The Journal of Experimental Biology 207, 1479-1486
Published by The Company of Biologists 2004
doi:10.1242/jeb.00913

Immunohistochemical localization of *Papilio* RBP in the eye of butterflies

Motohiro Wakakuwa[1], Koichi Ozaki[2] and Kentaro Arikawa[1,*]

[1]*Graduate School of Integrated Science, Yokohama City University, Yokohama, Kanagawa 236-0027, Japan* and
[2]*Graduate School of Frontier Bioscience, Osaka University, Toyonaka, Osaka 560-0043, Japan*

*Author for correspondence (e-mail: arikawa@yokohama-cu.ac.jp)

Accepted 2 February 2004

Summary

We recently identified a novel retinoid binding protein, *Papilio* RBP, in the soluble fraction of the eye homogenate of the butterfly *Papilio xuthus*, and demonstrated that the protein is involved in the visual cycle. We now have localized the protein in the *Papilio* eye by light and electron microscopic immunohistochemistry using a monospecific antiserum produced against artificially expressed *Papilio* RBP. We found strong immunoreactivity in the primary as well as secondary pigment cells and in the tracheal cells. The pigment cells have long been regarded as an important site of the visual cycle, and this view is further supported by the present result. Interestingly, the cytoplasm and nuclei of these cells were equally labeled, indicating that the protein exists in both the cytoplasm and the nucleus. We conducted a survey for the existence of the *Papilio* RBP-like proteins in other insects including several species of butterflies, dragonflies, cicadas, grasshoppers and honeybees. Anti-*Papilio* RBP immunoreactivity was confirmed in the proteins isolated only from butterflies belonging to the superfamily Papilionoidea and not from other species. In all insects tested, however, fluorescing proteins were clearly detected, suggesting that these insects also have similar retinol-binding proteins.

Key words: butterfly, *Papilio xuthus*, Papilionoidea, retinoid binding protein, immunohistochemistry, 3-hydroxyretinol, pigment cell, tracheal cell, visual cycle.

Introduction

A visual pigment molecule is composed of a protein opsin with an 11-*cis* retinal attached to it. The 11-*cis* retinal absorbs light and changes its configuration to the all-*trans* form, which subsequently causes a conformational change of the protein into an active form, metarhodopsin. The activated visual pigment triggers the phototransduction cascade, whose end result is the change in membrane potential.

Visual pigments will be depleted by prolonged illumination unless they are regenerated and/or newly synthesized. A key process of the visual pigment regeneration, the visual cycle, is to generate 11-*cis* retinal from all-*trans* retinal, retinol and other retinoids. As a result of extensive studies of this process in recent decades, a number of retinoid binding proteins have been identified in both invertebrates and vertebrates (Hara and Hara, 1991; McBee et al., 2001; Ozaki et al., 1987; Saari, 1999; Stavenga et al., 1991). We recently identified a retinol binding protein, *Papilio* RBP, whose ligand is 3-hydroxyretinol, in the compound eye of the Japanese yellow swallowtail butterfly *Papilio xuthus* (Wakakuwa et al., 2003). The *Papilio* RBP is a novel protein, for it has little homology with any other retinoid binding proteins so far reported. We found that the isomer composition of the ligand of *Papilio* RBP changes between light- and dark-adaptation: the content of 11-*cis* isoform increases in eyes in the light. In addition, illumination significantly increases the amount of 11-*cis* isoform, especially in the distal part of the retinal layer (Wakakuwa et al., 2003). This observation fully agrees with the results described in a previous report, where retinoid composition was measured under various conditions of adaptation (Shimazaki and Eguchi, 1995). We thus concluded that the protein is involved in the regeneration of *Papilio* visual pigment whose chromophore is 11-*cis* 3-hydroxyretinal (Wakakuwa et al., 2003).

Where in the eye does the *Papilio* RBP function? Is this novel binding protein strictly specific to *Papilio*, or shared by other species? To answer these questions, we raised a specific antiserum against *Papilio* RBP. We then carried out light and electron microscopic immunohistochemistry in the *Papilio* retina to localize the protein in the retinal tissue. We also performed combined native PAGE and immunoblot analyses on several other insect species to evaluate the distribution of *Papilio* RBP-like protein among insects.

Materials and methods

Animals

Japanese yellow swallowtail butterfly *Papilio xuthus* L., was taken from a cultured population. The population was derived from eggs laid by females caught in the field. The hatched larvae were fed with fresh citrus leaves at 25°C under a light regime of 10 h:14 h light:dark. The pupae were stored at 4°C for at least 3 months and then allowed to emerge at 25°C.

For the comparative study, we used four papilionid species, *Papilio machaon*, *Papilio protenor*, *Papilio helenus* and *Graphium sarpedon*, a nymphalid *Vanessa indica*, a pierid *Pieris rapae*, and a hesperid *Parnara guttata*, captured in the field around the campus of Yokohama City University. We also investigated a dragonfly *Orthetrum albistylum speciosum* (Odonata, Libellulidae), a cicada *Graptopsaltria nigrofuscata* (Hemiptera, Cicadidae), a locust *Oedaleus infemalis* (Orthoptera, Acrididae) and a honeybee *Apis mellifera* (Hymenoptera, Apidae). These insects were captured in the field around the campus of Yokohama City University, except for *Apis mellifera*, which was taken from a hive culture.

