Dissection of the pathway required for generation of vitamin A and for Drosophila phototransduction

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Dietary carotenoids are precursors for the production of retinoids, which participate in many essential processes, including the formation of the photopigment rhodopsin. Despite the importance of conversion of carotenoids to vitamin A (all-trans-retinol), many questions remain concerning the mechanisms that promote this process, including the uptake of carotenoids. We use the Drosophila visual system as a genetic model to study retinoid formation from β-carotene. In a screen for mutations that affect the biosynthesis of rhodopsin, we identified a class B scavenger receptor, SANTA MARIA. We demonstrate that SANTA MARIA functions upstream of vitamin A formation in neurons and glia, which are outside of the retina. The protein is coexpressed and functionally coupled with the β, β-carotene-15′, 15′-monooxygenase, NINAB, which converts β-carotene to all-trans-retinal. Another class B scavenger receptor, NINAD, functions upstream of SANTA MARIA in the uptake of carotenoids, enabling us to propose a pathway involving multiple extraretinal cell types and proteins essential for the formation of rhodopsin.

Introduction

Retinoids (vitamin A and its derivatives) are critical for processes ranging from the immune response to neuronal plasticity, development, visual pigment generation, cell proliferation, and other essential physiological processes (for review see Lane and Bailey, 2005; Travis et al., 2007). In animals, all retinoids must be acquired from the diet either as preformed vitamin A (all-trans-retinol) or must be formed from the provitamin A precursor, carotenoids. The dietary carotenoids are synthesized in plants, certain fungi, and bacteria, and, to become biologically active, must first be absorbed and then delivered to the site in the body where they are converted to vitamin A (for review see von Lintig et al., 2005).

The β, β-carotene-15′, 15′-monooxygenase (BCO) is the key enzyme in vitamin A formation, which catalyzes the centric cleavage of β-carotene to yield retinaldehyde (all-trans-retinal; von Lintig and Vogt, 2000; Kiefer et al., 2001; Paik et al., 2001; Redmond et al., 2001; Fig. 1 A). However, until relatively recently, the identities of these enzymes in vertebrates and invertebrates were not known. In Drosophila, BCO is encoded by ninab (neither inactivation nor afterpotential B), and mutations in this gene disrupt retinoid production and phototransduction as a result of elimination of rhodopsin (von Lintig et al., 2001). As carotenoids are highly lipophilic molecules, specific proteins must exist to transport them to specialized target tissues and to absorb the provitamin A into cells.

It has been suggested that class B scavenger receptors may play important roles in the cellular uptake of carotenoids (for review see von Lintig et al., 2005). In Drosophila, mutations in the ninad gene (Johnson and Pak, 1986), which encodes a membrane protein homologous to the mammalian class B type I scavenger receptor (SR-BI; Acton et al., 1994), result in a defect in the uptake of carotenoids and synthesis of retinoids (Kiefer et al., 2002). SR-BI plays critical roles in cholesterol and high-density lipoprotein metabolism and in maintaining plasma cholesterol levels (Acton et al., 1996). SR-BI also mediates cellular uptake of free cholesterol (Acton et al., 1996), triglycerides (Stangl et al., 1999), phospholipids (Thuahmai et al., 2001), and vitamin E (Goti et al., 2001). Moreover, SR-BI is expressed in the human intestine (Hauser et al., 1998; Levy et al., 2004), where it is proposed to mediate absorption of dietary β-carotene (Reboul et al., 2005; van Bennekum et al., 2005). The combination of these studies suggests that class B scavenger receptors may function as carotenoid receptors. Although the molecular mechanism through which class B scavenger
receptors mediate absorption of carotenoids is not known, it might involve binding of carotenoid containing lipoproteins or micelles via the extracellular domain, separating the two transmembrane segments (Tao et al., 1996; Gu et al., 2000), followed by uptake of carotenoids through a process independent of endocytosis (Acton et al., 1996).

The photopigment, rhodopsin, consists of a seven-transmembrane protein, opsin, and a chromophore (3-hydroxy-11-cis retinal and 11-cis retinal in Drosophila and mammals, respectively), which is formed through metabolism of vitamin A (Montell, 1999; Travis et al., 2007). In Drosophila, light results in a cis- to trans-isomerization of the chromophore, and this transformation represents the only light-driven step during phototransduction. The all-trans-retinol is converted to 11-cis-retinal in pigment cells in a light-dependent, rather than an enzyme-dependent, manner (Wang and Montell, 2005), whereas the pathway leading from dietary carotenoids to all-trans-retinal takes place outside of retina tissues (Gu et al., 2004). Deprivation of vitamin A, either by depletion of dietary retinoids or as a result of mutations in the vitamin A pathway causes reductions in rhodopsin levels and defects in vision.

