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Altered expression of a novel adaptin leads to defective pigment granule biogenesis in the *Drosophila* eye color mutant *garnet*

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Drosophila **eye pigmentation defects have thus far been attributed to mutations in genes encoding enzymes required for biosynthesis of pigments and to ABC-type membrane transporters for pigments or their precursors. We report here that a defect in a gene encoding a putative coat adaptor protein leads to the eye color defect of *garnet* mutants. We first identified a human cDNA encoding δ-adaptin, a structural homolog of the α- and γ-adaptin subunits of the clathrin coat adaptors AP-1 and AP-2, respectively. Biochemical analyses demonstrated that δ-adaptin is a component of the adaptor-like complex AP-3 in human cells. We then isolated a full-length cDNA encoding the *Drosophila* ortholog of δ-adaptin and found that transcripts specified by this cDNA are altered in *garnet* mutant flies. Examination by light and electron microscopy indicated that these mutant flies have reduced numbers of eye pigment granules, which correlates with decreased levels of both pteridine (red) and ommachrome (brown) pigments. Thus, the eye pigmentation defect in the *Drosophila garnet* mutant may be attributed to compromised function of a coat protein involved in intracellular transport processes required for biogenesis or function of pigment granules.**
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Introduction

Membrane traffic between the organelles of the endocytic and secretory pathways is mediated by transport vesicles that shuttle between different compartments. Both the generation of transport vesicles and the selection of proteins for inclusion in these vesicles are dependent on the function of protein coats attached to the cytosolic aspect of the membranes (reviewed by Rothman and Wieland, 1996; Schekman and Orci, 1996). Although various protein coats have been described to date, those containing clathrin and the clathrin-associated adaptor complexes AP-1 and AP-2 are the best understood. Clathrin forms the scaffold of the coats and is thought to induce the deformation of the membranes required for vesicle budding, while AP-1 and AP-2 provide a physical link between clathrin and the membranes. AP-1 is associated with the *trans*-Golgi network (TGN) and is involved in biosynthetic transport of proteins to the endosomal/lysosomal system, whereas AP-2 is found at the plasma membrane and participates in the internalization of cell surface proteins (reviewed by Keen, 1990; Kirchausen, 1993; Robinson, 1994).

AP-1 and AP-2 are multisubunit complexes comprising two large chains, a medium chain and a small chain (named respectively γ-adaptin, β1-adaptin, μ1 and σ1 for AP-1, and α-adaptin, β2-adaptin, μ2 and σ2 for AP-2). The association of the adaptors with clathrin occurs mainly through β1-adaptin and β2-adaptin (Gallusser and Kirchausen, 1993; Shih *et al*., 1995), and the binding of the adaptors to putative docking molecules in the TGN or the plasma membrane is mediated by γ-adaptin or α-adaptin, respectively (Robinson, 1993; Page and Robinson, 1995; Seaman *et al*., 1996). In addition to mediating clathrin attachment to membranes, the adaptor complexes are also involved in protein sorting events. Both AP-1 and AP-2 have been found to recognize tyrosine-based and di-leucine-based sorting signals located in the cytosolic tails of integral membrane proteins (reviewed by Mellman, 1996; Marks *et al*., 1997). The recognition of tyrosine-based signals is attributable to a direct interaction with the μ chains of adaptor complexes (Ohno *et al*., 1995, 1996; Boll *et al*., 1996). Integral membrane proteins having this type of signal are thus recruited to sites of clathrin-coated vesicle formation.

The concept, exemplified by AP-1 and AP-2, that analogous events in different traffic pathways are mediated by structurally homologous components of the trafficking machinery is further supported by the discovery of other related coat proteins. COPI or coatomer is a seven-subunit complex found on non-clathrin-coated vesicles that mediate traffic within the endoplasmic reticulum (ER)/Golgi system (reviewed by Rothman and Wieland, 1996; Schekman and Orci, 1996). The β, δ and ζ chains of COPI exhibit sequence homology to the β, μ and σ chains of the clathrin adaptors, respectively. Recently, a neuron-specific complex with subunit composition similar to AP-1 and AP-2 has been described, with the β and μ chains (termed β-NAP and p47B, respectively) bearing sequence homology to the corresponding chains of AP-1 and AP-2 (Newman *et al*., 1995; Simpson *et al*., 1996). In addition, we have recently described a ubiquitous adaptor complex, AP-3, which is associated with the TGN and/or endosomes and may be a widely expressed version of the neuronal complex (Dell'Angelica *et al*., 1997a). (Note that the AP-3 complex is distinct from the AP-3 protein described previously by Murphy *et al*., 1991.) The AP-3 complex comprises the small chains σ3A or σ3B, the medium chain μ3A (originally termed p47A), the large chain β3A-adaptin (Dell'Angelica *et al*., 1997b) and a fourth subunit presumably related to the α- and γ-adaptins of AP-1 and AP-2.

We now report the identification of this fourth subunit of the human AP-3 complex that is homologous to α- and γ-adaptin, and which we have named δ-adaptin. The predicted amino acid sequence of δ-adaptin was found to have a high degree of identity with that of a partial sequence of a gene deposited in the GenBank database that maps to the *garnet* locus of *Drosophila* (Lloyd, 1995). We have isolated a full-length cDNA encoding *Drosophila* garnet which shows extensive amino acid homology to human δ-adaptin, and demonstrate that transcripts of this gene are altered in *garnet* mutant flies. Morphologic analysis of eyes of *garnet* mutant flies show reduced numbers of pigment granules, suggesting that the garnet protein is required for proper biogenesis of eye pigment granules in *Drosophila*. Since pigment granules are thought to derive from the TGN and/or endosomes, these observations suggest that δ-adaptin, and by extension the AP-3 complex, is involved in protein sorting events in biosynthetic or endocytic pathways.

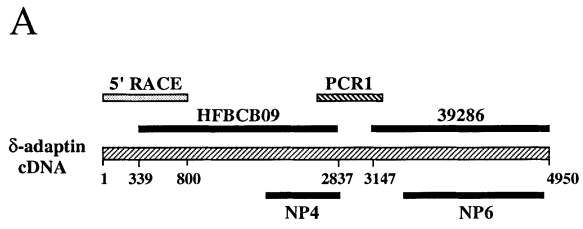
Results

Identification of a cDNA encoding human δ-adaptin, a structural homolog of α- and γ-adaptin

We have recently described a ubiquitous adaptor-like complex, AP-3, that is structurally homologous to the clathrin adaptors AP-1 and AP-2 (Dell'Angelica *et al*., 1997a). Of the four subunits in the AP-3 complex, three have been cloned and their cDNA sequences determined, namely, the small chain (σ3A or σ3B) (Watanabe *et al*., 1996; Dell'Angelica *et al*., 1997a), the medium chain (μ3A) (Pevsner *et al*., 1994) and the β-adaptin-related large chain (β3A-adaptin) (Dell'Angelica *et al*., 1997b). To identify the fourth subunit, which, by analogy with AP-1 and AP-2 is expected to be structurally related to α- and γ-adaptin, we searched the database of expressed sequence tags (dbEST) for candidate cDNAs bearing amino acid sequence similarity to these proteins. We have identified a human cDNA encoding a protein with structural homology to both α- and γ-adaptin, and have named it δ-adaptin. The complete cDNA sequence was derived from non-overlapping EST clones, PCR products containing the intervening sequence and 5′ RACE (see Figure 1A and Materials and methods for details).