Gel electrophoresis

For native PAGE, Laemmli's buffer system was used (Laemmli, 1970) but without SDS and 2-mercaptoethanol in the gel, running and sample buffer solutions. Unless otherwise stated, whole compound eyes were used for extraction. The compound eyes were homogenized in 63 mmol l^{-1} Tris-Cl buffer (pH 6.8), and the homogenate was centrifuged at 15 000 ***g*** for 30 min at 4°C. The supernatant was loaded onto a 10% polyacrylamide gel, and soluble proteins were electrophoretically separated. After electrophoresis, the gel was illuminated with UV light, which visualizes the RBP as a single fluorescing band. The RBPs were recovered from cut pieces of the gel containing the fluorescing band (purified RBP). When necessary, the gel was stained with Coomassie Brilliant Blue (CBB). Regular SDS-PAGE was also carried out using a 12% polyacrylamide gel (Laemmli, 1970). The gel was then stained with CBB.

Antiserum production

To produce antigen by expression, we first carried out overexpression of *Papilio* RBP. We prepared a pair of oligo nucleotide primer (ROLBP-forward, 5′-GTGAAGACATAT-GTCTTCACGAATATATCC-3′; ROLBP-reverse, 5′-GAA-CTCGAGTTCAACTTTTGCCCCAAATATTTTG-3′) based on the full-length cDNA sequence of *Papilio* RBP (Wakakuwa et al., 2003). Using these primers, the entire coding region of *Papilio* RBP (714 bp) was amplified. We subcloned the polymerase chain reaction (PCR) product into the pET-21 expression vector; the vector was designed to enable recombinant expression of the *Papilio* RBP as a fusion protein to which a 6 × His-tag was added at the C-terminal end for one-step purification by nickel chelate affinity chromatography.

Female Wistar rats were immunized intradermally with 0.1 mg of purified recombinant *Papilio* RBP in 200 µl of phosphate-buffered saline emulsified 1:1 with Freund's complete adjuvant (Difco Laboratories, Detroit, USA). The rats were boosted every 2 weeks in a similar manner using *Papilio* RBP in incomplete Freund's adjuvant (Difco Laboratories): the rats were injected antigen six times in total. Immune serum was obtained 7 days after the final boost. Monospecificity of the antiserum was confirmed by immunoblot analysis (see below).

Immunoblot

Water-soluble extracts of retinal homogenates or the RBP purified from the native PAGE gel were separated by 12% SDS-PAGE. The protein samples were then blotted onto a polyvinylidene difluoride (PVDF) membrane. Nonspecific binding sites were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), followed by overnight incubation with the antiserum. After washing with PBS, the PVDF membrane was incubated with alkaline phosphatase-conjugated secondary antibody. After washing with PBS, the PVDF membrane was incubated in 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) in alkaline phosphatase buffer (100 mmol l^{-1} Tris-HCl, pH 9.5, 100 mmol l^{-1} NaCl and 5 mmol l^{-1} $MgCl_2$), until adequate stain intensity was obtained.

Immunohistochemistry

For light microscopic immunohistochemistry, isolated compound eyes were fixed in 2% paraformaldehyde and 0.2% picric acid in 0.1 mol l^{-1} phosphate buffer, pH 7.4 (PB) for 30 min at room temperature. After a brief wash with 0.1 mol l^{-1} PB, the eyes were then dehydrated in a graded ethanol series, infiltrated with xylene and embedded in paraffin. Thin sections (8 µm) mounted on slides were incubated in 0.3% H_2O_2 in water for 5 min to quench endogenous peroxidase activity (Larsson, 1988). Non-specific binding sites for antibodies were blocked by treating the sections with 10% normal goat serum in PBS for 30 min, and then the sections were incubated in the anti-*Papilio* RBP in 1% BSA in PBS overnight at 4°C. The sections were subsequently reacted with biotinylated secondary antibody for 30 min, and further incubated with Vectastain ABC Reagent (Vector Laboratories, Burlingame, USA). After washing with PBS, the sections were incubated in peroxidase substrate solution (0.2% 3,3′-diaminobenzidine in 50 mmol l^{-1} Tris-HCl, pH 7.4) until adequate stain intensity was obtained.

For electron microscopic immunohistochemistry, isolated eyes were prefixed by immersing in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 mol l^{-1} cacodylate buffer, pH 7.3 (CB) for 30 min on ice. After a 10 min wash with 0.1 mol l^{-1} CB, the eyes were postfixed in 2% OsO_4 in 0.1 mol l^{-1} CB for 30 min on ice. After dehydration with graded methanol series, the eyes were embedded in LR White resin. Ultrathin sections, mounted on nickel grids, were treated with 4% BSA in PBSG (0.25% fish gelatin in PBS) for 20 min to block non-specific antibody binding sites, and then incubated with anti-*Papilio* RBP in PBSG overnight at 4°C. After washing with PBSG, the sections were reacted with secondary antibody-conjugated 15 nm colloidal gold particles for 1 h. After washing with PBSG, the sections were stained with 4% uranyl acetate in distilled water, and observed in a transmission electron microscope (JEM 1200EX; Tokyo, Japan).

