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ORIGINAL PAPER

C. Mullins · L. M. Hartnell · J. S. Bonifacino

Distinct requirements for the AP-3 adaptor complex in pigment granule and synaptic vesicle biogenesis in *Drosophila melanogaster*

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Abstract The AP-3 adaptor protein complex has been implicated in the biogenesis of lysosome-related organelles, such as pigment granules/melanosomes, and synaptic vesicles. Here we compare the relative importance of AP-3 in the biogenesis of these organelles in *Drosophila melanogaster*. We report that the Drosophila pigmentation mutants *orange* and *ruby* carry genetic lesions in the $\sigma3$ and $\beta3$-adaptin subunits of the AP-3 complex, respectively. Electron microscopy reveals dramatic reductions in the numbers of electron-dense pigment granules in the eyes of these AP-3 mutants. Mutant flies also display greatly reduced levels of pigments housed in these granules. In contrast, electron microscopy of retinula cells reveals numerous synaptic vesicles in both AP-3 mutant and wild-type flies, while behavioral assays show apparently normal locomotor ability of AP-3 mutant larvae. Together, these results demonstrate that Drosophila AP-3 is critical for the biogenesis of pigment granules, but is apparently not essential for formation of a major population of synaptic vesicles in vivo.

Key words AP-3 · Biogenesis · Pigment granule · Synaptic vesicle · *Drosophila*

Introduction

The biogenesis and maintenance of organelles of the endocytic and secretory pathways depend upon transport vesicles that ferry cargo proteins from one membrane-bound compartment to another. Formation of these vesicles starts with recruitment of coat proteins from the cytosol to specific sites on the donor organelles. The coat proteins congregate into large supramolecular assemblies that both drive vesicle budding and select cargo for transport. Among the coat proteins that function in the endocytic and late secretory pathways are the heterotetrameric adaptor protein (AP) complexes AP-1, AP-2, AP-3 and AP-4 (Hirst and Robinson 1998; Bonifacino and Dell'Angelica 1999; Kirchhausen 1999).

Our laboratory has been particularly interested in understanding the function of AP-3 in protein trafficking and organelle biogenesis. Like other AP complexes, AP-3 is composed of two large subunits (δ and $\beta3$), a medium-sized subunit ($\mu3$), and a small subunit ($\sigma3$) (Dell'Angelica et al. 1997, 1997b; Simpson et al. 1997; Cowles et al. 1997; Stepp et al. 1997). Mammals, but not yeast, express two isoforms (A and B) each of $\beta3$, $\mu3$ and $\sigma3$. AP-3 is associated with the *trans*-Golgi network (TGN) and/or endosomes (Dell'Angelica et al. 1997a; Simpson et al. 1997), and is recruited to these organelles in a reaction regulated by the small GTP-binding protein ARF1 (Faundez et al. 1998; Ooi et al. 1998). Each subunit of AP-3 is thought to play a specialized role within the complex. By analogy with subunits of other AP complexes (Owen et al. 1999), δ is thought to interact with molecules that regulate coat formation (Rehling et al. 1999). Mammalian $\beta3$, binds the scaffolding protein clathrin (Dell'Angelica et al. 1998), although it remains to be determined whether AP-3 functions as part of a clathrin coat. $\mu3$ binds tyrosine-based sorting signals found in the cytosolic tails of integral membrane proteins, with sequence preferences characteristic of lysosomal targeting signals (Ohno et al. 1998). The complete AP-3 complex has also been shown to interact with dileucine-based sorting signals from the lysosomal membrane protein LIMP-II and the melanosomal protein tyrosinase (Höning et al. 1998). The function of $\sigma3$ is still unknown.

The signal-binding preferences of AP-3 are thus suggestive of a role for this complex in protein sorting to lysosomes and lysosome-related organelles such as

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C. Mullins · L. M. Hartnell · J. S. Bonifacino (✉)
Cell Biology and Metabolism Branch,
National Institutes of Child Health and Human Development,
National Institutes of Health, Building 18T/Room 101,
Bethesda, MD 20892, USA
E-mail: juan@helix.nih.gov
Tel.: +1-301-4966368; Fax: +1-301-402-0078

melanosomes. This notion has received strong support from genetic analyses of AP-3 function in various eukaryotic organisms. Defects in δ (Ooi et al. 1997; Simpson et al. 1997) and μ3 (Mullins et al. 1999) have been linked to abnormalities in visual pigment granules in the *Drosophila melanogaster* mutants *garnet* (*g*) and *carmine* (*cm*), respectively. Mutations in δ and β3A in the mouse strains *mocha* (Kantheti et al. 1998) and *pearl* (Feng et al. 1999), respectively, have been associated with defects in lysosome-related organelles, such as melanosomes and platelet dense granules. Similarly, mutations in β3A have been found to cause Hermansky-Pudlak syndrome (HPS) type 2, a human genetic disorder characterized by defective melanosomes and platelet dense granules (Dell'Angelica et al. 1999; Shotelersuk et al. 2000). Cells from *mocha* and *pearl* mice, as well as from HPS-2 patients, exhibit increased trafficking of lysosomal membrane proteins via the cell surface (Dell'Angelica et al. 1999, 2000), suggesting that mis-sorting of these proteins may underlie some of the defects in lysosome-related organelles. The same lysosomal protein mis-sorting phenotype was observed in cultured human cells in which AP-3 levels were lowered using an antisense RNA methodology (Le Borgne et al. 1998). Finally, mutations in AP-3 subunit orthologs in the yeast *Saccharomyces cerevisiae* have been shown to result in defective sorting of a subset of proteins to the vacuole, the yeast equivalent of the metazoan lysosome (Cowles et al. 1997; Stepp et al. 1997). Taken together, these findings suggest that AP-3 plays important roles in common sorting pathway(s) utilized by lysosomes and lysosome-related organelles.

In addition to its role in the biogenesis of lysosome-related organelles, AP-3 has been proposed to participate in synaptic vesicle (SV) biogenesis in mammals (Faundez et al. 1998; Kantheti et al. 1998; Salem et al. 1998). AP-3 is required for formation of synaptic-like microvesicles (a type of SVs) from endosomes in vitro (Faundez et al. 1998; Lichtenstein et al. 1998). SV formation in this assay is dependent on a neuronal form of the AP-3 complex comprising brain-specific isoforms of β3 and μ3, termed β3B and μ3B (Faundez et al. 1998). AP-3-dependent SV biogenesis requires ARF1, but not clathrin or dynamin (Faundez et al. 1998), and involves an interaction of AP-3 with VAMP-2 (Salem et al. 1998). An alternative pathway of SV biogenesis from the plasma membrane involving AP-2, clathrin and dynamin has also been described (Takei et al. 1996; Gonzalez and Jäckle 1997; Dornan et al. 1997; Shupliakov et al. 1997; Schmidt and Huttner 1998; Shi et al. 1998). *mocha* mice, which are deficient in expression of AP-3 δ, display hyperexcitability and a tendency to seizures; this has been attributed to impaired sorting of the zinc transporter ZnT-3 to SVs (Kantheti et al. 1998). It is currently unclear, however, to what extent this phenotype is due to a general defect in SV biogenesis.

