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Light-dependent Structural Change of Chicken Retinal Cryptochrome4*

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Background: Photoreaction and localization of vertebrate cryptochrome are not well understood.
Results: We found that chicken cryptochrome4 is expressed in the retina. The carboxyl terminus of chick retinal cryptochrome4 bound with a specific antibody in a light-dependent manner.
Conclusion: Molecular accessibility of the carboxyl-terminal region of chick retinal cryptochrome4 changes upon light illumination.
Significance: Nonmammalian vertebrate cryptochromes are likely involved in light-dependent physiology in the retina.

Animals have several classes of cryptochromes (CRYs), some of which function as core elements of circadian clockwork, circadian photoreceptors, and/or light-dependent magnetoreceptors. In addition to the circadian clock genes *Cry1* and *Cry2*, nonmammalian vertebrates have the *Cry4* gene, the molecular function of which remains unknown. Here we analyzed chicken CRY4 (cCRY4) expression in the retina with *in situ* hybridization and found that cCRY4 was likely transcribed in the visual pigment cells, cells in the inner nuclear layer, and retinal ganglion cells. We further developed several monoclonal antibodies to the carboxyl-terminal extension of cCRY4 and localized cCRY4 protein with immunohistochemistry. Consistent with the results of *in situ* hybridization, cCRY4 immunoreactivity was found in visual pigment cells and cells located at the inner nuclear layer and the retinal ganglion cell layer. Among the antibodies, one termed C1-mAb had its epitope within the carboxyl-terminal 14-amino acid sequence (QLTRDDADDPMEMK) and associated with cCRY4 in the retinal soluble fraction more strongly in the dark than under blue light conditions. Immunoprecipitation experiments under various light conditions indicated that cCRY4 from the immunocomplex formed in the dark dissociated from C1-mAb during blue light illumination as weak as 25 μW/cm^2 and that the release occurred with not only blue but also near UV light. These results suggest that cCRY4 reversibly changes its structure within the carboxyl-terminal region in a light-dependent manner and operates as a photoreceptor or magnetoreceptor with short wavelength sensitivity in the retina.

Cryptochrome/photolyase family genes have been found in a wide variety of living organisms such as bacteria, fungi, plants, and animals (1–3). Cryptochromes (CRYs)[2] in plants and invertebrates are shown to play important roles in photomorphogenesis and circadian photoreception, respectively (1, 2). In *Drosophila* circadian photoreception, illumination triggers interaction between CRY (dCRY) and TIMELESS (dTIM), both of which are important factors for circadian clock oscillation and resetting (4). Recent studies have shown that dCRY serves a purpose in both photoresponse in neural cells (5) and light-dependent magnetoreception (6), implying that there are multiple dCRY-dependent sensory systems in *Drosophila*. Contrary to the fruit fly, the photoreceptive or magnetoreceptive properties of cryptochromes remain elusive in vertebrate species. Vertebrates commonly retain two cryptochrome types, CRY1 and CRY2, both of which inhibit transactivation by CLOCK and BMAL through E-box cis-elements in clock-controlled genes (7). Neither mammalian CRY1 nor CRY2 is likely involved in photoreception, but nonmammalian CRYs are implied to have photoreceptive properties as well as transcriptional inhibitory functions: chicken CRY1 and CRY2 (cCRY1 and cCRY2) are suggested to operate a circadian clock oscillator in the pineal gland (8) as well as light-dependent pupillary contraction in the embryonic chick iris (9), although little is known about the downstream phototransduction pathway. These previous investigations suggest that CRY1 and CRY2 lost its photoreceptive functions in the course of vertebrate evolution.

Our focus has been on the *Cry4* vertebrate cryptochrome found in many nonmammalian vertebrates, and elucidation of its function. We previously analyzed the mRNA levels of chicken *Cry4* (*cCry4*) in a variety of chick tissues using RT-PCR and revealed that cCRY4 is highly expressed in photosensitive neural tissues, the pineal gland and retina (10). Anti-cCRY4 polyclonal antibody revealed a soluble nature in cCRY4 and localized cCRY4 immunoreactivities in the pineal gland (10), and it was suggested that cCRY4 might operate as a photoreceptor in the pineal gland together with another pineal photo-

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[2] The abbreviations used are: CRY, cryptochrome; CCT, cryptochrome carboxyl-terminal region; cCRY, chicken CRY; dCRY, *Drosophila* CRY; INL, inner nuclear layer; LED, light-emitting diode; MBP, maltose-binding protein; RGC, retinal ganglion cell; VPC, visual pigment cell.

receptor pinopsin (11). Although the pineal cells are suitable for the study of circadian biology and its input pathways for intrinsic photoreceptive ability (12), the retina is better suited for protein analysis of cCRY4 when size is a consideration. Furthermore, the mRNA expression level of *cCry4* in the retina is higher than that of the pineal gland (10).

In the present study, we also prepared a monoclonal antibody (mAb) to cCRY4, estimated the expression level of cCRY4 protein in the retina, and investigated the cellular localization of cCRY4 mRNA and protein in the retina. The monoclonal antibody enabled us to detect light-dependent structural change of cCRY4 in chick retinal soluble fraction. Localization of the epitope of mAb suggested a structural change in the cCRY4 carboxyl-terminal region upon photoreception.

EXPERIMENTAL PROCEDURES

Animals and Tissues—All studies were approved by the Committee for Animal Experimentation of the School of Science and Engineering at Waseda University (permission 08A02, 09A01, 10A01, WD10-91, WD11-84, 2011-A077). One-day-old male chicks were purchased from local suppliers. The chicks were housed under a 12-h light/dark cycle with a light intensity of approximately 1,000 lux and used for experiments within a week.

Immunoblot Analysis—Immunoblot analysis was performed as described previously (10) except that 1% skim milk (Difco) in TBS (50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM $MgCl_2$) was used instead of Block Ace (DS Pharma Biomedical) in some experiments. Signals were detected using either AttoPhos fluorescent substrate (Roche Applied Science) and Typhoon 9410 scanner (GE Healthcare) or using CDP-Star Reagent (NEB) and Hyperfilm ECL (GE Healthcare).

Monoclonal Antibody—MBP-CCT, a fusion protein consisting of maltose-binding protein and the CCT (cryptochrome carboxyl-terminal region) of cCRY4 (Asp^{470}–Thr^{529}) was used as an antigen for mouse immunization. Hybridoma cells from spleen cells of the immunized mice and P3U1 myeloma were screened using GST-CCT (a fusion protein consisting of glutathione *S*-transferase and the CCT region). Those cell lines producing anti-cCRY4 antibodies were further analyzed using immunoblot and ELISA.

