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Characterization of photolyase/blue-light receptor homologs in mouse and human cells

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ABSTRACT

We isolated and characterized mouse photolyase-like genes, mCRY1 (mPHLL1) and mCRY2 (mPHLL2), which belong to the photolyase family including plant blue-light receptors. The mCRY1 and mCRY2 genes are located on chromosome 10C and 2E, respectively, and are expressed in all mouse organs examined. We raised antibodies specific against each gene product using its C-terminal sequence, which differs completely between the genes. Immunofluorescent staining of cultured mouse cells revealed that mCRY1 is localized in mitochondria whereas mCRY2 was found mainly in the nucleus. The subcellular distribution of CRY proteins was confirmed by immunoblot analysis of fractionated mouse liver cell extracts. Using green fluorescent protein fused peptides we showed that the C-terminal region of the mouse CRY2 protein contains a unique nuclear localization signal, which is absent in the CRY1 protein. The N-terminal region of CRY1 was shown to contain the mitochondrial transport signal. Recombinant as well as native CRY1 proteins from mouse and human cells showed a tight binding activity to DNA Sepharose, while CRY2 protein did not bind to DNA Sepharose at all under the same condition as CRY1. The different cellular localization and DNA binding properties of the mammalian photolyase homologs suggest that despite the similarity in the sequence the two proteins have distinct function(s).

INTRODUCTION

Photolyase catalyses light-dependent repair of UV-induced DNA damage (1). Cyclobutane pyrimidine dimers (CPDs) have been known to be the substrates for photolyase (CPD photolyase), but recently, another photolyase, acting on 6-4 photoproducts (6-4PPs), was found (2). The gene encoding the photolyase for 6-4PPs (6-4 photolyase) showed similarity to the CPD photolyase genes (3). Besides photolyases, a plant photolyase-like gene was identified from *Arabidopsis thaliana* (4). Mutations of the gene resulted in a defective response of blue-light regulation for hypocotyl elongation of the plant. The gene product was named CRY1 from 'cryptochrome' previously proposed for 'cryptic' blue-light receptors (5). Very recently, another photolyase-like gene of the plant *Arabidopsis*, CRY2, was isolated and found to regulate flowering in response to blue light (6). A cry1 cry2 double mutant of *A.thaliana* was more impaired in blue-light responses such as anthocyanin accumulation and inhibition of hypocotyl elongation than cry1 single mutant suggesting some overlaps in their functions as the blue-light receptors (7,8).

Although CPD photolyases are distributed in various organisms ranging from bacteria, yeast, insect and many vertebrates including aplacental mammals, a human photolyase gene has not yet been isolated (9,10). However, recently in human cDNA data bases, two photolyase-like genes were found, which are highly similar to 6-4 photolyase (3,11,12). A special feature of the human photolyase-like genes is the presence of a C-terminal extension, which is not present in photolyases, but found in CRY1 and CRY2 of *Arabidopsis*, though the sequence and the length of the extended region completely differ among the genes. The recombinant human photolyase-like gene products were found to possess an FAD and a pterin cofactor but showed no photolyase activity (12). Because of the absence of photolyase activity in the recombinant photolyase homologs, it has been speculated that human photolyase-like genes may encode human blue-light photoreceptors. Accordingly, it was proposed to call the genes hCRY1 and hCRY2 (12).

To date, the absence of photolyase activity has only been shown for recombinant plant or human CRY proteins (12,13). Furthermore, knowledge about the characteristics of the native proteins including the subcellular localization or the DNA binding activity of CRY proteins are completely lacking, which should be the important clues for the understanding of their functions. Here we report the cloning of two mouse CRY genes (mCRY1 and mCRY2) and the characterization of their gene products in mouse liver and human cells. Using specific antibodies, we show that the mammalian CRY1 and CRY2 proteins differ in their subcellular localization and DNA binding characteristics.

MATERIALS AND METHODS

cDNA source and cDNA cloning

The following degenerate primers, designed from conserved sequences among human hCRY1, *Arabidopsis* CRY1 and *Chlamydomonas* photolyase homolog, were synthesized and

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used for cloning of the mouse homolog mCRY1; MB-51, d(TGG TTC CGI AAG GGG/C CTC CG) for WFRKGLR sequence and MB-31: d(CCA IGG A/GA/TA GCC IGT CCG ICC) for GRTGF/YPW sequence, where I indicates inosine base. Three mRNA sources of brain, liver and a keratinocyte cell line of mouse were used for cloning and complete cDNA sequence determination. 5′ Sequence of the gene was obtained from a mouse liver cDNA library (Invitrogen) by 5′ RACE and 3′ sequence was obtained by 3′ RACE of cDNA prepared from mRNA of the mouse keratinocyte cell line PAM. The final sequence was determined by independent cloning and sequencing of several cDNAs prepared from PAM cells by PCR using primers containing the putative start and stop codons. To determine the C-terminal sequences of other rodent species, CRY1 homolog genes were isolated from cDNA of rat liver (CLONTECH) or cDNA of Chinese hamster prepared from an ovary cell line, UV210, respectively.