In contrast to mammals, in Drosophila, retinoids are not required for viability but appear to be required exclusively in the retina (Harris et al., 1977). As such, Drosophila represents a highly tractable animal model to study the metabolism of vitamin A in vivo. The ninaB gene encodes a BCO, which functions outside the retina for conversion of carotenoids to all-trans-retinal (Stephenson et al., 1983; von Lintig et al., 2001; Kiefer et al., 2002). Thus, a key question concerns the identity of the scavenger receptor that is functionally coupled to NINAB for the uptake of carotenoids. It has been suggested that the class B scavenger receptor, NINAD, is the protein that functions in concert with NINAB (Fig. 1 A; Gu et al., 2004). However, ninaD expression is enriched in bodies, whereas ninaB expression is reported to be enriched in heads (von Lintig et al., 2001; Kiefer et al., 2002), which questions how the two differentially expressed gene products are coupled.

In the present study, we describe the isolation of the santa maria (scavenger receptor acting in neural tissue and majority of rhodopsin is absent) locus, which encodes a new member of class B scavenger receptor family. Mutation of santa maria profoundly affected the visual response and production of rhodopsin, both of which were restored by providing all-trans-retinal to the diet. We found that santa maria functioned downstream of ninaD, in a step required for the conversion of carotenoids to vitamin A. The santa maria gene functioned outside of the retina and appeared to display a similar expression pattern as ninaB in fly heads. We provide evidence that santa maria and ninaB function in the same cells in vivo. Based on these results, we propose that the class B scavenger receptor, SANTA MARIA, is functionally coupled with the BCO enzyme, NINAB, in the conversion of carotenoids to retinaldehyde. In contrast to NINAB and SANTA MARIA, we show that the other class B scavenger receptor, NINAD, functions in the uptake of carotenoids primarily in the midgut. Combined with our previous demonstration that the retinoid binding protein, PINTA (PDA [prolonged depolarization afterpotential] is not apparent), functions in the retinal pigment cells in the final step in the generation of the chromophore, we propose a pathway involving the NINAB, NINAD, PINTA, and SANTA MARIA proteins acting in multiple cell types in the conversion of carotenoids to the rhodopsin chromophore.

Results

A mutant defective in the generation of rhodopsin

To identify new genes in Drosophila that functioned in the generation of rhodopsin and other aspects of phototransduction, we conducted a screen of chromosome 2 for homozygous viable mutations that caused a defect in electroretinogram (ERG) recordings (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200610081/DC1). ERGs are extracellular recordings that measure the summed retinal response to light. In Drosophila, the chromophore stays bound to the light-activated metarhodopsin, and a second photon of light is required for the reconversion of the metarhodopsin to the inactive rhodopsin (Pak, 1979; Montell, 1999). The major rhodopsin (Rh1) responds to either orange or blue light, whereas metarhodopsin responds effectively to orange light only. As a consequence,
blue light causes stable activation of metarhodopsin, resulting in a PDA (Fig. 1 B). The PDA requires a molar excess of the active form of the metarhodopsin over the available arrestin, which is required to arrest the activity of the metarhodopsin (Dolph et al., 1993). Thus, when the Rh1 level is decreased, as occurs upon mutation of the structural gene for the Rh1 opsin (ninaE), a PDA is not produced (Fig. 1 C; O’Tousa et al., 1985; Zuker et al., 1985).

One of mutant lines isolated in the ERG screen displayed a PDA-defective ERG phenotype, similar to that observed in ninaE (ninaE<sup>232b</sup>) flies (Fig. 1 D). Mutations in three second-chromosomal genes, ninaA, ninaC, and ninaD, are known to reduce or eliminate the PDA (Matsumoto et al., 1987; Montell and Rubin, 1988; Schnewly et al., 1989; Colley et al., 1991; Kiefer et al., 2002). The new mutation complemented ninaA, ninaC, or ninaD (unpublished data). Therefore, this mutation disrupted a new gene required for the generation of the PDA, which we refer to as santa maria.

As the PDA phenotype is usually due to a reduction in the level of Rh1, we checked the Rh1 concentration and found that it was severely reduced in the santa maria<sup>1</sup> mutant, as was the case in ninaE<sup>234b</sup> and ninaD<sup>226b</sup> flies (Fig. 1 E). We also checked ninaE (rh1) mRNA expression in santa maria<sup>1</sup> using Northern blots and found that it was not reduced compared with wild type (Fig. 1 F). Thus, the reduction in Rh1 protein was not due to disruption in expression or stability of the ninaE mRNA.

