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Expression and Functional Analysis of the Cellular Retinoic Acid Binding Protein from Silkworm Pupae (*Bombyx mori*)

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Abstract Cellular retinoic acid binding protein (CRABP) is a member of intracellular lipid-binding protein (iLBP), and closely associated with retinoic acid (RA) activity. We have cloned the CRABP gene from silkworm pupae and studied the interaction between *Bombyx mori* CRABP (BmCRABP) and all-trans retinoic acid (atRA). The MTT assay data indicated that when BmCRABP is overexpressed in Bm5 cells, the cells dramatically resisted to atRA-induced growth inhibition. Conversely, the cells were sensitive to atRA treatment upon knocking down the BmCRABP expression. Subcellular localization revealed that BmCRABP is a cytoplasm protein, even when treated with atRA, the CRABP still remained in the cytoplasm. These data demonstrated that the function of BmCRABP have an effect on the physiological function of atRA. J. Cell. Biochem. 102: 970–979, 2007.

Key words: *Bombyx mori*; cellular retinoic acid binding protein; all-trans retinoic acid; subcellular localization

Retinoic acid (RA), the metabolite of vitamin A, influences the proliferation and cellular differentiation of a wide variety of cell types by modulating the transcription of numerous target genes, and thus plays critical role in embryonic development, growth, and remodeling of adult tissues. RA also can induce cellular apoptosis by activating the transcription of apoptosis-related genes, including ubiquitin-activating enzyme E1-like protein, CCAAT/enhancer binding protein ε, tumor necrosis factor (TNF)-related apoptosis-inducing ligand, caspase 7 and caspase 9 [Park et al., 1999; Altucci et al., 2001; Kitareewan et al., 2002; Donato and Noy, 2005]. Natural and synthetic RA derivatives, collectively known as retinoids, have been shown to have inhibitory activity in some carcinomas, and are used as chemotherapeutic and chemopreventive agents in a variety of malignancies such as head and neck cancers, promyelocytic leukemias, and cancers of the respiratory and digestive tracts [Lotan, 1996; Hong and Sporn, 1997; Miller, 1998; Shin et al., 2000; Papadimitrakopoulou et al., 2002]. RA was also suggested to be efficacious in the treatment of breast cancer. However, long-term usage of RA could result in RA-resistance [Dencker et al., 1990; Warrell, 1993a,b].

The cellular retinoic acid binding proteins (CRABPs), the retinoic acid receptors (RARs) and retinoid X receptors (RXRs) were centered in RA signal transduction, in which RARs responded to all-trans and 9-cis-isomers of RA, and the RXRs were activated by 9-cis-RA exclusively. CRABPs are small (~14 kDa)

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soluble proteins, which are members of the intracellular lipid binding proteins family and bind RA with high affinity and specificity [Dong et al., 1999]. There are two CRABPs (CRABP-I and CRABP-II) in vertebrates and only one in invertebrates. In general, CRABP-I was believed to arrest RA and help to metabolite RA by enhancing the activity of an enzyme(s) that catalyzes RA degradation [Boylan and Gudas, 1991, 1992] in the aqueous space of the cytosol. However, CRABP-II is believed to protect RA and deliver RA to its nuclear receptor through direct protein–protein interactions between the binding protein and the receptor [Budhu et al., 2001]. There is no nuclear localization signal (NLS) in the primary sequences of CRABP-II. But when CRABP-II interacts with RA to form holo-CRABP-II, a "classical" NLS occurs in the three-dimensional (3-D) configuration of CRABP-II [Sessler and Noy, 2005], and makes the CRABP-II-RA complex enter into the nucleus. In the presence of RA, the CRABP-II-RAR complex is a short-lived intermediate. They interact transiently and rapidly transfer RA to its receptor [Budhu and Noy, 2002], thereby enhancing the transcriptional activity of the receptor [Dong et al., 1999; Budhu et al., 2001; Budhu and Noy, 2002]. RAR cannot transcribe genes by itself. Instead, it forms heterodimers with the retinoid X receptor. RXR–RAR heterodimers bind onto regulatory regions of target genes and enhance transcriptional rates upon binding of RA [Mangelsdorf et al., 1995]. RXRs can form homodimers and activate retinoid X response elements or form heterodimers with other members of the steroid receptor family to activate transcription, thus providing opportunities for cross-talk among different signaling pathways [Chambon, 1996]. However, RAR usually transcribes genes through heterodimerization with RXR [Yu et al., 1991; Durand et al., 1992; Hallenbeck et al., 1992; Leid et al., 1992]. All RA responded genes contain a specific DNA sequence termed retinoic acid response element (RARE), which is composed of two direct repeats of the consensus sequence PuG (G/T) TCA, separated by either 2 bp (DR-2) or 5 bp (DR-5) [The et al., 1990; Chambon, 1996].

