**Drosophila** NinaB and NinaD Act Outside of Retina to Produce Rhodopsin Chromophore*

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The *Drosophila* **ninaA** gene encodes a **β,β**-carotene-15,15'-oxygenase responsible for the centric cleavage of **β**-carotene that produces the retinal chromophore of rhodopsin. The **ninaD** gene encodes a membrane receptor required for efficient use of **β**-carotene. Despite their importance to the synthesis of visual pigment, we show that these genes are not active in the retina. Mosaic analysis shows that **ninaB** and **ninaD** are not required in the retina, and exclusive retinal expression of either gene, or both genes simultaneously, does not support rhodopsin biogenesis. In contrast, neuron-specific expression of **ninaB** and **ninaD** allows for rhodopsin biogenesis. Additional directed expression studies failed to identify other tissues supporting **ninaB** activity in rhodopsin biogenesis. These results show that nonretinal sites of **ninaA** **β,β**-carotene-15,15'-oxygenase activity, likely neurons of the central nervous system, are essential for production of the visual chromophore. Retinal or another **C** 40 carotenoid, not members of the **β**-carotene family of **C** 40 carotenoids, are supplied to photoreceptors for rhodopsin biogenesis.

Mutants in eight *Drosophila* genes, designated **ninaA** through **ninaH**, are characterized by reduced rhodopsin levels in photoreceptors and altered electroretinograms (1). The opsin protein component of rhodopsin is coded by the **ninaE** gene (2, 3). The low rhodopsin phenotypes observed in other **nina** mutants are caused by deficits in the post-translational rhodopsin maturation process. For example, **ninaA** encodes a molecular chaperone required for movement of newly synthesized rhodopsin from the endoplasmic reticulum to the photosensitive rhodobacter membranes (4, 5). In the case of **ninaB** and **ninaD**, defective rhodopsin production is the result of a failure to generate the chromophore of rhodopsin, 3-OH retinal (6).

Animal species usually obtain retinals in their food. Plants and microorganisms produce **C** 40 carotenoids such as **β**-carotene and zeaxanthin, which animals metabolize to **C** 20 retinoids. In *Drosophila*, the **ninaD** gene encodes a membrane “scavenger” receptor proposed to mediate the cellular uptake of carotenoids (7). The **ninaB** gene encodes a **β,β**-carotene-15,15'-oxygenase (BCO) responsible for the centric cleavage of **β**-carotene to form retinal (8). This enzyme was originally named as a **β**-carotene dioxygenase. It has now been renamed BCO (9) in light of recent data showing related enzymes act as monooxygenases (10).

In vertebrates, vitamin A is essential for development and differentiation processes as well as its role in vision. The availability of vitamin A for metabolic processes is governed by multiple factors, including dietary absorption, transport, metabolism, and storage (11). A human BCO is expressed in the retinal pigment epithelium and also in the kidney, intestine, liver, brain, stomach, and testis (12, 13), suggesting that the processing of dietary carotenoids occurs in a variety of vertebrate tissues. Additional studies show that centric cleavage of **β**-carotene plays a major role in the processing of carotenoids (14, 15).

In *Drosophila*, vitamin A is only needed for vision, as animals grown on medium lacking vitamin A show only nonlethal visual deficits (16). Thus, the *Drosophila* experimental system is easily amenable to the genetic and molecular analysis of the components and cellular sites responsible for vitamin A metabolism. In this report, we used targeted expression studies to show that the **ninaB** and **ninaD** genes did not act within photoreceptors to produce rhodopsin chromophore. Rather, only nonretinal neuronal cells were identified as being capable of supporting the conversion of **β**-carotene to retinal. These results showed that, despite a requirement for retinal only within photoreceptors, other cell types are responsible for carotenoid metabolism.

**MATERIALS AND METHODS**

*Drosophila* Maintenance—Unless specified, all flies were reared on standard corn medium at 25 °C in a 12-h light/dark cycle. For medium supplementation experiments, **β**-carotene or all-trans-retinal (Sigma) was dissolved in EtOH to create 10 mM solutions, and then 200 μl was added to vials of ∼5 ml standard medium. Adult flies were maintained on the supplemented medium for 3 days prior to examination. For the experiments involving heat shock treatment, adult flies were placed at 37 °C for 1 h.