In addition to Rh1, there are four minor rhodopsins (Rh3–6) expressed in the retina (Montell, 1999). These minor opsins are spatially localized in nonoverlapping subsets of the smaller R7 and R8 cells (Montell et al., 1987; Zuker et al., 1987; Chou et al., 1996; Huber et al., 1997; Papatsenko et al., 1997). To address whether the santa maria<sup>1</sup> mutation reduced the expression of an opsin other than Rh1, we checked the protein levels of Rh4 and found that the concentration of this protein was also diminished (Fig. 1 E). The levels of other photoreceptor proteins, such as the eye-enriched PLC (NORPA [no receptor potential A]) and the transient receptor potential channel, did not change (Fig. 1 E).

Therefore, the santa maria<sup>1</sup> mutation caused a reduction in the concentration of rhodopsins but did not result in a general defect in the expression of photoreceptor cell proteins.

**The santa maria gene encodes a class B scavenger receptor**

To identify the gene responsible for the santa maria phenotype, we mapped the site of the mutation to the 27F4 to 28A2 region (Fig. 2 A; see Materials and methods), which included 10 known or predicted genes (http://flybase.bio.indiana.edu) spanning the region between CG5261 and CG6630. Among these 10 genes, the predicted amino acid sequences of three genes suggested...
that they were excellent candidates for encoding SANTA MARIA. Two of them (CG5958 and CG5973) encode putative retinoid binding proteins, and the third (CG12789) encodes a homologue of class B scavenger receptors. The predicted CG12789 protein shares 33% identity with the human SR-BI (hSR-BI; Calvo and Vega, 1993; Acton et al., 1994); 26% identity with mouse CD36, the founding member of this family (Endemann et al., 1993); and 30% identity with the Drosophila scavenger receptor NINAD, which also functions in rhodopsin biosynthesis (Johnson and Pak, 1986; Kiefer et al., 2002; Fig. 2 B). Class B scavenger receptor family are suggested to consist of two transmembrane domains and cytoplasmic N- and C-termini (Tao et al., 1996).

To find out which of the three candidates was the santa maria gene, we introduced transgenes encoding CG5958, CG5973, and CG12789 into santa maria1 flies. CG12789, expressed under the control of the heat-shock protein 70 promoter (hs-CG12789), restored a wild-type PDA (Fig. 2, C–E) and increased the level of the Rh1 protein in the mutant flies (santa maria1;hs–santa maria1/+; Fig. 2 F). The level of rhodopsin in these flies was lower than in wild-type, possibly because of the relative weakness of the heat-shock protein 70 promoter in some cell types. In contrast, neither the CG5958 nor the CG5973 transgenes rescued the PDA defect or increased Rh1 levels in the santa maria1 flies (unpublished data). Therefore, CG12789, which encodes a predicted class B scavenger receptor, is the santa maria gene.

**santa maria functions outside of the retina**

Some gene products that are essential for production or transport of the chromophore function in the retina, whereas others play roles outside the retina. The two proteins required in the retina are the retinoid binding protein, PINTA, which functions in pigment cells for chromophore synthesis (Wang and Montell, 2005), and a oxidoreductase, NINAG, which is required in the compound eye for chromophore synthesis (Sarfare et al., 2005). In contrast, two gene products that have been reported to operate outside of the retina for carotenoid metabolism are ninaD, which encodes a class B scavenger receptor (Pak, 1979; Kiefer et al., 2002), and ninaB, which encodes a BCO (Pak, 1979; von Lintig and Vogt, 2000; Kiefer et al., 2001). These findings raise the question as to the tissue and cellular requirements for santa maria.

To determine whether santa maria was required in the compound eye, we used two approaches. First, we generated mosaic flies using a mitotic recombination approach that leads to the generation of fully homozygous mutant eyes in otherwise heterozygous animals (Stowers and Schwarz, 1999). We found that the mosaic flies expressed normal levels of Rh1 (Fig. 3 A), indicating that santa maria was not required in the compound eye. Second, we tested for rescue of the santa maria1 phenotype, after expressing wild-type santa maria in the retina, using the GAL4/UAS (upstream activator sequence) system (Brand and Perrimon, 1993). This approach results in expression of genes that are linked 3′ to the UAS, to occur specifically under the control of the GAL4 transcription factor. Therefore, we generated UAS–santa maria transgenic flies, and introduced GAL4 transgenes into these flies that direct expression of santa maria in different retinal cells. Normal Rh1 levels or a wild-type PDA were not restored in santa maria1 upon expression of UAS–santa maria throughout the eye (GMR-GAL4) or exclusively in pigment cells (CG7077-GAL4) or photoreceptor cells (ninaE-GAL4; Fig. 3, B and D–G; and Fig. S2, A–C, available at http://www.jcb.org/cgi/content/full/jcb.200610081/DC1). The lack of rescue was not due to a problem with the UAS–santa maria transgene, as the santa maria1 phenotype was reversed in flies containing a santa maria–GAL4 in combination with the