Here we compare the requirement for AP-3 for the biogenesis of visual pigment granules and SVs in Drosophila in vivo. Initially, we identify cDNAs encoding the AP small-chain (σ) subunits σ1, σ2 and σ3. We find defective expression of the genes encoding AP-3 subunits σ3 and β3 in the Drosophila pigmentation mutants *orange* (*or*) and *ruby* (*rb*), respectively. Together with the AP-3 δ and μ3 mutants *g* and *cm*, respectively, these newly identified mutants allow us to assess the functional importance of all four subunits of the AP-3 complex. Electron microscopy and biochemical analyses of *or* and *rb* mutants reveal dramatic reductions in both the number of visual pigment granules and their total pigment contents. Electron microscopy of synaptic terminals from brains of *or* and *rb* mutants, on the other hand, reveal SVs that are apparently normal in both number and morphology. In addition, Drosophila AP-3 mutants do not exhibit serious neurological defects like those reported for mutants in components of the AP-2-dependent pathway of SV biogenesis, such as α-adaptin (Gonzalez and Jäckle 1997; Dornan et al. 1997) or dynamin (*shibire*, Poodry et al. 1973; Poodry and Edgar 1979; Kosaka and Ikeda 1983) mutants. These findings thus suggest that AP-3 is more critical for pigment granule biogenesis than for SV biogenesis in Drosophila.

Materials and methods

Identification of Drosophila σ chains and cytological mapping of the genes encoding them

A homology search of the Berkeley Drosophila Genome Project (BDGP) EST database identified a clone (LD14109, Accession No. AA439518) encoding a polypeptide that is highly homologous to mouse σ1. This clone was obtained from Genome Systems (St. Louis, Mo.) and sequenced to obtain the full-length Drosophila σ1 cDNA sequence. We also identified a sequence of genomic DNA (Accession No. AC008308) encoding a polypeptide that is highly homologous to mouse σ2. In addition, we identified a sequence encoding a polypeptide with homology to amino acids 55–141 of mammalian σ3 in Drosophila genomic DNA P1 clone DS02867 (Accession No. AC004642). This sequence was used to design oligonucleotide primers for 5′- and 3′-RACE (rapid amplification of cDNA ends) using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, Calif.) and a Drosophila cDNA library (Ooi et al. 1997). Sequence derived from 5′ and 3′ RACE products was used to derive the complete σ3 cDNA sequence. To map the σ1 gene, a high-density P1 filter from Genome Systems was hybridized with a full-length σ1 cDNA labeled with ^{32}P and examined by autoradiography. The cytological position of the σ1 gene was then derived by the use of conversion tables supplied by Genome Systems and information available from the BDGP web server (http://fruitfly.berkeley.org/). The map positions of the Drosophila σ3, σ2, and β3 (Accession No. AJ011778) genes were obtained from the BDGP database.

Drosophila strains

The *D. melanogaster* CantonS (wild-type) stock, the pigmentation mutants *purpleoid* (*pd*, allele pd^1), *or* (alleles or^1 and or^{49h}), *g* (allele g^3), and *rb* (allele rb^1), and the dynamin mutant *shi* (temperature sensitive allele shi^1) were obtained from the Bloomington Stock Center (Bloomington, Ind.).

Northern blot analyses

To examine $\sigma3$ and $\beta3$ expression in wild-type and mutant flies, total RNA was extracted using TRIZOL (Life Technologies, Gaithersburg, Md.). RNA samples (10 μg) were fractionated, transferred to GeneScreen Plus nylon membrane (New England Nuclear, Boston, Mass.), and hybridized as previously described (Mullins et al. 1999). The hybridization shown in Fig. 2 was performed using a ^{32}P-labeled probe corresponding to the full-length $\sigma3$ cDNA sequence. Hybridizations in Fig. 3 were performed using ^{32}P-labeled probes corresponding to the full-length $\beta3$ cDNA sequence, the $\beta3$ sequence encoding residues 1–210 with 17 nucleotides of the 5′ untranslated region (5′ probe), and the $\beta3$ sequence encoding residues 941–1160 with 9 nucleotides of the 3′ untranslated region (3′ probe). A probe corresponding to the TATA binding protein (TBP) cDNA was used as a loading control. Hybridized filters were examined by autoradiography.

Electron microscopy and spectrophotometric analysis of Drosophila eye pigments

For examination of pigment granules, eyes from 2- to 3-day old females of the Drosophila strains rb^1, or^1, or^{49h} and CantonS were dissected, fixed, processed and embedded in Spurr resin as previously described (Mullins et al. 1999). Thin sections were obtained from 10 flies of each strain and contrasted using 3% uranyl acetate/lead citrate solutions. Sections were then examined and photographed on a Philips CM10 electron microscope. For examination of SVs from retinula cells, heads from 2- to 3-day old females of Drosophila strains rb^1, or^1, or^{49h}, shi^1 and CantonS were dissected and fixed whole in 2% formaldehyde, 2% glutaraldehyde in 100 mM HEPES buffer (pH 7.4) at room temperature for 30 min. A slit was then made near the proboscis and fixation allowed to continue overnight at room temperature. Prior to fixation, shi^1 flies were incubated at 30 °C for 10 min, dissected and immediately transferred to fixative prewarmed to 30 °C. Sample processing continued as above. Thin sections from 3–6 heads of each strain were obtained from areas rich in retinula cells and contrasted and examined as above. Extraction and quantitation of the red (pteridine) and brown (ommochrome) pigments from adult eyes was performed as previously described (Ooi et al. 1997; Mullins et al. 1999).

Drosophila behavioral assays

For the mobility assay, approximately 50 Drosophila females were isolated from each stock tested, placed in culture bottles, and allowed to lay eggs for 48 h. The adults were then removed, and bottles were incubated for an additional 4 days at 25 °C. For *rb* and *shi* mutants, incubation of males and females together during the egg collection phase was found to provide larger numbers of larvae. Larvae (primarily 3rd instar) were removed by the addition of a 15% sucrose solution and gentle agitation with a spatula. The larvae, which float to the surface due to their buoyancy in the sucrose, were collected using a pipette and transferred to distilled water, and allowed to settle to the bottom of the vessel. Larvae were washed twice in distilled water and removed using a pipet. Excess water was wicked away and larvae were divided into groups of approximately 50–100. Groups of larvae were placed in the center of assay plates consisting of 10 ml of hardened 1.1% agarose. Assay plates were immediately placed in a darkened box to rule out any influence of external cues, and the larvae were allowed to disperse for 2 min at room temperature (23 °C). Strains tested for temperature sensitivity were incubated at 30 °C or 37 °C for 10 min, then transferred to prewarmed plates and incubated in a darkened incubator (at 30 °C or 37 °C) for 2 min. Plates were then placed on dry ice to halt migration. Larvae were immediately scored for migration from the center to one of 4 concentric rings (numbered 1–4, with No. 4 being the outermost) drawn on the assay plate. A response index (RI) was calculated using the formula $(0 \times C) + (1 \times 1st) + (2 \times 2nd) + (3 \times 3rd) + (4 \times 4th)$/total number of larvae, with C, 1st, 2nd, 3rd, and 4th being the numbers of larvae counted in the center, 1st, 2nd, 3rd, and 4th concentric rings of the assay plate, respectively.

The olfactory dose-response assay was performed essentially as described previously (Monte et al. 1989; Lilly and Carlson 1990). Briefly, propionic acid (Sigma, St. Louis, Mo.) was applied to one filter disk and the same volume of distilled water was spotted on the control disk. Larvae were allowed to migrate for 5 min at room temperature (23 °C). After incubation, assay plates were placed on ice and dividers were inserted into the agar to bisect the plates. Larvae were counted and an RI was calculated using the formula $RI = (S - C)/(S + C)$, where S and C represent the number of larvae found on the stimulus and control halves, respectively.

