Archives of Biochemistry and Biophysics xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Review

Recent progress in molecular genetic studies on the carotenoid transport system using cocoon-color mutants of the silkworm

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ARTICLE INFO

Article history:
Received 7 November 2014
and in revised form 19 December 2014
Available online xxxx

Keywords:
Selective transfer of carotenoid
Lipophorin receptor
CD36
SR-BI
START protein
Silkworm

ABSTRACT

The existence of tissue-specific delivery for certain carotenoids is supported by genetic evidence from the silkworm *Bombyx mori* and the identification of cocoon color mutant genes, such as *Yellow blood* (*Y*), *Yellow cocoon* (*C*), and *Flesh cocoon* (*F*). Mutants with white cocoons are defective in one of the steps involved in transporting carotenoids from the midgut lumen to the middle silk gland via the hemolymph lipoprotein, lipophorin, and the different colored cocoons are caused by the accumulation of specific carotenoids into the middle silk gland. The *Y* gene encodes carotenoid-binding protein (CBP), which is expected to function as the cytosolic transporter of carotenoids across the enterocyte and epithelium of the middle silk gland. The *C* and *F* genes encode the *C* locus-associated membrane protein, which is homologous to a mammalian high-density lipoprotein receptor-2 (Cameo2) and scavenger receptor class B member 15 (SCRB15), respectively; these membrane proteins are expected to function as non-internalizing lipophorin receptors in the middle silk gland. Cameo2 and SCRB15 belong to the cluster determinant 36 (CD36) family, with Cameo2 exhibiting specificity not only for lutein, but also for zeaxanthin and astaxanthin, while SCRB15 seems to have specificity toward carotene substrates such as α-carotene and β-carotene. These findings suggest that Cameo2 and SCRB15 can discriminate the chemical structure of lutein and β-carotene from circulating lipophorin during uptake. These data provide the first evidence that CD36 family proteins can discriminate individual carotenoid molecules in lipophorin.

Introduction

The silkworm *Bombyx mori* eats mulberry leaves, which are rich in lutein and β-carotene, therefore, lutein and β-carotene occur in the silkworm [1]. During feeding, carotenoids are transported from the midgut to the middle silk gland, which produces a silk protein and deposits carotenoids [2].

Considering the difficulty of moving hydrophobic carotenoids in the aqueous biological environment, special systems support their movement in the body. Lipophorin is the major lipoprotein found in insect hemolymph, which contains diacylglycerol as the predominant lipid with lesser amounts of cholesterol, hydrocarbons, and carotenoids in the core [3–5]. Two apoproteins and phospholipids cover the surface of nearly spherical lipophorin particle [6–9]. The key to understanding lipophorin metabolism came with the discovery that it functions as a reusable shuttle [4,10,11]. Lipophorin can deliver core lipids to tissues, or accept core lipids from tissue, without being internalized into the tissue [8]. Another important property of lipophorin is its ability to bring about the tissue-specific and selective delivery of certain lipids. For example, hydrocarbons and carotenoids are delivered mainly to the cuticle in the tobacco hornworm *Manduca sexta*, and carotenoids are delivered mainly to the middle silk gland of *B. mori* [12].

Neither of these crucial aspects of lipophorin metabolism has been characterized fully. Although the receptors can presumably hold lipophorin at the external surface of the cell and allow lipid delivery without internalization, other cell surface components are probably necessary to transfer lipids from lipophorin to the cell. One of these components might be lipid transfer particles (LTPs)[1] [13–18]. LTPs from *B. mori* are member of the apoB/large lipid transfer protein family that have a higher molecular weight (650 kDa) than lipophorin [19,20]. Although LTPs catalyze the transfer of lipids between a lipophorin [21–25], and LTPs are involved in the transfer of lipids from tissue to lipophorin [26,27] or vice versa