CRABP is closely associated with carcinoma. Overexpression of CRABP-I in F9 teratocarcinoma cells alters both the amounts and types of atRA metabolites in these cells and shortens the intracellular half-life of RA [Boylan and Gudas, 1992]. Transfection of CRABP-I resulted in the resistance of head and neck squamous carcinoma (HNSCC) cells to RA because the transfected CRABP-I increased CYP26-mediated catabolism of RA, which decreased the amount of RA that may access to the nuclear receptors [Won et al., 2004]. However, compared to normal cells, the level of CRABP-II is downregulated in some carcinoma cells, such as prostate cells [Okuducu et al., 2005] and renal cell carcinoma (RCC) cells [Goelden et al., 2005]. On the other hand, CRABP-II-transfected neuroblastoma cell lines showed an increase in cancer gene transcription and cell motility. Hence, CRABP-II has been proposed to be a potential diagnostic marker for some cancers [Okuducu et al., 2005; Gupta et al., 2006].

Here we reported the investigation of the biological activity of CRABP from *Bombyx mori*. We have cloned CRABP gene from silkworm pupae and studied the function of this protein. We showed that *B. mori* Bm5 cells were sensitive to RA-induced growth inhibition. Overexpression of the BmCRABP makes the cell resistant to RA while inhibition of BmCRABP expression sensitizes the cell to RA-induced growth inhibition. The subcellular localization analysis indicated that regardless of the presence of RA, the CRABP locates to the cytoplasm all the time.

MATERIALS AND METHODS

Materials

4′-6-Diamidino-2-phenylindole (DAPI) was purchased from Sigma. All-trans retinoic acid (atRA, Sigma) was dissolved in dimethyl sulfoxide (DMSO, Sigma) and stored at −80°C. Bm5 Cells (gift from Prof. Zhi-Fang Zhang), silkworm ovary cell line, was cultured in TC-100 medium (Sigma) supplemented with 10% fetal calf serum (FCS, Gibco BRL) at 27°C.

Reverse Transcription PCR

The total RNA was extracted from silkworm pupae using the Trizol reagent (Invitrogen) according to the kit instruction. The total RNA was reverse transcribed into the first-strand of cDNA using the First-Strand cDNA Synthesis Kit (Amersham). The BmCRABP gene was cloned by PCR amplification using the first-

strand of cDNA as template, and the primers complemented to the flank sequences of the open reading frame (ORF) of the gene containing *Bam*H I and *Hin*d III recognition sites were used as follows:

5′-TAGGGATCCATGGAATTCGTAGGCA-3′,
5′-GTGAAGCTTTTACTGGACCTTGTAG-3′

PCR product was analyzed by a 1% agarose gel electrophoresis.

Proteins and Polyclonal Antibody

The ORF of BmCRABP was prepared by PCR amplification. Purified PCR product was subcloned into the pGEM-T easy vector (Promega) and sequenced using Genetic Analyzer (3100-Avant, Applied Biosystems) (Accession No. EF112401). Then the CRABP gene was inserted into the *Bam*H I-*Hin*d III sites of the bacterial expression vector pET-28a (+) to construct the pET-28a (+)-CRABP plasmid. The pET-28a (+)-CRABP plasmid was transferred into *Escherichia coli* BL21 strain to express the protein. *E. coli* cells harboring the vector were grown at 37°C to an A_{600} about 0.6 and protein expression was induced with 0.1 mM IPTG. Cells were grown for an additional 5 h period, and pelleted by centrifugation. Following lysis, protein was purified by gel filtration chromatography (sephadex G-200, Amersham) and then metal-chelating affinity chromatography (Ni^{2+}-Sephadex™ G-25 Superfine, HiTrap™ Desalting Column, Amersham). Purified BmCRABP was analyzed by SDS–PAGE, and polyclonal antibody was prepared using New Zealand rabbit [Hu et al., 2002].