Results

Identification of Drosophila AP complex small chains $\sigma1$, $\sigma2$ and $\sigma3$

To identify genes for AP complex small (σ) chains in *D. melanogaster*, a sequence similarity search of the expressed sequence tag (EST) database of the Berkeley Drosophila Genome Project (BDGP) was performed using sequences of the mammalian σ chains as query sequences (Materials and methods). From this search DNA sequences encoding polypeptides showing high sequence homology to mammalian AP-1 subunit $\sigma1$ and AP-2 subunit $\sigma2$, respectively, and a partial genomic DNA sequence encoding a polypeptide with homology to an internal region of the AP-3 subunit $\sigma3$ were identified. Conceptual translation of the ORFs from these sequences revealed $\sigma1$ and $\sigma2$ orthologs with predicted molecular weights of 19.5 kDa and 17 kDa, respectively (Fig. 1). The cDNA sequence of $\sigma3$ was obtained through 5′ and 3′ RACE (Materials and methods). Sequencing and conceptual translation of the ORF revealed a $\sigma3$ ortholog with a predicted molecular weight of 21.7 kDa (Fig. 1). Comparative sequence and phylogenetic analyses demonstrate a high degree of sequence identity between Drosophila $\sigma1$, $\sigma2$ and $\sigma3$ chains and their respective orthologs in mammals and *Caenorhabditis elegans*, and somewhat less homology to those in *S. cerevisiae* (Table 1). Our searches of the Drosophila database failed to reveal an ortholog to mammalian $\sigma4$ or, in fact, any other AP-4 subunit.

The cytological location of the $\sigma1$ gene was determined by hybridization of specific probes to Drosophila high-density P1 filters. The $\sigma1$ locus was found to map to 95C8–95D2. The map location of $\sigma2$ was identified as 93D based on the description of locus AC008309 available on the BDGP website. A search of these locations using information available on the BDGP and FlyBase (http://flybase.bio.indiana.edu/) websites failed to reveal any mutations related to protein sorting. The $\sigma3$ locus was mapped by the BDGP to cytological location 60A. A search of this location revealed two mutations, *purpleoid* (*pd*) and *or*, belonging to a class of eye-color mutations termed the granule group (Lloyd et al. 1998). This suggested to us that a defect in the gene encoding

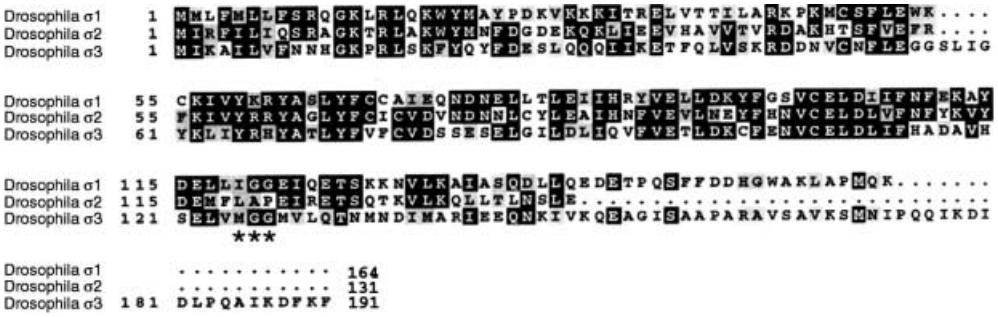


Fig. 1 Amino acid sequences of Drosophila σ chains. Amino acid sequences of Drosophila small chains $\sigma1$ (Acc. No. AF228054), $\sigma2$ (Acc. No. AF228055), and $\sigma3$ (Acc. No. AF228056) are presented. Identical residues are *shaded in black* and similar residues are *shaded in gray*. Alignments were produced using the ClustalW Multiple Sequence Alignment software (available at the European Bioinformatics Institute web address http: //www2.ebi.ac.uk/clustalw/) with shading performed using the BOXSHADE program (http://www.biophys.uni-duesseldorf.de/bionet/rt_1.html). The residues of $\sigma3$ that are missing in the or^1 mutant (see Fig. 2) are indicated by *asterisks*

AP-3 subunit $\sigma3$ may be responsible for the *pd* or *or* mutant phenotype.

Analysis of $\sigma3$ and $\beta3$ expression in Drosophila pigmentation mutants

To examine $\sigma3$ expression in the *pd* mutant, total RNA was isolated from pd^1 and CantonS flies, and subjected to Northern blot analysis using a radiolabeled probe corresponding to the full-length $\sigma3$ cDNA sequence. This analysis revealed no apparent difference in $\sigma3$ mRNA size or expression level between pd^1 and wild-type flies (data not shown). In addition, sequence analysis of the $\sigma3$ cDNA isolated from total pd^1 RNA revealed no mutations.

Northern analysis was then performed on total RNA from the *or* mutants or^1 and or^{49h}, and wild-type flies. Hybridization with a radiolabeled probe corresponding to the full-length $\sigma3$ cDNA revealed no apparent difference in $\sigma3$ mRNA size or levels between wild-type (Fig. 2, lane 1) and or^1 (Fig. 2, lane 2) flies. However, the level of the $\sigma3$ mRNA was greatly reduced in or^{49h} (Fig. 2, lane 3) relative to wild type. The $\sigma3$ cDNA was then amplified from total RNA isolated from or^1 and or^{49h} flies and subjected to sequence analysis. A deletion of the nucleotides encoding amino acids 125–127 (Met-Gly-Gly) was revealed in the $\sigma3$ mRNA of or^1 (indicated by asterisks in Fig. 1). These residues are conserved in human and mouse $\sigma3$A and $\sigma3$B. The or^{49h} $\sigma3$ mRNA, though present in reduced levels, showed no mutations. Thus, two distinct defects in $\sigma3$ were detected in *or* flies: apparently wild-type levels of an mRNA encoding a protein with a three-amino acid deletion in the or^1 mutant, and reduced levels of a mRNA with wild-type sequence in the or^{49h} mutant.

Interestingly, the gene encoding the $\beta3$ subunit of AP-3 maps to a cytological location (4C6–7, Zhang and Broadie, Accession No. AJ011778) that harbors the granule-group mutation *rb*. This suggested that a defect in the gene encoding AP-3 subunit $\beta3$ might be responsible for the *rb* mutant phenotype. We therefore examined expression of the $\beta3$ gene in the *rb* mutant. For this analysis, total RNA was isolated from rb^1 and CantonS flies and subjected to Northern analysis using a radiolabeled probe corresponding to the full-length $\beta3$ cDNA. This analysis revealed a mRNA of ~4.4 kb in wild-type flies (Fig. 3A, lane 1), while a smaller species (~1.25 kb) was detected in rb^1 (Fig. 3A, lane 2). Further analysis using probes directed to the 5′ (Fig. 3A, lanes 3 and 4) and 3′ ends (Fig. 3A, lanes 5 and 6) of the $\beta3$ ORF revealed a deletion of the 3′ end of the $\beta3$ mRNA in rb^1 (Fig. 3B).