In Situ Hybridization—A 331-bp DNA fragment of the chicken *Cry4* cDNA (nucleotides 1159–1489 of AY300013) was amplified using PCR from the cCRY4 cDNA and subcloned into the pMD20-T vector (Takara Bio). A digoxigenin-labeled RNA probe was prepared using the DIG RNA Labeling Kit (Roche Applied Science). Retinal frozen sections (14–16 μm) were fixed with 4% paraformaldehyde and incubated with 10 μg/ml proteinase K (Merck) for 20 min at 37 °C. The sections were treated in 0.1 M triethanolamine (pH 8.0) for 5 min and 0.25% acetic anhydride/0.1 M triethanolamine for 10 min for acetylation. After washing with 0.1 M sodium phosphate (pH 7.4), the sections were dehydrated through a graded ethanol series. Hybridization buffer (50% formamide, 5% dextran sulfate, 0.2 mg/ml yeast tRNA (Sigma), 1× Denhardt's solution, 0.6 M NaCl, 0.25% SDS, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5)) was preincubated for 1 h at 65 °C and mixed with the digoxigenin-labeled RNA probe (3 μg/ml) for 30 min at 72 °C. The sections were hybridized in a humid chamber for 16 h at 60 °C in hybridization buffer containing the probe. Slides were then washed in 5× SSC (1× SSC; 150 mM NaCl, 15 mM sodium citrate (pH 7.0)) for 5 min at 60 °C, in 2× SSC/50% formamide for 30 min at 60 °C, in NTE buffer (0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA) for 10 min at 37 °C, and then in 10 μg/ml RNaseA/NTE buffer for 10 min at 37 °C; this was followed by wash in NTE buffer for 10 min at 37 °C, 2× SSC for 20 min at 60 °C, 0.2× SSC (two times), and NT buffer (150 mM NaCl, 100 mM Tris-HCl) for 5 min. The sections were blocked with blocking solution (1% blocking reagent (Roche Applied Science), 0.1 M maleic acid, 150 mM NaCl (pH 7.5)) for 60 min. The hybridized probe was detected with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Applied Science) and visualized with a color product from substrates 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

Immunohistochemistry—Retinas were collected under light conditions from chicks a few days old and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, and the retinal sections were analyzed as described previously (10, 13). Briefly, 10-μm thick retinal sections were treated with blocking solution (PBS (10 mM sodium phosphate (pH 7.4), 140 mM NaCl, 1 mM $MgCl_2$) containing 0.05% Triton X-100 and 1.5% horse normal serum) for 1 h at room temperature and then incubated for 16 h at 4 °C with the primary antibody (1 μg/ml C1-mAb or mouse IgG). After washing sections with the blocking solution at room temperature, the sections were successively incubated with a biotinylated anti-mouse IgG and avidin-biotin complex solution using the Vectastain Elite ABC kit (Vector Laboratories). Positive signals were visualized by incubating the slides for 5 min in TBS containing 0.1% diaminobenzidine and 0.02% H_2O_2.

Light Irradiation—The irradiation apparatus using light-emitting diodes (LEDs) and the spectroscopic characteristics were the same as described previously (14). Peak wavelengths were 405 nm (near UV), 471 nm (blue), 542 nm (green), and 634 nm (red).

Immunoprecipitation and Light-induced Elution of Chick Retinal cCRY4—Chick retinas were homogenized in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol, 10% (v/v) glycerol, 0.1% Tween 20, and Complete EDTA free protease inhibitor mixture (Roche Applied Sciences)) and centrifuged at 20,000 × *g* for 30 min to obtain chick retinal soluble fraction. This fraction contains a part of peripheral membrane proteins that weakly bound to lipid membranes as well as cytoplasmic soluble proteins. Anti-cCRY4 C1 antibody was conjugated to the Affi-Gel Hz beads (Bio-Rad) according to the manufacturer's instructions and mixed with the chick retinal soluble fraction. Unbound materials were removed after incubation at 4 °C, and the gel was washed with wash buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 0.1% Tween 20) five times. Bound materials were eluted using incubation under various conditions and analyzed with immunoblotting.

RESULTS

CRY4 Is Highly Expressed in the Chicken Retina—In a previous study, we investigated cCRY4 mRNA levels in a variety of

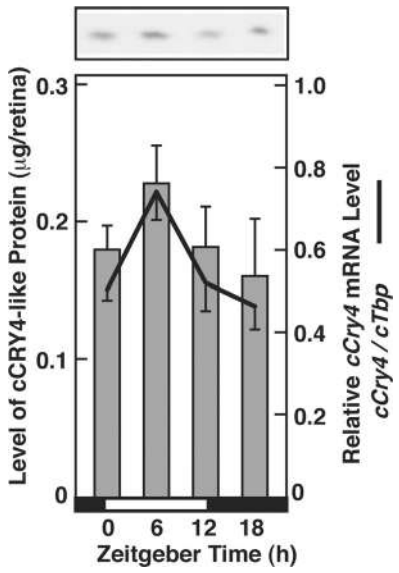

FIGURE 1. **Expression analysis of *cCry* transcripts and cCRY4 protein.** Temporal variation of levels of cCRY4-like immunoreactivity in the retina in LD. Chick retinal homogenates were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with an anti-cCRY4 CCT polyclonal antibody. BlockAce was used for blocking and dilution of antibodies. A representative blotting image is shown in the *upper panel*. *Bars* in the *lower panel* represent levels of cCRY4-like immunoreactivity (mean ± S.E. (*error bars*), $n = 3$) estimated by comparing their signals with those of various amounts of MBP-CCT fusion proteins. Proteins equivalent to 0.01 retina were loaded in each *lane*. For comparison, the chick retinal *cCry4* mRNA profile in LD was reproduced from Kubo *et al.* (10).

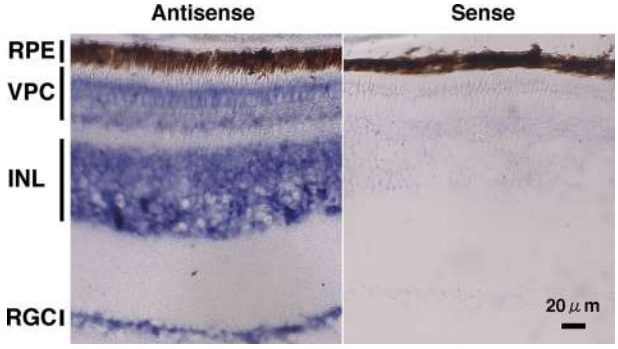

FIGURE 2. ***In situ* hybridization of *cCry4* in the chicken retina.** *Left*, antisense probe; *right*, sense-probe (control). *RPE*, retinal pigment epithelium.

chick tissues using real time quantitative RT-PCR analysis (10) and found that the levels of *cCry4* mRNA were highest in the retina when *cCry4* mRNA levels at different time points were averaged for each tissue.

In this study, we first quantified the levels of cCRY4 proteins in the total homogenate of the chick retina every 6 h under light-dark conditions (Fig. 1) using a polyclonal antibody to the CCT region of cCRY4 (Asp^{470}–Thr^{529}). This region corresponds to the carboxyl-terminal extension in which CRYs have highly diverged amino acid sequences. There was no significant daily change, but there was consistency with a profile similar to that of *cCry4* mRNA having a small peak at ZT6. The expression level of cCRY4 was estimated to be ~180 ng/retina when using different amounts of MBP-CCT fusion protein as a standard.