Multiple Amino Acid Sequence Alignment and 3-D Molecular Modeling

CRABP sequences which were used in multiple amino acid sequence alignment were retrieved by BLASTP search of the GenBank database at the NCBI. Multiple amino acid sequence alignment was performed by using the CLUSTALW method in BioEdit and the amino acid sequences were listed in Table I. 3-D Molecular Model was generated by SWISS-MODEL (http://swissmodel.expasy.org//SWISS-MODEL.html) using *Bos taurus* cytosolic fatty acid binding protein (FABP) chain A (Accession No. 1PMP_A), *Bos Taurus* FABP chain B (Accession No. 1PMP_B), *B. Taurus* FABP chain C (Accession No. 1PMP_C), *Schistocerca gregaria* FABP chain A (Accession No. 1FTP_A) and *S. gregaria* FABP chain B (Accession No. 1FTP_B) as the template [Peitsch, 1995; Guex and Peitsch, 1997; Schwede et al., 2003].

TABLE I. *B. mori* CRABP and Some Other CRABPs

Accession no.	Organism	Type
EF112401	Silkworm	CRABP
AAC24317	Tobacco hornworm	CRABP
BAF02663	Morning glory sphinx moth	CRABP
CAG33298	Human	CRABP-I
AAT38218	Zebrafish	CRABP-I
1CBR_A	House mouse	CRABP-I
NP_001869	Human	CRABP-II
AAO85530	Zebrafish	CRABP-II
AAA37452	House mouse	CRABP-II

Construction of pEGFP-C1-CRABP

The *Hin*d III and *Bam*H I sites were introduced into the two ends of the CRABP gene by PCR amplification. The primers were as follows:

5′-ACTGAAGCTTGTATGGAATTCGTAGGC-3′,
5′-CTTGGATCCCTTTTACTGGACCTTGT-3′

The PCR fragment was digested and subcloned into the pEGFP-C1 vector, and the constructed plasmid (pEGFP-C1-CRABP) was confirmed by sequencing.

Synthesis of BmCRABP dsRNA

The restriction fragment obtained from *Hin*d III/ *Bam*H I digestion of plasmid pEGFP-C1-CRABP was ligated into pET-28a (+) vector which was digested using the *Bam*H I and *Hin*d III too. In this recombinant, the CRABP gene was ligated into pET-28a (+) vector in the reverse orientation, named as pET-28a (+)-rCRABP. Then we used the T7 primer and the downstream primer (5′-ACTGAAGCTTGTATGGAATTCGTAGGC-3′) to do PCR amplification using pET-28a (+)-rCRABP as the template. Meanwhile we used the T7 primer and the downstream primer (5′-GTGAAGCTTTTACTGGACCTTGTAG-3′) and pET-28a (+)-CRABP as the template to do PCR amplification. The two PCR amplification products were separately purified and used to synthesis the dsRNA using T_7 RiboMAX™ Express RNAi System (Promega) according to the manufacture's instruction.

Transfection Assay

Bm5 cells were seeded on a 24-well plate and transfected with dsRNA, pEGFP-C1, or pEGFP-C1-CRABP. The transfection protocol was according to the kit (Cellfectin Reagent, Invitrogen) instruction. At 24 h after transfection, the same volume of DMSO (control, 1/1,000 of media) or atRA with different concentration (0, 10, 100, 1,000, 10,000 μM) was added (the volume of solvent = 1/1,000) to culture medium. The medium with atRA or DMSO was changed every 48 h. Four days after treatment, 1% of 0.5 mg/ml MTT reagent was added and incubated for an additional 4 h. After that, the culture was removed and 750 μl DMSO was added. Absorbance at 490 nm was measured using an enzyme-linked immunosorbent assay reader (SPECTRAmax PLUS, Molecular Devices), and absorbance at 630 nm was used as a reference.

Subcellular Localization

Bm5 cells were seeded arbitrarily in the dish (Bio-Line Instruments) which was used specially for Confocal Microscope. After 12 h the cells were treated with DMSO (control) or atRA (1 μM) for 3 h. Culture medium was removed, cells were rinsed twice with 1 ml phosphate-buffered saline (PBS), fixed in 3.7% formaldehyde at 25°C for 10 min, and treated in 0.2% Triton X-100 at 25°C for 10 min. Then the cells were blocked with 3% BSA at 25°C for 2 h. After that, the cells were incubated with anti-BmCRABP serum (dilution, 1/50) at 4°C for 12 h (the cells incubated with negative serum as control). After three washes in PBST (PBS + 0.05% Tween-20, 10 min each), cells were incubated with goat anti-rabbit antibody (Cy3 labeled, Promega) (dilution, 1/200) and 4′-6-Diamidino-2-phenylindole (dilution, 1/2,000) at 37°C for 2 h. Following three washes with PBST (10 min each), cells were analyzed by Nikon ECLIPSE TE2000-E Confocal Microscope with image analysis software EZ-C1.