For the cloning of the mouse CRY2 gene, mCRY1 and hCRY2 were used as PCR primers: PHL-54, d[GG(G/C) TT(T/C) CCI TGG AT(T/C/A GA(T/C) GCI AT(T/C/A) ATG] for GFPWI-DAIM; PHL-34, d[GGI TT(T/C) (T/G)CI TT(T/C) (A/G)AI CT(T/C) CGI CG] for PKRKLEAA; PHL1251–1255, d[GTI CA(C/T) TGG TT(C/T) (C/A)GI AA(A/C/T) GGI] for VHWFRKG; PHL23–2, d[(G/A)CA (G/A)TA (G/A)CA (G/A)TG (G/A)AA (G/A)AA (T/C)TG (T/C)TG (G/A)AA (G/A)AA] for FFQQFFHCYC. Using mouse liver cDNA library (Gibco), PCR with primers PHL-54 and CRY-34 yielded a 630 bp DNA fragment. Another PCR with primers CRY1251-5 and CRY23-2 yielded a 1.2 kb DNA fragment. Additional sequences at the 3′ end were obtained from mouse liver cDNA library (Gibco) by PCR. DNA sequencing was carried out by the dideoxynucleotide chain terminating procedure with fluorescence-labeled primers using a SHIMADZU DSQ-1000.

Chromosomal localization

Genomic DNA fragments of 10 and 3, and 4 and 3 kb, containing mCRY1 and mCRY2, respectively, were isolated by LA-PCR (TAKARA). Each DNA fragment was biotin-labeled, and labeled DNA fragment was used for *in situ* hybridization. After incubation with avidin D-FOTC (Vector, USA), the probes were visualized by FISH as reported previously (11).

Northern blot analysis

A tissue northern blot containing 2 µg of poly(A)$^+$ RNA per lane (Clontech mouse and human multiple tissue northern blot) was hybridized to ^{32}P-labeled DNA probes containing the isolated coding region of each cDNA, mCRY1, mCRY2 and hCRY2, by using hybridization buffer (Clontech Express Hyb) for 1 h at 68°C. Membrane was washed twice in 2× SSC (0.1% SDS) for 15 min at room temperature and subsequently twice in 0.1× SSC (0.1% SDS) for 15 min at 55°C. Hybridized membrane was analyzed with a Fujix BAS2000 (Fuji Photo Film) image analyzer.

Purification and refolding of recombinant mCRY1 protein

A DNA fragment containing the whole cDNA of the mouse mCRY1 gene was introduced into pGEX4T-2 (Pharmacia) for production of recombinant proteins fused with glutathione-*S*-transferase in *Escherichia coli* host cells. The transformed cells were grown at 37°C in 1 l LB medium with 0.1 mM ampicillin, until an A_{600} of 0.6 was reached. Expression was induced by the addition of a final concentration of 1 mM IPTG, and growth was continued at 37°C for 3 h. Cells were collected and suspended in a lysis buffer 50 mM Tris–HCl pH 7.6, 2 mM 2-mercaptoethanol, 1 mM PMSF, 10% glycerol containing 100 mM KCl). Cell extract was prepared by sonication, followed by centrifugation at 32 000 *g* for 30 min. GST–mCRY1 fusion protein was obtained as a complex with *E.coli* GroEL, a molecular chaperonin. Eluted fusion protein–GroEL complex was digested with thrombin protease and applied to p-aminobenzamidine Sepharose 6B (Sigma) gel to remove the thrombin from the sample. To refold and release the recombinant mCRY1, the complex was incubated with 2.5 µM GroES, 5 mM ATP, 20 mM $MgCl_2$, 1mM FAD at 25°C for 45 min before application to a double strand (ds)DNA Sepharose. The mCRY1 was eluted with a step gradient 0.1–1.2 M KCl.

Antibody production and purification

Rabbit polyclonal antibody to the mCRY1 protein was raised against a GST–mCRY1 fusion protein. Anti-mCRY1–CT antibody was purified through a Sepharose column prepared with cyanogen bromide activated Sepharose (Pharmacia) conjugated with a fusion protein between GST and the amino acids residues between 506 and 607 from mCRY1. Antibody specific against mCRY2 was raised against the peptide SGPASPKRKLEAAE, which is present in the C-terminal part of mCRY2. Anti-mCRY2–CT was purified by a Sepharose column containing maltose-binding protein (MBP) fusion of the mCRY2 C-terminal sequence of 69 amino acids, which was prepared by fusion of the sequence to MBP in pMal-c2 vector (NEB). Since the same sequence of this peptide is present in human hCRY2, anti-mCRY2–CT recognizes hCRY2 as well.