**FLP/FRT Mosaic Analysis—** **ninaB** P728 mosaic flies were created using the FLP/FRT (flippase recombinase/fliipase recombinase target) system (17) with the following components: P*tyr* hs-neo-FRT28B (18), eyFLP (19), and GMRHid (20). The genotype of the **ninaB** mosaic was y w eyFLP; P*tyr* hs-neo-FRT82B *ninaB*P263 (P*tyr*P*1* 2.2) = neoFRT82B P*(w* + ac) = GMR-hid;SS4, 1(3)C-LR. The **ninaD**P23 mosaic was similar. The **ninaD**P51 and **ninaD**P102 mosaics were created in an analogous manner with strains containing FRT40A (18). The procedures for this analysis have been described by Stowers and Schwarz (20) and Stowers et al. (21).

**Transgenic Drosophila Strains—** The *pUAST-ninaB* plasmid was constructed by inserting a sequence (5' NotI-ninaB open reading frame XbaI 3') into *pUAST* (22). The *hs-ninaB* (heat shock promoter **ninaB**) plasmid was constructed in a similar way using the *pCaSpeR*-Ha plasmid (23). All constructed plasmids were verified by restriction enzyme analysis and DNA sequencing. The plasmids were used to create transgenic *Drosophila* stocks by standard P element transformation methodology (24). The transgenes were chromosome-mapped and moved into the appropriate mutant background using conventional genetic crosses.
are also consistent with the postulated role of form needed for rhodopsin biogenesis (25). The current results gene encoding BCO (26), because retinal is the product of this all-retinal when reared in a 12-h light/dark cycle. The trans rhodopsin chromophore when reared in the dark but could use mental of light to convert all-trans-cis-retinal to the 11-

tary supplementation of these mutants (6, 8) and the require-

tions. The top two panels show all-trans-retinal rescues rhodopsin levels

tants by dietary supplementation and light exposure. Compari-

tions. The middle with β-carotene has no effect irrespective of light conditions but not when flies are reared in the dark. The bottom two panels show ninaD is rescued by supplementation with either β-carotene or all-trans-retinal when flies are reared in light but not when reared in the dark.

Other transgenic strains were obtained from the Drosophila Stock Center, Bloomington, IN.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—Proteins from two adult heads were extracted in 0.125 M Tris, pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, and 0.004% bromphenol blue, separated by 10% SDS-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes (Amersham Biosciences). Rhodopsin proteins were detected using the polyclonal rabbit anti-rhodopsin antibody. The immunoreactive proteins were visualized using horseradish peroxidase-conjugated goat anti-rabbit IgG followed by ECL detection (Amersham Biosciences). In many experiments, the rhodopsin blots were stripped and rebotted with anti-actin antibody to be sure similar amounts of protein were present in each lane.

RESULTS

Rescue of ninaB and ninaD by Dietary Vitamin A—We examined the rhodopsin content in ninaB and ninaD mutant flies fed on diets supplemented with either β-carotene or all-trans-retinal as the form of vitamin A (Fig. 1). The ninaB mutant was unable to use β-carotene or all-trans-retinal as the source of rhodopsin chromophore when reared in the dark but could use all-trans-retinal when reared in a 12-h light/dark cycle. The ninaD mutant can use both sources to synthesize rhodopsin, again with the requirement that the flies are exposed to light. These findings are consistent with earlier results showing dietary supplementation of these mutants (6, 8) and the requirement of light to convert all-trans-retinal to the 11-cis retinal form needed for rhodopsin biogenesis (25). The current results are also consistent with the postulated role of ninaB as the gene encoding BCO (26), because retinal is the product of this reaction and, therefore, the enzyme would not be required if retinal was supplied in the diet. The rescue of the ninaD mutant by both β-carotene and all-trans-retinal is also consistent with the postulated role as a β-carotene membrane receptor (7), with the assumption that high levels of dietary β-carotene allow sufficient entry of this compound into cells, even in the absence of the NinaD receptor.