RESULTS

Expression of CRABP Gene of *B. mori*

A cDNA sequence was obtained from our laboratory's silkworm pupae cDNA database. After blasting with GenBank and SilkBase (http://papilio.ab.a.u-tokyo.ac.jp/silkbase/), it was identified as the CRABP gene. The ORF of this gene was obtained using RT-PCR and blasted with the database online. The identity between this ORF sequence and *B. mori* CRABP mRNA (Accession No. NM_001043899 NM_001043900, AB267807) is 100%. It is also conserved with *Agrius convolvuli* CRABP mRNA (Accession No. AB267808) that the identity is 85%, *Manduca sexta* CRABP mRNA (Accession No. U75307) is 84% and *Plutella xylostella* CRABP mRNA (Accession No. AB180450) is 86%. Therefore, we concluded that this cDNA is a CRABP gene of silkworm.

Recombinant BmCRABP was purified by gel filtration chromatography and metal-chelating affinity chromatography. After the gel filtration step, proteins with about 18kDa appeared as distinct bands. Except for 18kDa protein, other proteins were mostly removed by the affinity chromatography. The protein in each chromatographic step was analyzed by SDS–PAGE (Fig. 1), and polyclonal antibody was prepared using New Zealand rabbit. The titration of the polyclonal antibody exceeded 1:12,800.

Multiple Amino Acid Sequence Alignment and 3-D Molecular Modeling

The key amino acids, which are necessary and sufficient for ligand binding, of CRABP-I are

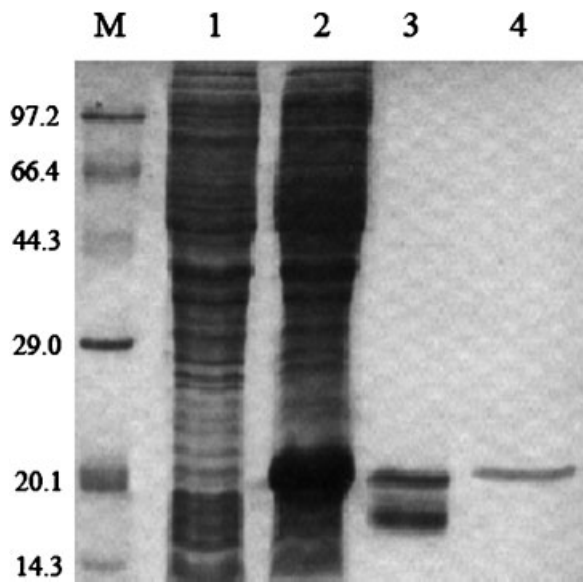

Fig. 1. SDS–PAGE analysis of BmCRABP. **Lane M**, marker proteins. **Lane 1**, protein sample from pre-IPTG-induced bacterial cells. **Lane 2**, protein sample from IPTG-induced bacterial cells. **Lane 3**, protein sample from sephadex G-200 gel filtration chromatography. **Lane 4**, protein sample from metal-chelating affinity chromatography.