**Figure 3.** santa maria is required for carotenoid metabolism outside of the retina. [A] santa maria functions outside of the compound eye for production of Rh1. The level of the Rh1 protein was normal in santa maria mosaic eyes: santa maria1 FRT/1[P[GMR-hid]G1 P[neoFRT]40 12CI-L; P[GAL4-ey-H]SS5 P[UAS-FLP1.D]JJD2. The samples were prepared from flies ~2 d after eclosion, and the blot was probed with anti-Rh1 and anti-tubulin antibodies. [B] Expression of santa maria in different sets of retinal cells using the GAL4/UAS system (Brand and Perrimon, 1993) did not restore Rh1 levels. Expression of UAS–santa maria was driven ubiquitously in the eye using the GMR-GAL4 (Freeman, 1996) or specifically in pigment or photoreceptor cells using the CG7077-GAL4 (not depicted) or the ninaE-GAL4, respectively. To examine the level of Rh1, we prepared head extracts from flies ~2 d after eclosion, and a Western blot was probed with anti-Rh1 and anti-tubulin antibodies. [C] Normal Rh1 levels were restored in santa maria1 flies by feeding all-trans-retinal but not by feeding with β-carotene. The ninaB15; ninaD24; and santa maria1 flies were fed either 0.2 mM all-trans-retinal or 0.2 mM β-carotene. To perform the Western blot, we prepared extracts from flies ~4 d after eclosion and probed the filter with anti-Rh1 and anti-tubulin antibodies. [D–I] ERGs using a series of orange (O) and blue (B) light stimuli as described in Fig. 1 B (see event markers at the bottom of G–I). All the flies were in a w1118 background. Flies were fed normal food unless indicated otherwise. (D) wild-type; (E) santa maria1; (F) santa maria1;UAS–santa maria; santa maria–GAL4/+; (G) santa maria1;UAS–santa maria;GMR-GAL4/+; (H) all-trans-retinal fed santa maria1; (I) β-carotene fed santa maria1.
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A

B

C

D

E

F

Figure 4. *santa maria* but not *ninaD* appear to be functionally coupled with *ninaB*. [A–C] Western blots containing head extracts prepared from flies ~2 d after eclosion were probed with anti-Rh1 and anti-tubulin antibodies. (A) Rh1 protein levels in *ninaB* flies after expression of UAS-*ninaB* under control of *ninaB-GAL4*, ninaD-GAL4, or *santa maria-GAL4*. (B) Rh1 protein levels in *ninaB* flies after expression of UAS-*ninaD* under the control of *ninaB-GAL4*, ninaD-GAL4, or *santa maria-GAL4*. (C) Rh1 protein levels in *santa maria* flies after expression of UAS-*santa maria* under control of *ninaB-GAL4*, ninaD-GAL4, or *santa maria-GAL4*. (D–F) ERG paradigm to test for a PDA, as described in Fig. 1B. All flies were in a *w*1118 background. (D) *santa maria*, UAS–*santa maria*; *santa maria-GAL4*; (E) *santa maria*, UAS–*santa maria*; ninaB-GAL4; (F) *santa maria*, UAS–*santa maria*; ninaD-GAL4.

UAS–*santa maria* (Fig. 3, B and F). These results demonstrate that *santa maria* is required outside the retina for biosynthesis of rhodopsin.

Dietary all-trans-retinal but not β-carotene rescues the *santa maria* phenotype

Neither flies nor mammals can synthesize β-carotene but must obtain this vitamin A/chromophore precursor from the diet. Both *ninaB* and *ninaD*, whose activities are required outside of the retina for rhodopsin biogenesis, function in the pathway from β-carotene to all-trans-retinal because both mutant phenotypes are rescued by supplementation of the food with all-trans-retinal (Stephenson et al., 1983; Gu et al., 2004; Fig. 3C and Fig. S2, D, E, G, and H). In contrast, PINTA and NINAG are required in the compound eyes and function subsequent to the generation of all-trans-retinal, as supplementation with all-trans-retinal does not restore Rh1 to wild-type levels in these mutants (Wang and Montell, 2005; Ahmad et al., 2006). Because *santa maria* acts outside of the compound eyes, it may also function in a step necessary for the conversion of β-carotene to vitamin A. Therefore, we checked whether the *santa maria* phenotype could be rescued by addition of all-trans-retinal to the diet. As was the case with *ninaB* and *ninaD*, we found that supplementation of the food with all-trans-retinal (0.2 mM) restored the Rh1 levels and the PDA in *santa maria* flies (Fig. 3, C and H).