Nonretinal Expression of ninaB—To test the requirement of ninaB activity within photoreceptors, genetic mosaics were constructed in which the eye tissues, but not other tissues of the fly, were mutant for ninaB. Fig. 2A assesses the rhodopsin content in a set of control genotypes and the ninaB mosaic. The controls establish Rh1 rhodopsin specificity of the protein blot (compare wild type with the Rh1 null) as well as the presence of ninaB on the FRT-ninaB chromosome. In the ninaB mosaic, animals (Fig. 2A, at the far right, labeled nina patch), the retinal tissue is mutant for ninaB, but the rest of the animal is ninaB+. These mosaic flies possess normal levels of rhodopsin, showing that NinaB BCO activity is not required in the photoreceptors or other cell types of the retina for normal rhodopsin expression. The lower blot in Fig. 2A shows a control experiment carried out with a ninaE mutation. The ninaE gene is also on chromosome arm 3R, and therefore the same procedures and parent stocks were used in the construction of these mosaics. However, the results show that, unlike ninaB, the ninaE gene must be present within photoreceptors to generate rhodopsin expression. This is the expected result, because the ninaE gene codes for the protein moiety of rhodopsin. For the ninaB and ninaE mosaics, we also assayed the original phenotype used to characterize the nina mutants, that is, the lack of the prolonged depolarizing afterpotential (PDA) in the electroretinogram response (27). These results, displayed in Fig. 2B, show that the ninaB mosaic has a wild type photore-

FIG. 1. Rescue of rhodopsin levels in ninaB and ninaD mutants by dietary supplementation and light exposure. Comparison of the top two panels show all-trans-retinal rescues rhodopsin levels in ninaB mutants when flies are reared under 12-h light/dark (L/D) conditions but not when flies are reared in the dark. Supplementation of the medium with β-carotene has no effect irrespective of light conditions. The bottom two panels show ninaD is rescued by supplementation with either β-carotene or all-trans-retinal when flies are reared in light but not when reared in the dark.

FIG. 2. NinaB BCO activity is not required in retina for the rescue of rhodopsin levels. A, in the upper panel, protein blot analysis shows a mosaic fly (lane on far right) lacking retinal ninaB activity has high rhodopsin levels. The lower panel shows a similar experiment with ninaE, the gene encoding the rhodopsin protein. In this case, ninaE expression in retinal cells is essential for rhodopsin production. B, ninaB and ninaE flies lack the PDA in electroretinogram analysis. In ninaB mosaics, the PDA is restored despite the lack of retinal ninaB activity. In contrast, the lack of ninaE within retinal tissues is responsible for the lack of PDA in ninaE. The electroretinogram protocols used 5-S stimuli of orange (Or) and blue (B) light.

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ceptor response, consistent with the conclusion that NinaB BCO activity is not required within the photoreceptors. The complementary experiment is to express ninaB solely within photoreceptors to determine whether photoreceptors were able to serve as a site of NinaB BCO activity. This was accomplished by generating transgenic animals that carried the ninaB coding sequence under control of a UAS GAL4 promoter (UAS-ninaB). The expression of this construct was restricted to R1–R6 photoreceptors by providing the pniaE-GAL4 element, Rh1-GAL4 (28). These animals were also homozygous for the ninaB mutant, therefore the UAS-driven ninaB was the only source of NinaB BCO protein. Fig. 3A shows the results of a protein blot assessing the rhodopsin content of experimental and control animals. The control animals include siblings carrying only the UAS-ninaB to determine the basal level of expression from this transgene. The results show there is no increase in rhodopsin production over the basal levels when pniaE-GAL4 is present to elicit NinaB BCO expression in the R1–R6 photoreceptor cells. Two other retinal-specific GAL4 driver strains confirmed and extended this result. The first of these is the enhancer trap line P(Gaw-B)LL54, in which GAL4 is expressed exclusively in the retinal pigment cells (29). A second, P(GAL4-ninaE.GMR)/12, expresses GAL4 in all cells of the retina (30). In both of these experiments, no rescue of the ninaB mutant phenotype was observed. Thus, retinal cell types are not able to support NinaB BCO activity.

As a control for these experiments, the same ninaB open reading frame was placed under the heat shock promoter. Even in the absence of heat shock, there is partial rescue of rhodopsin in ninaB mutant flies carrying hs-ninaB (Fig. 3B), showing...
that there is a basal level of hs-ninaB expression in the absence of heat shock treatment. However, heat shock treatment during the adult stage dramatically enhanced the rhodopsin content of these flies. Thus, the restriction of ninaB expression to adult tissues does not explain the failure of photoreceptors to serve as a site for NinaB BCO activity.