Cry4 mRNA Is Transcribed in Multiple Layers of Chick Retinal Cells—The cellular localization of cCRY4 was examined by investigating its mRNA expression. The retina is composed of multiple types of neural and non-neural cells, and through *in situ* hybridization, strong signals were detected in the retinal ganglion cell (RGC) layer, inner nuclear layer (INL), and visual pigment cells (VPCs, Fig. 2).

Monoclonal Antibodies to Chicken CRY4 Protein—We established several hybridoma cell lines producing monoclonal antibodies to the CCT region using the MBP-CCT antigen. To verify the availability of these monoclonal antibodies, they were subjected to immunoblot analysis using C-terminally tagged cCRYs (cCRY1-1D4, cCRY2-1D4, and cCRY4-1D4; Fig. 3*A*, *lanes 4–6* and *10–12*) or nontagged cCRYs expressed in cultured mammalian HEK293 cells (Fig. 3*A*, *lanes 1–3*). The 1D4-tagged proteins were expressed to compare the expression levels of cCRY1, cCRY2, and cCRY4 in HEK293 cells (Fig. 3*A*, α-1D4, *lanes 10–12*). An antibody termed C1 (C1-mAb) showed a strong immunoreactive band with the mobility of ~61 kDa and three weaker bands below the main band. Because these bands were not detected by C1-mAb in cCRY1-1D4- or cCRY2-1D4-transfected cells (*lanes 4* and *5*) and because the predicted molecular mass of cCRY4 (61,021 Da) coincided with the mobility of the main band, C1-mAb specifically reacted with cCRY4 and did not cross-react with either cCRY1 or cCRY2. The weak immunoreactive bands below the main band were considered to be decomposed products of cCRY4. C1-mAb detected a single band at the predicted mobility of cCRY4 (61 kDa) in the chick retinal homogenate (Fig. 3*B*), indicating high specificity of this antibody. Then, we analyzed the epitope region for C1-mAb using competitive ELISA (Fig. 3*C*), in which an immunocomplex of GST-CCT fusion protein and C1-mAb was treated with a partial peptide for the CCT-region (Fig. 3*D*, CT1–CT9). The immunoreaction to GST-CCT was strongly suppressed when the immunocomplex was treated with CT5 peptide (YQLTRDDADDPMEMK), indicating that the CT5 peptide contained C1-mAb epitope and competitively bound to C1-mAb.

CRY4 Protein Is Expressed in Multiple Types of Chick Retinal Cells—The high specificity of C1-mAb demonstrated in immunoblot analysis (Fig. 3*B*) further prompted us to utilize this antibody in the immunohistochemical analysis of chick retinas (Figs. 4 and 5). When reacted with C1-mAb, a cCRY4-like immunoreaction was detected in many cells in the visual pigment cells, inner nuclear layer, and ganglion cell layer (Figs. 4 and 5), but the signal intensities for cells in the ganglion cell layer were relatively strong compared with those for cells in the INL. Most cells, likely retinal ganglion cells, in the retinal ganglion cell layer seemed to exhibit cCRY4-like immunoreaction, but the precise identity of the immunopositive cells requires further analysis. Inner segments of many photoreceptor cells that are likely to be rods were weakly but significantly immunopositive in the VPC layer (Fig. 4). Immunoreaction was suppressed when the antibody was preincubated with the CT5 epitope peptide, but not with the CT5-shuffled peptide (Fig. 5).

Light-dependent Structural Change of CRY4—In the preliminary experiment on immunoprecipitation using chick retinal soluble fraction, we noticed that cCRY4 immunoreactivities in the precipitate varied depending on experimental light condi-