Results

Antibody specificity (immunoblot)

Monospecificity of the produced polyclonal antiserum

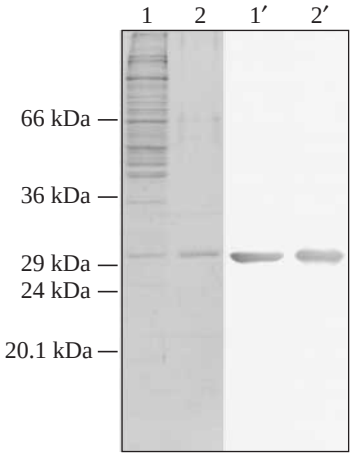

Fig. 1. SDS-PAGE (lanes 1, 2) and immunoblot (lanes 1′, 2′) analyses of the soluble fraction of the *Papilio* compound eye homogenate (lanes 1, 1′) and purified *Papilio* RBP (lanes 2, 2′). Anti-*Papilio* RBP antiserum monospecifically detected *Papilio* RBP at about 31 kDa.

against purified recombinant *Papilio* RBP was confirmed by immunoblot analysis. Fig. 1 shows the results of SDS-PAGE and the immunoblot. Immunoblot labeled with the antiserum revealed a single band of about 31 kDa, in both the retinal homogenate and the purified *Papilio* RBP, which was immunoreactive to the antiserum. The band was not detected by non-immune serum (data not shown). These results indicate that the antiserum detects *Papilio* RBP monospecifically. We therefore refer to the antiserum as anti-*Papilio* RBP.

Localization of Papilio *RBP in the eye of* Papilio xuthus

We studied the possible localization of *Papilio* RBP *via* the distribution of anti-*Papilio* RBP immunoreactivity in the compound eye (Fig. 2). An ommatidium of a *Papilio* eye consists of a dioptric apparatus, the corneal facet lens and crystalline cone, and a retinula, containing nine photoreceptor cells (Fig. 2A). The photoreceptor cells together construct a photoreceptive rhabdom, which is a long, slender cylinder in the center of the ommatidium. Neighboring ommatidia are optically separated by the primary and secondary pigment cells, both containing dark-brown pigment granules. The primary pigment cells wrap the crystalline cones, whereas the secondary pigment cells are located between the ommatidia along the entire length of the retinal layer. We found that anti-*Papilio* RBP labeled these pigment cells but not the photoreceptor cells (Fig. 2B,C). The anti-*Papilio* RBP strongly labeled the cytoplasm of the tracheal cells, proximal of the basement membrane, forming the fenestrated layer between the retina and the lamina, i.e. the first optic ganglion (Fig. 3D). Non-immune serum gave no labeling (Fig. 3B inset).

The subcellular localization of *Papilio* RBP was further studied by electron microscopic immunohistochemistry. The results fully confirmed the light microscopical findings. Fig. 3 shows five pairs of electron micrographs, each consisting of

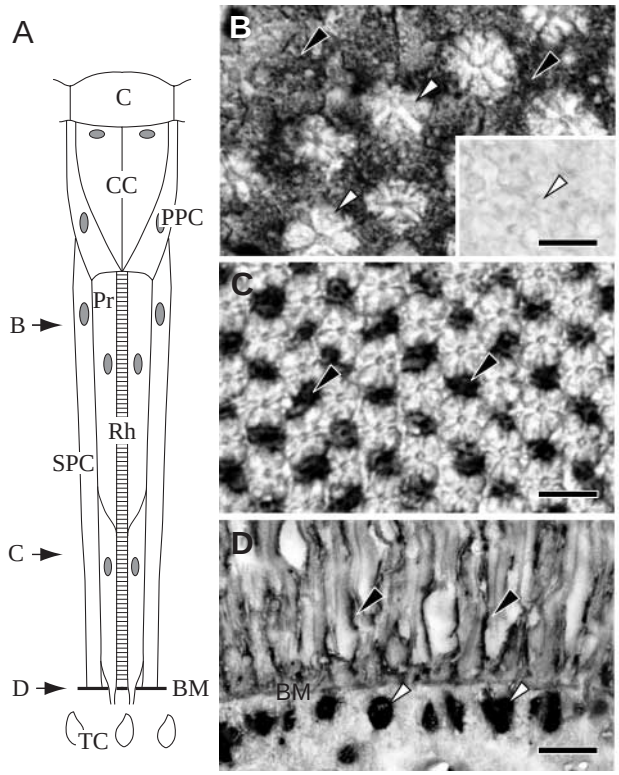

Fig. 2. Light microscopic immunohistochemistry using the anti-*Papilio* RBP in the retina of *Papilio xuthus*. (A) Schematic drawing of an ommatidium. Longitudinal view. Arrows on the left indicate the approximate locations where sections B–D were obtained. BM, basement membrane; C, corneal facet lens; CC, crystalline cone; PPC, primary pigment cell; Pr, photoreceptor; Rh, rhabdom; SPC, secondary pigment cell; TC, tracheal cell. (B) Cross section through the distal portion of the retina. The pigment cells (black arrowheads) were labeled. Photoreceptors (white arrowheads) were not labeled. Non-immune serum gave no labeling (inset). (C) Transverse section through the proximal part of the retina. The secondary pigment cells (black arrowheads) were labeled, but no photoreceptors were labeled. (D) Longitudinal section around the basement membrane (BM). The tracheal cells (white arrowheads) as well as the secondary pigment cells (black arrowheads) were strongly labeled. Scale bars, 30 μm.

