWS1 pigments responsible for the evolution of VS in primates.

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