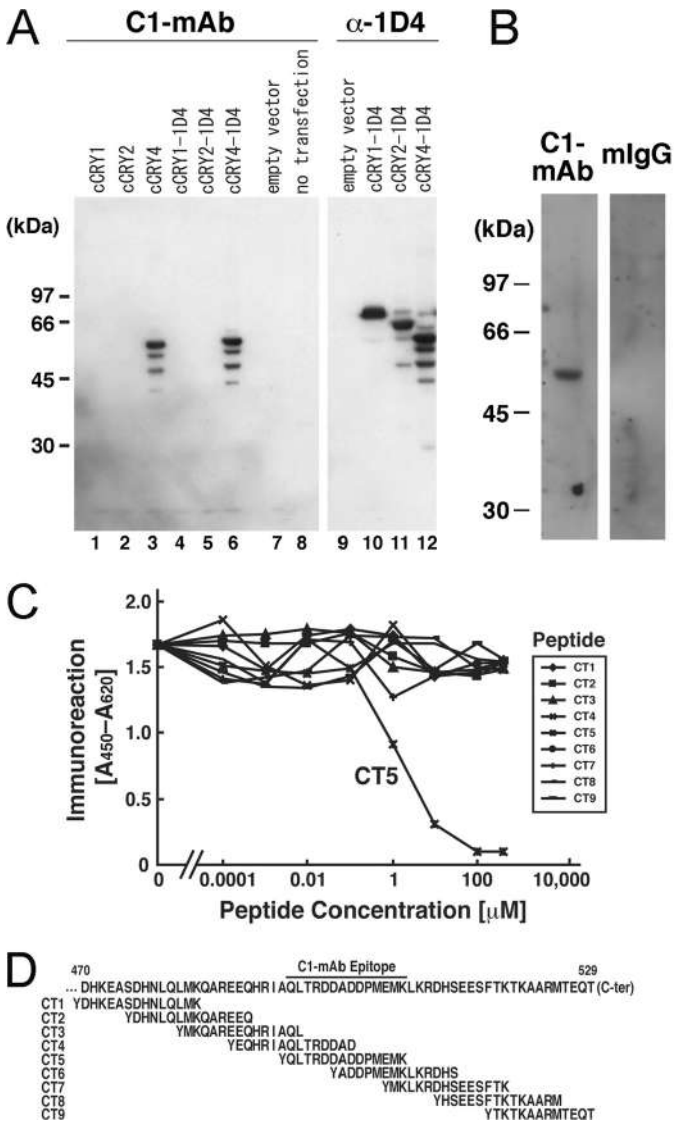

FIGURE 3. **Specificity and epitope of anti-cCRY4 C1-mAb.** *A*, immunoblot analysis of cCRY1, cCRY2, and cCRY4 expressed in HEK293 cells. *cCry1*, *cCry2*, and *cCry4* cDNAs were subcloned into the pcDNA3.1 vector. A 1D4 tag (23) was added at the C terminus of the CRYs (termed cCRY1-1D4, cCRY2-1D4, and cCRY4-1D4, respectively) to detect the expression of cCRY proteins. cCRYs with or without the 1D4 tag were overexpressed in HEK293 cells as described previously (10) and analyzed using C1- and 1D4-mAbs. *B*, immunoblot analysis of chick retinal proteins by C1-mAb. Whole chick retinal proteins (equivalent to 0.01 retina) were separated on 9% SDS-PAGE and visualized with immunoblotting using C1-mAb or control mouse IgG (0.1 μg/ml). BlockAce was used for the blocking and dilution of antibodies in immunoblot analysis (*A* and *B*). *C*, determination of the epitope region of C1-mAb using ELISA. ELISA microplate wells were coated with GST-CCT antigen, blocked with 1% skim milk, reacted with C1-mAb, and the unreacted mAb was washed out. The wells were incubated with 1% skim milk containing various concentrations of peptides (CT1-CT9, see *D*), followed by detection of the remaining C1-mAb through use of an HRP-labeled secondary antibody. *D*, sequence of the CCT region of cCRY4 and peptides used in ELISA. A tyrosine residue was added to the amino terminus of each peptide for quantitation using UV spectroscopy.

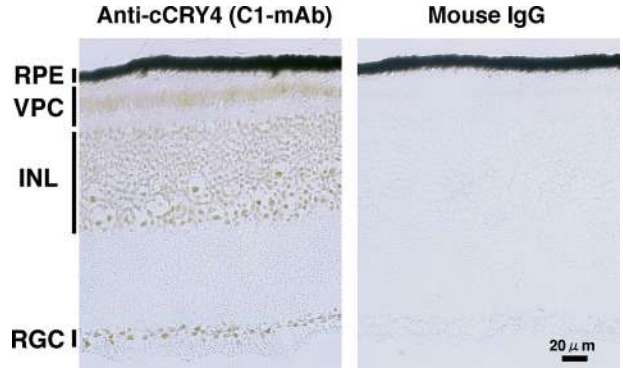

FIGURE 4. **Immunohistochemical localization of cCRY4 in the retina using C1-mAb, which recognizes CCT region of cCRY4.** Mouse IgG (1 μg/ml, *right panel*) was used as the primary antibody instead of C1-mAb (1 μg/ml, *left panel*). RPE, retinal pigment epithelium.

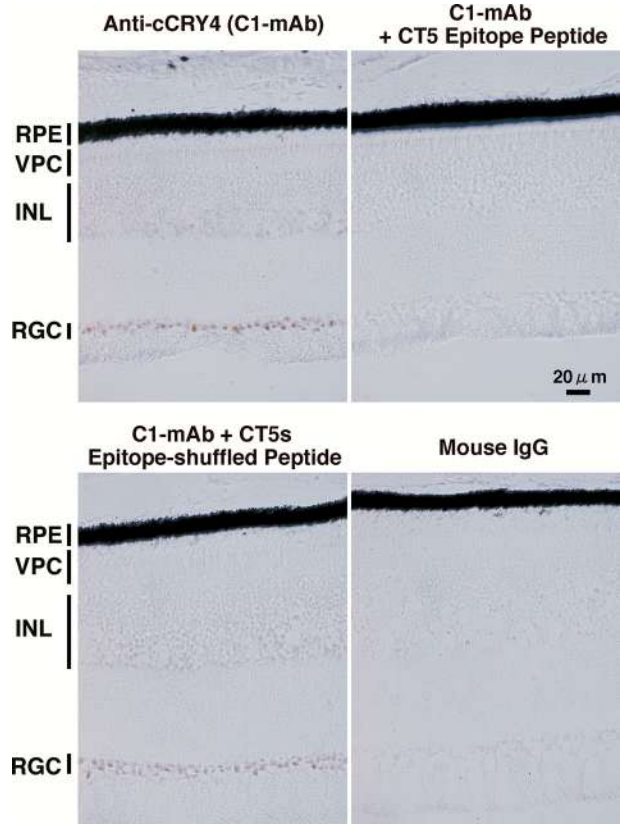

FIGURE 5. **Immunohistochemical localization of cCRY4 in the retina and specificity of the C1-mAb.** The primary antibodies (C1-mAb) were incubated overnight at 4 °C in blocking solution either with a competitor peptide (100 μM; CT5 epitope peptide (YQLTRDDADDPMEMK) or CT5s epitope-shuffled peptide (YQDMRPDMDDETALK)) or without competitor (for C1-mAb and mouse IgG). RPE, retinal pigment epithelium. The signals are weaker than Fig. 4 because of the shorter incubation time for detection reaction.

tions; the immunoreactivity in precipitant was higher in dark *versus* light conditions when we used C1-mAb. To test the hypothesis that the interaction between C1-mAb and cCRY4 varied depending on lighting conditions, C1-mAb was conjugated to Affi-Gel Hz beads, and the C1-mAb-conjugated gel (C1-mAb-gel) was mixed with chick retinal soluble fraction under different light conditions (Figs. 6–8). At first, the gel was preincubated under dark or blue light conditions to ascertain whether blue light illumination inactivated C1-mAb-gel. After preincubation, the gel was mixed with the retinal soluble fraction, incubated under blue light or dark conditions, washed to remove weakly bound materials, and subjected to immunoblot analysis (Fig. 6). Strong bands for cCRY4 were observed in the precipitants of samples incubated under dark conditions (Fig. 6*B*, *arrowheads*, DD and LD), whereas only weak bands were observed in those samples incubated under blue light condi-