one at low magnification and one at high magnification. In the retinal layer the gold particles, which indicate the localization of anti-*Papilio* RBP, were exclusively found in the pigment cells and not in the photoreceptor cells (Fig. 3A–H). Interestingly, the anti-*Papilio* RBP labeled the nuclei as well as the cytoplasm of the pigment cells at similar density (Fig. 3E–H). Electron microscopy revealed that the strongly labeled structures proximal to the basement membrane (Fig. 2D) are the nuclei and the cytoplasm of the tracheal cells (Fig. 3I,J). The photoreceptor axons, which pass through the basement membrane, were not labeled.

We quantified the labeling density in different regions of the eye (Fig. 4). The labeling of the pigment cells is significantly higher than the background labeling of the photoreceptor cells (*, $P<0.01$, one-way ANOVA, Tukey test). In the primary and

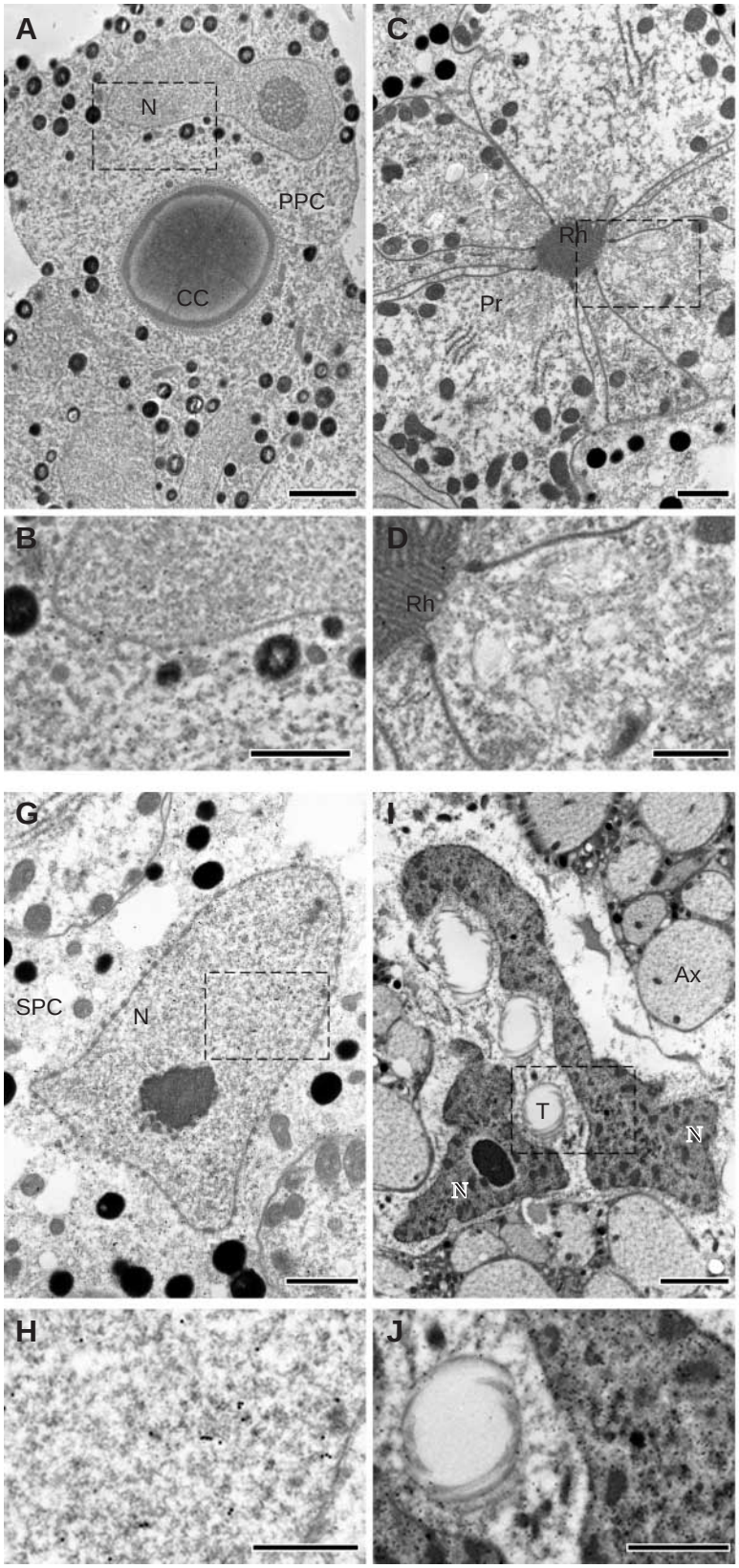

Fig. 3. Electron miscroscopic immunohistochemistry using anti-*Papilio* RBP secondary antibody conjugated to 15 nm colloidal gold. The areas marked by broken squares in A, C, E, G and I are magnified in B, D, F, H and J, respectively. (A,B) Primary pigment cells (PPC) surround the crystalline cone (CC). Both the cytoplasm and the nucleus (N) were densely labeled. (C,D) Photoreceptor cells (Pr). No labeling was found in the cell body of the photoreceptor or in the rhabdom (Rh). (E,F) Secondary pigment cells (SPC) fill the space between photoreceptors (Pr). The cytoplasm of the secondary pigment cell was strongly labeled. (G,H) The nucleus (N) of the secondary pigment cell was also strongly labeled. (I,J) Tracheal cells. Labeling was found both in the cytoplasm surrounding the tracheole (T) and in the nucleus (N). Photoreceptor axons (Ax) were not labeled. Scale bars, 2 μm (A,I); 1 μm (B,C,E,G,J); 500 nm (D,F,H).