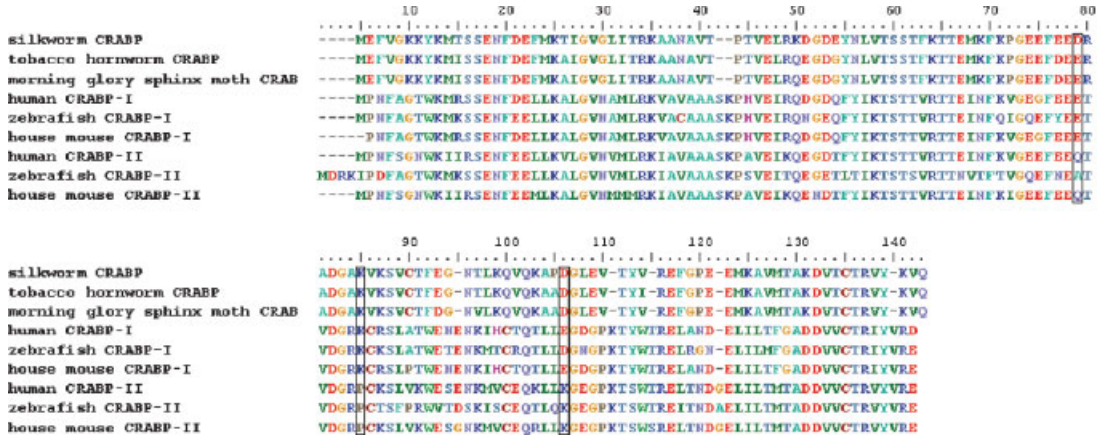

Fig. 2. Multiple amino acid sequence alignment. Several invertebrate and vertebrate CRABP sequences were aligned with BmCRABP sequence. According to the result of multiple amino acid sequence alignment and the key amino acids, which are necessary and sufficient for ligand binding, of CRABP-I and CRABP-II, we proposed that the key amino acids of BmCRABP may be D73, K79, D99 (shown with open boxes).

E75, K81, E102, and of CRABP-II are Q75, P81, K102 [Budhu et al., 2001]. According to the result of multiple amino acid sequence alignment and the key amino acids of CRABP-I, -II, we proposed that the key amino acids of BmCRABP might be D73, K79, and D99 (Fig. 2). Since the key amino acids between BmCRABP and CRABP-I are similar, we thought that the function of BmCRABP may be similar to CRABP-I, which it correlates with the degradation of RA. 3-D molecular model of BmCRABP (Fig. 3) was processed by SWISS-MODEL. The image indicated that this protein is a cylinder, and mainly composed of 10 β-strand sheets and α-helices.

Transfection Assay of dsRNA in Bm5 Cells

There was 200 μl culture medium in each well. When the concentration of transfection reagent was 2 μl/ml and dsRNA was 3.5 μg/ml, the MTT assay data indicated that the cell growth-inhibition rate was the biggest after 100 nM of atRA treatment for 4 days (Fig. 4). This experiment demonstrated that knocking down the expression of BmCRABP would sensitize the cells to RA-induced growth inhibition.

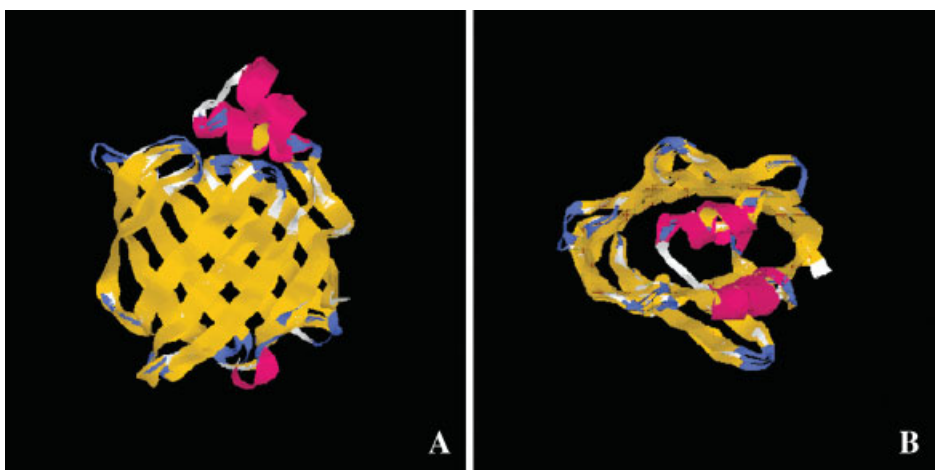

Fig. 3. The analysis of the 3-D molecular modeling structure of BmCRABP. **A** and **B** are elevation and plan, respectively. The BmCRABP are mainly composed of 10 β-strand sheets and two helices, the retinoic acid binding pocket are in the core.