Table 1. *santa maria* functions together with *ninaB* in neural cells

| Mutant          | GAL4   | UAS  | Rh1 levels |
|-----------------|--------|------|------------|
| ninaB*2246      | No     | No   | −          |
| ninaD*2246      | ninaB-GAL4 | UAS-ninaB | +        |
| ninaB*2246      | ninaD-GAL4 | UAS-ninaB | −        |
| ninaB*2246      | santa maria–GAL4 | UAS-santa maria | +        |
| ninaB*2246      | repo-GAL4 | UAS-ninaB | +        |
| ninaB*2246      | elav-GAL4 | UAS-ninaB | −        |
| ninaD*2246      | No     | No   | −          |
| ninaD*2246      | ninaB-GAL4 | UAS-ninaD | −        |
| ninaD*2246      | ninaD-GAL4 | UAS-ninaD | +        |
| ninaD*2246      | santa maria–GAL4 | UAS-santa maria | −        |
| ninaD*2246      | repo-GAL4 | UAS-ninaD | −        |
| ninaD*2246      | elav-GAL4 | UAS-ninaD | +        |
| ninaD*2246      | drm-GAL4 | UAS-ninaD | −        |
| ninaD*2246      | ninaD-GAL4 | UAS–santa maria | −        |
| ninaD*2246      | ninaD-GAL4 | UAS–SR-BI | −        |
| santa maria*    | No     | No   | −          |
| santa maria*    | ninaB-GAL4 | UAS–santa maria | +        |
| santa maria*    | ninaD-GAL4 | UAS–santa maria | −        |
| santa maria*    | santa maria–GAL4 | UAS–santa maria | −        |
| santa maria*    | repo-GAL4 | UAS–santa maria | +        |
| santa maria*    | elav-GAL4 | UAS–santa maria | −        |
| santa maria*    | drm-GAL4 | UAS–santa maria | −        |
| santa maria*    | santa maria–D-GAL4 | UAS-ninaD | −        |
| santa maria*    | santa maria–D-GAL4 | UAS–SR-BI | −        |

The summary of Rh1 levels is based on the data in Figs. 3–7. Plus signs indicate wild-type Rh1 (rhodopsin) levels, and minus signs indicate reduced Rh1 levels.

Both *ninaB* and *ninaD* function in the generation of retinoids from carotenoids; however, only the *ninaD* phenotype, not the *ninaB* phenotype, is rescued by high doses of β-carotene (Fig. 3C and Fig. S2, F and I; Stephenson et al., 1983; Gu et al., 2004). ninAB is an essential enzyme necessary for all-trans-retinal production, whereas NINAD is a scavenger receptor, which promotes the uptake of carotenoids. This latter function can be bypassed by large concentrations of dietary carotenoids. Based on these data, it has been proposed that *ninaB* functions downstream of *ninaD* (Gu et al., 2004). To test whether the *santa maria* phenotype was rescued by carotenoids, we fed the mutant flies 0.2 mM β-carotene. We found that addition of β-carotene did not rescue the *santa maria* phenotype (Fig. 3I).

SANTA MARIA and NINAB appear to be functionally coupled

NINAB is a BCO, which converts β-carotene to all-trans-retinal; therefore, NINAB would need to be coexpressed with a β-carotene receptor, to promote influx of β-carotene into the cells. The two class B scavenger receptors, SANTA MARIA and NINAB, are candidate proteins that could be functionally coexpressed with NINAB, and serve this role. To address whether NINAB or SANTA MARIA function in the same cells as NINAB, we used the GAL4/UAS system (Brand and Perrimon, 1993). To conduct these experiments, we generated *ninaB-GAL4*, *ninaD-GAL4*, and *santa maria–GAL4* transgenic flies (see Materials and methods) and introduced them into the *ninaB* , *ninaD*, and *santa maria* mutant backgrounds,
along with UAS-ninaB, UAS-ninaD, and UAS–santa maria. Each of these GAL4 lines was effective because the ninaB<sup>p115</sup>, ninaD<sup>226</sup>, or santa maria<sup>1</sup> phenotype was rescued by the GAL4/UAS transgenes corresponding to the same genes (Fig. 4 and Table I).

We found that the ninaB<sup>p115</sup> and santa maria<sup>1</sup> phenotypes were rescued by expression of one gene under the control of the other GAL4 line. Specifically, expression of UAS-ninaB using the santa maria–GAL4 restored Rh1 levels in ninaB<sup>p115</sup> flies (Fig. 4 A and Table I), whereas expression of UAS–santa maria under control of the ninaB-GAL4 rescued the santa maria<sup>1</sup> phenotype (Fig. 4, C and E; and Table I). Indistinguishable results were obtained using two independent santa maria–GAL4 and three ninaB-GAL4 lines (unpublished data). These data indicate that santa maria is functionally coexpressed with ninaB in the same cells.