the secondary pigment cells, both nuclei and the cytoplasm were equally labeled. The strongest labeling was found in the tracheal cells, where the calculated particle density was even higher in the nucleus than in the cytoplasm. This is in fact due to the uneven labeling pattern in the cytoplasm: labeling was rather confined to the region where more electron-dense

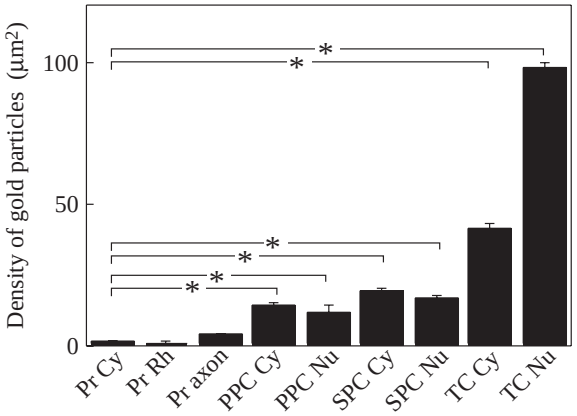

Fig. 4. Comparison of the labeling density quantified by particle density in the electron miscroscopic immunohistochemistry. Values are means ± S.E.M. of the particle density in nine separate regions. *Statistically significant difference (P<0.01, one-way ANOVA, Tukey test). For abbreviations, see Fig. 2. Cy, cytoplasm; Nu, nucleus.

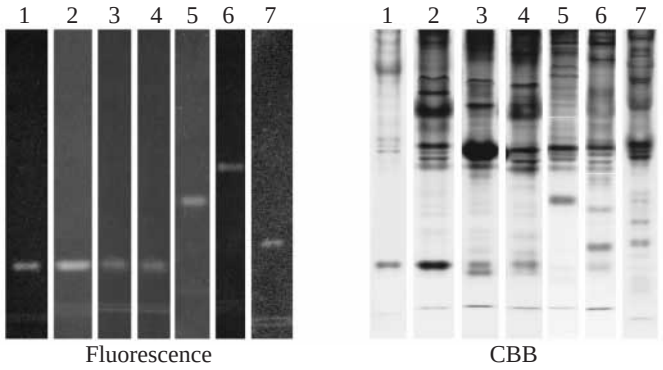

Fig. 5. Native PAGE of crude retinal extract of lepidopteran species belonging to the superfamily Papilionoidea. Fluorescence under UV light (left); Coomassie Brilliant Blue (CBB) staining (right). Lane 1, *Papilio xuthus* (Papilionidae); 2, *Papilio machaon* (Papilionidae); 3 *Papilio protenor* (Papilionidae); 4, *Papilio helenus* (Papilionidae); 5, *Graphium sarpedon* (Papilionidae); 6, *Vanessa indica* (Nymphalidae); 7, *Pieris rapae* (Pieridae).

materials are concentrated (data not shown). Local labeling densities in the nucleus and the cytoplasm were comparable (see for example Fig. 3J). Although the labeling density is higher in tracheal cells than in all other cells observed (Fig. 4), this does not contradict our previous results that the *Papilio* RBP is mainly distributed in the distal retinal layer (Wakakuwa et al., 2003): the total volume of the pigment cells is overwhelmingly large compared to that of the tracheal cells.

Papilio *RBP-like proteins in other insect species*

Fig. 5 shows the results of the native PAGE analysis of crude retinal extracts from seven butterfly species belonging to the superfamily Papilionoidea. We identified a single band emitting whitish fluorescence under UV illumination in all species tested (Fig. 5). The mobility of the fluorescing proteins in the native gel seems genus dependent. Mobilities in species of the genus *Papilio* are similar, whereas those of *Graphium* (Papilionidae), *Vanessa* (Nymphalidae) and *Pieris* (Pieridae) are distinctly lower.

Fig. 6 shows results of immunoblot analysis of these proteins. We first cut out the gel piece containing the fluorescing band. The proteins were then extracted from the gel pieces and separated by SDS-PAGE. The anti-*Papilio* RBP revealed a single band in all species. In the Papilionid species the molecular mass of these proteins was about 31 kDa and, in *Vanessa* and *Pieris* it was 25 kDa. Although in *Vanessa* three bands were evident in the SDS-PAGE, the anti-*Papilio* RBP detected only one of them at 25 kDa.

