Isolation, Characterization, and cDNA Sequence of a Carotenoid Binding Protein from the Silk Gland of Bombyx mori Larvae*

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A carotenoid binding protein (CBP) has been isolated from the silk glands of Bombyx mori larvae. The protein has an apparent molecular mass of 33 kDa and binds carotenoids in a 1:1 molar ratio. Lutein accounts for 90% of the bound carotenoids, whereas α-carotene and β-carotene are minor components. Immunological analysis demonstrated the presence of CBP only in the yellow-colored tissues of the silk gland, midgut, testis, and ovary. Several phenotypes of B. mori mutants linked to carotenoid transport have been utilized to characterize CBP. The Y (yellow hemolymph) gene controls uptake of carotenoids from the midgut lumen into the midgut epithelium, and larvae with the +Y gene lack this property. Immunoblotting analysis confirmed the presence of CBP in mutants with the dominant Y gene only. Immunohistochemistry verified the localization of CBP in the villi of the midgut epithelium, indicating that CBP might be involved in absorption of carotenoids. A cDNA clone for CBP encoding a protein of 297 amino acids has been isolated from the B. mori silk gland cDNA library. The deduced amino acid sequence revealed that CBP is a novel member of the steroidogenic acute regulatory (STAR) protein family with its unique structural feature of a STAR-related lipid transfer domain, known to aid in lipid transfer and recognition. Lutein-binding capacity of the recombinant CBP (rCBP) determined by incubating rCBP with lutein followed by immunoprecipitation using anti-CBP IgG conjugated to protein A-Sepharose, demonstrated the formation of a lutein-rCBP complex. Sequence analyses coupled with binding specificity suggest that CBP is a new member of the STAR protein family that binds carotenoids rather than cholesterol.

Carotenoids play important and diverse roles in insects. For example, a deficiency of carotenoids in Drosophila melanogaster causes a decrease in visual sensitivity (1). Phototactic responses are lost when the silkworm Bombyx mori larvae are reared on a carotenoid-free diet (2). The green color, a product of yellow and blue bile pigments, serves a camouflage role in insects (3). The production of a yellow cocoon in B. mori is dependent on the availability of carotenoids in the silk gland site of silk production. How dietary carotenoids are transferred from the midgut to the silk gland via the hemolymph is unclear.

The existence of cellular carrier proteins that facilitate this transfer process has been predicted for many years (4). A lutein-binding protein (LBP)1 was purified from B. mori midgut (5). LBP is a 35-kDa, water-soluble protein found in the midgut, testis, and fat body. However, the role of this protein in lutein transfer and its sequence has not been reported. In mammalian systems many carotenoid-specific binding proteins have been identified (6–8). Three cytosolic retinol binding proteins, type I, II, and III, and two cytosolic retinoic acid binding proteins have been described (9). Retinoid binding proteins belong to the lipocalin family, the members of which share a very low sequence identity. The ligand-binding sites of these lipocalins differ in their structural details providing distinct ligand selectivity (9).

In insects, transport of absorbed carotenoids by hemolymph lipoprotein, lipophorin, has been documented by many investigators (10, 11). Lipophorin serves as a reusuable shuttle, which moves lipids from one tissue to another without itself entering the site (12, 13). Transfer of carotenoids from midgut to lipophorin or from lipophorin to silk gland occurs at the interface between cell plasma membrane and hemolymph. Lipophorin selectively deposits certain lipids at specific tissues. For example, during the larval stage lipophorin delivers diacylglycerol mainly to fat body, hydrocarbon to integument, and carotenoids to silk gland and reproductive tissues. The observed selectivity is not fully understood or characterized, although some of the proteins such as lipid transfer particle (14) and the lipophorin receptor (15) involved in lipid mobilization have been identified. It is conceivable that other cell surface components are present to facilitate the selective lipid delivery.

