Defective Pigment Granule Biogenesis and Aberrant Behavior Caused by Mutations in the Drosophila AP-3β Adapin Gene ruby

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ABSTRACT

Lysosomal protein trafficking is a fundamental process conserved from yeast to humans. This conservation extends to lysosome-like organelles such as mammalian melanosomes and insect eye pigment granules. Recently, eye and coat color mutations in mouse (mocha and pearl) and Drosophila (garnet and carmine) were shown to affect subunits of the heterotetrameric adaptor protein complex AP-3 involved in vesicle trafficking. Here we demonstrate that the Drosophila eye color mutant ruby is defective in the AP-3β subunit gene. ruby expression was found in retinal pigment and photoreceptor cells and in the developing central nervous system. ruby mutations lead to a decreased number and altered size of pigment granules in various cell types in and adjacent to the retina. Humans with lesions in the related AP-3β gene suffer from Hermansky-Pudlak syndrome, which is caused by defects in a number of lysosome-related organelles. Hermansky-Pudlak patients have a reduced skin pigmentation and suffer from internal bleeding, pulmonary fibrosis, and visual system malfunction. The Drosophila AP-3β adaptor also appears to be involved in processes other than eye pigment granule biogenesis because all ruby allele combinations tested exhibited defective behavior in a visual fixation paradigm.

TRAFFICKING of integral membrane proteins within the eukaryotic cell occurs through transport vesicles that form by the assembly of coat proteins onto the cytoplasmic face of precursor membranes. The coat is required for budding of the vesicle and for cargo selection (Waters et al. 1991; Schekman and Orci 1996; Robinson 1997). Clathrin, the first coat to be identified, assembles with adaptor protein complexes that provide the link between the membrane and the clathrin scaffold. The adaptor protein complexes provide membrane specificity for clathrin assembly by recognizing sorting motifs in the cytoplasmic domains of membrane proteins and by interacting with putative docking molecules (reviewed in Heilker et al. 1999). Originally, two homologous adaptor protein complexes were identified: AP-1 for sorting at the trans-Golgi network (TGN) during biosynthetic transport to the endosomal/lysosomal system and AP-2 for endocytosis at the plasma membrane. More recently, two further complexes, AP-3 (Dell’Angelica et al. 1998) and AP-4 (Dell’Angelica et al. 1999b) were characterized. For AP-3, the question of clathrin coat association is controversial (Dell’Angelica et al. 1998). AP-4 was not found in association with clathrin-coated vesicles (Del l’Angelica et al. 1999b). All AP complexes are heterotetrameric proteins built from homologous subunits. They consist of two large chains (γ, α, δ, and ε, and β1-β4, respectively, of 90–130 kD), one medium chain (μ1-μ4 of ~50 kD), and one small chain (σ1-σ4 of ~20 kD). The interaction of AP-1 and AP-2 with clathrin is mediated by the β subunits (Schröder and Ungewickel 1991; Shih et al. 1995).

Evidence for the function of the "classical" complexes AP-1 and AP-2 was predominantly gained from biochemical experiments. In the case of AP-3, genetics is providing information on its role in an apparently conserved transport pathway to lysosome-like organelles (Dell’Angelica et al. 1998). In Saccharomyces cerevisiae, AP-3 subunits were identified as factors involved in the transport of alkaline phosphatase from the TGN to the vacuole. This pathway is independent of the function of AP-1 and AP-2 or clathrin (Cowles et al. 1997). AP-3 in mouse and humans consists of neuronal and non-neuronal isoforms. The latter complex consists of β3A and μ3A and the ubiquitous δ, α3A, or α3B subunits (Dell’Angelica et al. 1997a,b; Simpson et al. 1997). In neuronal cells, AP-3 contains β3B, μ3B, and the ubiquitous subunits (Neu mann et al. 1995; Pev sner et al. 1995; Simpson et al. 1996). Mutations in the two large mouse AP-3 subunit genes, AP-3β in mocha (Kantheti et al. 1998) and AP-3β in pearl (Feng et al. 1999), were recently identified as the cause of a related set of syndromes.