Analysis of visual pigment granules in *or* and *rb* mutant flies

To determine whether the pigmentation defects observed in *or* and *rb* mutants were due to defects in pigment

Table 1 Percent amino acid identity for σ proteins[a]

	Human σ1A	Human σ1B	*C. elegans* σ1	*S. cerevisiae* APS1p
D. melanogaster σ1	76	81	84	51
	Human σ2		*C. elegans* σ2	*S. cerevisiae* APS2p
D. melanogaster σ2	91		91	47
	Human σ3A	Human σ3B		*S. cerevisiae* APS3p
D. melanogaster σ3	74	74		35

[a] GenBank Accession Nos.: human σ1A, BAA33391; human σ1B, BAA33392; *C. elegans* σ1, 2315354; *S. cerevisiae* APS1p, 461518; human σ2, 1703323; *C. elegans* σ2, AAA96207; *S. cerevisiae* APS2p, 231554; human σ3A, X99458; human σ3B, X99459; *S. cerevisiae* APS3p, Z49299

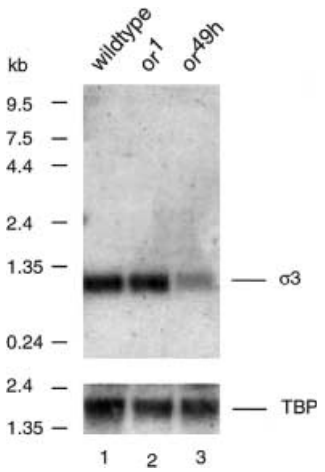


Fig. 2 Expression of the $\sigma 3$ mRNA in wild-type and *or* mutant flies. Whole RNA was isolated from the wild-type strain CantonS and or^1 and or^{49h} homozygous mutants and subjected to gel electrophoresis (10 μg/lane) and Northern analysis using a radiolabeled probe corresponding to the full-length wild-type $\sigma 3$ cDNA sequence. The hybridized filter was examined by autoradiography, stripped and rehybridized with a radiolabeled probe corresponding to the full-length cDNA for the Drosophila TATA binding protein (TBP), to control for loading. Relative positions of $\sigma 3$ and TBP mRNAs and RNA size markers (in kb) are indicated

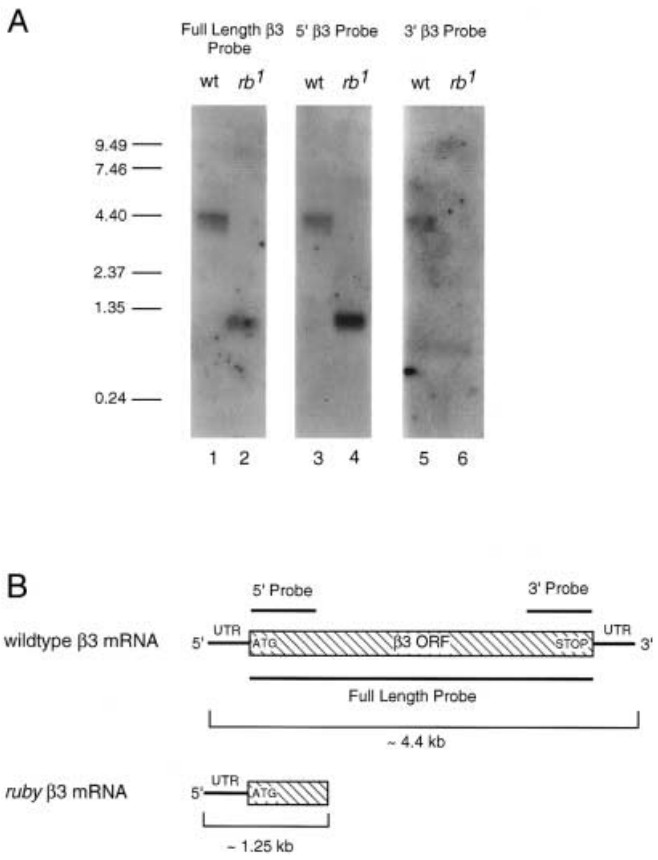


Fig. 3A, B Expression of the $\beta 3$ mRNA in wild-type and *rb* mutant flies. **A** Whole RNA was isolated from the wild-type strain CantonS and the rb^1 homozygous mutant and subjected to gel electrophoresis (10 μgs/lane) and Northern analysis. The filter was hybridized using radiolabeled probes corresponding to the full-length $\beta 3$ cDNA sequence (lanes 1–2), a 5′ region of the $\beta 3$ coding sequence (lanes 3 and 4), and a 3′ region of the $\beta 3$ coding sequence (lanes 5 and 6). Relative positions of RNA size markers (in kilobases) are indicated. **B** Diagrammatic representation of the relative sizes of the $\beta 3$ mRNA in wild-type and rb^1 mutant flies. The size of the wild-type $\beta 3$ mRNA was derived from information on $\beta 3$ submitted to GenBank (Acc. No. AJ011778) and the migration of the mRNA observed in **A**. The size of the rb^1 $\beta 3$ mRNA is estimated from **A**. Relative sequence lengths representing the $\beta 3$ full-length, 5′, and 3′ probes are indicated. The $\beta 3$ ORF is indicated by the *hatched box* with the untranslated regions (UTRs) indicated by the *lines*. The diagram is drawn to scale

granules, we examined eye sections by electron microscopy. *En face* sections of wild-type eyes (Fig. 4A) show sheaths of electron-dense secondary and tertiary pigment granules (S/TPG) surrounding groups of photoreceptor cells, each containing a photosensitive rhabdomere oriented towards the center of the ommatidium (for a review of Drosophila eye structure see Cagan and Ready 1989). Examination of mutant rb^1 (Fig. 4B) and or^1 (Fig. 4C) eyes reveals a dramatic decrease in the numbers of electron-dense pigment granules. The or^{49h} allele showed an equivalent defect to the or^1 allele in this analysis (data not shown). The R7 (Lloyd et al. 1998) level is depicted here, though identical results were seen upon examination of sections ranging from the apical surface (R7 level) to the distal base (R8 level) of the ommatidia.

Pteridine (red) and ommochrome (brown) pigments are synthesized in the fly via a series of enzymatic steps; the final maturation step is believed to occur within the lumen of the pigment granule itself (Hearl and Jacobson 1984). Measuring pigment levels is therefore one way to assess defects in pigment granule biogenesis. For this measurement pteridine and ommochrome pigments were extracted from the eyes of wild-type, *or* and *rb* flies and their absorption spectra were measured throughout the visible range. This analysis revealed dramatic reductions in both pteridine and ommochrome pigments in the or^1, or^{49h} and rb^1 mutants (Fig. 5) relative to wild type. These results are, therefore, consistent with the electron microscopy findings and further document the pigment granule biogenesis defect in the AP-3 mutants.