Nonretinal Expression of ninaD—ninaD is postulated to encode the scavenger receptor responsible for β-carotene cellular uptake (7). The cell-type specificity requirements of ninaD were determined using similar strategies as described previously for ninaB, except the effort involved the generation of chromosome arm 2L mosaic genotypes. ninaA, a chaperone required for rhodopsin transport through the secretion pathway, is on the same chromosome arm as ninaD. For this reason, ninaA was expected to require photoreceptor cell-autonomous expression and served as a convenient control for the mosaic construction protocols. ninaD, but not ninaA, was able to be rescued by gene expression outside of the retina (Fig. 4A). The protein blot results were confirmed by assessing the presence of the PDA in the electroretinogram response of these ninaD mosaics (Fig. 4B). These results indicate that, like ninaB, ninaD can act outside of the retina to promote rhodopsin biogenesis. Exclusive photoreceptor expression of ninaD was examined by placing the UAS-ninaD construct (7) and pniaE-GAL4 in ninaB mutant flies. No rescue of the ninaD phenotype was observed in this experiment (Fig. 4C). These results show that photoreceptors cannot serve as the sole site of ninaD activity.

Molecular analysis suggested sequential roles of ninaD and ninaB in the same cell. ninaD is involved in the cellular uptake of β-carotene and ninaB is responsible for the cleavage of β-carotene to generate the retinal chromophore. For this reason, we investigated whether photoreceptors failed to serve as the site of chromophore production in each mutant because both genes need to be simultaneously expressed. However, the results (Fig. 4D, upper panel) show that simultaneous expression of the ninaB and ninaD genes in photoreceptors failed to rescue the ninaB mutant. Further, results shown in the lower panel of Fig. 4D demonstrate that providing excess β-carotene in the diet sufficient to overcome the requirement for ninaD still did not permit formation of chromophore in ninaB mutants.

Drosophila Nervous System Tissues Support ninaB and ninaD Activity—The results presented above show that retinal cells are not required and cannot serve as the sole cell type supporting ninaB or ninaD activities. These results prompted us to identify the Drosophila tissues that would support ninaB or ninaD activities. To address this question, a set of genotypes in which GAL4 was expressed in discrete subsets of Drosophila tissues was created and analyzed. For analysis of ninaB, the genotypes were homozygous for ninaB, carried the UAS-ninaB transgene on the X chromosome, and carried one additional transgene that specified GAL4 expression in defined tissues. Each of these strains, with appropriate control strains, was
assessed for rhodopsin content. In Fig. 5A, these results show that GAL4 expression in some, but not all, nonretinal tissues will support the ninaB activity. The pan-neuronal driver elav-GAL4 element, C155 (31), showed complete rescue of ninaB with similar results observed for V55. The V55 element was previously reported to express GAL4 in the larval peripheral nervous system but also shows uniform expression within the adult central nervous system (data not shown). No rescue was observed when ninaB was expressed solely within the mushroom bodies of the central nervous system using the OK107 driver (32). Similarly, no rescue is observed with GAL4-twi.G and GAL4-hwAB two strains with broad expression of GAL4 within embryonic mesodermal cells (33, 34). Our analysis showed that these two strains express prominently in adult muscle and fat body cells (data not shown), which rules out these tissues as sites of chromophore production.

Thus, the analysis shows that productive ninaB activity is confined to a limited number of cell types and that only central nervous system neurons have been identified as possible sites for ninaB activity. To test whether ninaD showed a similar profile of rescue, we generated ninaD mutants carrying UAS-ninaD and several of these GAL4 drivers. These results are shown in Fig. 5B. As with ninaB, the C155 elav-GAL4 driver provided the best rescue, with AB1 also showing substantial rescue. OK107, allowing ninaD expression only in the mushroom bodies of the central nervous system, provided minimal rescue. Thus, the results are similar to those obtained in the ninaB studies, suggesting a nonretinal neuronal cell type as the site of action for both genes.