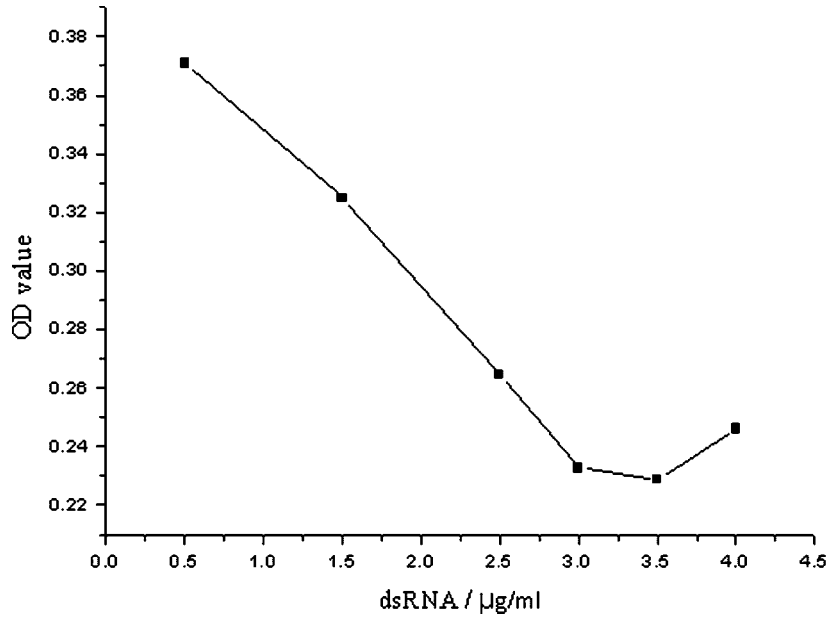

Fig. 4. The influence of dsRNA in Bm5 cells. Bm5 cells were transfected with different concentration of dsRNA. Then the cells were treated with 100 nM of atRA for 4 days. And the cytoactivity of cells were measured using the MTT assay (absorbance at 630 nm was used as a reference).

BmCRABP Rescues the Bm5 Cells From atRA-Induced Growth Inhibition

According to the similarity in the key amino acids, the function of the BmCRABP may be to arrest RA and help to degrade RA. We designed that one group of Bm5 cell was transfected with pEGFP-C1-CRABP and the other transfected with dsRNA, which resulted in cells that would overexpress or underexpress the CRABP

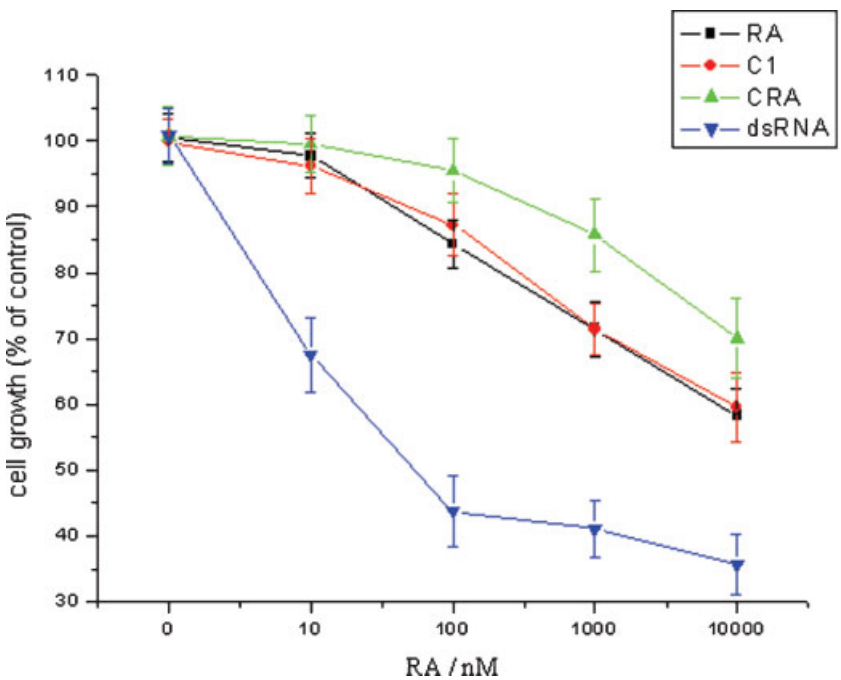

Fig. 5. BmCRABP rescues the Bm5 cells from atRA-induced growth inhibition. Parental Bm5 cells (RA) and their derivatives which transfected with pEGFP-C1 (C1), pEGFP-C1-CRABP (CRABP), dsRNA (dsRNA) were treated with atRA for 4 days, and the cytoactivity of cells were measured using the MTT assay. Data shown represent cell growth as a percent of vehicle-treated cell growth (control) and are the mean ± SEM (n = 5).

followed by atRA treatments at different concentrations. In order to eliminate the influence of DMSO, the same volume of DMSO was added to all wells. At the end of this experiment, the cytoactivity of cells was measured using the MTT assay. When the cells were transfected with pEGFP-C1-CRABP compared to parental cells, the growth rate was dramatically elevated (Fig. 5). But when knocking down the expression of CRABP using dsRNA, the growth rate greatly reduced (Fig. 5). The growth rate of the cells transfected with pEGFP-C1 plasmid (control) was similar to that of the parental cells (Fig. 5).