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[1] *Abbreviations used:* C, yellow cocoon gene; Cameo2, C locus-associated membrane protein homologous to mammalian HDL receptor 2; CBP, carotenoid-binding protein; CD36, cluster determinant 36; *F*, *Flesh color cocoon gene*; *I*, *Yellow blood inhibitor gene*; LTPs, lipid transfer particles; SCRB15, scavenger receptor class B member 15; SR-BI, scavenger receptor class B type I; WT, wild-type; *Y*, *Yellow blood gene*.

http://dx.doi.org/10.1016/j.abb.2014.12.029
0003-9861/

[28–31], the selectivity of lipid transport is not accomplished in LTPs only [32].

The existence of selective transport for certain lipids is supported by genetic evidence from *B. mori* [33–36]. A class of *B. mori* white cocoon mutants in which the normal yellow pigment of the cocoon, carotenoid, is absent. In one mutant, the transfer of lutein from lipophorin to the middle silk gland is inhibited, and so lutein remains within lipophorin in the hemolymph. The transfer of other lipids such as diacylglycerol, cholesterol [36], and β-carotene is normal [37], so only the transfer of lutein is affected. One possible explanation for this observation is that the mutant has an impaired lutein-specific transfer factor on the surface of cells. At least two components for lipid selective transport must be present: a certain lipid specific-transporter in the cytosol and a non-internalizing lipophorin receptor on the cell surface that transfers certain carotenoids from lipophorin to the cell. Furthermore, observations that certain tissues accumulate specific species of carotenoids and exclude others suggest that special systems ensure selective carotenoid transport.

Here we describe current knowledge regarding the carotenoid transport system of the domesticated silkworm *B. mori*, which were done by our laboratory. We also summarize recent progress in molecular genetic studies using cocoon-color mutants of the silkworm, which has elucidated aspects of the special systems for carotenoid transport in the silkworm.

Overview of the classic genetic knowledge of the carotenoid transport system in the silkworm

Genetic analysis of cocoon color of the silkworm began in the 1900s, and the obtained results confirmed Mendelian hypotheses [38–42]. During the history of sericulture over the last 4000 years, several mutants defective in parts of the carotenoid transport pathway have been identified and maintained in laboratories and industries. Several genetic loci (genes) responsible for these cocoon-color mutants defective in carotenoid transport have been identified using classic genetic mapping [43,44]. Dissection and analysis of the tissues of these mutants revealed which steps in the carotenoid transport pathway are affected. Three genes are mainly involved: *Yellow blood* (*Y*), which permits uptake of carotenoids from the diet into the midgut and from the hemolymph into the middle silk gland; *Yellow blood inhibitor* ($+^I$), which permits carotenoids to enter the hemolymph from the midgut; and *Yellow cocoon* (*C*), which permits lutein to enter the middle silk gland from the hemolymph. These observations suggest that genes *Y* and $+^I$ encode carotenoid-transfer proteins in the midgut and the middle silk gland. The wild-type (WT) allele of the *I* gene is the recessive allele $+^I$, which is dominant negative. Importantly, the transfer of other lipids from the midgut to lipophorin is normal in the *I* mutant, so only the transfer of carotenoid is affected [36]. The WT strain produces a yellow cocoon, yellow hemolymph, and its genotype is [Y $+^I$ C]. Three major mutant genotypes are as follow: [Y $+^I$ $+^C$], which has white cocoons and yellow hemolymph; [Y I C], with white cocoons and colorless hemolymph; and [$+^Y$ $+^I$ C], which has a white cocoon, colorless hemolymph, and a colorless midgut. In addition, the *Flesh cocoon* (*F*) gene produces creamier yellow-colored (also called flesh) cocoons. The genotype [Y $+^I$ $+^C$ F] results in the formation of flesh-colored cocoons. The strains that carry visible markers on the same chromosomes as the carotenoid transfer genes are constructed using a mating system that distinguishes between the WT and mutant. For example, the *Y* gene and $+^P$ are both located on chromosome 2 [45], the latter of which encodes an Apontic-like transcription factor that donates three types of black spots (normal) on larval skin [46]. Two strains [Y $+^P$] or [$+^Y$ P], which have normal skin and yellow blood or plain larval markings and colorless blood, respectively [47], are available from the National Bioresource project (silkworm) of the Ministry of Education, Culture, Sports, Science and Technology, Japan [48]