**DISCUSSION**

The series of experiments described here establish that the ninaB and ninaD genes, although required for production of visual pigment chromophore, are not active within photoreceptors. By simultaneously expressing both genes in photoreceptors, we showed that this is not simply due to a requirement that both activities be present in the same cell. Rather, the results suggest that photoreceptors lack specific molecular components or appropriate cellular compartments required for ninaB activity. Alternatively, it is possible that β-carotene is not efficiently transported to the retina, and hence no substrate is available even when ninaB and ninaD are expressed. This latter explanation is considered unlikely for the following three reasons. First, rescue is still not observed when these flies are fed high doses of β-carotene, which eliminates the need for ninaD activity. Second, a previous study suggests that expression of ninaD in the photoreceptors does allow accumulation of carotenoids within head tissues (7). Finally, the failure of other tissues to allow ninaB activity suggests specialized components are required that are not represented in most cell types.

Although photoreceptor expression is not effective, central nervous system expression of ninaB and ninaD is sufficient for production of chromophore. Previously, von Lintig and Vogt (26) showed that ninaB expression is limited to head tissue, consistent with the results here showing that ninaB is not active in a variety of tissues and may be expressed exclusively in central nervous system tissue. ninaD appears to have a broad expression pattern in embryos (7); larval and adult expression patterns have not been reported.

The model shown in Fig. 6 summarizes these results. Up-take, presumably through the digestive tract, allows transport of β-carotene to a nonretinal neuronal cell likely located within the central nervous system. The transport of β-carotene uptake into this cell by the NinaD scavenger receptor provides the substrate for processing of β-carotene to retinal by the NinaB BCO. We assume here that ninaD and ninaB are acting in the same cells based on the rescue with the same two GAL4 driver strains. However, because both of these GAL4 strains show broad central nervous system activity, the requirement for NinaD in additional or different neuronal cells cannot be ruled out. The requirement of β-carotene as the C40 carotenoid acted on by the NinaB BCO is suggested by the observation that bacterially expressed NinaB BCO does not process zeaxanthin or other hydroxylated C40 carotenoids to the C20 3-OH retinal (26). Similar specificity was observed for mammalian BCO homologs (12, 13). If *Drosophila in vivo* conditions of NinaB BCO activity are the same, the successful use of zeaxanthin or lutein (16) as a dietary source for the 3-OH retinal requires that these hydroxylated C40 carotenoids are first converted to β-carotene. The cellular location of these processes is not known and could not be addressed in the current study. After cleavage of β-carotene in the "ninaB cell," the retinal is hydroxylated to form 3-OH retinal. The location of this reaction is also not known; therefore retinal or 3-OH retinal may be the compound transported to photoreceptors for use in vision.

This view of *Drosophila* β-carotene metabolism is remarkably different from the current view of vertebrate metabolism. In vertebrates, β-carotene processing occurs in a variety of different tissues including the intestines, liver, testis, and lungs. The identified β,β-carotene-15,15-oxygenase (12, 13) has an expression profile in good agreement with these identified sites. In many cases, such as in the testis, the β-carotene is a source for retinoic acid (35, 36) acting in cellular differentiation. The lack of retinoic acid signaling in *Drosophila* likely limits the cell types required to process β-carotene. The reason the processing occurs within a subset of neuronal cells needs to be determined. One possibility is a requirement for retinoids in the extra-retinal noncryptochrome photoreceptors involved in circadian clock activity (37, 38).

Further work is needed to determine the identity and the properties of the nonretinal neuronal cells supporting NinaB BCO activity. The failure to rescue ninaB by expression in photoreceptors, salivary glands, fat body and muscle cells suggests these cells lack specific molecular components or appropriate cellular compartments. At least for photoreceptors, the only cell type tested in this regard, the expression of the NinaD receptor is not sufficient. For this reason, NinaD cannot be the only missing component in other components need to be identified. Mammalian BCO activity also may be restricted to particular cell types, as the majority of the intestinal activity comes from jejunal enterocytes (39). Thus it is possible that mammals also retain cellular specializations supporting β-carotene metabolism in a limited number of cell types.

In summary, the work described here establishes that photoreceptors are not the site of dietary vitamin A processing for use in vision. A subset of neuronal cells in the central nervous system are the only cells identified as capable of supporting this reaction, and the results show that a limited number of cell types supports the production of retinal from β-carotene. The reason for this restriction is not known, as activity of the NinaB BCO enzyme from *E. coli* expression has been achieved (26). One experimental approach that will address this question is the identification and characterization of the cell types, probably a subset of nonretinal neuronal cells within the central nervous system, responsible for the ninaB activity.

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