In contrast to these results, the NINAB BCO did not function together with the other scavenger receptor, NINAD, as previously proposed (Gu et al., 2004). Expression of UAS-ninaB or UAS–santa maria under control of either of two ninaD-GAL4 lines did not reduce the severity of the ninaB<sup>p115</sup> and santa maria<sup>1</sup> phenotypes, respectively (Fig. 4, A, C, and F; Table I; and not depicted). To determine the sensitivity of this analysis, we conducted a dilution experiment and found that we could detect 2% the wild-type levels of Rh1 (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200610081/DC1). Because the levels of Rh1 produced in santa maria<sup>1</sup>; UAS–santa maria flies, either in the presence or absence of ninaD-GAL4, were both at the threshold for detection (≤2% wild-type levels; Fig. S3), we conclude that if there was any rescue with the ninaD-GAL4, it was ≤2%. Furthermore, within the resolution of our analysis, expression of UAS-ninaD using the ninaB-GAL4 or santa maria–GAL4 did not increase Rh1 levels in ninaD<sup>226</sup> flies (Fig. 4 B and Table I). These results were not due to ineffectiveness of the ninaD-GAL4 or the UAS-ninaD, as the ninaD<sup>226</sup> phenotype was rescued by coinjection of these transgenes into the mutant flies (Fig. 4 B). Thus, ninaD was not functionally coexpressed with ninaB, consistent with the proposal that SANTA MARIA is the critical scavenger receptor operating in combination with NINAB.

**ninaB and santa maria were both required in neurons and glia**

To find out which cell types express ninaB and santa maria, we first tested whether expression of these genes was enriched in bodies or heads, using a GFP reporter. We prepared extracts from the heads and bodies of ninaB-GAL4/UAS-GFP and santa maria–GAL4/UAS-GFP flies and probed Western blots with anti-GFP antibodies. In ninaB-GAL4/UAS-GFP flies, GFP was detected exclusively in the heads, whereas in santa maria–GAL4/UAS-GFP flies, the GFP was found in both heads and bodies (Fig. 5 A and not depicted). Because ninaB and santa...
maria appear to be functionally coexpressed, the two gene products may collaborate primarily in fly heads for the generation of all-trans-retinal.

To address the cell types in adult heads expressing reporters under control of the ninaB and santa maria enhancer/promoters, we performed double-labeling experiments. We stained head sections obtained from ninaB-GAL4/UAS-GFP.nls and santa maria–GAL4/UAS-GFP.nls flies with anti-GFp antibodies, in combination with glial (anti-REPO) or neuronal markers (anti-ELAV). A GFP with a nuclear localization signal was used in ninaD/F26; ninaD–GAL4 flies. The Western blot, processed as indicated in Fig. 5 C, was probed with anti-Rh1 and anti-tubulin antibodies.

FIGURE 6. ninaD and santa maria cannot substitute for each other. (A) Expression of neither ninaD nor SR-BI restored Rh1 expression in santa maria1 flies. The indicated UAS transgenes were expressed in santa maria1; santa maria–GAL4 flies. The Western blot, processed as indicated in Fig. 5 C, was probed with anti-Rh1 and anti-tubulin antibodies. (B) The reduced Rh1 expression in ninaD/F26 flies was not rescued by expression of either santa maria or SR-BI. The indicated UAS transgenes were expressed in ninaD/F26; ninaD–GAL4 flies. The Western blot, processed as indicated in Fig. 5 C, was probed with anti-Rh1 and anti-tubulin antibodies.

Because NINAD and SANTA MARIA share considerable amino acid homology (30% identity) and both are required for the generation of retinoids from carotenoids, the two scavenger receptors might have the same molecular functions. To address whether NINAD and SANTA MARIA can functionally substitute for each other, we tested whether UAS–santa maria could rescue the ninaD/F26 phenotype if it was expressed in those cells that normally express ninaD. In addition, we performed the reciprocal experiment by expressing the Santa maria–GAL4 in santa maria1 flies. However, Rh1 levels were not restored either in santa maria1 flies containing the santa maria–GAL4/UAS–ninaD transgenes or in ninaD/F26 flies containing the ninaD–GAL4/UAS–santa maria transgenes (Fig. 6).

A mammalian homologue of NINAD and SANTA MARIA, SR-BI, has been suggested to function in the uptake of a variety of lipids, including β-carotene (van Bennekum et al., 2005). Therefore, we considered the possibility that SR-BI may have the same molecular function as either NINAD or SANTA MARIA. To test this proposal, we introduced a UAS–SR-BI transgene and expressed SR-BI under control of the ninaD–GAL4 or the santa maria–GAL4 in ninaD/F26 or santa maria1 flies, respectively. However, expression of SR-BI did not rescue either the santa maria1 or ninaD/F26 phenotypes (Fig. 6, A and B; Table I; and Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200610081/DC1).