Cell extract preparation of mouse liver cells and western analysis

Mitochondria and nuclear fractions were prepared from the livers of five mice by the standard method (14), which is briefly described below. All subsequent procedures were carried out at 0–4°C. Tissues were washed in PBS, swollen in 2 vol of 0.25 STM buffer (50 mM Tris–HCl pH7.6, 25 mM KCl, 5 mM $MgCl_2$, and 0.25 M sucrose) containing 1 mM PMSF and 10 mM 2-mercaptoethanol, and disrupted in a Dounce homogenizer with 6–7 strokes. The homogenate was centrifuged at 700 *g* for 10 min to obtain a crude nuclear fraction and a post-nuclear supernatant. The crude nuclear pellet was suspended in the same volume of 2.2 STM buffer (50 mM Tris–HCl pH7.6, 25 mM KCl, 5 mM $MgCl_2$ and 2.2 M sucrose) using a loose-fitting Dounce homogenizer and centrifuged at 100 000 *g* for 30 min. The pellets of nuclei were washed with 0.25 STM buffer. The purified nuclei was consecutively extracted twice with a buffer (50 mM Tris–HCl pH 7.6, 2 mM 2-mercapto-ethanol, 10% glycerol) containing 0.35 and 2.2 M KCl. The post-nuclear supernatant was layered on 0.35 M STM buffer and centrifuged at 700 *g* for 10 min. The supernatant was collected and this procedure was repeated. The supernatant was centrifuged at 8000 *g* for 10 min and pellet was suspended in 0.25 M STM buffer. This procedure was repeated to obtain purified mitochondrial fraction.

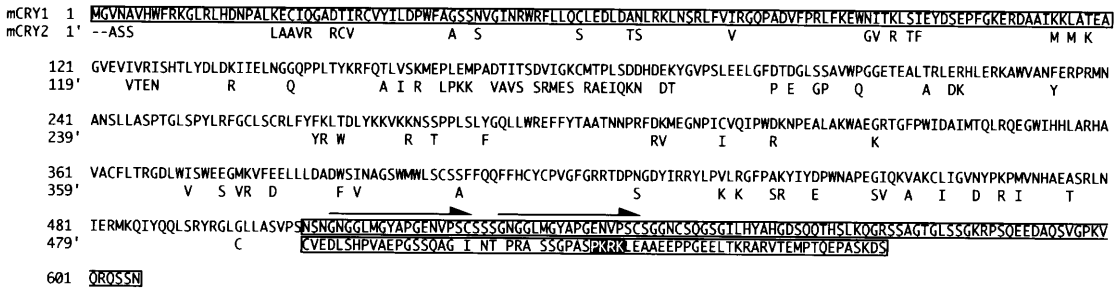

Figure 1. Amino acids sequence alignment of mouse mCRY1 and mCRY2. Regions fused with GFP are indicated by boxes. Arrows indicate repeat sequence in mCRY1. The putative nuclear localization signal sequence in mCRY2 is shown by white letters on a black background.

Identification of the purity of nuclear and mitochondrial fractions

A monoclonal antibody against PCNA (CALBIOCHEM), which stains nuclear and cytosol fractions, and a monoclonal antibody against the mitochondrial cytochrome C (PharMingen) were used to identify the origin and the purity of the prepared fractions from cell extracts.

Fluorescence detection of the expressed fused gene products in transfected cells

The N-terminal sequence of the mCRY1 cDNA (the first 120 amino acids of the gene product shown in Fig. 1) was fused behind the GFP at *Xho*I (5′ end) and *Kpn*I (3′ end) sites in pEGFP-N1 plasmid (CLONTECH). The mCRY2 C-terminal 69 amino acids (Fig. 1) was fused in front of the GFP sequence at *Xho*I (5′ end) and *Kpn*I (3′ end) sites in pEGFP-C1 plasmid (CLONTECH). Mouse C3H10T1/2 cells were cultured on glass coverslip in MEM medium supplemented with 10% FBS and 2 mM L-glutamine at 37°C. Cells were washed with serum-free medium and then incubated at 37°C for 5 h in Opti MEM I (Gibco BRL) containing 2 µg of DNA and 20 µg of Lipofectamin (Gibco BRL) for transfection. MEM with 20% FBS was added and incubated at 37°C for 18–24 h. Then cells were replaced in the MEM with 10% FBS medium and incubated at 37°C for 24 h. Cells were washed once with PBS and then fixed with 4% paraformaldehyde in PBS for 30 min at room temperature before viewing with a Leica DMLB microscope using L4 filter cube.