The δ-adaptin cDNA comprises 4950 bp (sequence deposited with DDBJ/EMBL/GenBank, accession code AF002163) and contains an open reading frame (ORF) encoding a protein of 1153 amino acids with a predicted molecular mass of 130 kDa. The first ATG codon of the ORF is preceded by a sequence in good agreement with the Kozak consensus sequence for translation initiation (Kozak, 1984). The 5′ untranslated region has 311 bp, while the 3′ untranslated region is 1177 bp long and contains a polyadenylation signal 19 bp upstream of the poly(A) tail (not shown). In addition, we also found three variants of the cDNA sequence with in-frame deletions; one EST clone (HUKAP21) was missing codons 170–260, while two PCR products lacked codons 117–285 and codons 746–877, respectively. These species may represent products of alternative splicing of the mRNA.

The predicted amino acid sequence of δ-adaptin and its comparison with the N-terminal 'head' domains (~480 amino acids) of α- and γ-adaptin (Robinson, 1989, 1990)



B

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δ     MALKMVKGSH DRMFDKNLQD LVRGIRNHK. .EDEAKYISQ CIDEIKQELK   48
α     .....MPAVS KGDGMRGLAV FISDIRNCKS KEAEIKRINK ELANIRSKFK
γ     .....MPAPI R......LRE LIRTLRTART QAEEREMLQK ECAAIRSSFR

δ     QD...NIAVK ANAVCKLIYL QMLGYDISWA AFNIIEVMSA SKFTFKRIGY   95
α     GDKALDGYSK KKYVCKLLFI FLLGFDIDFG HMEAVNLLSS NRYTEKQIGY
γ     EE...DNTYR CRNVAKLLYM HMLGYPAHFG QLECLKLIAS QKFTDKRIGY

δ     LAASQSFHEG TDVIMQTTNQ IRKDLSSPSQ YDTGVALTGL SCFVTPDLAR  145
α     LFISVLVNSN SELIRLINNA IKNDLASRNP TFMGLALHCI ANVGSREMAE
γ     LGAMLLLDER QDVHLLMTNC IKNDLNHSTQ FVQGLALCTL GGMGSSEMCR

δ     DLANDIMTLM ..SHTKPYIR KKAVLIMYKV FLKYPESLRP A..PPRLKEK  191
α     AFAGEIPKIL VAGDTMDSVK QSAALCLLRL YRTSPDLVPM GDWTSRVVHL
γ     DLAGEVEKLL KTSNSY..LR KKAALCAVHV IRKVPELMEM ..FLPATKNL

δ     LPDPDPGVQS AAVNVICELA RRNP...... KNYLSLAPLF PKLMTSST..  233
α     LNDQHLGVVT AATSLITTLA QKNPE...EF KTSVSLAVSR LSRIVTSAST
γ     LNEKNHGVLH TSVVLLTEMC ERSPDMLAHF RKLVPQLVRI LKNLIMSGYS

δ     .......... .NNWVLIKTI KLFGALTPLE PRLGKKLIPP LTNLIH....  268
α     DLQDYTYYFV PAPWLSVKLL RLLQCYPEPD PAVRGRLTPC LETILNKAQE
γ     PEHDVS..GI SDPFLQVRHL RLLRILGRND DDSSEAMNDI LAQV...ATN

δ     .STSAMSLLY ECVNTVIAVL ISLSSGMPNH SASIQLCVQK IRILIEDSDQ  317
α     PPKSKKVQHS NAKNAVLFEA ISLIIHHDSE PNLLVRACNQ LGQFLQHRET
γ     TETSKNVG.. ...NAILYET VLTIMDIKSE SGLRVLAINI LGRFLLNNDK

δ     NLKYLGLLAM SKI..LKTHP KSVQSHKDLI LQQLD.DKDE SIRLRALDLL  364
α     NLRVLALESM CTLASSEFSH EAVKTHIETV INALKTERDV SVRQRAVDLL
γ     NIRVVALTSL ..LKTVQTDH NAVQRHRSTI VDGLK.DLDV SIKRRAMELS

δ     YGMVSKKNLM EIVKKLMTHV DKAEGTTYRD ELLTKIIDIC SQSNYQYITN  414
α     YAMCDRSNAQ QIVAEMLSYL ETAD.YSIRE EIVLKVAILA E....KYAVD
γ     FALVNGNNIR GMMKELLYFL DSCE.PEFKA DCASGHFLAA E....KYAPS

δ     FEWYTSILVE LIRLLQTRHG HLIAAQMLDV AIRVKAIRKF AVSQMSALLD  464
α     YTWNVDTILN LIRIACDYVS EEVWYRVIQI VINRDDVQGY AAKTV.....
γ     KRWHIDTIMR VLTTACSYVR DDAVPNLIQL ITNSVEMHAY TVQRL.....

δ     SAHLLASSTQ RNGICEVLYA AAWICGEFSE HLQEPHHTLE AMLRPRVTTL  514
α     FEALQQAPACH EN....LVKV GGYHLGEF.. .......... ..........
γ     YKAILGDYSQ QP....LVQV AAWCIGEY.. .......... ..........