Conditions affecting human AP-3 subunits are of clinical relevance. A patient with autoimmunity against AP-
3βB suffered from degeneration of the cerebellum, where the protein is prominently expressed (Newman et al. 1995). Mutations in AP-3βA were recognized in a subset of patients suffering from the autosomal recessive disease Hermansky-Pudlak syndrome (HPS, Online Mendelian Inheritance in Man no. 203300) (Del’Angelica et al. 1999a). HPS is characterized by defects in a number of lysosome-related organelles. Patients exhibit subnormal eye and skin pigmentation due to aberrant melanosome development. Associated with this oculocutaneous albinism, patients suffer from impairment in visual acuity, misrouting of optic fibers, nystagmus, and underdeveloped fovea (Summer et al. 1988). Clinically more serious is a deficiency of platelet dense granules and a lysosomal malfunction in reticuloendothelial cells, which generally leads to early death. HPS is genetically heterogeneous (Hazelwood et al. 1997). In addition to patients affected in the human paril homolog, patients with mutations in the homolog of the mouse pale ear gene have been diagnosed with HPS (Oh et al. 1996; Gardner et al. 1997). A phenotype similar to HPS was described in 14 mouse mutants, all originally isolated by their eye/skin pigmentation defect (Odorizzi et al. 1998; Swank et al. 1998; Spritz 1999).

Pigmentation mutants of Drosophila offer another approach to the study of lysosome and AP-3 function (Lloyd et al. 1998). The eye color genes garnet and carmine were recently identified as the Drosophila homologs of AP-3β and AP-3μ, respectively (Ooi et al. 1997; Simpson et al. 1997; Mullins et al. 1999). We report here that the Drosophila AP-3β homolog is encoded by the eye color gene ruby (rb). Ruby flies, first described by Bridges in 1914 (Morgan et al. 1925), show a reduced number of eye pigment granules. The expression of rb throughout ontogenesis and behavioral aberrations of adult flies indicate that the gene plays a role beyond eye pigmentation. Ruby therefore can provide a model for studying the role of AP-3-mediated vesicular trafficking in development and in adult central nervous system function.

**MATERIALS AND METHODS**

Fly stocks and genetic screens: rb2 was generated in this study by γ-irradiation. Df(1)ovo1G7 was obtained from Dr. A. P. Mahowald (Chicago). rb1, rb2, Df(1)rb1, Df(1)rb4, In(1)rb2, and Dp(1;2)A1125 from Dr. W. L. Pak (Purdue University), and rb5 and brb cx from the Mid-American Drosophila Stock Center (Bowling Green, OH). The cytological breakpoints of the deficiencies are listed in Pfugfelder et al. (1990). Dp(1;2)A1125, also known as Tb(1;2)rb2-1125 (Lindsley and Zimm 1992), covers the chromosomal region from 3F3 to SE8. rb5 contains a second site lethality not covered by Dp(1;2)A1125 (Poeck 1992).

**Molecular methods:** Southern and Northern blot analysis, cloning, and sequencing were performed as described (Pfugfelder et al. 1990). cDNAs were isolated from a head cDNA λgt11 library (Zinsmaier et al. 1990). More recent sequence analysis employed an ABI Prism Dye terminator cycle sequencing kit (Perkin-Elmer, Norwalk, CT) and an automated ABI 373 sequencer.

**In situ hybridization:** Frozen sections of pupal and adult tissue were fixed and hybridized as described in Poeck et al. (1993). For whole mounts, adult brains were dissected on ice in phosphate-buffered saline (PBS, pH 7.5) and fixed in 4% paraformaldehyde/PBS overnight at 4°C. Hybridization was carried out as above in a volume of 50 μl. Whole mounts were stained for 8-16 hr, dehydrated in an ethanol series, and embedded in Epon for 1-μm serial sections.

**Tissue sections for light and electron microscopy:** Adult heads were prepared for light and electron microscopy as described in Renfranz and Benzer (1989). For light microscopy, 1-μm serial sections were cut and stained with 1% toluidine blue in 1% Borax. Ultrathin Epon plastic sections were poststained with 2% uranyl acetate, followed by Reynolds’ lead citrate, and stabilized for transmission electron microscopy by carbon coating. Examination was done with a Philips 201C electron microscope.