Indirect immunofluorescence

Indirect immunofluorescence was performed using Tyramide Signal Amplification as recommended by the manufacturer (NEN Life Science Products). Mouse C3H10T1/2 cells grown in coverslip were washed with TNT buffer (0.1 M Tris–HCl pH 7.5, 0.15 M NaCl, 0.05% Tween 20) and fixed with fresh 4% paraformaldehyde in PBS for 10 min at room temperature. The fixed cells were treated with 0.2% Triton X-100 in PBS for 2 min. The blocking reaction was performed by incubating cells with TNB buffer [0.1 M Tris–HCl pH 7.5, 0.15 M NaCl, 0.5% blocking reagent (NEN)] for 30 min. After cells were kept in the primary antibody for 1 h at room temperature, cells were washed with TNT buffer and incubated for 1 h with biotinylated goat anti-rabbit IgG. Cells were then washed in TNT buffer, incubated with streptavidin-HRP for 30 min, washed with TNT buffer, incubated with biotinyl tyramid (NEN) for 30 min, and subsequently incubated with streptavidin–FITC. Cells were viewed with a Leica DMLB microscope using L4 filter cube. For identification of mitochondria, MitoTracker Red CMX VOS probe (Molecular probe) was used.

dsDNA-affinity column chromatography

A dsDNA-affinity column was prepared by linking calf thymus dsDNA to CNBr-activated Sepharose (Pharmacia). Cell extracts or recombinant protein were loaded onto a 2 ml dsDNA-Sepharose column equilibrated with buffer A (50 mM Tris–HCl pH7.5, 2 mM PMSF) containing 0.1 M KCl. Column was washed with the same buffer and eluted with a step gradient using 0.2, 0.3, 0.4, 0.5, 0.6 and 1.2 M KCl in buffer A.

RESULTS

Mouse homologs of human CRY1 and CRY2

cDNAs of the mouse genes mCRY1 and mCRY2 were isolated based on their sequence similarity to human counterparts. Their deduced amino acids sequences are highly similar to each other as shown in Figure 1. However, there are apparent differences between human and mouse CRY genes. mCRY1 (sequence data is registered as mPHLL1 no. AB000777) contains an 18 amino acid repeated sequence in the C-terminus, shown by two arrows in Figure 1, which is not found in its human counterpart. This insertion was found in all the mouse cDNAs obtained from three different sources (brain, liver and a keratinocyte cell line). However, such insertion was not found in rat CRY1 prepared from rat brain or in Chinese hamster CRY1 prepared from a Chinese hamster ovary cell line (not shown), suggesting that this insertion appears to be a recent event. Concerning mCRY2, we think that the sequence shown in Figure 1 (based on the nucleotide sequence for mPHLL2 no. AB003433) lacks its N-terminal region, because the putative initiation codon for translation was not determined and the native mCRY2 gene product in mouse liver cells was identified as a protein of ~80 kDa by western blotting, in contrast to the protein of 70 kDa determined for its human counterpart (see below). Possibly due to a very stable secondary structure in mRNA of mCRY2 at 5′ end, the 5′ end of the mouse CRY2 mRNA is difficult to obtain.

Chromosomal localization of the mouse genes

In situ hybridization was done to determine the chromosomal localization of mCRY1 and mCRY2 genes. mCRY1 was mapped at chromosome 10, band C (Fig. 2A), and mCRY2 at chromosome 2, band E (Fig. 2C).