δ     PGHIQAVYVQ NVVKLYASIL QQKEQAGEAE GAQAVTQLMV DRLPQFVQSA  564

δ     DLEVQERASC ILQLVKHIQK LQAKDVPVAE EVSALFAGEL NPVAPKAQKK  614

δ     VPVPEGLDLD AWINEPLSDS ESEDERPRAV FHEEEQRRPK HRPSEADEEE  664

δ     LARRREARKQ EQANNPFYIK SSPSPQKRYQ DTPGVEHIPV VQIDLSVPLK  714

δ     VPGLPMSDQY VKLEEERRHR QKLEKDKRRK KRKEKEKKGK RRHSSLPTES  764

δ     DEDIAPAQQV DIVTEEMPEN ALPSDEDDKD PNDPYRALDI DLDKPLADSE  814

δ     KLPIQKHRNT ETSKSPEKDV PMVEKKSKKP KKKEKKHKEK ERDKEKKKEK  864

δ     EKKKSPKPKK KKHRKEKEER TKGKKKSKKQ PPGSEEAAGE PVQNGAPEEE  914

δ     QLPPESSYSL LAENSYVKMT CDIRGSLQED SQVTVAIVLE NRSSSILKGM  964

δ     ELSVLDSLNA RMARPQGSSV HDGVPVPFQL PPGVSNEAQY VFTIQSIVMA 1014

δ     QKLKGTLSFI AKNDEGATHE KLDFRLHFSC SSYLITTPCY SDAFAKLLES 1064

δ     GDLSMSSIKV DGIRMSFQNL LAKICFHHHF SVVERVDSCA SMYSRSIQGH 1114

δ     HVCLLVKKGE NSVSVDGKCS DSTLLSNLLE EMKATLAKC             1153
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Fig. 1. Molecular cloning and sequence analysis of human δ-adaptin cDNA. (**A**) Schematic representation of the full-length cDNA encoding human δ-adaptin. The relative positions of sequences derived from the two EST clones (HFBCB09 and 39286), the 5′ RACE product, and PCR1 are shown, as well as the locations of the probes used for Northern analysis (NP4 and NP6). Numbers indicate nt positions in the cDNA. (**B**) Predicted amino acid sequence of δ-adaptin: comparison with the head domains of α- and γ-adaptin. The cDNA sequence of δ-adaptin was obtained as described in Materials and methods, and deposited in the DDBJ/EMBL/GenBank database (accession code AF002163). The translated amino acid sequence of the ORF (1153 amino acids) is shown, aligned with the amino acid sequences of the head domains of α- and γ-adaptin. Sequence alignments were obtained using the PILEUP program. Boxed amino acids indicate identity with δ-adaptin.

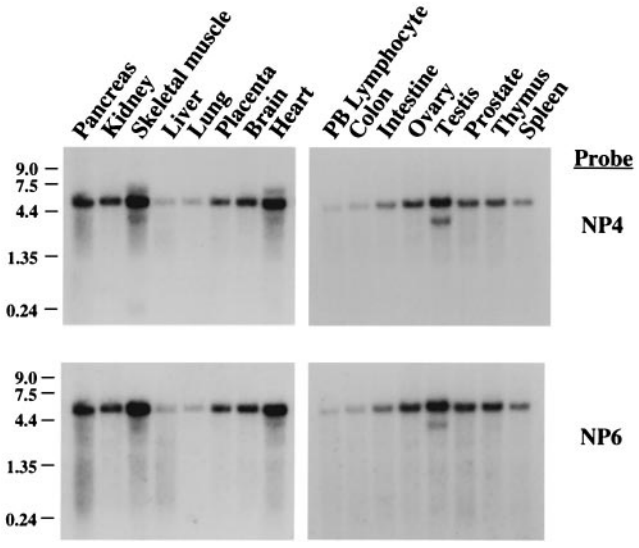

Fig. 2. Northern blot analysis of the expression of δ-adaptin mRNA in various human tissues. Multiple tissue Northern blots were analyzed using two different ^{32}P-labeled probes containing sequences derived from non-overlapping EST clones. The positions of the probes (NP4 and NP6) relative to the δ-adaptin cDNA sequence are indicated in Figure 1A. NP4 (nt 2062–2837) was a *Hin*dIII fragment of HFBCB09, while NP6 (nt 3315–4916) was obtained by PCR. The migration of RNA size markers (in kb) is indicated to the left.

are shown in Figure 1B. This is the region where δ-adaptin exhibits greatest similarity with α- and γ-adaptin, with ~25% identity in both cases. Although the extent of similarity to α- and γ-adaptin is the same, it is not due solely to invariant amino acids among all three species, i.e. in many cases different residues in α- and γ-adaptin exhibit identity to δ-adaptin. Beyond the 'head' domain, i.e. in the regions corresponding to the 'hinge' and 'ear' domains of α- and γ-adaptin (Robinson, 1989, 1990), the sequence of δ-adaptin diverges considerably. In addition, δ-adaptin has a C-terminal extension that results in the hinge ear domains being ~2-fold larger than those of α- and γ-adaptin. The extent of similarity between the head regions of δ-adaptin versus α- and γ-adaptin is comparable with that seen for the small and medium chains of AP-3 versus AP-1 and AP-2, which have ~30% identity overall (Pevsner *et al*., 1994; Dell'Angelica *et al*., 1997a). δ-adaptin also bears a more distant relationship to the other large subunit of AP-3 (β3A-adaptin; Dell'Angelica *et al*., 1997b), with 17% amino acid identity that extends throughout the entire molecule (not shown). This is reminiscent of the relationship between α-adaptin and β2-adaptin of AP-2, which have 20% identity that extends over the whole molecule (Duden *et al*., 1991).

The size and tissue distribution of δ-adaptin mRNA was assessed by multiple tissue Northern blot analysis. Hybridization with two separate probes corresponding to regions in the δ-adaptin ORF that were derived from the two non-overlapping ESTs (see Figure 1A) gave identical results (Figure 2). A 5 kb species was observed in all tissues examined; this size is consistent with the cDNA that we have sequenced. A minor 5.5 kb species was observed in skeletal muscle and heart, while a minor 4 kb species was found in testis. The expression of δ-adaptin was highest in skeletal muscle, heart, pancreas and testis, and thus parallels the pattern of tissue distribution of both σ3 and β3A-adaptin (Dell'Angelica *et al*., 1997a,b). Northern blot analyses of RNA from cell lines derived from several different tissues also showed the presence of δ-adaptin mRNA in all samples examined (not shown). This indicates that δ-adaptin is widely expressed.

δ-adaptin is a component of the AP-3 complex

We have previously shown that the AP-3 complex comprises subunits of 22 kDa (σ3A), 47 kDa (μ3A) and 140 kDa (β3A), as well as a 160 kDa species (Dell'Angelica *et al*., 1997a,b). To determine if the δ-adaptin cDNA encodes a protein that is part of the AP-3 complex, we raised an antibody (GA2) to a peptide comprising residues 783–800 of the predicted δ-adaptin amino acid sequence, and analyzed a purified fraction of AP-3 by immunoblotting. The complex was affinity-purified from Jurkat cell cytosol by using an antibody to σ3 covalently coupled to protein A–Sepharose beads. Immunoblot analysis of the purified material using a σ3 antibody yielded a major 22 kDa species as expected, while analysis with the GA2 δ-adaptin antibody yielded a 160 kDa species (Figure 3A). These bands were not observed when a similar experiment was performed using a control protein A–Sepharose column for adsorption of cytosol (not shown). Thus, δ-adaptin co-purifies with σ3 on affinity chromatography.