Genetic linkage mapping has identified three genes whose products control the uptake and transport of carotenoids in

1 The abbreviations used are: LBP, lutein-binding protein; CBP, carotenoid-binding protein; rCBP, recombinant CBP; STAR, steroidogenic acute regulatory protein; START, STAR-related lipid transfer; IPTG, isopropyl-1-thio-β-D-galactopyranoside; Y, yellow hemolymph; I, yellow inhibitor; C, golden yellow cocoon; BSA, bovine serum albumin; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; HPLC, high-performance liquid chromatography.
B. mori, and mutants of these genes are currently available (16) as follows: (i) The Y gene (yellow hemolymph) controls uptake of carotenoids from the midgut lumen into midgut epithelium, thus, larvae of mutants with the $y^{+/+}$ phenotype cannot absorb carotenoids from the midgut. (ii) The I gene (yellow inhibitor, suppresses Y phenotype) is required for the middle parts of the silk gland. Only larvae with the phe-no type I cannot absorb carotenoids from the midgut. (iii) The C gene (golden yellow cocoon) controls the uptake of carotenoids from hemolymph to the middle parts of the silk gland. Only larvae with the phenotype Y$^+$/C make white cocoons. All other gene combinations make white cocoons. Analyses of these mutants offer great promise for dissecting the pathway of carotenoids transport.

In the present study, we utilized the Y$^+$/C (N4) strain to purify a carotenoid binding protein (CBP) involved in the uptake of carotenoids into the midgut epithelium and silk gland. This protein is a new member of the STAR family that is able to specifically bind carotenoids.

**EXPERIMENTAL PROCEDURES**

**Materials**—Protease inhibitor mixture III and protease inhibitor mixture complete were purchased from Calbiochem (La Jolla, CA) and Roche Molecular Biochemicals (Germany). DEAE-Trisacryl M was obtained from Biosepra (France) and Sephadex G-75, PBE 96, Polybuffer, and Roche Molecular Biochemicals (Germany). DEAE-Trisacryl M was obtained from Biosepra (France) and Sephadex G-75, PBE 96, Polybuffer, and Roche Molecular Biochemicals (Germany). DEAE-Trisacryl M was obtained from Biosepra (France) and Sephadex G-75, PBE 96, Polybuffer, and Roche Molecular Biochemicals (Germany). DEAE-Trisacryl M was obtained from Biosepra (France) and Sephadex G-75, PBE 96, Polybuffer, and Roche Molecular Biochemicals (Germany). DEAE-Trisacryl M was obtained from Biosepra (France) and Sephadex G-75, PBE 96, Polybuffer, and Roche Molecular Biochemicals (Germany). DEAE-Trisacryl M was obtained from Biosepra (France) and Sephadex G-75, PBE 96, Polybuffer, and Roche Molecular Biochemicals (Germany). DEAE-Trisacryl M was obtained from Biosepra (France) and Sephadex G-75, PBE 96, Polybuffer, and Roche Molecular Biochemicals (Germany).

**Insects—**B. mori of the N4 strain were reared on an artificial diet (made from mulberry leaves, Yakult, Japan) at 25°C on a 12-h light/12-h dark photoperiod. We used four mutant strains of B. mori with the following four genotypes: YI, Y$^+$/I, Y$^++$/I, and Y$^+$/I. The YI strain cannot transfer carotenoids from the midgut epithelium to the lymph, whereas the Y$^+$ strain cannot. The I strain cannot transfer carotenoids from the midgut epithelium to the lymph, whereas the Y$^+$ strain can. All mutants were reared on fresh mulberry leaves.