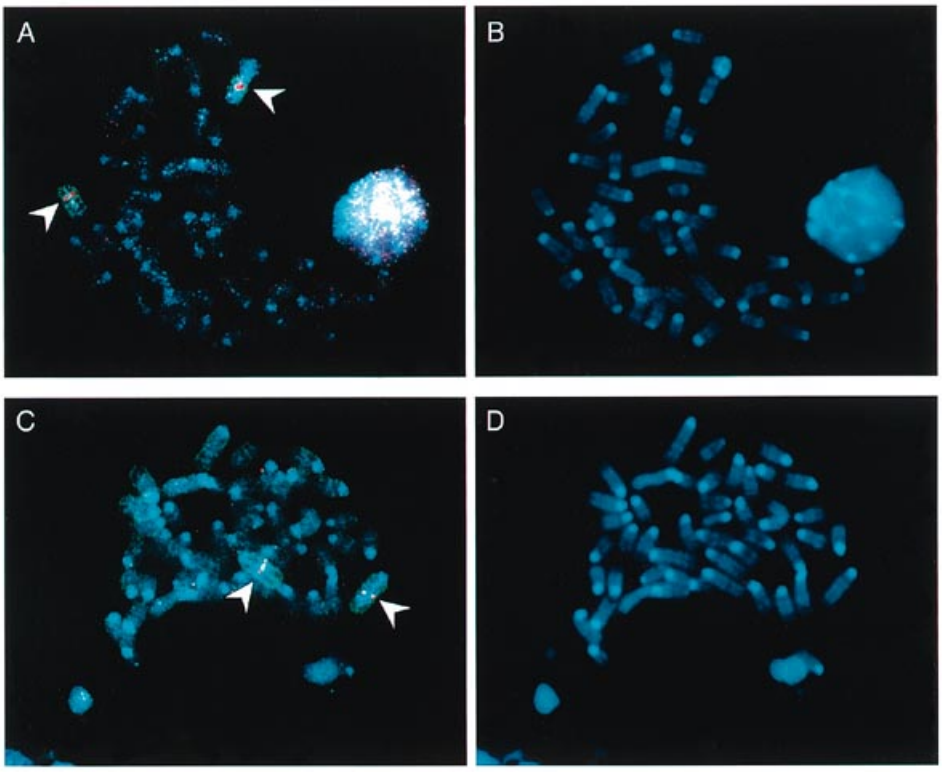

Figure 2. *In situ* hybridization of mouse metaphase chromosomes with biotinylated mouse CRY1 (**A**) and CRY2 (**C**) genomic DNA probes. The DAPI banding of the metaphase of (A) and (C) are shown in (**B**) and (**D**), respectively. The arrowheads indicate the hybridization signals localized on chromosome 10, band C for mCRY1, and chromosome 2, band E for mCRY2.

Northern analysis of the mouse genes

Figure 3A and B depict northern blot analysis of mCRY1 and mCRY2 mRNAs from various mouse tissues, respectively. Expression of mCRY1 was found in all tissues examined with the highest expression in the testes and liver. This expression pattern is very similar to that of its human counterpart already published (11). Two transcripts of 3.0 and 4.4 kb for mCRY1 were identified in all tissues of mouse as was the case for hCRY1 gene. The expression pattern of mCRY2 was not much different from that of mCRY1. Only the expression of mCRY2 in spleen was much lower than those in other tissues. To compare the expression of mCRY2 with that of hCRY2, hCRY2 expression was also analyzed using cDNA of hCRY2 as the probe (Fig. 2C). In contrast to the mouse blot, hCRY2 gene is expressed in spleen as much as in other tissues. Thus, mammalian CRY1 and CRY2 genes have the character of house keeping genes.

Western analysis of mouse proteins

To discriminate between mCRY1 and mCRY2 proteins having highly conserved sequences, we raised antibodies specific to the unique C-terminal sequence of each protein, anti-mCRY1–CT and anti-mCRY2–CT, respectively. Using these antibodies, we found that mCRY1 is present only in the mitochondrial extracts prepared from mouse liver cells, whereas mCRY2 was detected mainly in the nuclear extracts (Fig. 4). One should not overestimate the amount of mCRY2 in mitochondria from this western blot, because the same amount of proteins from mitochondria and nucleus were loaded on each lane for the western analysis. As a control for the purity of the fractions, antibodies against PCNA and cytochrome C were used to identify their presence in the nuclear and mitochondrial fraction, respectively.

Immunochemical identification of the cellular localization of the proteins and their targeting signals

Using indirect immunofluorescence microscopy, we visualized the localization of the proteins in mouse C3H10T1/2 cells. Anti-mCRY1–CT stained organelles outside of the nucleus in mouse cells (Fig. 5A1). Figure 5A2 shows the staining of the mitochondria of the same cells, which can be superposed in Figure 5A3, showing that mCRY1 is present in the mitochondria. In contrast to mCRY1, the target protein recognized by the anti-CRY2–CT resides mainly in the nucleus (Fig. 5B1), which does not overlap with spots of the mitochondrial immunostaining (Fig. 5B2 and B3).

Analysis of the deduced amino acid sequences suggests that the N-terminal sequence in mCRY1 (indicated by a box at the N-terminus of mCRY1 in Fig. 1) may serve as a signal sequence for transport of the protein into mitochondria. In mouse and human CRY2 there is a putative nuclear localization signal sequence, PKRK, in its C-terminal region (indicated by white letters on a black background of the mouse CRY2 in Fig. 1). This sequence was found neither in mouse nor in human CRY1 genes. We examined whether short DNA fragments containing these signal sequences are able to function as the targeting signals for transport of the proteins to each organelle. Therefore, genes

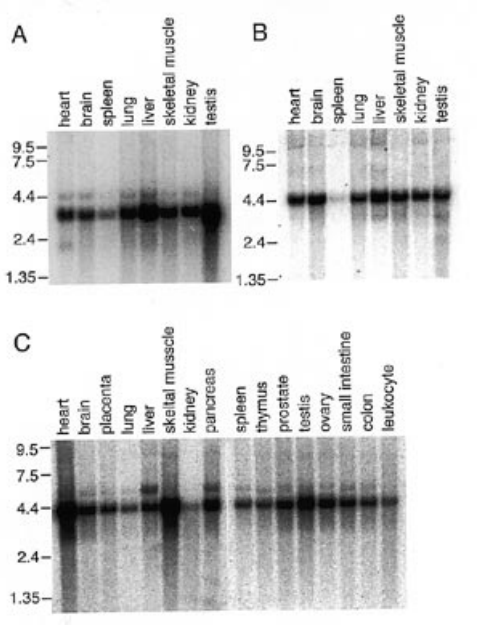

Figure 3. Northern blot analysis of different mouse and human tissues for mRNA expression of mCRY1 (**A**), mCRY2 (**B**) and hCRY2 (**C**). Northern blot analysis of human tissues by hCRY1 was already reported (9).