To confirm further that δ-adaptin is part of the AP-3 complex, we performed co-immunoprecipitation experiments. Human fibroblast M1 cells were metabolically labeled with [^{35}S]methionine, and the AP-3 complex from either whole-cell lysate or a cytosolic fraction immunoprecipitated with an antibody to σ3 as previously described (Dell'Angelica *et al*., 1997a). The proteins in the isolated immune complex were dissociated and denatured with SDS/DTT and heating, and then a second immunoprecipitation carried out with various antibodies. Antibodies to the σ3, μ3 and β3-adaptin subunits immunoprecipitated proteins of 22 kDa, 47 kDa and 140 kDa, respectively, as expected (Figure 3B; Dell'Angelica *et al*., 1997a). Re-precipitation with the δ-adaptin GA2 antibody yielded a 160 kDa band similar to that observed in the immunoblot analysis described above, as well as a minor ~200 kDa band (Figure 3B). Re-precipitation experiments using another δ-adaptin antibody, GA1, that recognizes a different peptide sequence in δ-adaptin (residues 29–48) also yielded 160 kDa and/or 200 kDa bands (not shown); these bands were not observed when re-precipitation with the GA1 antibody was carried out in the presence of competing peptide (not shown) or when the re-precipitation was carried out with antibodies to BSA (Figure 3B), α-adaptin, or γ-adaptin (not shown). The reason for the presence of higher-molecular weight forms of δ-adaptin is not known, but could reflect aggregation of δ-adaptin upon exposure to the conditions of the re-precipitation experiment. Indeed, we have noticed that the protein has a tendency to aggregate after denaturation (not shown). The results from the immunoblot and co-immunoprecipitation experiments indicate that δ-adaptin is specifically associated with the AP-3 complex.

We also analyzed the migration on sucrose gradients of the different subunits of the AP-3 complex. Fractions containing the AP-3 complex were identified by detection of σ3 upon immunoprecipitation with the σ3 antibody

(Figure 3C). δ-adaptin, as well as μ3A and β3A-adaptin peaked in the same fractions, as revealed by re-precipitation experiments with the respective antibodies (Figure 3C). The co-migration of these proteins on the sucrose gradient is further proof that they are components of the same AP-3 complex.

The AP-3 complex is ubiquitously expressed, and was previously shown to be present in several cell lines derived from different tissue types (Dell'Angelica *et al*., 1997a). Multiple tissue Northern analysis has shown that δ-adaptin is also ubiquitously expressed (Figure 2). Re-precipitation experiments (as described above) using cell lysates prepared from HeLa, RD4, SK-N-MC and SK-N-SH cells showed that δ-adaptin is also associated with the AP-3 complex in these other human cell lines (not shown).

Identification of a cDNA encoding a Drosophila homolog of δ-adaptin

A search of the GenBank database for other structural homologs of δ-adaptin revealed a high degree of identity to a portion of a *Drosophila* cDNA sequence that had been identified through the use of genomic sequences flanking a P-element in the *Drosophila garnet* g^P mutant (GenBank accession code DMU31351; Lloyd, 1995). Comparison of the predicted 784 amino acid sequence translated from the reported *Drosophila* cDNA to human δ-adaptin showed that their similarity was primarily in a region of only ~165 residues (residues 265–429 of δ-adaptin, 81% identity). Further analysis of the *Drosophila* sequence suggested that it most likely contained intronic sequences, and was incomplete at both the 5′ and 3′ ends. Since we wished to explore the possibility that garnet is a *Drosophila* ortholog of human δ-adaptin, we proceeded to obtain the complete cDNA sequence of *garnet* using DNA sequence information in the regions of high homology between the two species (see Materials and methods).

We identified a cDNA sequence of 3526 bp (sequence deposited with DDBJ/EMBL/GenBank, accession code AF002164) containing an ORF that encoded a protein of 1034 amino acids (predicted molecular mass 115 kDa), with a 186 bp 5′ untranslated region and 235 bp 3′ untranslated region preceding the poly(A) tail. Comparison of our cDNA sequence and its translated amino acid sequence with that in the GenBank database confirmed that the GenBank sequence was incomplete at both the 5′ and 3′ ends (921 bp and 823 bp missing, respectively) and contained a number of introns. The translated amino acid sequence of the *Drosophila* garnet protein and its comparison with that of human δ-adaptin is shown in Figure 4. The two proteins exhibit 81% amino acid identity over the first 491 residues which, by analogy to the other adaptins, should represent their head domains. Within this domain, there is a 200-residue stretch where the two proteins have 95% identity. Beyond the head domain they are less similar, having 37% identity from residues 500 to 840 and no significant homology in the remaining C-terminal part. There are, however, three analogous highly charged regions in the C-terminal half of the molecule. The overall high degree of structural homology between the two proteins, in particular in the head region, leads us to conclude that the garnet protein is most likely the *Drosophila* ortholog of either human δ-adaptin or a closely related protein.

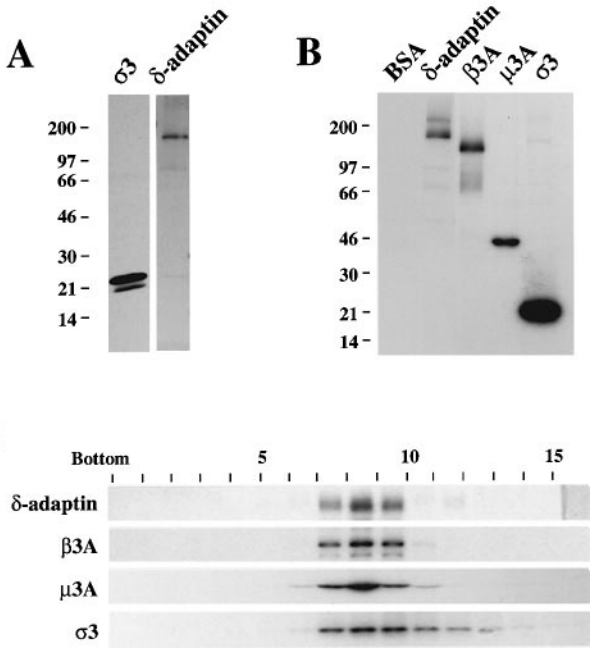

Fig. 3. δ-adaptin is a subunit of the ubiquitously expressed AP-3 complex. (**A**) Detection of δ-adaptin in affinity-purified AP-3 by immunoblot analysis. A purified fraction of AP-3 from Jurkat cell cytosol was prepared by immunoadsorption on a σ3 antibody column. Western blots of the AP-3 complex were probed using either the σ3 antibody or the δ-adaptin antibody GA2. (**B**) Co-precipitation of δ-adaptin with other subunits of AP-3. The AP-3 complex was first immunoprecipitated either from whole-cell lysate (δ-adaptin re-precipitation only) or the cytosol fraction of [^{35}S]methionine-labeled M1 cells. Following dissociation of the subunits with SDS/DTT at 95°C, a second immunoprecipitation using antibodies to either BSA, δ-adaptin (GA2), β3, μ3 or σ3 was carried out. Re-precipitated samples were analyzed by SDS–PAGE and fluorography. Similar results were obtained when whole-cell lysates were used for re-precipitation experiments performed with BSA, β3, μ3 or σ3 antibodies. The specificities of antibodies used for re-precipitation are indicated on top. Migration of molecular weight markers are indicated on the left. (**C**) Co-migration of δ-adaptin with other AP-3 subunits on a sucrose gradient. Whole-cell lysates from [^{35}S]methionine-labeled M1 cells were centrifuged on a linear 5–20% sucrose gradient. Fractions were collected (fraction numbers indicated at the top) and the re-precipitation experiment as described in (B) performed on each fraction. Antibodies used in the second precipitation are indicated on the left. The affinity-purified GA1 δ-adaptin antibody was used.