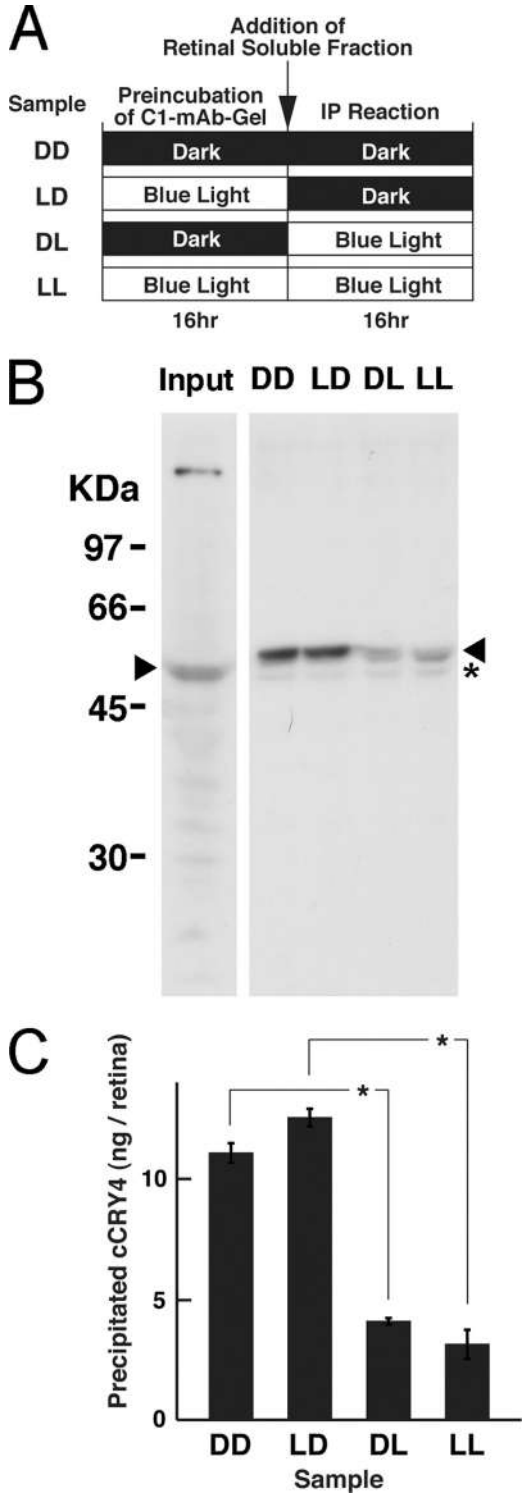

FIGURE 6. **Immunoprecipitation of cCRY4 under blue light or dark conditions and the effect on precipitation efficiencies in light conditions during the preirradiation of C1-mAb-conjugated gel.** *A*, schematic diagram of experimental procedure. *B* and *C*, cCRY4(-like immunoreactivities) eluted in the supernatants after incubation under various light conditions. C1-mAb-gel was incubated under dark (D) or blue light (L, 424 μW/cm^2, 1.0 $\times$ 10^{15} photon/cm^2/s) conditions for 16 h at 4 °C, mixed with chick retinal soluble fraction, incubated for 16 h at 4 °C under D or L conditions, and washed with wash buffer three times. The supernatants were then subjected to immunoblot analysis to estimate the relative amounts of cCRY4 protein eluted from the gel using C3 mAb (1 μg/ml), which recognizes the carboxyl-terminal CT8 region of cCRY4 (HSEESFTKTKAARM). Skim milk (1%) in TBS was used for blocking and dilution of antibodies. Proteins equivalent to those from 0.39 retina were loaded in each *lane*. Signals were detected using the AttoPhos fluorescent substrate (Roche Applied Science) and Typhoon 9410 scanner (GE Healthcare). A representative immunoblot image (one of the three replicates) is shown in *B*. *Error bars* represent the S.E. An *asterisk* in *B* denotes bands due to nonspecific binding of the secondary antibody. *Asterisks* in *C* denote $p < 0.01$.

tions. Quantitative analysis showed that the precipitated cCRY4 immunoreactivities were statistically less in blue light compared with dark conditions ($p < 0.01$, Fig. 6*C*). On the other hand, the blue light conditions during preincubation of C1-mAb-gel did not affect immunoprecipitation (compare DD and LD conditions, Fig. 6*C*), indicating that blue light irradiation had no impact on the binding capacity of C1-mAb-gel. Therefore, the reduced immunoprecipitation efficiency in C1-mAb under DL and LL conditions was most likely not due to any irreversible effects from light but instead ascribable to the photic reaction of cCRY4. Next, we examined whether the light-dependent change in cCRY4 in the retinal soluble fraction was reversible or irreversible by preincubating the retinal soluble fraction before incubation with the mAb-gel (Fig. 7). The retinal soluble fractions were preincubated in blue light or darkness for 16 h, mixed with the mAb-gel, and then incubated under blue light or dark conditions (Fig. 7*A*). The cCRY4 immunoreactivities were less in samples irradiated in blue light during the immunoprecipitation reaction (DL and LL, Fig. 7), whereas cCRY4 immunoreactivity was efficiently precipitated even when the retinal soluble fraction was irradiated before the immunoprecipitation reaction (Fig. 7, LD). Thus, these results strongly suggest that the structural change occurring in cCRY4 was reversible and that cCRY4 changed its form to a dark state during incubation in the dark.

Finally, we investigated whether light irradiation reduced the affinity between cCRY4 and the C1-mAb-gel even after cCRY4 had bound to the gel in the dark, and we also examined the intensity and wavelength dependences of the reaction (Fig. 8). The retinal soluble fractions were incubated in the dark with C1-mAb-gel and washed to remove unbound materials. Then the immune complexes were incubated in the dark or irradiated under various light conditions. After incubation, supernatants were subjected to immunoblot analysis to quantitate cCRY4 immunoreactivities that were released from immune complexes. Chicken CRY4 immunoreactions in the supernatants were significantly larger in those samples irradiated with 25 or 100 μW/cm^2 of blue light than those samples incubated in the dark (Fig. 8*B*). Irradiation with 6.25 μW/cm^2 of blue light did not induce the release of cCRY4, suggesting that the critical intensity for the reaction to occur was likely between 6.25 and 25 μW/cm^2. When the immune complex was irradiated at various light wavelengths, significant cCRY4 immunoreactivity was detected in the samples irradiated with near UV, blue, and green light but not red light (Fig. 8*C*).

DISCUSSION

In the present study, we investigated mRNA and protein expression of CRY4 in the chicken retina. The distribution of mRNA expression (Fig. 2) and immunoreaction (Fig. 4) overlapped in multiple layers of the chick retina, of which the immunoreactivity was strongest in the RGCs (Figs. 4 and 5). In other