We also searched for *Papilio* RBP-like proteins in the eye of insects other than Papilionoidea, namely a skipper *Parnara guttata* (Lepidoptera, Hesperiidae), a dragonfly *Orthetrum albistylum speciosum* (Odonata, Libellulidae), a cicada *Graptopsaltria nigrofuscata* (Hemiptera, Cicadidae) and a grasshopper *Oedaleus infemalis* (Orthoptera, Acrididae). We identified a fluorescing band in all of these species under UV illumination on native PAGE (Fig. 7). However, none of them cross-reacted with the anti-*Papilio* RBP on immunoblots. We also checked whether the honeybee *Apis mellifera* (Hymenoptera, Apidae) has a corresponding protein, but we could not detect any fluorescing band on the native gel and any protein reacting with the anti-*Papilio* RBP on the immunoblot (data not shown).

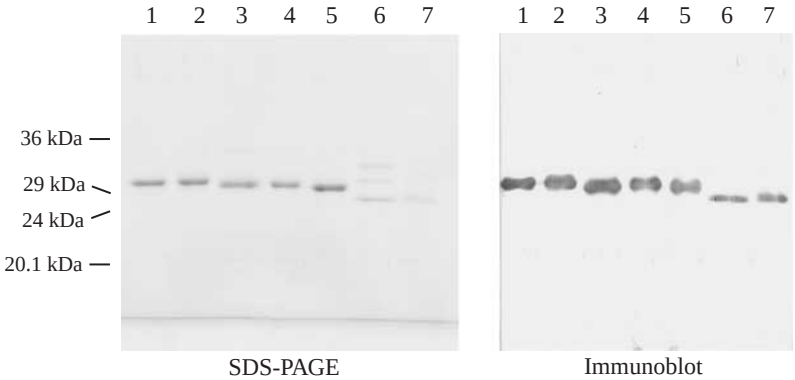

Fig. 6. SDS-PAGE (left) and immunoblot (right) analyses using purified fluorescing proteins from the native gel shown in Fig. 5. Lane 1, *Papilio xuthus*; 2, *Papilio machaon*; 3, *Papilio protenor*; 4, *Papilio helenus*; 5, *Graphium sarpedon*; 6, *Vanessa indica*; 7, *Pieris rapae*. Except for *Vanessa indica*, the purified fluorescing proteins appeared as single bands. The molecular masses of these proteins were between 25 kDa and 31 kDa, all of which were detected by the anti-*Papilio* RBP. In *Vanessa indica*, only the 25 kDa protein was detected by anti-*Papilio* RBP.

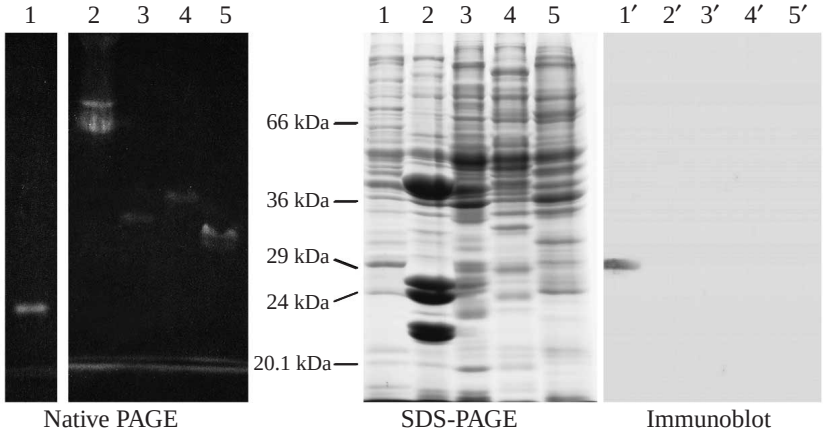

Fig. 7. Native PAGE (left), SDS-PAGE (middle) and immunoblot (right) analyses of the crude retinal extracts of various insect species. Lane 1, *Papilio xuthus* as the control; 2, *Parnara guttata* (Hesperidae, Lepidoptera); 3, *Orthetrum albistylum speciosum* (Libellulidae, Odonata); 4, *Graptopsaltria nigrofuscata* (Cicadidae, Hemiptera); 5, *Oedaleus infemalis* (Acrididae, Orthoptera). No proteins from insects other than *Papilio xuthus* cross-reacted with the anti-*Papilio* RBP.

Localization of Papilio *RBP-like protein in the eye of* Pieris rapae

Fig. 8 shows the localization of the anti-*Papilio* RBP imunoreactivity in the eye of *Pieris rapae*. As in *Papilio xuthus*, specific labeling was confined to the primary pigment cells, the secondary pigment cells and the tracheal cells proximal to the basement membrane. No labeling was detected in the photoreceptor cells.

Discussion

Localization and function of Papilio *RBP in the eye of butterflies*

We found a strong *Papilio* RBP-like immunoreactivity in the retinal primary and secondary pigment cells (Fig. 2). This finding supports the presently accepted view that the primary pigment cells are somehow involved in the visual cycle (Schwemer, 1989, 1993; Smith and Goldsmith, 1991). Of course, so far we have no direct evidence to indicate that the primary pigment cells are the site where the visual cycle is taking place in *Papilio*. However, the distal portion of the *Papilio* retina functions in the light-dependent oxidation of 3-hydroxyretinol and isomerization of 3-hydroxyretinal (Shimazaki and Eguchi, 1993). We have also demonstrated that *Papilio* RBP is involved in retinoid metabolism in the distal portion of the eye (Wakakuwa et al., 2003). Taken together, the primary as well as the secondary pigment cells are probably also involved in the visual cycle in the *Papilio* eye. Identification and localization of respective enzymes for the visual cycle, as in honeybees (Smith and Goldsmith, 1991), would provide further support for the view.