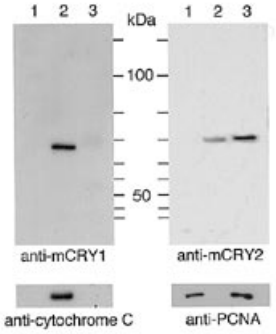

Figure 4. Western blot analysis of mCRY1 and mCRY2 proteins in the cytosolic (1), mitochondrial (2) and nuclear (3) fractions of mouse liver cell homogenates. Antibodies used are indicated in the figures. Anti-cytochrome C and anti-PCNA were used to estimate the relative purity of each fraction.

encoding the green fluorescence protein (GFP) which fused behind the N-terminal region of mCRY1 or in front of the C-terminal sequence of mCRY2 were transiently expressed in mouse C3H10T1/2 cells. Although expression of GFP alone gave a dim distribution of the fluorescence (Fig. 6, top panel), the N-terminal sequence of CRY1 [CRY1(NT)-GFP] transferred the GFP to the mitochondria-like organelles (Fig. 6, second panel). The C-terminal sequence of the CRY1 made GFP to a similar distribution as GFP alone (Fig. 6, third panel), while a clear nuclear localization of GFP fused with the CRY2 C-terminal sequence was obtained (Fig. 6, bottom panel). These data suggest that both terminal sequences function as the targeting signals for each protein.

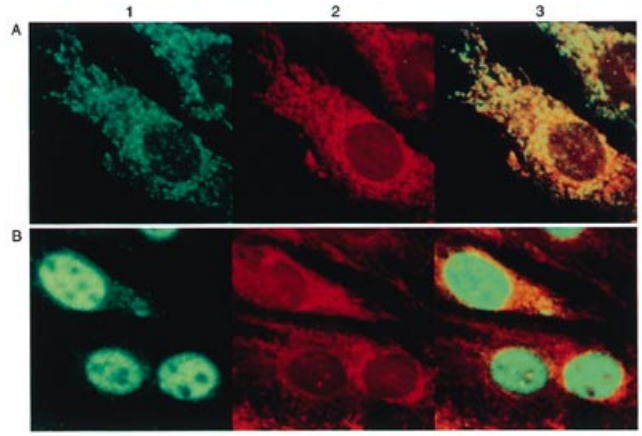

Figure 5. Subcellular localization of mouse CRY proteins identified by immunofluorescence microscopy in mouse C3H10T1/2 cells. CRY proteins were directly visualized by anti-mCRY1–CT (**A1**) or by anti-mCRY2–CT (**B1**). (A2) and (B2) show the fluorescence produced by MitoTracker. (A3) and (B3) represent the superposed figures of the panels 1 and 2.

Purification of a recombinant mCRY1 protein by dsDNA Sepharose

SDS gel of crude extracts from *E.coli* cells expressing recombinant GST-fused mCRY1 gene is shown in Figure 7, lane 1. The extract was applied to a glutathione Sepharose column and bound protein was eluted with glutathione (Fig. 7, lane 2). The eluate was then incubated with thrombin to cleave the fused protein and applied to Q-sepharose (Fig. 7, lane 3). Since the eluate contained a large amount of the molecular chaperonine GroEL protein of *E.coli* host cells, the eluate was further incubated with GroES in the presence of ATP. We found that the recombinant mCRY1 was able to be separated from the chaperonine proteins on dsDNA Sepharose column yielding homogeneously purified recombinant mCRY1 (Fig. 7, lane 4). This shows that the recombinant mCRY1 protein is able to bind dsDNA Sepharose.