**Eye pigment spectroscopy:** Eye pigments were extracted using a scaled-down variant of the method of Real et al. (1985). For the determination of red pigments (drosopelterins), 15 longitudinally split heads of male flies were used. For the extraction of brown pigments (xanthommatins), 100 male heads were homogenized. Spectra were measured in a Beckman (Fullerton, CA) DU40 spectrophotometer.

**Behavioral analysis:** Optomotor response of fixed flies walking on a styrofoam ball, which was floating on an air jet, was determined as in Buchner (1976) (24° pattern wave length, 1 Hz pattern frequency, light intensity 50 lux). The landing response was tested in suspended flight with a spiral stimulus (Braitenberg and Taddel-Ferretti 1966). Fixation in the walking mode was measured by releasing wing-clipped flies into the middle of an illuminated arena (29 cm diameter) that contained an upright and an inverted “T” at opposite sides of the vertical wall (line width 10°, height from bottom 19°, light intensity 3000 lux). The angle of initial approach to these visual stimuli was measured (Wehner 1972). In Buridan’s paradigm (Götz 1980), individual flies walked spontaneously between two opposing inaccessible landmarks (vertical black stripes on an otherwise uniformly white surround) on an elevated circular disk of 85 mm diameter surrounded by a water-filled moat. Wings were shortened to about one-third of their normal length under cold anesthetics (4°C). At least 4 hr of postoperative recovery time with access to water was given prior to testing. Fly positions were recorded at 5 Hz sampling rate with a video scanning device (Strauss and Pichler 1998).

**RESULTS**

**Genetic and molecular characterization of the ruby gene:** Ruby was mapped to cytological position 4C6 proximal to the gene optomotor-blind (omb) (Bang et al. 1986; Pfugfelder et al. 1990; Lindsley and Zimm 1992). Essential parts of the ruby gene were revealed by the 8.5-kb overlap of the deficiencies Df(1)rb16 and Df(1)ovo1G7 (Figure 1). Flies transheterozygous for these deficiencies were viable and had ruby-colored eyes (Poeck 1992). Probes from the 8.5-kb interval detected two transcripts of 5.4 and 5.2 kb in length in Northern blots (Figure 2). Both transcripts were confined to a 6-kb interval within the deficiency overlap, indicating the lack of large introns. The smaller transcript was only expressed in adult females and in early embryonic stages and is con-
Consider a maternal variant. Both transcripts were eliminated in rb mutant flies trans-heterozygous for Df(1)rb1 and rb10 [Df(1)rb1 eliminates the entire region and rb10 carries a 7-kb insertion in the rb transcription unit]. This allele combination is, therefore, null for rb function. Transcript size was slightly reduced in rb2 flies (data not shown). A 4.2-kb cDNA (pcD4.2) and several smaller cDNAs were isolated from a head cDNA library (Roth 1991). Use of pcD4.2 as a Northern blot probe confirmed the identity of this transcription unit as the rb gene (Figure 2). Ruby transcript abundance was relatively uniform throughout development. The apparent modulation of signal intensity in Figure 2A was mainly caused by unequal RNA load (compare Figure 2B). The direction of transcription was determined as proximal to distal by comparative restriction mapping of genomic and cDNA clones.