Subcellular Localization of BmCRABP

BmCRABP may be involved in RA degradation. To test this hypothesis, the subcellular localization of endogenous BmCRABP was determined using the Bm5 cells. Immunostaining indicated that BmCRABP localizes to the cytoplasm both in the presence and absence of atRA (Fig. 6).

DISCUSSION

This study was undertaken to obtain insights into the function of BmCRABP. Our data revealed that BmCRABP may correlate with the degradation of RA. According to the 3-D molecular model, compared to CRABP-II, BmCRABP shares higher similarity with CRABP-I in structure. In 3-D structure, CRABP is mainly composed of β-strand sheets and α-helices. However, BmCRABP and CRABP-I have two α-helices such as house mouse CRABP-I chain A (Accession No. 1CBI_A), cattle CRABP-I Chain A (Accession No. 2CBR_A), while CRABP-II have one helix such as human CRABP-II (Accession No. 1BLR). This may be the reason why the function of BmCRABP is similar to CRABP-I. Because there is only one domain, BmCRABP-RA complex may be similar to CRABP-I-RA complex as the substrate of RA-catalizese [Won et al., 2004].

So far, there is only one CRABP found in invertebrate. It may only correlate with the

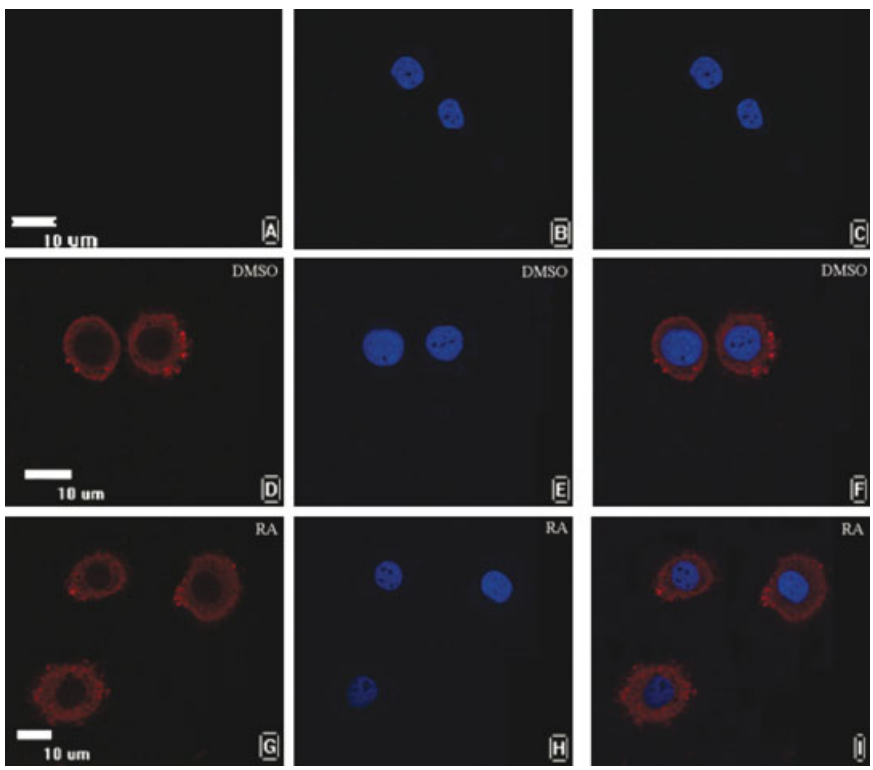

Fig. 6. The subcellular localizes of endogenous BmCRABP. Bm5 cells were treated with DMSO (**D**–**F**) or 1 μM RA (**G**–**I**) for 3 h, and (**A**–**C**) are negative control. (A,D,G) Immunostaining with BmCRABP antibodies; (B,E,H) Imaging of nuclei using the nucleic acid stain DAPI; (C,F,I) overlay of images of BmCRABP and nuclei.