**Purification of CBP**—During the development of the purification scheme, CBP was followed by its yellow color. Purification was moni-tored spectrophotometrically using the ratio of the carotenoid absorbance at 452 nm to protein absorbance at 280 nm. The silk glands from day 5 fifth instar B. mori larvae (N4 strain; Y$^+$/C) were dissected out and washed three times with ice-cold phosphate buffered saline (PBS; 10 mM phosphate, 150 mM NaCl, pH 6.5) containing 1 mM benzamidine. The silk glands were homogenized with 1300 ml of PBS and 1 ml of a protease inhibitor mixture III in a Polytron homogenizer for 30 s and centrifuged at 10,000 $\times$ g for 15 min. The supernatant was adjusted to 45% saturation in ammonium sulfate, equilibrated for 1 h, and then centrifuged for 15 min at 10,000 $\times$ g. Using the same procedure, the supernatant was adjusted to 75% saturation in ammonium sulfate. The precipitate was dissolved in 20 ml phosphate buffer, pH 6.5, dialyzed, and applied to a DEAE-Trisacryl M column equilibrated in the same buffer. Different fractions were eluted with a linear NaCl gradient (0–200 mM) at a flow rate of 30 ml/h. The flow-through fraction, containing the yellow protein, was applied to a Sephadex G-75 (2.5 × 110 cm) column equilibrated in 20 mM Tris-HCl (pH 7.5) containing 2 mM EDTA, 150 mM NaCl, and 1 mM benzamidine and was eluted with the same buffer at a flow rate of 15 ml/h. Fractions containing the yellow protein were pooled and dialyzed against 25 ml Tris-CH$_3$COOH buffer (pH 8.5) applied to a PBE96 chromatofocusing column and eluted with pH 5 Polybuffer at a flow rate of 36 ml/h following the manufacturer’s instructions. Fractions containing the yellow protein were pooled, dialyzed against 20 ml phosphate buffer, 2 mM EDTA, 1 mM benzamidine, pH 7.0, and applied to a hydroxyapatite column. The yellow protein was eluted with a linear NaCl gradient (0–300 mM) at a flow rate of 22 ml/h. The purity of the CBP was confirmed by 12.5% SDS-PAGE analysis.

**Amino Acid Sequence of CBP—**Purified CBP was loaded on 12.5% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and stained with Coomassie Blue. The band was cut and incubated with lysyl endopeptidase (17). Peptides were separated by reverse-phase liquid chromatography (HPLC). Amino acids were sequenced from three peptides using an HP G1005A protein sequencing system.

**Determination of Protein Concentration**—Protein concentration was determined by either the bichinchoninic acid assay (BCA) or Bradford (18) with bovine serum albumin (BSA) as a standard.

**Analysis of Carotenoid Composition**—All solutions were flushed with $N_2$ gas. Purified CBP (60 µg containing 24.8 µg of protein) was added to 1 ml of ethanol containing 0.5 ml of distilled water and vortexed for 30 s. Six milliliters of the n-hexane was added to the mixture followed by vortexing for 5 min. The organic phase was removed and purged with $N_2$ gas, and anhydrous sodium sulfate was added to remove water. Five milliliters of the organic phase was dried in a rotary evaporator, resolubilized in 0.2 ml of ethanol, and used for carotenoid analyses and quantification using HPLC. Extracted carotenoids were injected onto an Intersil ODS 3.5-µm column (4.6 × 150 mm) equilibrated with acetonitrile:methanol:hexane:water (58:38:7, v/v/v), with a flow rate of 1 ml/min. Eluted components were detected by a Shimadzu SPD-10AV detector and compared with a mixture of carotenoid standards: β-carotene, β-carotene, and lutein.

**Immuno-**—Antibody for immunoblotting was raised in Japanese White rabbits by subcutaneous injection of the CBP and adjuvant (monophosphoryl lipid A and trehalose dimycolate + cell wall skeleton material (Corixa Corp., Hamilton, MT)) mixture. The serum was stored at -80°C. To identify the presence of CBP in different tissues and mutants, protein samples were separated on SDS-PAGE, transferred to nitrocellulose filters using the method of Towbin et al. (19), and immunoblotted using rabbit anti-CBP antibody and goat anti-rabbit IgG-conjugated alkaline phosphatase. The filters were developed using a color-developing kit.