Sequencing of pcD4.2 revealed a 1071-amino-acid (aa) open reading frame (ORF) open at the 5' end. A search of the computer database showed strong sequence homology over the entire ORF to the human β3A and β3B adaptins and indicated that ~90 aa might still be lacking from the N terminus. Sequencing of genomic DNA upstream of pcD4.2 showed that the ORF extended for another 89 codons, including 6 ATG codons. A 5' expressed sequence tag clone (Berkeley Drosophila Genome Project GH25915) extended our cDNA by 544 bp and confirmed the continuation of the ORF. On the basis of the sequence homology between the ORFs of ruby, β3A, and β3B that extended all the way to the first start codon of the ruby ORF and the good fit of the first initiation codon environment to the Cavener consensus (Cavener 1987), we assume that ruby is translated from the first AUG codon to yield a 1160-aa protein (Figure 3). A genomic sequence encompassing the ruby ORF submitted by Zhang and Brodie under accession no. AJ011787 differs in only three (silent) positions from pcD4.2. Our cDNA extends 629 bp beyond the 3' end of the AJ011787 sequence and includes a polyA tail preceded by several potential polyadenylation signals, indicating the presence of a 1029-bp 3' untranslated region. Sequence identity between RUBY and human β3A and β3B is 51 and 54%, respectively, and extends over the entire length of the proteins (Figure 3). In the N-terminal two-thirds (trunk domain) sequence identity is 66 and 70%, respectively. At the beginning of the C-terminal domain, the sequence LLDDLDD (aa 875-880 in RUBY), which has been identified as a clathrin-binding motif (Dell'Angelica et al. 1998), is fully conserved. Sequence similarity to the mammalian β1, β2, and β4 proteins is much lower and is obvious only in parts of the N-terminal trunk domain. RUBY is similarly related to the rat β2 and to the Drosophila β1/2-type adaptin BAD-1 (Camidge and Pearse 1994) (36% in 572 aa of the N-terminal domain).

**ruby expression in the developing retina and central nervous system:** For the spatial analysis of the rb expression pattern, whole mount and cryostat head sections of stage 13 pupae (Bainbridge and Bownes 1981) and adults were analyzed by in situ hybridization. In the pupal eye/lamina complex, rb was strongly expressed in a distal zone of the retina extending up to the cornea.
corresponding to the cell body position of the retinal pigment and outer photoreceptor cells (Figure 4A and C). Expression in the photoreceptor cells was also visualized by the staining pattern of tangential eye sections, which in each ommatidium outlines the outer rhabdomeres (Figure 4E). In addition, we found expression in the cortex of the entire pupal brain (Figure 4A). This cortical expression declined with pupal maturation and was no longer found in the adult (Figure 4G). This decreasing cortical expression is not an artifact of low probe accessibility to the adult brain cortex because probes for a less abundantly transcribed gene yielded detectable signal under the same conditions (Poeck et al. 1993). In the adult visual system, rb expression was found in a layer of cells covering the retina basolaterally and within the retina (Figure 4G). The distal staining enwrapped the pseudocone and showed tapering extensions toward the basement membrane at ommatidial periodicity, indicative of expression in pigment cells (Figure 4H). Plastic sections of whole-mount stained head preparations identified the proximal expression in cells lying directly underneath the basement membrane, known as the fenestrated-layer glia (Saint Marie and Carlson 1983) or subretinal glial cells (Cagan and Ready 1989) (Figure 4I). In addition, we found expression in the cortex of the entire pupal brain (Figure 4A).

Reduced number of pigment granules and altered retinula-brain interface in rb\textsuperscript{+}/white\textsuperscript{-} larvae (St청 1936). Ruby-colored and white twin spots were generated at comparable frequency and appeared not to differ in size (data not shown). While we could not determine rb genotype independent of ruby probe accessibilty to the adult brain cortex because probes for a less abundantly transcribed gene yield detectable signal under the same conditions (Poeck et al. 1993). In the adult visual system, rb expression was found in a layer of cells covering the retina basolaterally and within the retina (Figure 4G). The distal staining enwrapped the pseudocone and showed tapering extensions toward the basement membrane at ommatidial periodicity, indicative of expression in pigment cells.