The principal function of the pigment cells is to optically isolate the ommatidia by absorbing off-axis incident light, so to optimize spatial resolving power. The light-driven isomerase, which may exist in the primary pigment cells (e.g. Smith and Goldsmith, 1991), therefore, occupies an ideal location for receiving light, so to serve the visual pigment regeneration cycle. The secondary pigment cells are elongated in shape, lying between photoreceptor cells along the entire length of the retinal layer (Fig. 2). Assuming that these cells

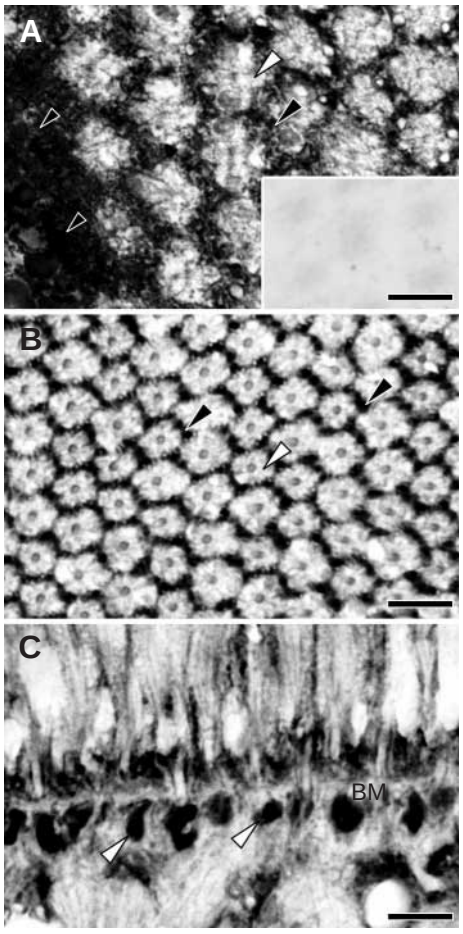

Fig. 8. Light microscopic immunohistochemistry using the anti-*Papilio* RBP in the retina of *Peris rapae*. (A) Transverse section of distal region of the retina. Both primary pigment cells (small arrowheads) and secondary pigment cells (arrowheads) were labeled. Photoreceptors (white arrowhead) were not labeled. Non-immune serum gave no labeling (inset). (B) Transverse section of the proximal region of the retina. The secondary pigment cells (black arrowheads) were labeled, but the photoreceptors were not (white arrowheads). (C) Longitudinal section around the basement membrane (BM). The tracheal cells (white arrowheads) were strongly labeled. Scale bars, 30 μm.

are also involved in the visual cycle, a possible function of the *Papilio* RBP then is to remove retinoid in the all-*trans* form from the photoreceptors and/or to supply retinoid in the 11-*cis* form back to the photoreceptors, to replenish visual pigment molecules.

The function of *Papilio* RBP in the tracheal cells is difficult to understand at present. Of course, the tracheae are not restricted to the eye, but exist throughout the body. We carried out a preliminary immunoblot analysis on the abdominal tracheae and found slight immunoreactivity (data not shown): maybe the *Papilio* RBP has some function specific to the tracheal system, or it may function in transporting retinoids from and to the haemolymph in turnover and *de novo* synthesis.

Even more conspicuously, *Papilio* RBP-like immunoreactivity was found in the nuclei of both the pigment cells and the tracheal cells. To the best of our knowledge, this is the first example of retinoid-binding proteins in cell nuclei.

Comparative aspects

Is *Papilio* RBP specific to *Papilio*, or is it shared by other species? This point is particularly important for elucidating a general scheme of the function of *Papilio* RBP in the visual cycle. With this question in mind, we carried out a comparative biochemical and immunohistochemical analysis in several other insect species.

In native PAGE, we detected a fluorescing band in all tested butterfly species. According to Seki et al. (1987), butterflies use 3-hydroxyretinal as the visual pigment chromophore and most of them also contain an excess amount of 3-hydroxyretinol. Presumably therefore, the fluorescing substance in tested butterfly species is 3-hydroxyretinol. The mobility of the fluorescing proteins in the native gel differs considerably, indicating that their surface charge, size and/or the three-dimensional structure rather vary. The proteins with lower mobilities may exist in certain polymerized forms. In addition to the fluorescence, the proteins were found to be immunoreactive to the anti-*Papilio* RBP in all butterflies, except for the skipper *Parnara guttata* (Hesperiidae). The molecular mass of the protein revealed by SDS-PAGE appeared to be similar in seven other butterflies (25–31 kDa; Fig. 6). In *Vanessa indica*, however, three bands were evident on SDS-PAGE (Fig. 6), probably due to overlap of three proteins in the location of the fluorescing protein (Fig. 5). Immunohistochemical localization of the *Papilio* RBP-like protein in the *Pieris* eye, using the anti-*Papilio* RBP, revealed that the distribution pattern is similar in *Pieris* and *Papilio*, indicating a similar function in both species (Figs 2 and 8).