Assessment of SVs in vivo

Having shown a critical role for AP-3, specifically the $\sigma 3$ and $\beta 3$ subunits, in the biogenesis of pigment granules, we next addressed the question of whether this complex is required for the formation of SVs in vivo. This was done by analyzing retinula cell termini from wild-type and AP-3 mutant brains by electron microscopy. The termini of retinula cells synapse on interneurons of the first optic ganglion (for a review of Drosophila retinula cell structure, see Summers et al. 1982). These cells have proven useful for studying various features of Drosophila neuroanatomy, including SV biogenesis (Koenig et al. 1983; Kosaka and Ikeda 1983). Our analysis of wild-type flies revealed the characteristics of normal retinula cells,

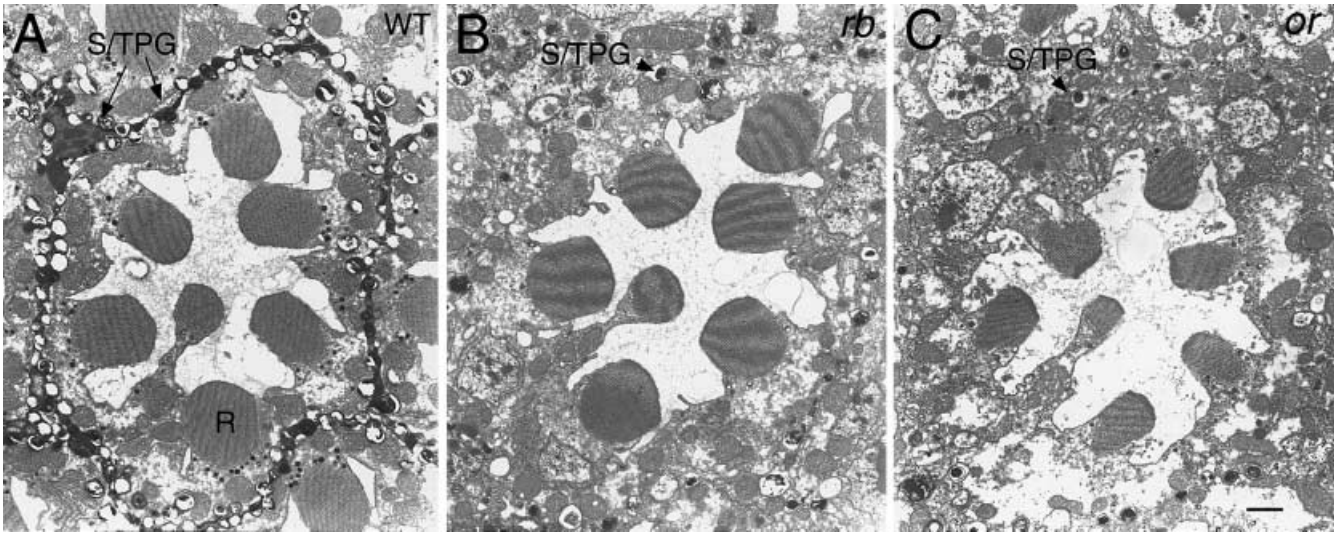


Fig. 4A–C Analysis of visual pigment granules in wild-type and AP-3 mutant flies. Electron micrographs depicting a tangential (*en face*) view of (**A**) CantonS (wild type) and (**B**) *rb* (rb^1 allele) and (**C**) *or* (or^1 allele) mutant eyes are shown. All sections analyzed were at the R7 level. Positions of secondary/tertiary pigment granules (S/TPG) and rhabdomeres (R) are indicated. The apparently fully formed but empty granules seen in **A** are an artifact resulting from the extremely brittle nature of fixed pigment granules, as previously reported (Stark and Sapp 1988). The *bar* represents 1.0 μm

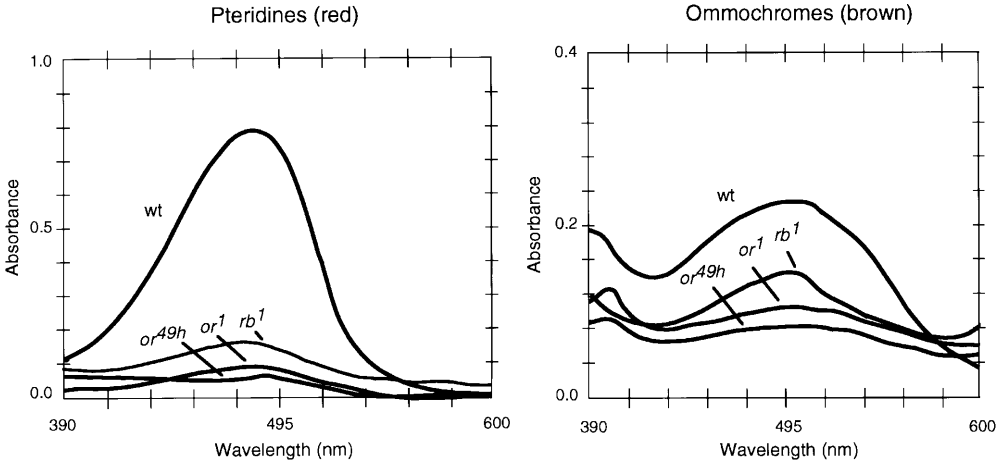
including numerous cytoplasmic SVs of approximately 50 nm; tubulovesicular or "sausage-shaped" membrane carrier vesicles (Tsukita and Ishikawa 1980); capitate projections from glial cells; smooth cisternae; and mitochondria (Fig. 6A). Examination of retinula cells from the rb^1 mutant revealed normal neuronal anatomy, including cytoplasmic SVs that were similar in number and appearance to those seen in wild-type cells (Fig. 6B). Similar observations were made for the $\sigma 3$ mutants or^1 and or^{49h} (data not shown).

To validate these observations, we examined retinula cell termini from the conditional mutant shi^1, which exhibits reversible paralysis due to an inhibition of endocytosis and SV recycling (Poodry and Edgar 1979; Koenig et al. 1983; Kosaka and Ikeda 1983; Kessell et al. 1989; Masur et al. 1990). This phenotype results from a temperature sensitive defect in dynamin, a GTPase that functions at the plasma membrane to allow formation of clathrin-coated vesicles (Chen et al. 1991; van der Bliek and Meyerowitz 1991). Following exposure of shi^1 flies to the non-permissive temperature of 30 °C, retinula termini showed a marked depletion of SVs (Fig. 6C), Analysis of shi^1 flies thus confirmed the ability of electron microscopy to detect depletion of SVs from retinula termini, and corroborated the inference that altered expression of AP-3 subunits has little or no effect on cytoplasmic populations of SVs in retinula cells.

Behavioral analyses of Drosophila AP-3 mutants

Abnormal SV biogenesis would be expected to result in sensory and/or motor defects, as is the case for *shi*. Other mutants in the protein trafficking machinery such

Fig. 5 Quantitation of red and brown pigments from wild-type and *or* and *rb* mutant flies. Red (pteridine) and brown (ommochrome) pigments were extracted from the eyes of CantonS (wild type) and *or* (or^1 and or^{49h} alleles) and *rb* (rb^1 allele) mutant flies (Ooi et al. 1997; Mullins et al. 1999). Pigments were then quantitated by measuring their absorption spectra in the 390–600 nm range