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\caption{Predicted amino acid sequence of RUBY (accession no. AF-247194) and the human \(\beta_3A\) and \(\beta_3B\) proteins (accession nos. U81504 and AF022152). Sequence alignment was performed with the program MegAlign from DNASTar; identical amino acids are boxed.}

\end{figure}
pigment cells, and in the subretinal glial cells (Cagan and Ready 1989). We have investigated the influence of rb1 on pigment granule number at two levels of the retinal subretinal complex. The eye color of rb1 or rb2 did not differ from that of the presumptive null allele combination Df(1)rb1/ Df(1)rb2. rb1 therefore can be considered as null or strongly hypomorphic for pigmentation function. Tangential sections through the eye below the level of the primary pigment cells and above the cone cell endfeet revealed an about twofold reduction in the number of pigment granules in secondary and tertiary pigment cells (Figure 5, A–D). At light microscopic resolution, only the large granules of the pigment cells can be recognized (Stark and Sapp 1988). Below the basement membrane, the reduction in granule number was more than threefold. The mutant granules appeared to have a larger diameter than wild type. The thickness of the pigment containing the fenestrated layer was reduced by \( \sim 40\% \) (Figure 5, E and F).

Both red (drosoperins) and brown pigments (xanthommatins) were reduced in ruby. In rb1 we found a reduction in drosoperin content to 25% of the wild-type level. The drosoperin content was similarly reduced in the g1 allele of garnet (which encodes the \( \delta \) subunit of Drosophila AP-3). In the rb1 g1 double mutant drosop-combination Df(1)rb1/ rb10, rbS1 thereupon can be considered as null or strongly hypomorphic for pigmentation function. Tangential sections through the eye below the level of the primary pigment cells and above the cone cell endfeet revealed an about twofold reduction in the number of pigment granules in secondary and tertiary pigment cells (Figure 5, A–D). At light microscopic resolution, only the large granules of the pigment cells can be recognized (Stark and Sapp 1988). Below the basement membrane, the reduction in granule number was more than threefold. The mutant granules appeared to have a larger diameter than wild type. The thickness of the pigment containing the fenestrated layer was reduced by \( \sim 40\% \) (Figure 5, E and F).
Figure 4.—ruby expression. Hybridization with sense and antisense RNA probes derived from pcD4.2 was performed on frontal cryostat sections (A–H) and on whole mounts (I). The latter were subsequently Epon-embedded and sectioned. Hybridization in A, C, E, G, H, and I was performed with antisense; in B, D, and F, with sense RNA. Both probes caused a strong background signal over the cornea tissue (compare A and B). (A–F) **rb** expression in the visual system of stage 13 pupae. A general **rb** expression was found in the entire brain cortex. The strongest signal was detectable in a layer of cells between the optic lobes and the retina (arrow in A) and in the distal retina (arrowheads in A and C). In the tangential section in E and F, subsequent ommatidial rows are cut at an increasingly proximal level (also evidenced by their decreasing diameter), revealing **rb** transcripts in the direct vicinity of the rhabdomeres. In the adult visual system, expression is most prominent in the pigment cells ensheathing the ommatidia (arrowhead in H) and around the basement membrane that separates the retina from the underlying brain tissue (G–I). The higher magnification in I shows hybridization predominantly brain-proximal to the basement membrane. Bar in A, 100 μm in A and B; in C–F and H, 50 μm; in G, 90 μm; in I, 30 μm. cb, central brain; ol, optic lobes; re, retina; la, lamina; mc, monopolar cells; fl, fenestrated layer.

detect brain malfunction. Four allelic combinations of **rb** were tested [(ln(1)rbD1/ rbS1, rb10/ rbS1, Df(1)rb1/ rbS1, and rb10/ Df(1)rb1) and responded normally to moving (landing and optomotor response) and stationary visual stimuli (pattern orientation; data not shown). The same mutants showed, however, abnormal behavior in Buridan’s paradigm, a test for alternating pattern fixation. Fixation of visual landmarks was significantly impaired in **rb** mutants. Unlike that in blind flies, this behavior was not random in **rb**. **rb** mutations also caused a highly significant increase in walking speed between the landmarks [17.6+/−0.9 (**rb**) vs. 13.4+/−0.5 (control) cm/sec; P<0.001]. Importantly, introduction of the duplication Dp(1;2)A1125 into the **rb** mutant background rescued both the eye color and the behavioral defects (Figure 7). The one tested allele g1 was similarly disturbed in both orientation behavior and walking speed (data not shown). As will be argued below, the altered eye pigmentation is unlikely to account for the highly specific behavioral phenotype.