Altered expression of garnet mRNA in Drosophila garnet eye pigmentation mutants

Drosophila garnet mutants exhibit pigmentation defects in the eye and other organs (Brehme and Demerec, 1942; Nolte, 1950, 1952; Lloyd, 1995). To ascertain if the pigmentation defects of *Drosophila garnet* mutant flies correlate with altered expression of the *Drosophila garnet* encoded by the cDNA sequence, we examined *garnet* transcripts from wild-type and mutant flies. Northern blot analyses were performed with RNA prepared from either Canton S wild-type flies, or flies with the g^3 and g^{50e} *garnet* mutant alleles. Two different DNA probes derived from either the 5′ or 3′ region of the coding sequence of *garnet* (probes NP7 and NP8, respectively; see Figure 5A) were used, as well as a control probe for the TATA binding protein (TBP). In wild-type flies, two mRNA

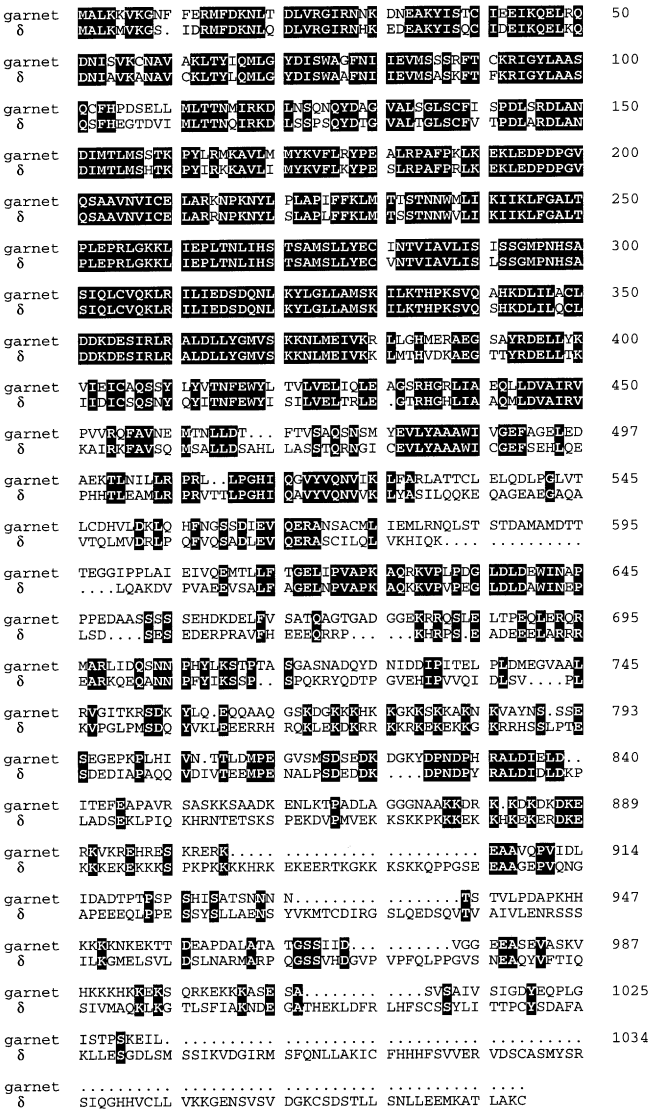

Fig. 4. Amino acid sequence of *Drosophila* garnet and its comparison with human δ-adaptin. The cDNA sequence of *Drosophila garnet* was obtained as described in Materials and methods, and deposited in the DDBJ/EMBL/GenBank database (accession code AF002164). The translated amino acid sequence of the ORF (1034 amino acids) is shown, aligned with the amino acid sequence of human δ-adaptin. Sequence alignments were obtained using the PILEUP program. Boxed residues indicate identity.

species were detected, migrating as a doublet of ~3.8 and 4 kb (Figure 5B). Both probes (NP7 and NP8) gave the same pattern (Figure 5B), indicating that these bands represented *garnet* mRNA. The 3.8–4 kb bands were barely detectable in the g^3 allele, which showed instead a highly expressed 1.5 kb species with the NP7 probe (Figure 5B). This 1.5 kb species was not observed with the NP8 probe, indicating that the g^3 mutant was expressing a truncated *garnet* transcript that was missing the 3′ half. Only a trace of the 3.8–4 kb transcripts was detected in the g^{50e} mutant with either *garnet* probe. Quantitation of bands by densitometry using the control TBP for normalization indicated that the *garnet* transcripts in the g^{50e} mutant were only present at 2% the level of that seen in wild-type flies.

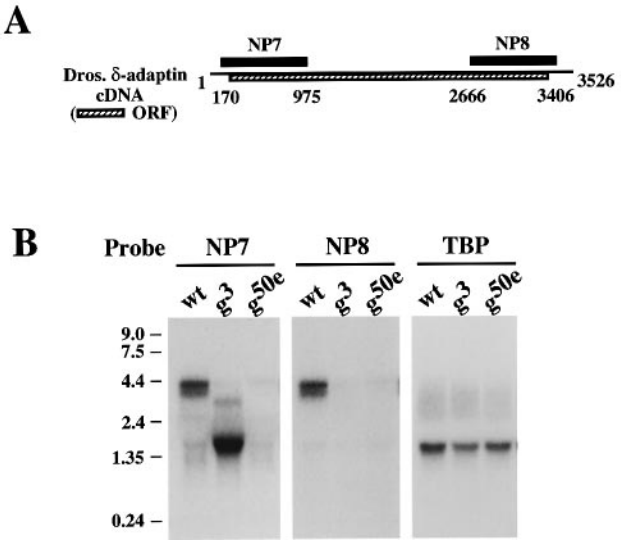

Fig. 5. Northern blot analysis of *Drosophila* δ-adaptin mRNA in wild-type and *garnet* mutants. (**A**) Schematic representation of *Drosophila* δ-adaptin showing the locations of the probes (NP7 and NP8) used for Northern analysis. Numbers indicate nt positions in the cDNA. Both probes were generated by PCR. (**B**) Northern blot analysis. RNA samples were prepared from either Canton S wild-type or *garnet* mutant flies (g^3 and g^{50e} alleles). Blots were analyzed using either NP7 or NP8 probes, as well as a probe derived from the TATA binding protein (TBP) cDNA as a control. Migration of RNA markers (in kb) are indicated on the left.