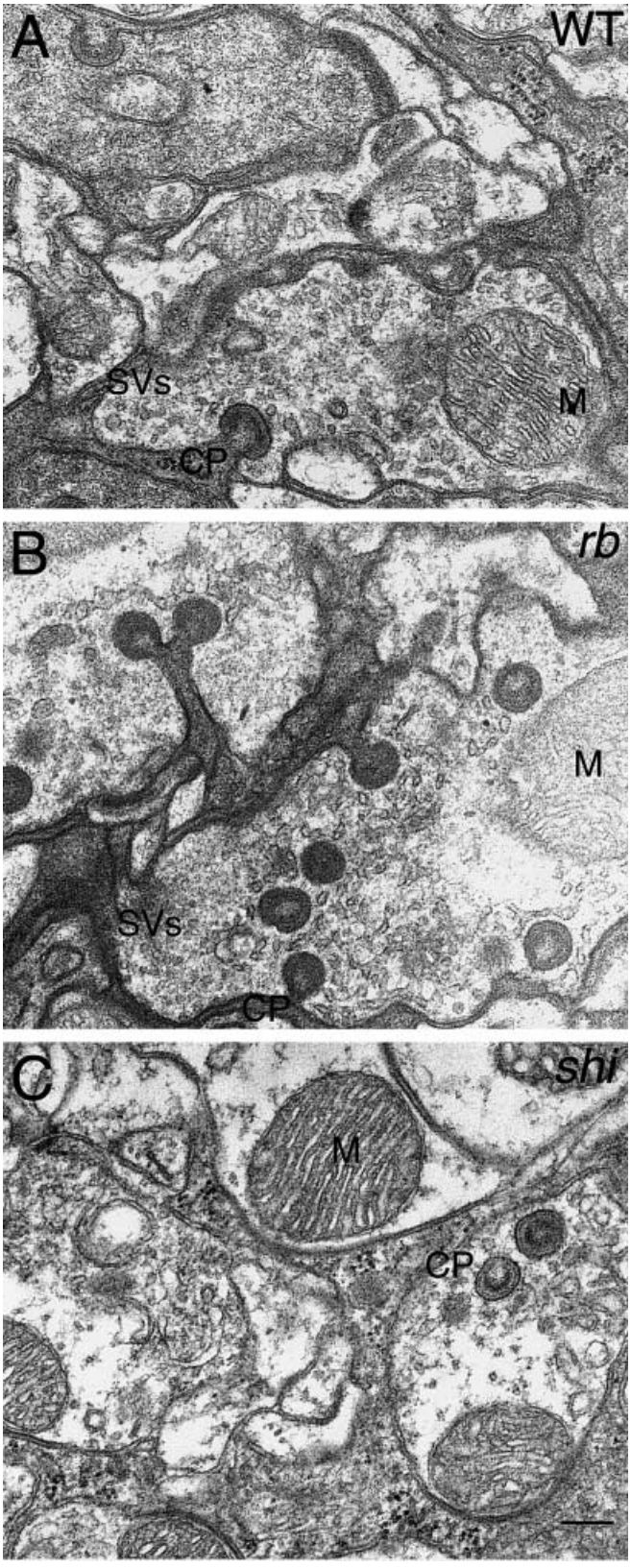


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Fig. 6A–C Examination of SVs in wild-type and AP-3 mutant flies. Electron micrographs depicting a tangential (*en face*) view of retinula cell axon terminals from brains of (**A**) CantonS (wild type), (**B**) the AP-3 $\beta 3$ mutant *rb* (rb^1 allele), and (**C**) the dynamin mutant *shi* (shi^1 allele). *shi* flies were shifted to 30 °C for 10 min prior to examination (see Materials and methods). A capitate projection of a glial cell (CP), SVs and mitochondria (M) are indicated. The *bar* represents 0.2 μm

as *comatose* (Siddiqi and Benzer 1976; Pallanck et al. 1995; Littleton et al. 1998) and *stoned* (Petrovich et al. 1993; Fergestad et al. 1999), also exhibit neurological dysfunction and abnormal SV biogenesis and/or recycling. As another way to examine a possible AP-3 requirement for SV biogenesis in vivo, we subjected AP-3 mutant flies to two different behavioral tests. These tests were selected because they do not depend on visual function, which would be expected to be impaired by the drastic reduction in visual pigments, as is the case for mice and humans with defects in AP-3 (Balkema et al. 1983; Shotelersuk et al. 2000). The first assay was developed in our laboratory and takes advantage of the natural migratory characteristics of Drosophila larvae (Pereira and Sokolowski 1993; Osborne et al. 1997). Larvae (primarily 3rd instar) were collected from wild-type and the AP-3 mutant strains or^1, or^{49h}, rb^1 and g^3, placed at the center of a plate, and tested for dispersion within 2 min (Fig. 7A). The short incubation time was chosen to prevent skewing of the results by larvae reaching the sides of the test plate and then turning back. Larvae remaining at the initial point of application (0) and reaching each of four concentric rings (numbered 1–4) were scored, and response indices (RIs) were calculated and averaged for multiple independent trials. AP-3 mutants all showed good locomotor activity in this assay, which was either similar (or^1, rb^1, g^3) or slightly reduced (or^{49h}) relative to wild type (Fig. 7B). To validate this test, AP-3 mutants were then compared to the temperature sensitive shi^1 mutant. shi^1 larvae were tested at 23 °C and following shifts to 30 °C and 37 °C. The temperature-dependent defect in shi^1 neurological function manifested itself here in a marked decrease in RIs following shifts to 30 °C and 37 °C relative to the AP-3 mutants or^{49h} and rb^1. Results with shi^1 thus demonstrated that deficiencies in SV biogenesis lead to loss of locomotor activity in this assay, confirming the absence of serious SV defects in AP-3 mutants.

The second behavioral assay takes advantage of the strong tendency Drosophila larvae display to migrate toward certain volatile chemicals, and was originally developed for the isolation and characterization of mutants defective in olfactory responses (Aceves-Pina and Quinn 1979; Monte et al. 1989; Lilly and Carlson 1990). This assay tests the ability of wild-type and AP-3 mutant larvae to migrate toward a source of the odorant propionic acid during a 5 min exposure to the compound (Fig. 8A). Normal performance in this assay requires appropriate synaptic transmission by both sensory and motor neurons. Larvae of wild-type, or^{49h}, and rb^1 strains were scored for migration toward the source of propionic acid versus a control of sterile water. Results showed a generally increasing migratory response with increasing concentrations of odorant for