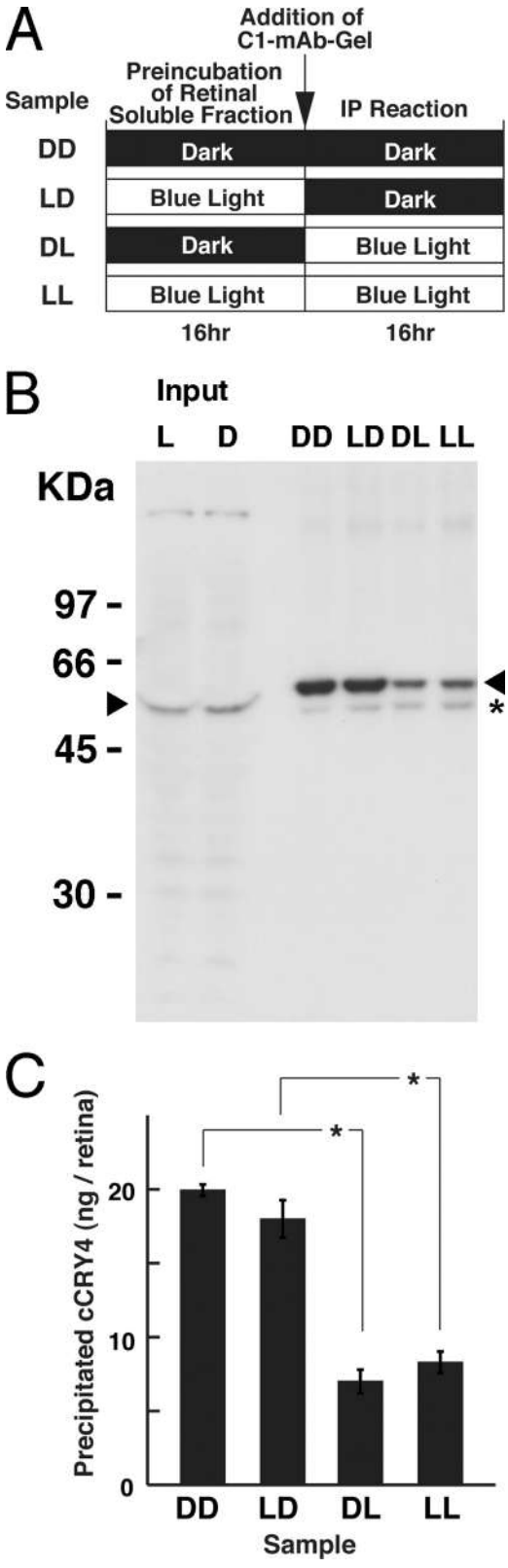

FIGURE 7. **Effects on the immunoprecipitation efficiencies of cCRY4 by blue light irradiation of retinal soluble fractions during and before immunoprecipitation.** *A*, schematic diagram of experimental procedure. *B* and *C*, cCRY4(-like immunoreactivities) eluted in the supernatants after incubation under various light conditions. Then, the chick retinal soluble fraction was divided into several aliquots, which were incubated in the dark (D) or illuminated with blue light (L, 424 μW/cm^2, 1.0 $\times$ 10^{15} photon/cm^2/s) for 16 h at 4 °C. They were mixed with the C1-mAb-gel and incubated for 16 h at 4 °C under dark (D) or blue light (L, 424 μW/cm^2, 1.0 $\times$ 10^{15} photon/cm^2/s) conditions, and the gel was washed with wash buffer three times. The supernatants were then subjected to immunoblot analysis to estimate the relative amounts of cCRY4 protein eluted from the gel using C3-mAb. Proteins equivalent to those of 0.39 retina were loaded in each lane. Skim milk (1%) in TBS was used for blocking and dilution of antibodies. Signals were detected as described in Fig. 6. A representative immunoblot image (one of the three replicates) is shown in *B*. An *asterisk* in *B* denotes bands due to nonspecific binding of the secondary antibody. *Error bars* represent the S.E. *Asterisks* in *C* denote $p < 0.01$.

retinal cell layers such as the INL (Fig. 2) and VPC (Fig. 2), strong signals were detected with *in situ* hybridization, and weak but significant signals were seen with immunohistochemistry. Because the total amounts of cCRY4 transcript and protein showed very weak rhythmicity and were nearly constant throughout the day in the retina (Fig. 1), the expression levels of cCRY4 in the retina did not seem to be strongly regulated by the circadian clock, in contrast to chicken red (iodopsin) (15) and melanopsin (16).

In chicken retinal cells, the expression of cCRY4 along with its light-dependent properties (Figs. 6–8) led us to believe that cCRY4 may be involved in circadian clock regulation and/or non-image-forming vision related to the photoreceptor melanopsin, and/or magnetoreception. A recombinant cCRY4 protein obtained in insect cells consistently retains FAD chromophore (17), and the retina has been suggested as a possible site for magnetoreception because of its spherical structure (18). These facts are consistent with a possible involvement of CRY4 in the magnetoreception in birds; however, CRY1-expressing large displaced ganglion cells within the INL (19) and CRY1-immunopositive UV/violet-sensitive cones (20) are suggested to play important role(s) in magnetoreception of migratory birds.

Anti-cCRY4 C1-mAb, which recognizes the carboxyl-terminal extension of cCRY4 (Figs. 3 and 9A), showed different binding affinity to cCRY4 immunoreactivity in the chick retinal soluble fractions under different light conditions (Figs. 6 and 7). The immunoreaction findings showed that C1-mAb had higher affinity to the dark form of cCRY4 than the light form (Fig. 9*B*). We cannot deny the possible involvement of factors other than cCRY4 in the light-dependent changes because SDS-PAGE analysis of proteins bound to the C1-mAb gel indicated the presence of at least two contaminating proteins in the immunoprecipitates (Fig. 10). We consider, however, that opsins are less likely to contribute to the photic change because we used the retinal soluble fraction (containing proteins solubilized with 0.1% Tween 20) in which membrane proteins such as opsins are likely not included. In this context, it should be noted that the spectral property of the photic release reaction of cCRY4 (Fig. 8*C*) roughly matched the putative blue light sensitivity of cCRY4 with FAD chromophore, yet also seemed to have a slightly wider to longer wavelength region (Fig. 8*C*, *green*). This apparent inconsistency may be ascribed to the wider emission spectrum of the green LED (14) and relatively strong light irradiation used in the present experiments. Vertebrate CRYs including cCRY4 have recently been obtained as recombinant proteins by using an overexpression system in insect cells (17), which will in turn facilitate detailed characterization of the photic properties of vertebrate CRYs in the near