**Discussion**

We have determined by genetic and molecular means the identity of the Drosophila **ruby** gene. High sequence homology throughout the protein coding region to the mammalian β3 adaptins and phenotypic similarity to mammalian β3A mutants suggests that **ruby** encodes a functional β3 adaptin homolog. In humans, two β3 variants with 61% aa sequence identity were described. The β1 and β2 adaptins constitute another pair of related proteins (83% aa sequence identity) (Dell’Angelica et al. 1997b; Simpson et al. 1997). Sequence conservation between the three human gene groups β1/2, β3A/B, and β4 lies between 21 and 28% aa sequence identity (Dell’Angelica et al. 1999b). RUBY is about equidis-
tant to human β3A and β3B (51 and 54%), but is clearly more closely related to these proteins than to β1/2 or β4 where sequence similarity is confined to N-terminal regions. Another Drosophila AP-β protein has previously been described (BAD-1, β-adaptin-Drosophila; Camidge and Pearse 1994). Its sequence is equidistant to human β1 and β2 (73% homology). We found no evidence (from low stringency Southern hybridizations or from the sequence database) for the existence of more β3-related genes in Drosophila. It appears, therefore, that the evolutionary split into β1 and β2 and into β3A and β3B took place after the separation of protostomes and deuterostomes and only in the deuterostome lineage. RUBY contains a putative clathrin-binding motif. In Drosophila, several mutations in clathrin heavy chain gene were described (Bazinet et al. 1993). The significance of the clathrin-binding motif can now be assessed in transgenic animals expressing mutant versions of the rb gene and by testing for genetic interactions.

*rb* is transcribed throughout development. Two transcripts differing only slightly in size were found in young embryos and in adult females. The smaller transcript appears to be a purely maternal form, not found in later embryonic stages or in adult males. Surprisingly, the larger transcript also was found during the first hour of embryonic development before the onset of zygotic transcription. In contrast to *garnet* where female sterility and to *carmine* where lethality was reported for some alleles, all rb mutants and allele combinations were fertile. *garnet* also differs from rb in that strong alleles have an orange eye color (Lindsley and Zimm 1992).

If all subunits of a multiprotein complex were equally necessary for its function, the phenotype of mutants in all subunit genes should be the same. This appears to be the case for yeast AP-3 (Cowles et al. 1997; Panek and St 1997). The difference in the severity of strong rb, g, and cm phenotypes could mean that β3, δ, and μ3 differ in the degree with which their function can be substituted by related proteins. There are observations that support this hypothesis. In mammalian AP-3 subunits, a higher constraint on δ vs. β3 function is evident from the sequence conservation between mouse and humans: 98% for δ and 88% for β3A (Kantheti et al. 1998; Feng et al. 1999). Mutation of AP-3δ in the mocha mouse leads to a nearly complete loss of the three other subunits of the complex; mutation of AP-3β in the pearl mouse has a more subtle effect on the complementary subunits (Kantheti et al. 1998). In transfected Cos cells, the β1/2-type Drosophila BAD-1 protein can be accommodated in AP-1 and AP-2 complexes without disturbing their targeting to the TGN or plasma membrane (Camidge and Pearse 1994). Similarly, in the mammalian AP-3 complex, one δ subunit can interact with different isoforms of β, μ, and α (Odorizzi et al. 1998).