NINAD was expressed and required in the midgut

Because the class B scavenger receptor, NINAD, is not functionally coexpressed with NINAB, it would appear that ninaD is required in cells distinct from those in which ninaB and santa maria function. The expression of ninaD has been detected in the midgut primordia in embryos (Kiefer et al., 2002), raising the possibility that NINAD may function in the midgut for absorption of carotenoids into animals. To test whether ninaD is expressed in the midgut, we used a GFP reporter. We found that GFP fluorescence in ninaD–GAL4/UAS–GFP flies was detected almost exclusively in the midgut (Fig. 7 A). To address whether
ninaD expression was enriched in gut, we prepared extracts from ninaD-GAL4/UAS-GFP fly heads, bodies, dissected guts, and bodies without guts and probed a Western blot with anti-GFP antibodies. To aid in the comparison, the extracts were prepared in a constant volume consisting of the same actual numbers of dissected guts and bodies, rather than the same total mass. GFP was detected in bodies and dissected guts, but not in heads (Fig. 7 B). Moreover, the GFP signal was dramatically reduced in bodies after removal of the guts. The results further suggested that the ninaD-GAL4 transgene is expressed and functioned in the gut. Extracts were prepared from the heads, bodies, guts, and bodies without guts of ninaD-GAL4/UAS-GFP flies and from the heads and bodies of UAS-GFP flies. The total proteins in each lane was detected by staining with Ponceau S (not depicted). The Ponceau S staining in the gut lane was lighter than in the other lanes because the same number of bodies and guts (rather than the same mass) was loaded into each lane. (C) Expression of UAS-ninaD using a midgut GAL4 line (drm-GAL4) rescued the ninaDβ246 phenotype. The Western blot, which contained head extracts prepared from flies ~2 d after eclosion, was probed with anti-Rh1 and anti-tubulin antibodies.

Discussion

In Drosophila, a reduction in rhodopsin levels results from mutations affecting either the synthesis or transport of the opsin or chromophore subunits (Harris et al., 1977; Stephenson et al., 1983). As such, genetic screens for mutations that affect rhodopsin levels provide an excellent opportunity to identify and characterize the roles of gene products required for production of the opsin, vitamin A, and the chromophore. Several genes required for rhodopsin biosynthesis have been previously reported, including those that are essential for steps involved in the synthesis or transport of the opsin (O’Tousa et al., 1985; Zuker et al., 1985; Colley et al., 1991; Rosenbaum et al., 2006), all-trans-retinal from β-carotene (von Lintig et al., 2001; Kiefer et al., 2002), and the chromophore from vitamin A (Sarfare et al., 2005; Wang and Montell, 2005). However, there remained many questions concerning the cellular sites for the various steps in vitamin A/chromophore synthesis, the nature of the proteins that participate in the uptake, transport, and synthesis of the intermediates, and the identities of receptors and enzymes that functioned coordinately in the same cells.

In animals, ranging from flies to humans, dietary β-carotene is the substrate for production of vitamin A, and the vitamin A is subsequently converted into the chromophore. The critical step in the conversion of β-carotene to vitamin A is the centric cleavage by BCO, which in Drosophila is encoded by the ninaB gene (von Lintig et al., 2001). The key question concerns the identity of the receptor protein that operates in concert with NINAB and is necessary for the uptake of carotenoids in the ninaB expressing cells. It has been suggested that the class B scavenger receptor encoded by ninaD serves this function (Gu et al., 2004). However, we have found that the ninaD phenotype was not rescued by expression of wild-type ninaD in ninaB expressing cells. Moreover, we found that ninaB was expressed primarily in the heads, whereas ninaD was only detected in the bodies.

As ninaD does not operate in concert with ninaB, there would appear to be another receptor that serves this function. In the current work, we identify a new class B scavenger receptor, SANTA MARIA, and provide evidence that it is functionally coupled to the NINAB BCO. In support of these conclusions, we found that SANTA MARIA is homologous to known class B scavenger receptors and mutations in santa maria disrupt the biogenesis of rhodopsin. Moreover, both ninaB and santa maria are expressed in fly heads and function in neurons and glia. Most important, expression of ninaB under control of the santa maria promoter rescued the ninaB phenotype and expression of santa maria using the ninaB promoter rescued the santa maria phenotype. Therefore, we suggest that carotenoids are taken up from circulation by SANTA MARIA, thereby providing the substrate for processing of carotenoids to all-trans-retinal by the NINAB BCO.
Interestingly, CD36 also appears to be expressed in RPE cells, which are well known to function in the generation of the chromophore and for the uptake and centric cleavage of β-carotene. All-trans-retinal from carotenoids may occur in RPE cells, which are involved in the synthesis of vitamin A (Sarfare et al., 2005; Bhatti et al., 2003), suggesting that the biosynthesis of all-trans-retinal in RPE cells. However, the association of β-carotene in the gut or in the retina does not appear to be metabolized in the gut or in the retina, respectively. The SANTA MARIA scavenger receptor and the NINAB BCO function coordinately in neurons and glia, for the uptake and centric cleavage of β-carotene. The all-trans-retinal is then metabolized into vitamin A and transferred to the retinal pigment cells, where it is converted into the chromophore, through a process involving the PINTA retinoid binding protein (Wang and Montell, 2005). The NINAG oxidoreductase also participates in the production of the chromophore, in a step subsequent to the formation of vitamin A (Sarfare et al., 2005; Ahmad et al., 2006), although it remains to be determined whether it functions in the retinal pigment cells or in photoreceptor cells.