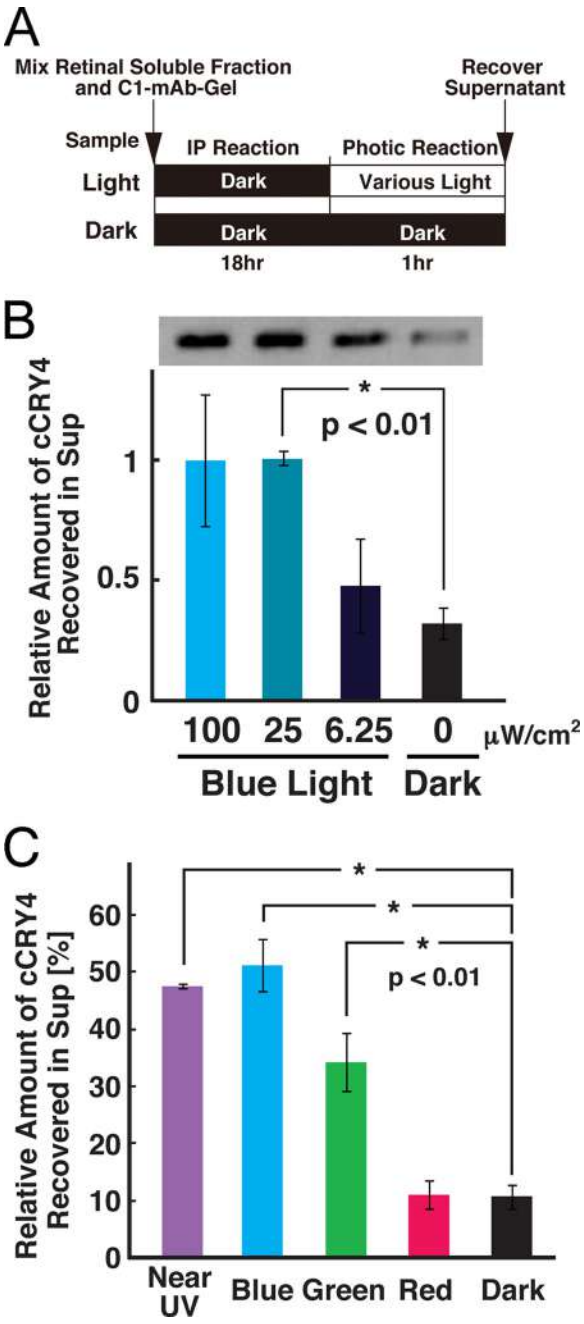

FIGURE 8. **Effects on the immunoprecipitation efficiencies of cCRY4 by varying intensity and at different wavelengths of light irradiation.** *A*, schematic diagram of experimental procedure. *B*, light dependence and the release of cCRY4 from the C1-mAb-gel. The chick retinal soluble fraction was mixed with C1-mAb-gel, incubated for 18 h at 4 °C, and washed with wash buffer five times. Then the gel was divided into several aliquots, which were incubated in the dark or illuminated by various intensities of blue light at 25 °C for 1 h. A representative immunoblot image (one of the three replicates) is shown in the *upper part* of the *panel*. *C*, wavelength dependence and the release of cCRY4 from the C1-mAb-gel. Chicken CRY4-bound gel was prepared as above, and the aliquots were incubated in the dark or irradiated with LED lights at a constant photon density (1.0×10^{15} photons/cm^2 per s) but different spectral characteristics at 25 °C for 1 h. The supernatants were then subjected to immunoblot analysis using C1-mAb (0.1 μg/ml) to estimate the relative amounts of cCRY4 protein eluted from the gel. Proteins equivalent to those from 0.08 retina were loaded in each lane. Skim milk (1%) in TBS was used for blocking and dilution of antibodies. Signals were detected as described in Fig. 6. *Error bars* represent the S.E.

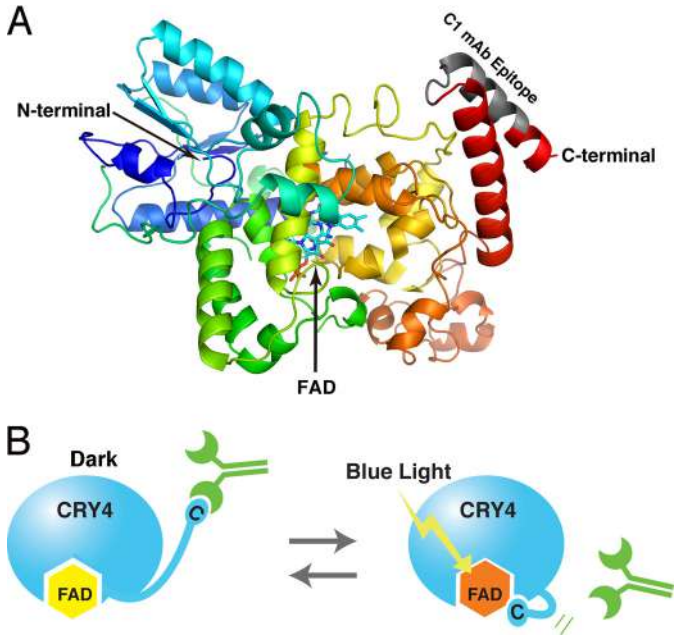

FIGURE 9. **Models for light-dependent structural change cCRY4.** *A*, model for cCRY4 and C1-mAb epitope within the carboxyl-terminal extension. A simple sequence replacement model of cCRY4 (Arg^4–Glu^{514}) with FAD chromophore was constructed based on *Arabidopsis thaliana* photolyase (Protein Data Bank ID code 3FY4) with the aid of MATRAS server (24) and drawn using the PyMOL Molecular Graphics System (MacPyMOL, Schrödinger, LLC). The model lacks some loop regions and amino- and carboxyl-terminal short amino acid stretches. The C1-mAb epitope within the carboxyl-terminal extension is colored in *gray*. *B*, model for the blue light-dependent interaction between cCRY4 and C1-mAb.

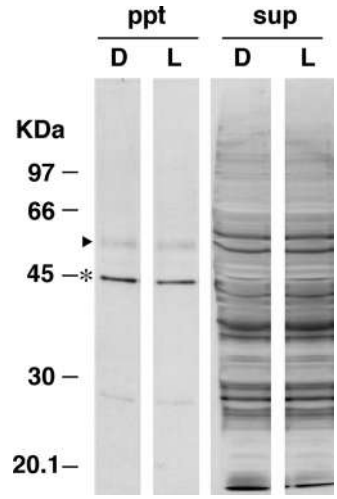

FIGURE 10. **Silver staining of immunoprecipitated proteins in the retinal soluble fractions.** C1-mAb-gel was mixed with chick retinal soluble fraction, incubated for 7 h at 4 °C under dark or blue light (424 μW/cm^2) conditions, and washed with wash buffer three times. The precipitates and supernatants were then subjected to SDS-PAGE followed by silver staining. Proteins equivalent to those from 0.05 retina were loaded in each *lane*. We observed no band for cCRY4 in the precipitate probably due to the low content. An *arrowhead* and *asterisk* indicate the protein bands for tubulin and β-actin, which were identified by MALDI-TOF MS (AXIMA CFR plus; Shimadzu). These two proteins showed the similar binding to a control IgG-conjugated gel, indicating their nonspecific binding to the C1-mAb-gel.

future. We believe, however, that the present study is of great importance in that this is the first direct investigation of a light-dependent structural change of vertebrate CRY obtained from native tissue.

Our immunoprecipitation experiments under various light schedules (Fig. 7) indicated that the light form of cCRY4 could change into a dark form after dark incubation for 16 h, suggesting that the dark and light forms of cCRY4 were interconvertible. Another important property of cCRY4 was that very low intensity blue light (471 nm) as weak as 25 $\mu W/cm^2$ (approximately 5.9×10^{13} photons/cm^2 per s, 15.5 lux) induced its structural change (Fig. 8*B*). Such high photosensitivity of cCRY4 is of biological significance and should be appreciated from the viewpoint of biotechnology.