DNA binding of CRY1 and CRY2 proteins in human and mouse cell extracts

We analyzed DNA binding activity of endogenous CRY proteins using a dsDNA Sepharose column. The mitochondrial extracts from mouse liver were applied on dsDNA Sepharose and bound proteins were eluted by buffers with gradient KCl concentrations. The presence of mCRY1 and mCRY2 was identified by their specific antibodies. Figure 8A shows that mCRY1 protein in the mitochondrial extracts was mainly eluted by buffers containing 0.3 and 0.6 M KCl concentration, whereas mCRY2 was found only in the pass-through fraction of the column. The binding profiles of both proteins did not change, if single stranded (ss)DNA Sepharose or, more importantly, UV-irradiated DNA Sepharose instead of dsDNA Sepharose was used (not shown). The recombinant mCRY1 also bound tightly to the column, although the elution profile of the recombinant protein showed the main peaks at 0.2 , 0.5 and 1.2 M KCl concentration, which was slightly different from that of the endogenous protein. The native protein may bind DNA Sepharose as a complex with other protein(s), which influences the binding characteristics to DNA

Figure 6. Localization of GFP-fused terminal sequence of mouse CRY proteins. GFP alone [GFP(control)]; GFP-fused N-terminal sequence of mCRY1 [CRY1(NT)-GFP]; GFP-fused C-terminal sequence of mCRY1 [GFP-CRY1(CT)] and GFP-fused C-terminal sequence of mCRY2 [GFP-CRY2(CT)] were transfected into C3H10T1/2 and GFP fluorescence was visualized.

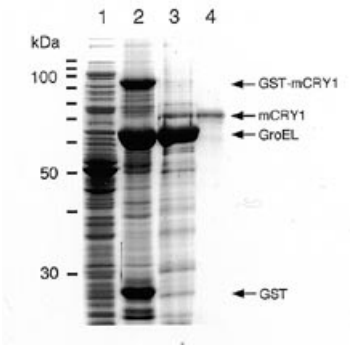

Figure 7. Purification of recombinant mouse mCRY1 protein from *E.coli* extracts. Crude extracts (lane 1), eluate from glutathione column (lane 2), eluate from Q-Sepharose after treatment of the eluate with thrombin (lane 3) and eluate from dsDNA Sepharose (lane 4) are shown.

Sepharose. Furthermore, since the recombinant mCRY1 was produced in *E.coli* in a tight complex with GroEL (Fig. 7), the protein folding of the recombinant CRY1 may be changed and influenced the binding profile. We used the same method for the whole cell extracts from HeLa cells. The elution pattern with the elution peaks at 0.3 and 0.6 M KCl was obtained for hCRY1 (Fig. 8B), which was the same as that of mouse mitochondrial extracts. The human CRY2 from the whole HeLa cell extracts was found exclusively in the through fraction of the DNA Sepharose column, as was the case for the mouse mitochondrial extracts. These results indicate that, in contrast to CRY1 protein, both nuclear and mitochondrial mammalian CRY2 proteins did not bind to the DNA Sepharose column.

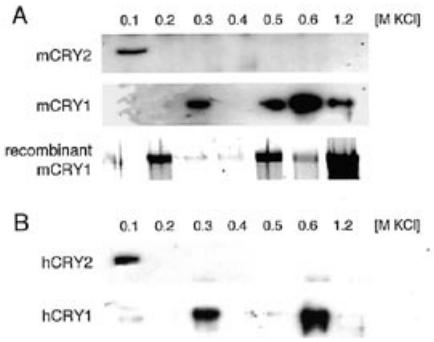

Figure 8. Identification of mouse and human CRY proteins by DNA Sepharose chromatography. (**A**) Mitochondrial fraction from mouse liver cells was applied on dsDNA sepharose and the presence of mCRY1 and mCRY2 proteins were identified by western analysis. Purified recombinant mCRY1 was also identified in eluted fraction from the column. (**B**) Total HeLa extracts were applied on dsDNA Sepharose and eluate was identified by the antibodies against mCRY1 and mCRY2, which cross-react their human counterparts.

DISCUSSION

There are contradicting results about the photolyase activity in human cells. While the presence of photoreactivating activity against CPD was shown in human blood cells (15), another report indicated that HeLa cells and human white blood cells do not possess any CPD photolyase activity (16). Despite the discussions, two human photolyase-like genes have been identified in databases and the complete sequences were obtained (3,11,12). Both genes encode proteins, which are highly homologous (~50% identity of amino acid sequence) to the recently identified 6-4 photolyases from *Drosophila* and *Arabidopsis* (3,17) but differ significantly in the primary sequence from the class II CPD photolyases found in aplacental mammals and higher eukaryotes, or from the class I CPD photolyases found in a number of microorganisms. Therefore, the finding of the human photolyase homologs does not answer the longstanding question whether human possesses CPD photolyase or not. However, neither CPD nor 6-4 photolyase activity was detected in the recombinant human photolyase homologs (12). Since the recombinant proteins from *E.coli* are highly contaminated with bacterial chaperonines (Fig. 7) and were only analyzed as fusion proteins, characterization of the native mammalian proteins was necessary. Therefore, we cloned the mouse homologs of the human genes, raised specific antibodies to each protein and determined two essential characters of the proteins, subcellular localization and DNA binding property.