**Tissue distribution of CBP** was determined on the following samples: the midgut, silk gland, hemolymph, fat body, malpighian tubules, integument, testis, muscle, and ovaries from day 4 of 5th instar larvae. Distribution of the CBP in the anterior, middle, and posterior portion on the silk gland and the midgut were also determined. All tissues were weighed and homogenized in 5 volumes of PBS using a Polytron homogenizer and centrifuged at 10,000 $\times$ g for 10 min. Samples of the supernatant (25 µg of protein) were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-CBP antibody following the procedure described above. Presence or absence of CBP in the midguts and silk glands from four tissues with the following phenotypes YI, Y$^+$/I, Y$^+$/I, and Y$^+$/I were confirmed by immunoblotting using anti-CBP antibody.

**Identification and Sequence Analysis of a cDNA of CBP**—A direction cDNA library was constructed by utilizing 2 µg of mRNA obtained from the silk gland of days 3–5 of 5th instar larvae for cDNA synthesis. The cDNA was ligated into λExCell EcoRI/CIP and packaged.

**TABLE I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein</th>
<th>$A_{452}/A_{280}$</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant from silk gland homogenate</td>
<td>1736.8</td>
<td>0.0566</td>
<td>1</td>
</tr>
<tr>
<td>45–75% ammonium sulfate fraction</td>
<td>663.8</td>
<td>0.0080</td>
<td>1.4</td>
</tr>
<tr>
<td>DEAE fraction</td>
<td>172.4</td>
<td>0.0113</td>
<td>2.0</td>
</tr>
<tr>
<td>Sephadex G-75 Fraction</td>
<td>18.9</td>
<td>0.1351</td>
<td>27.1</td>
</tr>
<tr>
<td>Chromatofocusing Fraction</td>
<td>3.4</td>
<td>0.6374</td>
<td>113.8</td>
</tr>
<tr>
<td>Hydroxyapatite Fraction</td>
<td>0.5</td>
<td>1.0769</td>
<td>192.3</td>
</tr>
</tbody>
</table>

**TABLE II**

<table>
<thead>
<tr>
<th>Carotenoids Composition of CBP</th>
<th>Lutein</th>
<th>β-Carotene</th>
<th>α-Carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/mg CBP</td>
<td>(88%)$^a$</td>
<td>(9%)$^b$</td>
<td>(3%)$^b$</td>
</tr>
<tr>
<td>23.38</td>
<td>2.61</td>
<td>0.87</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ As protein, determined by BCA assay.
$^b$ Parentheses represent carotenoids composition (%).
using the Gigapack III Gold packaging kit. The phage were plated onto NZYM plates with E. coli strain NM322 as the host and then transferred to nitrocellulose filters pretreated with isopropyl-1-thio-D-galactopyranoside (IPTG). The filters were probed with rabbit anti-CBP antibody, alkaline phosphatase-conjugated anti-rabbit IgG goat serum, and an alkaline phosphatase color development system. Bacteriophage containing CBP clones were used to infect E. coli strain NP66, which enables in vitro release of pExCell, autonomously replicating phagemid. The pExCell DNA was purified using a Plasmid Midi kit.

The obtained positive clone was sequenced by the dideoxy-chain termination procedure using a Time Saver sequencing kit using Sp6 and T7 universal primers. DNA sequencing was performed on an automated sequencer.

5’-Rapid Amplification of cDNA Ends—The 5’-terminal cDNA ends was amplified using the 5’-Full RACE Core Set according to the supplier’s instructions. The antisense strand synthesis was directed with anti-CBP antibody. A. Coomassie Blue staining of the separated proteins, B. Western analysis of different protein samples. Lane M represents molecular weight markers, and lane CBP represents purified CBP as reference.