The proposed pathway is not yet complete. Because NINAD appears to operate upstream of NINAB/SANTA MARIA, there may be additional proteins that facilitate the uptake of carotenoids into the gut and in the transport to the neurons and glia that express NINAB/SANTA MARIA. In addition, there may be yet-to-be-identified dehydrogenases, as well as other proteins that participate in the uptake of the chromophore into photoreceptor cells. Given the evolutionary conservation of the known components that are required for vitamin A/chromophore production, these yet-to-be-identified Drosophila proteins are also likely to participate in the carotenoid-metabolic pathway in mammals.

Materials and methods

Ethyl methanesulfonate (EMS) mutagenesis

The santa maria’ mutant was isolated by performing EMS mutagenesis and screening for second-chromosome mutations affecting the PDA. To perform the screen (Fig. S1), we mutagenized an isogenized w1118 stock with EMS as we have described recently (Wang et al., 2005). The mutagenized flies were mated to DTS91, Sco/Cyo flies, and the homozygous viable F3 progeny were screened by performing ERGs, using blue and orange light, as we have described recently (Wang et al., 2005). The Bloomington Stock Center was the source for the second-chromosome deficiency kit and the following stocks: Df(2R)ED479, Df(2R)Exel7031, y w, P{GMR-hid}G1, P{neoFRT}40 (I2Cl1)/CyO; P{GAL4-ey.H}/SS5, P{UAS-FLP1.DJ1}/D2, GMR-GAL4, dm-GAL4, UAS-EGFP, UAS-GFPnls, P{Sgs3-GAL4.PD3}, P{sp2-GAL4.HpT1}, P[w+;m(w)=Switch1]106, P{GawB,JHETD}pim, P[GawB(JH)66-7], elav-GAL4, and nos2-GAL4. To W. Pak (Purdue University, West Lafayette, IN) provided ninaD^{P132}, ninaD^{P146}, and SANTA MARIA REQUIRED FOR PRODUCTION OF VITAMIN A
ninaB[3][1], J. O’Toole [University of Notre Dame, Notre Dame, IN] provided UAS-ninaB and UAS-ninaD flies; and C. Desplan [New York University, New York, NY] supplied the ninaE–GAL4.

The fly stocks generated were as follows: Fig. 2, santa maria[1]; hs-santa maria[1][2]/++; Fig. 3, 1) [P{GMR-hsp68}G1{neoRT140}I(2)CCL/ P{neoRT140}I(2)drl] santa maria[1]GAL4;eya-H555 P{UAS-flp}D(2)D;[2], santa maria[1]/UAS–santa maria[1];GMR–GAL4/+; [3] CG7707 (pigment cell–GAL4); UAS–santa maria[1]/UAS–santa maria[1], [4] santa maria[1]/UAS–santa maria[1];ninaE–GAL4, [5] santa maria[1]/UAS–santa maria[1];repo–GAL4;[4, 6] ninaB–GAL4/++; [7] ninaD–GAL4/+; [8] ninaBP315, (6) GAL4[1](+); [9] ninaBP315/ninaDP246;repo–GAL4, (7)

Deficiency mapping the santa maria mutation

The santa maria mutation was crossed to the fly stocks that comprised the second/chromosome deficiency kit. The mutation was uncovered by Df(2L)EJ517[1], which deleted 27F2 to 28D1, but not by other deficiency lines in the kit. To map the santa maria locus further, we used smaller deficiencies in the region and found that the mutation was uncovered by Df(2L)EJ479 (27F4 to 28B1) and Df(2L)EJ7031 (27F3 to 28A1); Fig. 2 A). Based on these data, we localized the mutation responsible for the santa maria phenotype to 27F4 to 28A1. This interval is ~30 kb and included 10 genes spanning from CG5261 to CG6630 (Fig. 2 A).

ERG recordings

ERG recordings were performed as previously described [Wes et al., 1999]. In brief, two glass microelectrodes filled with Ringer’s solution were inserted into small drops of electrode cream placed on the surfaces of the compound eye and the thorax. A Newport light projector (model 765) was used to project light through the cuticle onto the retina, and the reflected light was detected using a charge coupled detector (CCD). The fluorescent signals were transferred into Photoshop 7.0 (Adobe) to assemble the figures.

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