The *Y*, *I*, *C* and *F* genes affect the carotenoid transport system in a different manner, suggesting the existence of multiple key molecules. Three genes, *Y*, *C*, and *F* were identified recently, as described below.

Identification of the *Y* gene

The *Y* gene, located on chromosome 2, controls the carotenoid transport pathway. A *Y* mutant ($+^Y$), homozygous for the recessive $+^Y$ allele of the *Y* gene exhibits colorless hemolymph defective in carotenoid content that produces a white cocoon. The involvement of the *Y* gene in transport from the midgut lumen to the midgut cell is confirmed by the colorless phenotype in the midgut and the hemolymph of the mutant $+^Y$.

The involvement of the *Y* gene in transport from the hemolymph to the middle silk gland cell was elucidated by Harizuka. He crossed a strain homozygous for the $+^Y$ allele with a hereditary mosaic strain that contains a WT *Y* allele of the *Y* gene. The hemolymph of the resulting mosaic larvae was yellow, and the middle silk gland was a mosaic of yellow-colorless cells [49,50]. Implantation experiments of the middle silk gland also support the involvement of the *Y* gene in carotenoid uptake in the middle silk gland [51,52].

The *Y* gene product was identified using biochemical strategies rather than a forward genetics. Because carotenoids are hydrophobic, carotenoid-binding proteins that cover the hydrophobic surface of carotenoids are required in the hydrophilic environment in the body. Therefore, studies were performed to isolate carotenoid-binding protein to elucidate the molecular mechanisms of the carotenoid transport system. The purification strategy used for carotenoid-binding proteins was conceptually simple. Proteins from carotenoid-rich tissues were separated under conditions in which carotenoids were expected to remain bound to carotenoid-binding proteins. Yellow-colored fractions were then considered to contain carotenoid-binding protein. Several studies have attempted to isolate carotenoid-binding proteins from the silkworm [53–55].

The purification and cloning of a carotenoid-binding protein was first achieved by our laboratory [56]. We purified carotenoid-binding proteins from the silk gland of a yellow cocoon-producing strain and obtained three yellow fractions, one of which was selected and purified further. The resulting 33-kDa protein, termed carotenoid-binding protein (CBP), was obtained and its cDNA was cloned.

Homology searches revealed that CBP contains a steroidogenic acute regulatory protein (StAR)-related lipid transfer domain (START domain) as a lipid-binding domain. StAR may play a critical role in the rapid translocation of cholesterol across the outer and inner mitochondrial membrane [57–62]. CBP is first member of START protein family which binds carotenoid other than cholesterol. Armed with this information, the identification of the human macular lutein-binding protein was performed by Bernstein group [63,64]. Screening of 15 human START proteins was analyzed using database search, Western blotting and immunohistochemistry. The CBP homology protein, human StarD3 (also known as MLN64), was identified as a human macular lutein-binding protein [65].

Next, the link between CBP and cocoon-color mutant genes was examined. Western blotting and immunohistochemistry indicated that the expression of CBP was associated with the genotype of the *Y* gene, and not with the genotypes of the *C* gene and *I* gene (Fig. 1) [66]. CBP was expressed predominantly in the midgut, middle silk gland, ovary and testis. Conversely, CBP was completely absent in the *Y* mutant homozygous for the $+^Y$ allele.

Please cite this article in press as: K. Tsuchida, T. Sakudoh, Arch. Biochem. Biophys. (2015), http://dx.doi.org/10.1016/j.abb.2014.12.029