The blue light-dependent change in the accessibility of C1-mAb to its epitope (Figs. 6 and 7) implies the occurrence of an enzymatic modification or conformational change near the epitope region. As an initial survey of an involvement of protein phosphorylation in the light-dependent change, we treated the immunoprecipitated cCRY4 with a λ phosphatase, but we did not observe either the release from the C1-gel or mobility change in the immunoblot analysis (data not shown). Although this result does not exclude the possible involvement of phosphorylation or other modification(s) under the native conditions in the light-dependent accessibility, hyperphosphorylation is unlikely to occur in cCRY4. The C1-mAb epitope is likely located within one of two helices in the carboxyl-terminal extension (Gln^{494}–Lys^{507}; QLTRDDADDPMEMK, Figs. 3*D* and Fig. 9*A*) previously identified as playing an important role in the phototransduction of *Arabidopsis* and *Drosophila* CRYs (1, 4, 21). These considerations together with the structural model of cCRY4 (Fig. 9) led us to postulate a model on dynamic change of the intramolecular configuration of cCRY4, one in which the carboxyl-terminal epitope region is exposed outside the molecule in darkness and moves inside or attaches to cCRY4 itself upon illumination (Fig. 9*B*). This model should be verified in future studies including determination of crystal structure of cCRY4. A recent crystal structure analysis of dark-state dCRY showed that the carboxyl-terminal helix of dCRY docks in an analogous groove that binds DNA substrates in photolyases (22). Because dCRY and cCRY4 share relatively low sequence identity (30–40%), comparison of crystal structures of cCRY4 and dCRY would be of great importance to infer the divergence and convergence of intramolecular phototransduction mechanisms among CRY proteins. After the photic structural change, cCRY4 might transmit photon signals to the other proteins like plant and insect CRYs. The anti-cCRY4 mAbs obtained in this study will help us to perform immunoaffinity purification of the cCRY4-containing complexes, which may provide a means toward identifying a possible phototransduction pathway downstream of cCRY4.

REFERENCES

1. Li, Q. H., and Yang, H. Q. (2007) Cryptochrome signaling in plants. *Photochem. Photobiol.* **83,** 94–101
2. Oztürk, N., Song, S. H., Ozgür, S., Selby, C. P., Morrison, L., Partch, C., Zhong, D., and Sancar, A. (2007) Structure and function of animal cryptochromes. *Cold Spring Harb. Symp. Quant. Biol.* **72,** 119–131
3. Bayram, O., Braus, G. H., Fischer, R., and Rodriguez-Romero, J. (2010) Spotlight on *Aspergillus nidulans* photosensory systems. *Fungal Genet. Biol.* **47,** 900–908
4. Busza, A., Emery-Le, M., Rosbash, M., and Emery, P. (2004) Roles of the two *Drosophila* cryptochrome structural domains in circadian photoreception. *Science* **304,** 1503–1506
5. Fogle, K. J., Parson, K. G., Dahm, N. A., and Holmes, T. C. (2011) Cryptochrome is a blue-light sensor that regulates neuronal firing rate. *Science* **331,** 1409–1413
6. Gegear, R. J., Casselman, A., Waddell, S., and Reppert, S. M. (2008) Cryptochrome mediates light-dependent magnetosensitivity in *Drosophila*. *Nature* **454,** 1014–1018
7. Ueda, H. R., Hayashi, S., Chen, W., Sano, M., Machida, M., Shigeyoshi, Y., Iino, M., and Hashimoto, S. (2005) System-level identification of transcriptional circuits underlying mammalian circadian clocks. *Nat. Genet.* **37,** 187–192
8. Yamamoto, K., Okano, T., and Fukada, Y. (2001) Chicken pineal *Cry* genes: light-dependent up-regulation of *cCry1* and *cCry2* transcripts. *Neurosci. Lett.* **313,** 13–16
9. Tu, D. C., Batten, M. L., Palczewski, K., and Van Gelder, R. N. (2004) Nonvisual photoreception in the chick iris. *Science* **306,** 129–131
10. Kubo, Y., Akiyama, M., Fukada, Y., and Okano, T. (2006) Molecular cloning, mRNA expression, and immunocytochemical localization of a putative blue-light photoreceptor CRY4 in the chicken pineal gland. *J. Neurochem.* **97,** 1155–1165
11. Okano, T., Yoshizawa, T., and Fukada, Y. (1994) Pinopsin is a chicken pineal photoreceptive molecule. *Nature* **372,** 94–97
12. Okano, T., and Fukada, Y. (2003) Chicktacking pineal clock. *J. Biochem.* **134,** 791–797
13. Kasahara, T., Okano, T., Yoshikawa, T., Yamazaki, K., and Fukada, Y (2000) Rod-type transducin α-subunit mediates a phototransduction pathway in the chicken pineal gland. *J. Neurochem.* **75,** 217–224
14. Akiyama, M., Okano, K., Fukada, Y., and Okano, T. (2009) Macrophage inhibitory cytokine MIC-1 is up-regulated by short-wavelength light in cultured normal human dermal fibroblasts. *FEBS Lett.* **583,** 933–937
15. Pierce, M. E., Sheshberadaran, H., Zhang, Z., Fox, L. E., Applebury, M. L., and Takahashi, J. S. (1993) Circadian regulation of iodopsin gene expression in embryonic photoreceptors in retinal cell culture. *Neuron* **10,** 579–584
16. Chaurasia, S. S., Rollag, M. D., Jiang, G., Hayes, W. P., Haque, R., Natesan, A., Zatz, M., Tosini, G., Liu, C., Korf, H. W., Iuvone, P. M., and Provencio, I. (2005) Molecular cloning, localization and circadian expression of chicken melanopsin (Opn4): differential regulation of expression in pineal and retinal cell types. *J. Neurochem.* **92,** 158–170
17. Ozturk, N., Selby, C. P., Song, S. H., Ye, R., Tan, C., Kao, Y. T., Zhong, D., and Sancar, A. (2009) Comparative photochemistry of animal type 1 and type 4 cryptochromes. *Biochemistry* **48,** 8585–8593
18. Ritz, T., Adem, S., and Schulten, K. (2000) A model for photoreceptor-based magnetoreception in birds. *Biophys. J.* **78,** 707–718
19. Mouritsen, H., Janssen-Bienhold, U., Liedvogel, M., Feenders, G., Stalleicken, J., Dirks, P., and Weiler, R. (2004) Cryptochromes and neuronal-activity markers colocalize in the retina of migratory birds during magnetic orientation. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 14294–14299
20. Niessner, C., Denzau, S., Gross, J. C., Peichl, L., Bischof, H.-J., Fleissner, G., Wiltschko, W., and Wiltschko, R. (2011) Avian ultraviolet/violet cones identified as probable magnetoreceptors. *PLoS ONE* **6,** e20091
21. Ozturk, N., Selby, C. P., Annayev, Y., Zhong, D., and Sancar, A. (2011) Reaction mechanism of *Drosophila* cryptochrome. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 516–521
22. Zoltowski, B. D., Vaidya, A. T., Top, D., Widom, J., Young, M. W., and Crane, B. R. (2011) Structure of full-length *Drosophila* cryptochrome. *Nature* **480,** 396–399
23. MacKenzie, D., Arendt, A., Hargrave, P., McDowell, J. H., and Molday, R. S. (1984) Localization of binding sites for carboxyl-terminal specific anti-rhodopsin monoclonal antibodies using synthetic peptides. *Biochemistry* **23,** 6544–6549
24. Kawabata T. (2003) MATRAS: A program for protein 3D structure comparison. *Nucleic Acids Res.* **31,** 3367–3369