Expression of Recombinant CBP cDNA in E. coli—The coding region for the putative mature protein was amplified by PCR (forward prime containing a sequence encoding ten histidines, 5’-CCGCGGATCCATCG-ACCATACCATCACATCAGTGCGACTCTACGTCCGA- AAACCG-3’; reverse primer, 5’-CCGGTCGACTGAGATTTCGGCTCT- GGCGCTTGGCTG-3’). The amplified region was cloned into the pGX-4T-3 vector containing a sequence encoding ten histidines, 5’-CGCGGATCCATC-ACCATACCATCACATCAGTGCGACTCTACGTCCGA- AAACCG-3’. Samples containing 25 μg and 50 μg lutein (dissolved in ethanol) were incubated in 1 ml of binding buffer for 3 h at 25 °C. Control experiments were performed using the same procedure in the absence of purified rCBP or the presence of BSA. Anti-CBP rabbit IgG-protein A-Sepharose was added to the reaction mixture and incubated for 4 h at 4 °C. Protein A-Sepharose-rCBP-lutein complex was collected by centrifugation at 13,000 × g for 10 s. To remove the unbound lutein, the pellets were washed three times with 500 μl of 20 mM Tris-HCl (pH 7.6) containing 150 mM NaCl, 1% Nonidet P-40, protease inhibitor mixture, and 1 mg/ml BSA. Binding of lutein to rCBP was confirmed by the presence of the yellow colored precipitate.

Fig. 1. Tissue distribution of CBP. Protein samples (25 μg) from different tissues were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-CBP antibody. A. Coomassie Blue staining of the separated proteins. B. Western analysis of different protein samples. Lane M represents molecular weight markers, and lane CBP represents purified CBP as reference.

Fig. 2. Distribution of CBP in different sections of the silk gland and the midgut. Protein samples were prepared from the anterior, middle, and posterior sections of the silk gland (A) and midgut (B). Samples (25 μg) were separated by SDS-PAGE and transferred to nitrocellulose. Panels I and III were stained with Coomassie Brilliant Blue R-250. Panels II and IV were Western analyses against anti-CBP antibody (refer to methodology for further details). Lane M represents molecular weight markers.
Immunohistochemistry—Histochemical staining was used to determine the localization of CBP. Midgut from day 4 of 5th instar larva was dissected in PBS, washed in the same buffer, and cut into small pieces. A drop of Tissue-tek was added to the pieces prior to freezing in n-hexane with acetone/dry ice mixture at $-70^\circ$C. The frozen samples were cut in sections of 4-$\mu$m thickness using Cryostat. The cryostat sections were fixed in acetone at $-10^\circ$C for 10 min, air-dried, washed in PBS, and incubated with normal rabbit serum diluted 1:10 in PBS containing 0.5 mg/ml BSA for 10 min at 37°C. The sections were further incubated with anti-CBP rabbit antibody (diluted 1:500 in PBS) for 30 min and then washed three times in PBS. Fixed and mounted sections of the midgut were incubated with anti-rabbit IgG goat serum coupled to peroxidase (diluted 1:500) for 30 min, washed three times in PBS, and developed for 10 min at room temperature using a substrate solution (0.005% H$_2$O$_2$, 0.02% 3,3’-diaminobenzidine in 0.1M Tris-HCl buffer, pH 7.5, containing 0.15 m NaCl) and then counterstained in Mayer’s hematoxylin. Images were photographed.

RESULTS AND DISCUSSION

Cocoons of B. mori are found with a wide range of colors, yellow, orange, and red, all of which are linked to absorbed carotenoids (16). In white cocoon mutants, the normal yellow carotenoid pigment is absent due to abnormal carotenoid transport (4). The Y gene controls absorption of carotenoids from the midgut lumen into the midgut epithelium, and mutants with the recessive Y gene cannot take up carotenoids, thus their cocoon is white.

Purification of CBP—We purified a carotenoid binding pro-
tein from the silk gland of N4 B. mori larvae using a combination of ammonium sulfate precipitation, DEAE-, Sephadex G-75-, chromatofocusing, and hydroxyapatite chromatography.