Eye pigmentation in Drosophila proceeds through
many steps. Low-molecular-weight pigment precursors are synthesized in the fat body and Malpighian tubules and are taken up by the future pigmented cells during pupal development. Precursor transport across the pigment cell membrane is accomplished by heteromeric ABC transporters encoded by white, brown, and scarlet (Dreesen et al. 1988; Tearle et al. 1989). In contrast to mutations in genes encoding precursor-synthesizing enzymes, mutations affecting uptake functions or pigment granule biogenesis are autonomous. Autonomy of the ruby eye color phenotype is suggested by our mosaic experiments, by the existence of the variegating allele rb<sup>mut</sup>, and by the pigment autonomy of rb<sup>eye</sup> disks transplanted into wild-type hosts (Beadle and Ephrussi 1936; Lindley and Zimm 1992). Ruby reduces the level of both types of eye pigments, the drosoperferins and ommochromes (Nolte 1950, 1952; our data). This property is shared by many of the more than 85 eye pigmentation mutants known in Drosophila, most of which have not yet been cloned. In addition to rb, the sequence and mutant phenotype of four pigmentation genes currently suggests their involvement in vesicle transport: garnet, deep orange, light, and carnat (Lloyd et al. 1998; Sevrioukov et al. 1999). Carnation was recently shown to be the constituent of a protein complex unrelated to AP but also involved in protein transport to lysosomes and pigment granules (Sevrioukov et al. 1999). Carnation, carmine, g, and rb were collectively termed "ruby group" based on the similarity of their pigmentation phenotype (Nolte 1952). Carmine, g, and rb have by now been identified as genes encoding AP-3 components. Since carnat does not encode the α3 subunit, mutations in the α3 gene will probably differ in eye phenotype from the members of the "ruby group."

Allele combinations null for rb function still contain pigment granules and have ruby-colored eyes. This can be explained by various hypotheses. As discussed above, Drosophila β-type adaptin function may be partially redundant. Another possibility is that alternative pathways for pigment granule biogenesis can be activated in rb or are constitutively active at a low level. This is in agreement with observations in yeast, where inactivation of AP-3 only partially eliminates transport of alkaline phosphatase to the vacuole (Cowles et al. 1997; Stemp et al. 1997). Lysosomes and melanosomes can receive proteins through both the biosynthetic route from the TGN and via endocytosis from the plasma membrane (Lloyd et al. 1998). Inactivation of AP-3 by mutations in β3A or with antisense oligonucleotides leads to an increased expression of lysosomal proteins on the surface of mammalian cells (LeBorgne and Hofack 1998; Dell'Angelica et al. 1999a). A similar effect was observed in AP-3 mutants in yeast (Cowles et al. 1997; Stemp et al. 1997). The coexistence of alternative pathways appears to be a general characteristic of membrane trafficking (Mostov et al. 1999). Our observation of an increased granule size in rb (Figure 5) may be an indication of an alternative biogenic pathway. Similar observations have previously been made in several Drosophila pigmentation mutants, including rb (Nolte 1950; Shoup 1966; Stark and Sapp 1988). Redundancy at the β-adaptin or pathway level also appears to exist in the mice where the pearl and mocha mutants have dilute, but not albino, pigmentation (Kantheti et al. 1998). As in Drosophila, altered melanosome size or shape was observed in several mouse pigmentation mutants, including the two HPS models pearl and pale ear (Mangini et al. 1985; Gardner et al. 1997).

In rb, the thickness of the pigmented layer under the retinal basement membrane was found to be reduced (Figure 5). Similar findings were described by Nolte (1950) at the resolution of light microscopy for rb and several other pigment mutants. Since pigment granules appear to take up a considerable fraction of

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**Figure 6.** Spectral analysis of pigment content. Drosoperferin (A) and xanthommatin (B) were extracted from heads of flies reared under identical conditions. The genotypes are indicated next to the curves. The small deflection in the curves below 380 nm is caused by the ultraviolet to visible wavelengths transition of the spectrophotometer. In A, the curves for rb<sup>+</sup> and g<sup>+</sup> were nearly superimposable. Only the rb curve is therefore shown. WT, wild type; rb, ruby; g, garnet.
the cellular volume, the volume reduction could be a consequence of the reduction in granule number. There are similarities in the structural organization of the interfaces between the vertebrate photoreceptor cells and the retinal pigment epithelium and between the dipteran fly retina and the optic lobes. In both cases pigmented glia cells extend apical processes in close contact with the photoreceptor cells (Saint Marie and Carlson 1983; Bok 1985; Williams et al. 1985). The pigment granules/melanosomes in these glial cells appear to be similarly affected in the pearl mouse and the \( rb \) fly. They are reduced in number and increased in size (Mangini et al. 1985; Williams et al. 1985).