Pigment granule defects in the eyes of garnet mutants

Our results thus far have indicated that the *Drosophila* eye pigmentation gene *garnet* encodes a protein which, based on its homology to human δ-adaptin, is likely to be part of an adaptor-like protein complex. How would compromised function of such a complex lead to a defect in eye pigmentation? One possibility is that the *garnet* protein, as an adaptin and a component of an organellar coat, is involved in the biogenesis or maintenance of intracellular pigment storage granules in the *Drosophila* eye. To investigate this possibility we examined wild-type and *garnet* mutant eyes for the presence of pigment granules. In the *Drosophila* eye, the majority of pigment granules are found in the secondary pigment cells that form a sheath around each ommatidium (Nolte, 1950; Shoup, 1966; Wolff and Ready, 1993). These granules contain both pteridine (red) and ommachrome (brown) pigments. The photoreceptor cells have some ommachrome granules, but these are fewer in number (Wolff and Ready, 1993). By light microscopy of unstained sections of wild-type eyes, we could visualize the pigment granules of secondary pigment cells, evident as brownish dotted structures encircling a group of photoreceptor cells (Figure 6A). In the g^3 mutant, granules were present but seemed to be somewhat reduced in number, and in the g^{50e} mutant even fewer granules were visible (Figure 6B and C).

To obtain better visualization of pigment granules, we examined wild-type and mutant eyes by transmission electron microscopy. The pigment granules of the secondary pigment cells of wild-type eyes were seen as ~100 small circular structures, some of which were darkly stained and others which appeared ‘empty’, surrounding

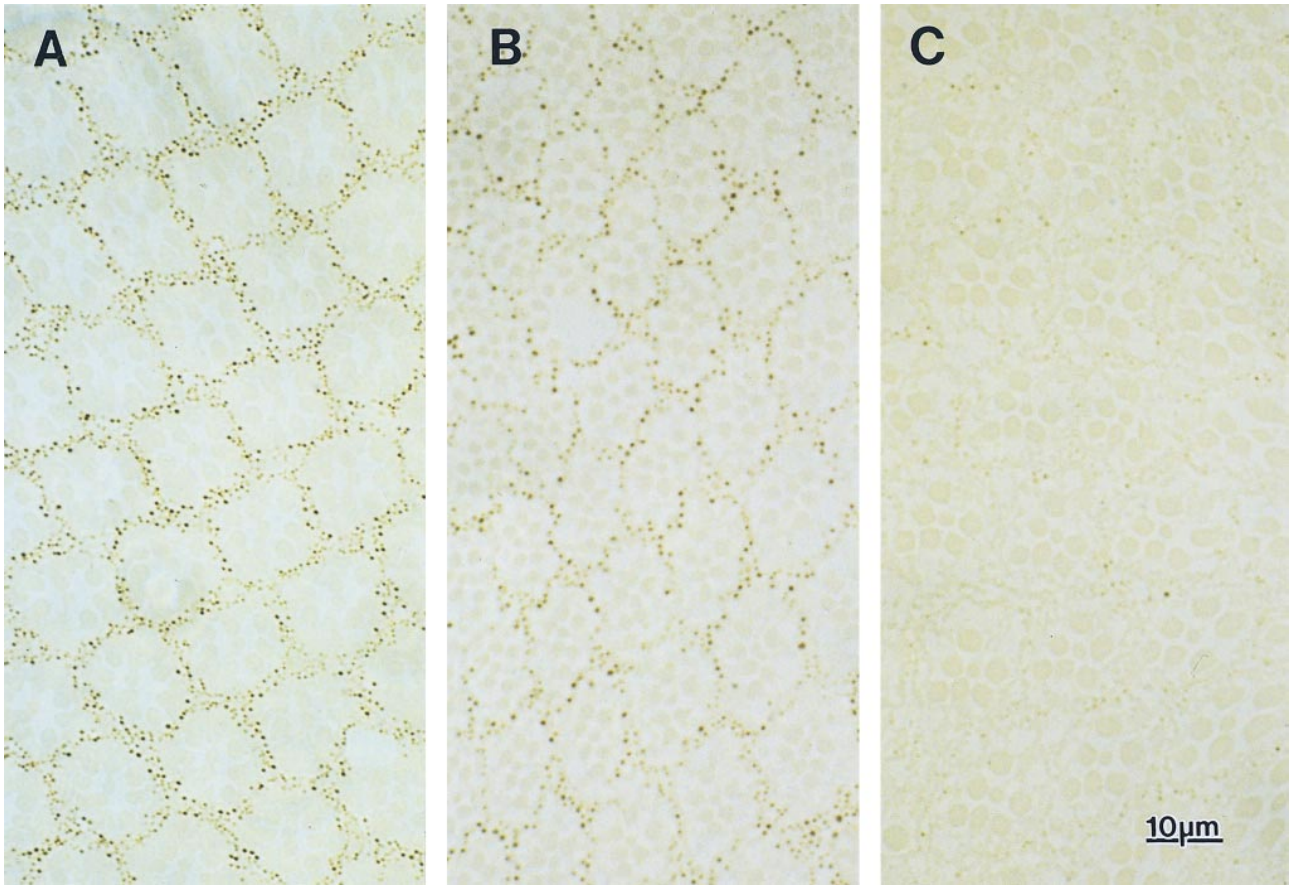

Fig. 6. Analysis of the eyes of wild-type and *garnet* flies by light microscopy. Whole *Drosophila* eyes dissected from wild-type (**A**) and mutants g^3 (**B**) and g^{50e} (**C**) were prepared as described in Materials and methods. Unstained sections were visualized by light microscopy. Pigment granules in the secondary pigment cells are seen as dark dots encircling each group of seven photoreceptor cells; rhabdomeres of the latter cells are visible as faint spots. Rhabdomeres at the R7 level can be seen in all three panels, and some at the R8 level are seen as well in (B).

each set of seven photoreceptor cells (Figure 7A). The empty granules could arise from incomplete retention of pigments within the granules during fixation of cells. The photoreceptor cells contained smaller granules that were fewer in number: these appeared as small dark dots in the cytoplasmic region adjacent to the rhabdomere (Figure 7A). In the g^{50e} mutant eyes, which showed the more significant phenotypic defect by light microscopy, much fewer pigment granules (~20% of wild-type) were observed in the secondary pigment cells (Figure 7B). The small granules of the photoreceptor cells appeared only somewhat reduced in number in this mutant (Figure 7B). The pigmentation defects and the reduced number of pigment granules in the g^3 and g^{50e} mutant eyes observed by light and electron microscopy correlated with lower levels of both pteridine and ommachrome pigments (Figure 8), as previously reported (Nolte, 1952; Lloyd, 1995). From these experiments we concluded that abnormal expression of the *garnet* mRNA leads to defects in the number of pigment granules and the total content of both pteridine and ommachrome pigments in the eye.