CBP was followed by its yellow color and monitored by the absorbance at 452 nm (Table I). Three yellow fractions were obtained after DEAE ion exchange chromatography. The flow-through fraction of the DEAE column, with the highest carotenoid-to-protein ratio along with a characteristic /H9252-carotene spectrum, was used in the subsequent purification steps. The purified CBP has a molecular mass of 33,000 Da on SDS-PAGE. The yield of CBP was 0.53 mg from about 2500 pairs of silk gland (wet weight, 260.3 g). Absorption spectrum of CBP is characterized by three absorbance maxima in the visible region at 436, 461, and 493 nm, apart from a protein peak at 280 nm. This spectrum is not unique to CBP, other invertebrate carotenoproteins, depending on the color of the chromophore, exhibit absorption maxima between 370 to 680 nm (5). The absorbance maxima of the CBP represent a significant red shift of 22 nm compared with lutein spectrum in hexane. It is interesting to mention that CBP exhibits identical absorption spectra to LBP isolated from the midgut of B. mori (5).

**Carotenoids Composition of CBP**—To analyze the CBP-associated yellow chromophore, the carotenoids were extracted from the purified CBP, analyzed, and quantified by HPLC (Table II). The bound chromophore, identified as lutein (88%), β-carotene (9%), and α-carotene (3%) is consistent with the carotenoids composition of lipophorin and the artificial diet contained mulberry leaves (11). Thus, dietary carotenoids are absorbed and transferred from the midgut lumen to the silk gland via lipophorin without any metabolic modifications. The 1:1 molar ratio of carotenoids to protein is in accordance with that reported for the vertebrate cellular /H9252-carotene binding protein (7) and not that of LBP (5).

**Tissue Distribution of CBP**—Immunoblotting analyses demonstrated that CBP is found in the silk gland, midgut, testis, and ovary and not in the hemolymph, fat body, malpighian tubules, muscle, and integument (Fig. 1). The presence of CBP in the carotenoid-containing yellow tissues emphasizes the tissue specificity of CBP. Distribution analyses of CBP within the silk gland and midgut indicated the presence of CBP only in the middle part of the silk gland (Fig. 2A, panel II). Furthermore, the middle part of the midgut exhibited the highest distribution of CBP with minor amounts in the anterior and posterior (Fig. 2B, panel IV). These results show that carotenoids might absorb from the middle part of midgut. These results are consistent with the hypothesis that carotenoids are only incorporated from lipophorin into those cells that contain a specific cytoplasmic binding protein such as CBP (21).
FIG. 8. Expression and purification of rCBP. CBP was cloned in the pgX-4T-3 expression vector. The rCBP was purified from lysed bacteria cells by sonication with His-Trap purification kit. 2 μg of rCBP was loaded on 12.5% SDS-PAGE and stained with Coomassie Blue (A). Immunoblotting of the rCBP using anti-CBP antibody (B). Lane 1 represents the molecular weight marker, and lane 2 represents purified rCBP.

FIG. 9. Binding assay of rCBP. Purified recombinant CBP (25 μM) was incubated with 50 μM lutein (dissolved in ethanol) in 1 ml of binding buffer at 25 °C. After 3 h the anti-CBP IgG conjugated to protein A-Sepharose was added to immunoprecipitate the complex (refer to methodology for more details). Tube 1 contains rCBP, tube 2 contains lutein without rCBP, and tube 3 contains rCBP plus lutein. The yellow color was detected by visual inspection at the bottom of the centrifuge tube after centrifugation.

Detection of CBP in the C mutant—The product of the C gene (present in the N4 strain) has been proposed to regulate carotenoid uptake from hemolymph into the middle silk gland (16). To determine whether CBP is the product of the C gene, protein samples from midguts and silk glands of the wild type (C) and the mutant (+/−) larvae were analyzed by immunoblotting using anti-CBP antibody (Fig. 3). CBP was found in the midgut and silk gland of both the C and +/− strains, suggesting CBP is neither the product of nor regulated by the C gene.

Detection of CBP in the Y Mutant—Morphological and genetic linkage mapping of the B. mori indicate that the product of the Y gene controls uptake of carotenoids from the midgut (16). A mutation in the Y gene produces insects with faint yellow-colored midguts and white cocoons compared with yellow midguts and cocoons of the wild type. To examine whether CBP is the product of the Y gene, midguts and silk glands from four strains with different genotypes: YI, Y+/I, +/Y, and +/− were used for immunoblotting (Fig. 4). Data clearly demonstrate the presence of the CBP in both midgut and silk gland of only the dominant Y gene (YI and Y+/−), suggesting that the Y gene might control the expression of the CBP gene or that CBP is the product of the Y gene. Our data do not reveal the connection between CBP and the Y gene but clearly demonstrate that CBP is not limited to the midgut tissue as has been suggested for the Y gene (16).