Pearl mice show a defective optokinetic nystagmus, a defect \( perl \) shares (with variation) with several mouse hypopigmentation mutants. This defect appears to correlate with a reduced numerical density of melanosomes in the retinal pigment epithelium. \( perl \), like many other hypopigmented mammalian mutants, displays a reduced ipsilateral retinal projection, the developmental origins of which are not yet clear (Marcus et al. 1996).

We did not observe pathfinding defects in the visual system of \( rb \) flies. Indeed, ruby flies responded normally to moving visual stimuli (optomotor and landing response). They were, however, abnormal in alternating pattern fixation in Buridan’s paradigm. In a different pattern fixation paradigm, in which only the first target approach was evaluated, \( rb \) flies performed normally. In agreement with previous investigations of various eye color mutants (Hengstenberg and Götz 1967), the normal performance of \( rb \) mutants in several visual tests indicates that the altered eye pigmentation has little effect on the processing of visual signals in the distal periphery of the visual system. The pattern fixation test in which \( rb \) flies performed normally requires a slightly higher visual acuity than Buridan’s paradigm, indicating that the behavioral defect in the latter is not caused by an influence of eye pigmentation on visual acuity. This suggests that the development or function of a higher coordination center is disturbed in \( rb \). The two behavioral defects, affecting orientation and walking speed, may map to different foci. Intriguingly, in a mocha mouse mutant an altered neocortical activity was observed (Noebels and Sidman 1989). Our in situ hybridization data showed \( rb \) expression in the central nervous system (CNS) cortex during pupal development but not in the

Figure 7.—Behavior analysis in Buridan’s paradigm. (A–C) Traces of single flies walking for 15 min between inaccessible visual landmarks (symbolized by solid bars flanking the circles). The genotypes are as indicated. In B, the \( rb \) phenotype is rescued by the duplication Dp(1;2)A1125. The wild-type trace in C is taken from Strauss and Pichler (1998). (D) Quantitative evaluation of 12 flies for each genotype. The angle of orientation between fly and approached target was measured every 0.2 sec (4500 recordings per fly). Angles between 0° and 5°, 5° and 10°, etc., were pooled and plotted against the normalized frequency with which they were observed. The error bars are smaller than the symbols. The difference in orientation behavior between the two genotypes is highly significant (t-test, two-tailed, \( P < 10^{-4} \)). The curve for random orientation was calculated as in Strauss and Pichler (1998).
adult stage. This indicates a developmental role of rb in the CNS, rather than a function in neuronal activity. An adult neuronal function is fulfilled by an AP-2-related Drosophila adaptor complex that is involved in synaptic vesicle recycling from the presynaptic cell membrane (González-Gaitán and Jäckle 1997), possibly in conjunction with another clathrin adaptor protein (Zhang et al. 1998, 1999). In vitro experiments have implied mammalian AP-3 in synaptic vesicle recycling from endosomes (Faundez et al. 1998). Whether AP-3 has a synaptic function in Drosophila can now be studied electrophysiologically in mutants of the identified AP-3 subunit genes.

There is indirect evidence for the developmental function of two other Drosophila eye pigmentation genes in the brain. The combination of the two viable mutations carntation and light leads to synthetic lethality. By mosaic analysis, the focus of this lethal interaction is on the function of two other Drosophila eye pigmentation proteins in Hermansky-Pudlak syndrome due to mutations in the trophysiologically in mutants of the identified AP-3 subunit genes.

There is indirect evidence for the developmental function of two other Drosophila eye pigmentation genes in the brain. The combination of the two viable mutations carntation and light leads to synthetic lethality. By mosaic analysis, the focus of this lethal interaction was localized to the brain, which is abnormal in double-mutant larvae (McCartney 1980). The high penetrance of the aberrant behavior revealed in Buridan's paradigm will allow an investigation of AP-3's neurobiological function. The spatial and temporal requirements of rb function for normal behavior can be determined using Gal4 (Brand and Perrimon 1993) and flipase (Golic and Lindquist 1989) technology.

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LITERATURE CITED


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