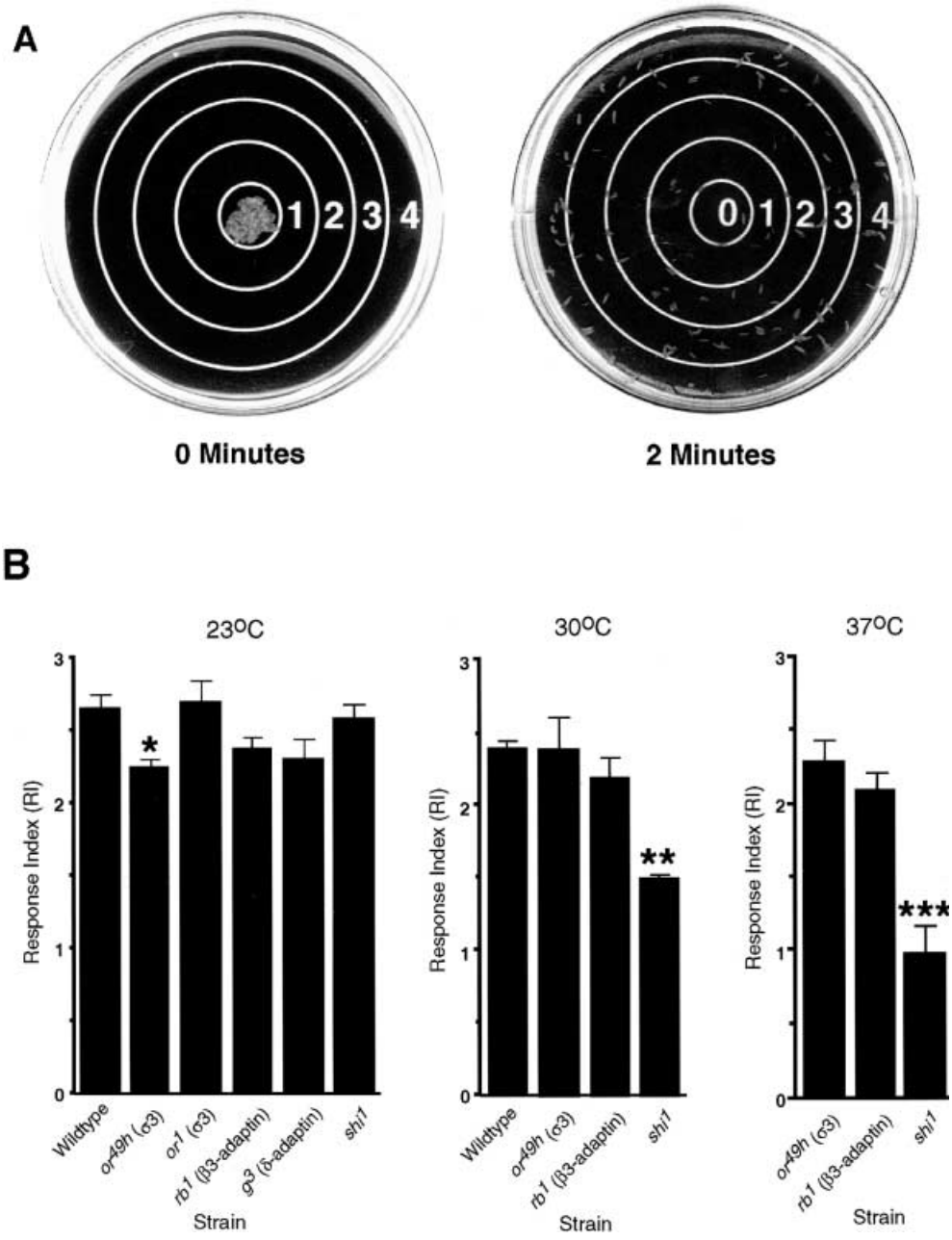

Fig. 7A, B Assay for larval motility. **A** The larval motility assay plate is pictured after initial placement of larvae (0 min) and following larval migration (2 min) (see Materials and methods). The numbers of larvae remaining in the center of the plate (denoted as 0) and found in each of 4 concentric rings (denoted as 1–4) were scored and used to calculate a response index (RI; see Materials and methods). **B** Relative migration rates for CantonS (wild type) and *or* (or^1 and or^{49h} alleles), *rb* (rb^1 allele), *g* (g^3 allele) and *shi* (shi^1 allele) mutant larvae at 23 °C, 30 °C, and 37 °C. The RI scale ranges from 0 (no migration from the center) to 4 (complete migration to area of ring 4) (Y-axis is depicted from 0–3 for reasons of space). RIs at 23 °C are represented as the means of seven trials for each strain (±SEM). RIs are represented as the means of 4–7 trials for strains at 30 °C and the means of 5–7 trials for wild-type and AP-3 mutants, and three trials for *shi*, at 37 °C (±SEM). RIs of wild-type and mutant strains at various temperatures were compared using Student's t-Test and *P* values were calculated. Statistical significance is indicated as follows: $P < 0.01$ (*), $P < 0.001$ (**), and $P < 0.0001$ (***)

wild-type and mutant strains (Fig. 8B). Although responses seemed slightly lower for the AP-3 mutants or^{49h} and rb^1 relative to wild type, the differences were not statistically significant.

Results from both behavioral assays thus show that AP-3 mutants perform as well, or almost as well, as the wild-type strain, which is consistent with the presence of apparently normal SVs in the mutant flies. Taken together, the electron microscopy analyses and behavioral assays suggest that AP-3 does not play a major role in SV biogenesis in Drosophila. This is in contrast to dynamin, the deficiency of which results in marked depletion of SVs and severe neurological dysfunction. The slight reduction in motor responses observed for the AP-3 mutants in some of the behavioral assays, however, leaves open the possibility that AP-3 might play a minor role in SV biogenesis or some other aspect of nervous system function.

Discussion

With this work, mutations in all four subunits of the Drosophila AP-3 complex have now been identified and linked to defects in the biogenesis of visual pigment granules. The $\sigma3$ mutant *or* and $\beta3$ mutant *rb* described here, and the previously identified δ mutant *g* (Ooi et al. 1997) and μ3 mutant *cm* (Mullins et al. 1999), all exhibit decreased numbers of pigmented granules in the eye and reductions in the levels of both pteridine and

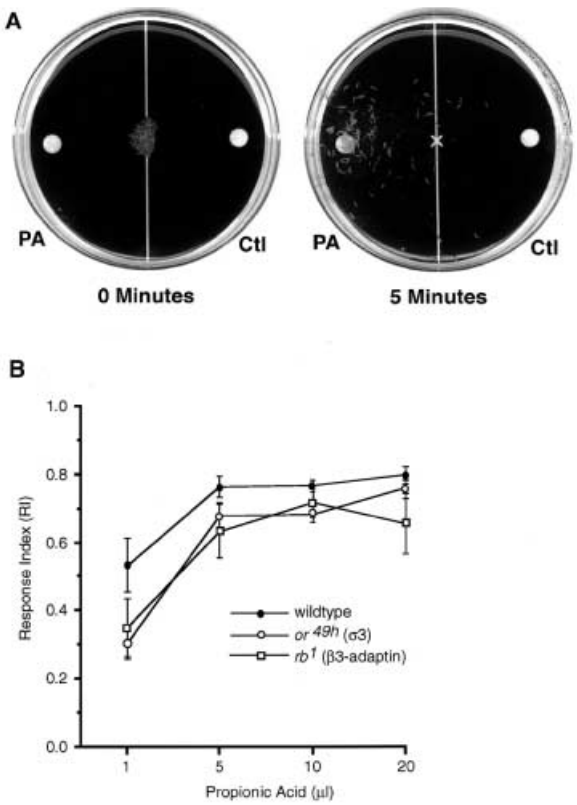

Fig. 8A, B Assay for larval migration upon olfactory stimulation. **A** The larval assay plate is pictured after initial placement of the larvae (0 min) and following larval migration (5 min). The odorant propionic acid (20 µl) was placed on the stimulus filter disk (denoted as PA), while an equal aliquot of sterile, distilled water is placed on the control filter disk (denoted as C). The numbers of larvae migrating to each half of the plate are scored and a response index (RI) is calculated (see Materials and methods). The number of larvae that failed to migrate from the initial point of placement was negligible. **B** Dose-response curves for CantonS (wild type) and *or* (*or49^h* allele) and *rb* (*rb^1* allele) mutant larvae. Larvae were tested for the ability to migrate in response to 1 µl, 5 µl, 10 µl, and 20 µl of propionic acid. RIs are represented as the means of 4–9 trials (±SEM). The RI scale ranges from 0 (random migration) to 1 (complete migration to stimulus half). RIs of mutant strains at each concentration of propionic acid were compared to that of the wild type using Student's t-Test and *P* values were calculated. No values equal to or less than 0.01 were found

ommochrome visual pigments. To date, the only analyses of the composition of the metazoan AP-3 complex have been done by co-precipitation using antibodies raised to the predicted sequences of the four putative subunits (Simpson et al. 1996, 1997; Dell'Angelica et al. 1997a, 1997b; Ooi et al. 1997). Our observations that altered expression of either δ, β3, µ3, or σ3 result in similar phenotypes provide compelling genetic evidence for their being components of the same AP-3 complex. Although each subunit of the AP-3 complex is thought to fulfill a different role, the similar phenotypes resulting from mutations in any of them could be explained by destabilization of the complex and partial degradation of the remaining subunits (Dell'Angelica et al. 1999; Zhen et al. 1999).