Comparison of the Lutein Binding Protein to CBP—Fig. 5 shows the Western analysis of the silk gland and midgut using anti-LBP or anti-CBP antibodies. Lack of cross-reactivity between CBP and LBP was demonstrated by the inability of either anti-LBP antibody or anti-CBP antibody to recognize the purified CBP (Fig. 5A) and LBP (Fig. 5B), respectively, demonstrating that the two proteins are different. LBP was present in all four mutants: YI, +/Y, Y+/I, and +/− (Fig. 5C), whereas CBP was present only in the Y gene containing mutants (Fig. 5D).

Both LBP and CBP purified from B. mori are characterized by their typical carotenoid-bound spectra. The similarity between these proteins is limited to their lipid composition, with lutein being the main bound carotenoids. However, the difference between LBP and CBP is conspicuous at different levels: 1) SDS-PAGE analysis demonstrated that CBP is a smaller (33 kDa) than LBP (36 kDa); 2) LBP is present in the fat body, whereas CBP is not; 3) LBP is present in all four mutants, but CBP is present only in the Y gene containing mutants; 4) The isoelectric point of LBP is 5.3 compared with 6.5 for CBP; 5) The ratio of lutein to protein is 3:1 for LBP and 1:1 for CBP; and 6) In addition, the antibodies of CBP or LBP do not cross-react with LBP and CBP, respectively. At the present time, it is not known whether CBP and LBP share the same physiological role.

Identification and Sequence Analysis of CBP cDNA—A cDNA library from the silk gland of B. mori N4 strain was prepared in λgt11 and screened using anti-CBP antibody. One positive clone was identified after a primary screen of around 200,000 plaques. This clone contains an 1874 bp insert encoding 296 amino acid residues (Fig. 6). After the termination codon, TGA there were a further 983 bases of untranslated sequence. The 3′-terminus contained a putative polyadenylation signal sequence, AATTAAA with the poly (A) tail beginning at position 2199. However, the 5′-terminus of the cDNA did not contain an ATG start site. A 520 bp product was obtained by 5′ RACE using gene specific primers. The
cDNA encoded a putative 5′-untranslated sequence of 345 bp, an ATG start site, and an open reading frame at position 346 and extending to position 1239. The deduced amino acid sequence of the putative CBP encoded a 297-residue polypeptide with a molecular weight of 33,636 and an isoelectric point of 6.53.

To verify that the obtained cDNA encodes the purified CBP, three peptides acquired from the digestion of the purified CBP with lysyl endopeptidase were sequenced. Sequence analyses of the peptides confirmed their presence in the deduced amino acid sequence of the CBP at positions 12–21, 141–150, and 247–254, respectively (Fig. 6, underlined sequences). Thus, the isolated putative cDNA encodes the sequence of the purified CBP.

A computer search of the Swiss-Prot data base revealed that the highest homology to CBP is the human, mouse, and bovine steroidogenic acute regulatory (StAR) proteins, and mouse MLN64 (Fig. 7). Sequence identity between B. mori CBP and the human StAR protein (22) and the mouse MLN64 protein (23) is 25 and 29%, respectively. The mammalian 30-kDa StAR protein plays a crucial role in the transport of cholesterol from the cytoplasm into the inner mitochondrial membrane, the rate-limiting step in steroidogenesis (24–26).