Most interestingly, we found by fractionation of cell extracts as well as by immunocytochemistry that the two mammalian CRY proteins reside in different organelles: mCRY1 protein is localized in the mitochondria, while mCRY2 was mainly found in the nucleus. GFP-fused peptides showed that the presence of mCRY2 in the nucleus is probably due to a functional nuclear localization signal (NLS) sequence in the extended C-terminal region of the mCRY2 gene. This NLS is also found in hCRY2, but not in mCRY1 and hCRY1 proteins. In case of CRY1 protein, there is a functional mitochondrial transport signals at the N-terminal region of the protein. Unfortunately, we could not identify a similar mitochondrial transport signal in mCRY2

protein, because the N-terminus of the cDNA is still incomplete. However, our cell fractionation experiments (Fig. 4) indicate that while the majority of CRY2 is transported into the nucleus due to the above-mentioned NLS signal, a small portion of CRY2 is imported into mitochondria. The same results as the mouse proteins were obtained for the human proteins (not shown). In conclusion, the mammalian mitochondria contain both CRY proteins, whereas in the nucleus only CRY2 is present.

We found marked differences not only in the subcellular localization of the CRY proteins, but also in their ability to bind DNA. In contrast to CRY2, which was not retained on dsDNA Sepharose column, the native mCRY1 binds to the column and eluted at two distinct peaks at KCl concentrations around 0.3 and 0.6 M (Fig. 8A). Since it cannot be excluded that mCRY1 binds to DNA via associating proteins, we have also performed DNA binding studies with purified recombinant mCRY1. The binding of the protein to dsDNA Sepharose did not differ much from that of the endogenous CRY1 protein (Fig. 8A), suggesting that DNA binding is a property of mCRY1 itself. Since the amino acid sequence of the photolyase-core portion of the CRY1 is highly similar to that of the CRY2 and photolyases are well known DNA binding proteins, the observed difference in DNA binding characteristics may result from differences in the C-terminal sequences of the proteins. We think that the carboxyl terminus of the CRY2 protein may prevent the core portion from binding to DNA. The use of UV-irradiated DNA Sepharose neither enhanced the binding of CRY1 to the column, nor caused CRY2 to bind the column (data not shown), suggesting that the native mammalian CRY proteins do not function as UV damage binding proteins. Binding activities of CRY proteins to specific DNA structure or to DNA damaged by other than UV remain to be determined. CPD photolyase of the yeast *Saccharomyces cerevisiae* was reported to bind DNA treated with *cis*-platinum or alkylated agents and modify the nucleotide excision repair of the damage (18).

The C-terminal extension of the Arabidopsis CRY1 was shown to be important for the blue-light response, because mutations in that region resulted in functionally altered CRY1 gene product (4). We have shown here that in case of mammalian CRY genes the C-terminal sequences determine the subcellular localization and possibly also other characteristics of the protein. Very recently, it was shown *in vitro*, that the *Arabidopsis* CRY1 protein binds to phytochrome A through the C-terminal region of the CRY1 (19). By gel filtration of the mouse mitochondrial fraction we found that mCRY1 was eluted as molecules of various sizes between 68 and 450 kDa (not shown), suggesting that CRY1 is in a complex(es) with other protein(s). We also identified CRY2 protein in a nuclear fraction over 450 kDa by gel filtration of mouse liver cells. A recently reported human protein, a phosphatase, may be one of the associating proteins, though it binds to both CRY1 and CRY2 at their photolyase-like core portions (20). However, since we showed in this report that CRY1 is a mitochondrial protein, the interaction between the nuclear phosphatase and CRY1 protein may not occur *in vivo*. Further identification of the associating proteins is very important for the understanding of the function of the CRY proteins.

Recently, Miyamoto and Sancar reported that mRNAs of the mouse CRY genes are specifically expressed in the retinal ganglion cells and inner nuclear layers of the mouse retina. In addition, mRNA of CRY1 was found to be expressed at high level in the suprachiasmatic nucleus (SCN) in a circadian manner (21). These observations led the authors to propose that the photolyase homologs function as a photoreceptor in the circadian clock. The characteristics of CRY proteins reported in this paper may be important to explain the possible function of the CRY1 protein in the circadian clock. There were a number of reports describing the relationship between mitochondria and the circadian clock (see 22 for review). Several inhibitors of mitochondrial functions lead to large phase shifts in the rhythms of plants, microorganisms and animals. Studies using cells of the filamentous fungi *Neurospora crassa* suggested that some part of the oscillator may be localized to the mitochondria, or alternatively, that the clock mechanism may be influenced by mitochondrial function (23). Although the expression patterns of the mouse CRY genes in the retina and the SCN are suggestive, there is no direct evidence that the mammalian CRY proteins are involved in the circadian rhythms. The expression of both CRY genes in all the mouse and human organs may suggest other (or additional) house-keeping functions of the mammalian photolyase homologs. Further studies using gene-disrupted animals or cell lines are required to identify the real function(s) of these photolyase-like proteins.

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