The mechanistic deficiency that is responsible for the pigmentation defects in Drosophila AP-3 mutants probably lies in decreased sorting of structural and/or enzymatic proteins to the forming granules. Since these granules have characteristics of lysosomes (Schraermeyer and Dohms 1993), the pigmentation defect may be the most visible manifestation of impaired sorting to lysosomes. Deficiencies in the biogenesis of lysosome-related organelles are observed in patients with Hermansky-Pudlak syndrome (HPS) type 2 (Dell'Angelica et al. 1999; Shotelersuk et al. 2000) and in the mouse coat-color mutant *pearl* (Feng et al. 1999; Zhen et al. 1999); mutations in the AP-3 subunit β3A, the mammalian ortholog of Drosophila *rb* are found in both of these conditions. Similar deficiencies are observed in the mouse coat-color mutant *mocha* (Kantheti et al. 1998), which carries a defect in the gene for AP-3 subunit δ, the mammalian ortholog of *g*. Sorting defects probably lead to abnormalities in melanosomes and platelet dense granules. While greatly reduced in numbers, some apparently fully formed pigment granules are observed in the Drosophila AP-3 mutants *rb* and *or* (Fig. 4), as well as in *g* (Ooi et al. 1997) and *cm* (Mullins et al. 1999) flies. This suggests that trafficking of cargo to a number of granules is still proceeding at some low level. In the case of the *or* mutants, this could be due to the presence of mutated or partially assembled AP-3 complexes that retain limited function. Another possibility is that alternative pathways of pigment granule biogenesis exist, which to a small extent compensate for the absence of AP-3.

Based on their phenotypic characteristics, the four Drosophila AP-3 mutants have previously been classified as belonging to the "granule group" (Lloyd et al. 1998). In addition, mutations in the Drosophila orthologs of yeast Vps18p (Shestopal et al. 1997), Vps41p (Warner et al. 1998) and Vps33p (Sevrioukov et al. 1999) have been found in the granule-group mutants *deep orange*, *light*, and *carnation*, respectively. The Drosophila *deep orange* and *carnation* proteins have recently been shown to form a complex and function in vesicular trafficking to lysosomes (Sevrioukov et al. 1999). Genetic interactions between a number of granule-group members have also been described (Lucchesi 1968). These findings provide strong support for the notion that granule-group mutants define a set of genes involved in sorting to, and biogenesis of, lysosome-related organelles. They also emphasize the strong evolutionary conservation of the basic protein sorting machinery across huge phylogenetic distances. The genetic defects in several members of the granule group of Drosophila pigmentation mutants remain to be identified. These include *claret*, *lightoid*, *pink* and *purpleoid*. It is likely that the defective gene products in these mutants will also prove to be constituents of the protein sorting machinery. Describing the defects in these remaining granule group mutants will not only shed additional light on the biogenesis of lysosome-related organelles, but also suggest other candidate genes that may be mutated in human HPS patients.

Although Drosophila AP-3 mutants display dramatic reductions in the numbers of pigmented granules in the eye, the number and appearance of SVs in retinula cells of AP-3 mutants are similar to those in wild-type flies. This does not seem to be due to the existence of neuronal isoforms of the AP-3 subunits, as our searches of the Drosophila genome did not reveal isoforms to any AP-3 subunit such as the neuron-specific β3B (β-NAP) (Newman et al. 1995) and μ3B (Pevsner et al. 1994) identified in mammals. Rather, our results suggest that AP-3 function is more important for pigment granule biogenesis than for SV biogenesis. Thus, alternative pathways seem less able to compensate for the loss of AP-3 in the pigment granule than in the SV pathway.

SV formation in neuronal cells requires many components of the machinery involved in generating transport vesicles and sorting protein cargo between organelles of the endocytic and late-secretory pathways (for a review see Hannah et al. 1999). Two alternative mechanisms for SV biogenesis have been described (Shi et al. 1998). The first mechanism depends on AP-2, clathrin and dynamin, while the second pathway requires AP-3 and ARF1. Evidence for the first mechanism comes from studies using cell-free systems, which showed a requirement for AP-2 and clathrin in formation of synaptic-like microvesicles from the plasma membrane (Shi et al. 1998). In addition, mutations in the α subunit of AP-2 or dynamin result in SV depletion from synaptic terminals in vivo (Dornan et al. 1997; Gonzalez and Jäckle 1997). The second mechanism was suggested by in vitro assays of SV biogenesis and entails formation of SVs from endosomes. This mechanism requires AP-3 and ARF1 but not AP-2, clathrin or dynamin (Faundez et al. 1998; Shi et al. 1998). Each mechanism might participate in a different pathway for SV biogenesis. Based primarily on studies of temperature-sensitive Drosophila mutants, such as *shi* (Koenig et al. 1983; Koenig and Ikeda 1996) and *stn* (Fergestad et al. 1999), two pathways for recycling have been proposed. These are termed the "fast" pathway, which involves SV formation directly from the presynaptic plasma membrane, and the "slow" pathway, which involves fusion of vesicles (derived from a site on the plasma membrane more distant from the active site) with an intermediate endosomal compartment, followed by budding of mature SVs from this compartment. Vesicles emanating from the fast pathway are localized to the presynaptic active site, while the larger SV population formed by the slow pathway are dispersed throughout the cytoplasm (Koenig and Ikeda 1996). The fast and slow pathways may ensure a readily releasable and a reserve pool of neurotransmitters, respectively (Koenig and Ikeda 1996). Based on their recycling, endosome-derived synaptic-like microvesicles from endocrine cells may therefore be analogous to the slow population of neuronal SVs (Faundez et al. 1998). The fact hat we observe no apparent depletion of a major population of cytoplasmic SVs in retinula cells, and no serious neurological defects in our AP-3 mutants, suggests that a slow pathway requiring AP-3 could account for the formation of only a small population of SVs in Drosophila under the conditions of our experiments. Distinguishing this small population from other more prominent populations of SVs may be beyond the sensitivity of the assays used here. The importance of this population may become manifest under conditions of exhaustive stimulation or other stresses. It is also possible that the AP-3-dependent pathway plays a more important role in SV biogenesis in mammals than it does in Drosophila.

The critical link between efficient SV biogenesis and neurological function is seen in the many Drosophila mutants deficient in these processes. For example, *shibire* (Poodry and Edgar 1979; Koenig et al. 1983; Kosaka and Ikeda 1983; Kessell et al. 1989; Masur et al. 1990), *stoned* (Petrovich et al. 1993; Fergestad et al. 1999), and *comatose* (Siddiqi and Benzer 1976; Pallanck et al. 1995; Littleton et al. 1998) mutants are defective in SV formation/recycling and exhibit temperature-induced paralysis. The *synaptotagmin* (*sty*) mutant is also defective in SV recycling and displays a loss of muscle coordination (Reist et al. 1998). Drosophila behavioral assays focusing on task performance, such as larval locomotion, flight, and learning have been instrumental in characterizing numerous mutants deficient in various aspects of neuromolecular function (for reviews see Ferrus and Canal 1994). Here we utilized behavioral assays designed to monitor larval locomotor ability to assess neurological function – and by inference the efficiency of SV formation/recycling – in various AP-3 mutants. In these tests, we observed that AP-3 mutants did not manifest any severe neurological defects such as paralysis or uncoordinated movements. The slightly reduced activity of the AP-3 mutants in some of the assays, however, hints at a possible role of AP-3 in the biogenesis of a minor subclass of SVs. Alternatively, AP-3 could be involved in some other trafficking event important for optimal function of the nervous system.

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