Analogous to six members of the StAR and MLN64 proteins, the carboxyl terminus of CBP contains a StAR-related lipid transfer domain (START) known to aid in lipid transfer/recognition (27). The conserved amino acid sequences EPNW at position 339–342 and PRDFV at position 364–368 of CBP are found in all six StAR proteins (Fig. 7, boldfaced sequences). This domain plays a role in binding cholesterol to StAR protein (28, 29). START domain is not unique to cholesterol transport proteins, but it occurs in proteins involved in lipid metabolism, signal transduction, and transcriptional regulation (30). It is present in a putative human acyl-CoA thiosterase, and different phosphatidylinositol transfer proteins (26, 31).

Recently, the crystal structure of the START domain of MLN64 has been determined, and a putative lipid-binding tunnel has been identified that shuttles cholesterol through the intermembrane space of the mitochondria (30). START consists of nine-stranded twisted antiparallel β-sheets, 4-α-helices, and 2-Ω-loops. The hydrophobic tunnel that extends nearly the entire length of the protein is the most interesting feature of its structure. The presence of different amino acids in the hydrophobic tunnel confers its distinct ligand-binding selectivity (32). It is conceivable that the CBP START domain exhibits similar biological activity as the MLN64 with the exception that CBP shuttles carotenoids instead of cholesterol. Actually this hydrophobic feature allowed us to follow CBP during its purification and was demonstrated when the yellow rCBP-lutein complex was immuno-noprecipitated as discussed below.

Characterization of the Recombinant CBP—To characterize the biochemical properties of CBP, we utilized the pGX4-4T-3 expression vector and E. coli strain BL21 as the host to express CBP. The cloned DNA included cDNA encoding the CBP mature protein, ten histidines at the amino terminus, and glutathione S-transferase. After cloning was accomplished, the presence of CBP cDNA was verified by colony-directed PCR using gene-specific primers. rCBP was purified from lysed bacteria cells with His-Trap purification kit. SDS-PAGE analysis demonstrated that rCBP had an apparent molecular mass of 62 kDa (Fig. 8A). This is the predicted size for the CBP plus the ten-histidine extension and the attached glutathione S-transferase. Recombinant CBP was further identified by immunoblotting the 62 kDa rCBP against anti-CBP antibody (Fig. 8B).

Lutein binding capacity of CBP was determined by incubating rCBP with lutein under different conditions and immuno-precipitated using anti-CBP IgG conjugated to protein A-Sepharose (Fig. 9). Immunoprecipitation of the lutein-rCBP formed a yellow complex (Fig. 9, tube 3). In contrast, no yellow precipitate was observed when lutein was added to BSA (Fig. 9, tube 2) or when rCBP in the absence of lutein was incubated in binding buffer followed by immunoprecipitation (Fig. 9, tube 1). The observed yellow precipitate was specific to rCBP-lutein complex, because this complex failed to form in the presence of BSA and lutein or in the presence of lutein alone (data not shown). These data strongly suggest that rCBP displays lutein binding capacity.

Immunohistochemistry—The midgut from day 4 of 5th instar larvae (wild type, N4), corresponding to the time when the tissue becomes most yellow in color and the point of maximal tissue size, was used for immunohistochemistry (Fig. 10). The frozen sections of the midgut were slide-mounted, fixed in acetone, and incubated with anti-CBP antibody. The dark blue stains represent the nuclei. Positive staining of the midgut (light brown) was predominant in the villi, demonstrating that CBP is present in cells along the midgut. Once absorbed, lutein would be captured by CBP, transported to epithelium, and exported to the hemolymph carried by lipophorin. These findings confirm the proposed function of CBP in aiding carotenoids absorption and transport from the lumen to the epithelia cells of the midgut.

Summary—In this report we described the purification, characterization, and sequence analysis of a carotenoid-binding protein from the wild type of silk gland of B. mori. This cystolic protein represents a new member of the StAR protein family with unique features of binding carotenoids, lutein. Lutein is specifically and stoichiometrically bound to CBP with a ratio of 1 mol of lutein per 1 mol of CBP. Site-directed mutagenesis and CBP-transient infection of the silk gland cells of white cocoon mutants will be investigated in our future studies.

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Isolation, Characterization, and cDNA Sequence of a Carotenoid Binding Protein from the Silk Gland of Bombyx mori Larvae
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