Discussion

We have identified a novel human protein called δ-adaptin which is homologous to α- and γ-adaptins. The homology to α- and γ-adaptin is confined to the head domain, as is the case with homologies between the β chains of AP or coat complexes (i.e. β-adaptins, β-COP and β-NAP) (Kirchausen *et al*., 1989; Ponnambalam *et al*., 1990; Duden *et al*., 1991; Newman *et al*., 1995). Homology in this region might reflect a requirement for similarly folded structures involved in interaction with other subunits in the complex. We have shown by various biochemical analyses that human δ-adaptin is part of the ubiquitous AP-3 complex. Although the function of AP-3 has not been elucidated, its proximity to the TGN and endocytic structures (Newman *et al*., 1995; Simpson *et al*., 1996; Dell'Angelica *et al*., 1997a) suggests a role in traffic pathways related to these compartments. Possible functions include biogenesis of specialized organelles derived from the TGN or endosomes, such as antigen-processing compartments (e.g. MIIC) or storage granules (e.g. melanosomes). In addition, the ubiquitous expression of AP-3 indicates that it may have general housekeeping functions related to transport from the TGN or endosomes, such as trafficking to the basolateral plasma membrane of polarized epithelial cells, or from early to late endosomes. The ability of the μ3A subunit to interact with tyrosine-based sorting signals (Dell'Angelica *et al*., 1997a) suggests a role for AP-3 in sorting of integral membrane proteins along these pathways.

The finding that the *Drosophila* eye pigmentation gene *garnet* encodes a putative ortholog of δ-adaptin provides

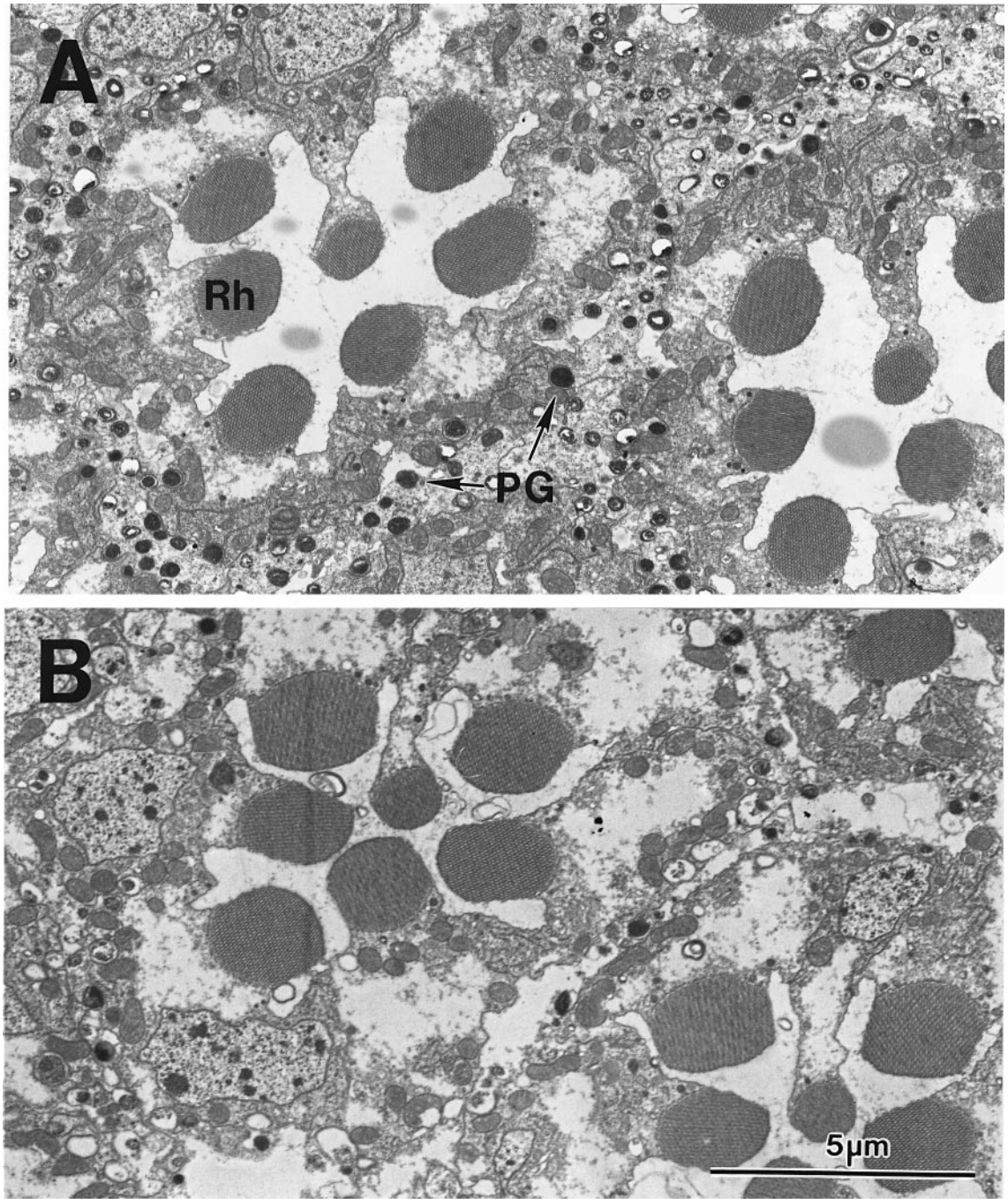

Fig. 7. Analysis of the eyes of wild-type and *garnet* flies by electron microscopy. Thin sections of *Drosophila* eyes from wild-type (**A**) or mutant g^{50e} (**B**) flies were prepared for EM as described in Materials and methods. Pigment granules of secondary pigment cells (PG) are indicated by arrows, and the rhabdomere of a photoreceptor cell is labeled (Rh).

us with an opportunity to further elucidate some of the functions of δ-adaptin. The first *Drosophila garnet* mutant was discovered in 1915 (Bridges, 1916). Since then, over 80 eye color mutant genes have been documented (Lindsley and Zimm, 1992; Lloyd, 1995); however, the function of only a few have been established. These genes encode mainly enzymes of the biosynthetic pathway for eye pigments, and ABC-type transporters that are probably responsible for uptake of pigments or their precursors into intracellular compartments (Dreesen *et al*., 1988; Tearle *et al*., 1989). Our studies indicate that the eye pigmentation gene *garnet* encodes a novel adaptin, which in mammalian cells is likely to be a coat protein required for biogenesis of organelles derived from the TGN or endosomes.