Our data on the *Papilio* RBP-like protein in other insects is still preliminary. For example although we found a fluorescing protein band in the gel (Fig. 7), we have not yet identified the fluorescing materials themselves. These insects have actually been shown to have significant amounts of retinol and/or 3-hydroxyretinol in the eye (Seki et al., 1987, 1989). Therefore the proteins detected here probably have the ability to bind retinols, although they do not bind to the anti-*Papilio* RBP.

We conclude that the *Papilio* RBP-like protein is shared by butterflies belonging to the superfamily Papilionoidea. In addition, native PAGE indicated that other insects possess their own RBPs. These findings suggest that insects may share a basic pathway of visual cycle to regenerate rhodopsin. Nevertheless, the molecular characteristics of RBP could not be identical between species (or at least beween genuses) (Figs 5 and 7). Such variability in the protein properties may reflect structural variability in the rhabdom among species, because the visual cycle involves the removal and incorporation of visual pigments from and to the rhabdom. In fact many butterflies have apposition eyes with photoreceptors with small rhabdoms, whereas the eyes of nocturnal species are of the superposition type containing photoreceptors with large rhabdoms (Eguchi, 1978). Different rhabdoms would require a somewhat different mechanism for removal and incorporation of the visual pigment molecules. At any rate, elucidation of the function of the *Papilio* RBP and *Papilio* RBP-like proteins in the visual cycle requires further study, including immunohistochemical localization and the uncovering of other enzymes involved in the process.

We thank to Dr Doekele G. Stavenga for critical reading of the manuscript. This work was supported by the Sasagawa Grant for Scientific Research from the Japan Science Society to M.W., Grants-in-Aid for Scientific Research from Japan Society for the Promotion of Science (JSPS) to K.A. and to K.O.

References

Eguchi, E. (1978). Comparative fine structure of lepidopteran compound eyes, especially skippers (Hesperioidea). *Zool. Mag.* **87**, 32-43.

Hara, T. and Hara, R. (1991). Retinal-binding protein: function in a chromophore exchange system in the squid visual cell. In *Progress in Retinal Research*, vol. 10 (ed. N. Osborne and G. J. Chader), pp. 179-206. Oxford-New York: Pergamon Press.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Larsson, L. (1988). *Immunocytochemistry: Theory and Practice*. Boca Raton-Florida: CRC Press.

McBee, J. K., Palczewski, K., Baehr, W. and Pepperberg, D. R. (2001). Confronting complexity: the interlink of phototransduction and retinoid metabolism in the vertebrate retina. *Prog. Ret. Eye Res.* **20**, 469-529.

Ozaki, K., Terakita, A., Hara, R. and Hara, T. (1987). Isolation and characterization of a retinal-binding protein from the squid retina. *Vision Res.* **27**, 1057-1070.

Saari, J. C. (1999). Retinoids in mammalian vision. In *Retinoids: The Biochemical and Molecular Basis of Vitamin A and Retinoids Action*, vol. 139 (ed. H. Nau and W. S. Blaner), pp. 563-588. Berlin: Springer-Verlag.

Schwemer, J. (1989). Visual pigments of compound eyes – structure, photochemistry, and regeneration. In *Facets of Vision* (ed. D. G. Stavenga and R. C. Hardie), pp. 112-133. Berlin-Heidelberg: Springer-Verlag.

Schwemer, J. (1993). Visual pigment renewal and the cycle of the chromohphore in the compound eye of the blowfly. In *Sensory Systems of Arthropods* (ed. K. Wiese and F. G. Gribakin). Berlin-Heidelberg: Springer-Verlag.

Seki, T., Fujishita, S., Ito, M., Matsuoka, N. and Tsukida, K. (1987). Retinoid composition in the compound eyes of insects. *Exp. Biol.* **47**, 95-103.

Seki, T., Fujishita, S. and Obana, S. (1989). Composition and distribution of retinal and 3-hydroxyretinal in the compound eye of the dragonfly. *Exp. Biol.* **48**, 65-75.

Shimazaki, Y. and Eguchi, E. (1993). Synthesis of 3-hydroxyretinal in the cytosol of the butterfly compound eye. *Vision Res.* **33**, 155-163.

Shimazaki, Y. and Eguchi, E. (1995). Light-dependent metabolic pathway of

3-hydroxyretinoids in the eye of a butterfly, *Papilio xuthus*. *J. Comp. Physiol. A* **176**, 661-671.

Smith, W. C. and Goldsmith, T. H. (1991). Localization of retinal photoisomerase in the compound eye of the honeybee. *Vis. Neurosci.* **7**, 237-249.

Stavenga, D. G., Schwemer, J. and Hellingwerf, K. J. (1991). Visual pigments, bacterial rhodopsins, and related retinoid-binding proteins. In *Photoreceptor Evolution and Function*, vol. 139 (ed. M. G. Holmes), pp. 261-349. London: Academic Press.

Wakakuwa, M., Arikawa, K. and Ozaki, K. (2003). A novel retinol-binding protein in the retina of the swallowtail butterfly, *Papilio xuthus*. *Eur. J. Biochem.* **270**, 2436-2445.