degradation of RA protecting cells from excess of RA. But when the RA is insufficient, CRABP cannot help RA to play its biological functions efficiently. So in the evolutionary process, CRABP have evolved into CRABP-I and CRABP-II. They play different functions to sequester RA, help to metabolize RA or transport RA to nucleus. However, this is a postulation needed further investigations. RARs have not been found in invertebrates [Giguere, 1994], and RXR are activated by 9-cis-RA exclusively. Then how can *B. mori* Bm5 cells respond to atRA-induced growth inhibition? Is there RAR in *B. mori* or can RXR bind to atRA to activate gene transcription? We are prone to think that there is no RAR in *B. mori* and that RXR in lower organism can be activated by a variety of retinoids, such as atRA. This may be the need of evolution.

The analysis of amino acid sequence of BmCRABP indicated that the function of BmCRABP may be similar to that of CRABP-I. We aligned the amino acid sequence of BmCRABP and several other CRABPs using the CLUSTALW method in BioEdit (Fig. 2). The key amino acids, which are necessary and sufficient for ligand binding, of CRABP-I are E75, K81, E102,and of CRABP-II are Q75, P81, K102 [Budhu et al., 2001]. Then we postulated that the key amino acids of BmCRABP may be D73, K79, D99. However, there are two differences in the key amino acids between CRABP-I and BmCRABP. Both of them are acidic amino acids, which may have little influence on the function of proteins. The two proteins share high similarity in 3-D structure. So we hypothesized that their function may be similar in the binding of RA as the substrate for other enzymes such as CYP26, which is a cytochrome retinoic acid hydroxylase [Won et al., 2004].

If BmCRABP does affect the degradation of RA, altering the expression of BmCRABP will influence the RA's growth-inhibition to cells. So we first established the right transfection conditions for dsRNA transfection in Bm5 cells and then studied the effects upon RA treatment (Fig. 4). This experiment not only verified that knocking down the expression of BmCRABP will sensitize the cells to RA-induced growth inhibition, but also validated the transfection. When the Bm5 cells were transfected with pEGFP-C1-CRABP at appropriate conditions, the cells displayed an atRA resistance (Fig. 5). Only when treated with high dose of RA, the cytoactivity of cells began to reduce. A-498 RCC cells which were stably transfected with CRABP-I consequently displayed substantial resistance to all-trans and 9-cis RA compared to vector controls lacking CRABP-I [Pfoertner et al., 2005]. On the other hand, Bm5 cells were dramatically sensitized to atRA-induced growth inhibition when transfected with dsRNA (Fig. 5). The data indicated that the BmCRABP weakens the physiological function of atRA. There is one domain in BmCRABP which binds RA and the BmCRABP-RA complex maybe the substrate of RA-catalizese. Then the RA's signal transduction may stop in this way. It is possible that RA enters into the nucleus by simple diffusion because of the absence of CRABP-II in *B. mori*. This may also protect lower organism from the teratogenicity of high dose of RA.

In mammary cells CRABP-I is associated with the degradation of RA. When treated with RA, CRABP-I localizes to cytoplasm. CRABP-II mainly transports RA to nucleus in the presence of RA [Budhu and Noy, 2002]. The subcellular localization of CRABP-I depends on the first 123nt of CRABPI 3′UTR which also influences the localization of CRABP-I mRNA [Martin et al., 2006]. An NLS occurs after CRABP-II bound RA [Sessler and Noy, 2005]. In *B. mori*, there is only one binding protein. Where will it localize when the cells are treated with RA? We have postulated that BmCRABP correlates with the degradation of RA. Thus, BmCRABP should be in cytoplasm after binding RA. Our data support this scenario (Fig. 6). Therefore, BmCRABP correlates with the degradation of RA. But this is only the postulation based on an indirect evidence, needing further validation. If we can find the metabolite of RA, elucidate the relationship between BmCRABP and RA, it will be better founded.

Overall, these data have shown that Bm5 cells are resistant to atRA-induced growth inhibition when cells overexpress CRABP, and sensitive to atRA when knocking down the expression of CRABP. This function is similar to the biological activity of CRABP-I in invertebrate. The subcellular localization is consistent with this: BmCRABP localizes to the cytoplasm in the presence or absence of atRA. So we believe that BmCRABP mainly weakens the physiological function of atRA. However, there is no direct evidence to prove that BmCRABP

accelerates the degradation of atRA, which is the next aim of our study.

ACKNOWLEDGMENTS

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