The observation that *garnet* mutants show a defect in biogenesis or maintenance of an organelle (in this case pigment granules) is consistent with the above postulated functions of AP-3/δ-adaptin. Thus, in *Drosophila*, δ-adaptin/garnet is probably a coat protein involved in intracellular transport routes leading to formation of specialized structures like pigment granules. Early electron microscopic work has suggested that eye pigment granules originate from vesicular structures derived from the Golgi region (Shoup, 1966), and other studies have suggested

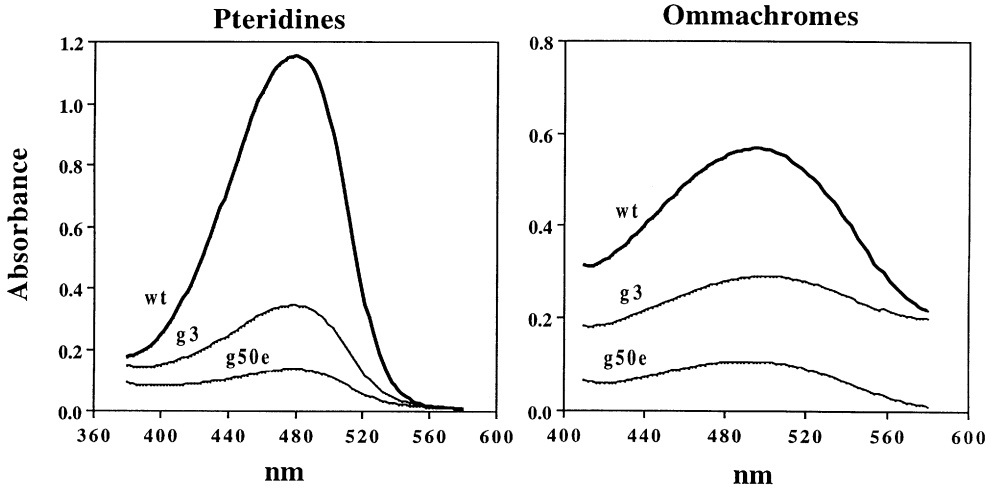

Fig. 8. Analysis of eye pigments in wild-type and *garnet* flies. Red (pteridine) and brown (ommachrome) pigments were extracted from Canton S wild-type (wt) and *garnet* mutant flies (g^3 and g^{50e} alleles), and the absorption spectra in the visible range measured.

that melanosomes (mammalian pigment granules) may interconnect with the endosomal/lysosomal system (Orlow, 1995; Schraermeyer, 1995). The presumed garnet-containing coat complex may be involved in membrane trafficking in these pathways, perhaps also performing sorting functions such as the delivery of biosynthetic enzymes or pigment/precursor transporters to these organelles. Consistent with the latter role, it has been reported that *garnet* interacts genetically with the *scarlet* gene and with certain alleles of the *white* gene (Nolte, 1952; Lloyd, 1995), both of which are presumed transporters for pigments or their precursors.

The altered eye color of *garnet* mutants could result from quantitative and/or qualitative modifications of the two eye pigments (Nolte, 1950, 1952). We have observed reduced numbers of pigment granules in the eyes of *garnet* mutants compared with wild-type flies, leading to reduced amounts of both red and brown pigments. The two mutant alleles that we have examined exhibit different defects in expression of *garnet* transcripts, and result in different morphologic phenotypes. The g^3 allele, which has a shorter transcript and probably expresses a truncated garnet protein, shows a somewhat reduced number of pigment granules in the secondary pigment cells. The g^{50e} allele, which has only 2% the level of transcripts seen in wild-type flies and probably expresses correspondingly less garnet protein, has significantly fewer secondary pigment cell granules. In addition to having less total pigment than wild-type cells, the *garnet* mutants may also have an altered ratio of red to brown pigments (see Figure 8), which could also contribute to a change in eye color. It is conceivable that the pigments in these mutants are also qualitatively modified. The pigment granules are thought to be not merely a repository for pigments, but also to be the site where completion of pigment biosynthesis takes place (Hearl and Jacobson, 1984). Mislocalization of biosynthetic enzymes in *garnet* mutants could result in qualitatively modified pigments, causing a change in eye color.

The defect in *garnet* mutants appears to be a general pigmentation defect since other pigmented organs, namely the Malpighian tubules and testes sheath, are also affected (Brehme and Demerec, 1942; Lloyd, 1995). Like the δ-adaptin mRNA, the *garnet* mRNA is likely to be ubiquitously expressed (Lloyd, 1995); thus, this protein may also be involved in more general trafficking pathways. However, no defect in other intracellular transport pathways has been observed to date. The two *garnet* mutants characterized in this study are not null mutants, and likely result in the production of either a truncated protein (g^3) or lower levels of the normal protein (g^{50e}). It is thus possible that in these mutants there is enough residual δ-adaptin activity to support the general functions of the protein in most cells and that only specialized functions that require higher levels of activity are compromised.

Precedent exists for a connection between pigmentation and intracellular transport defects. Two human diseases, the Chediak–Higashi syndrome and the Hermansky–Pudlak syndrome, are characterized by albinism as well as intracellular traffic disorders. In both diseases, cells exhibit abnormalities in endosomal/lysosomal trafficking and have defects in various cytoplasmic organelles including melanosomes, platelet granules and azurophilic granules (Scriver, 1995). The genes for both these syndromes have recently been cloned, but show no obvious homology to any known protein (Barbosa *et al*., 1996; Kingsmore *et al*., 1996; Nagle *et al*., 1996; Oh *et al*., 1996; Perou *et al*., 1996). Thus, identification of the *Drosophila garnet* gene as an adaptin provides the first link between an identifiable intracellular transport protein and a pigmentation phenotype.

Materials and methods

Identification of the cDNA sequence of human δ-adaptin

The full-length cDNA sequence of δ-adaptin is a composite of sequences obtained from two non-overlapping EST clones, 5′ RACE and RNA PCR (PCR1) (see Figure 1A) (all EST clones purchased from distributors assigned by the IMAGE Consortium). One EST clone, HFBCB09, was identified from a search for proteins with amino acid homology to α- and γ-adaptin using the BLAST algorithm (Altschul *et al*., 1990). It was from a human fetal brain cDNA library, and contained a 2.5 kb insert. Although the DNA sequence showed an in-frame stop codon near the 3′ end of the clone (at nt 2448), we observed that this stop codon was not present in three other EST clones encoding the same protein (clones HUKAP-21, 85375 and 45451). The GenBank database indicated the existence of a bovine cDNA encoding a bovine ortholog of δ-adaptin (88% amino acid identity over the C-terminal 348 residues of HFBCB09)

which contained an additional 2 kb beyond what corresponded to the 3′ end of HFBCB09 (Ban *et al*., 1993, 1994). A search of the dbEST database revealed several human cDNA clones that corresponded to this C-terminal extension, but none of which overlapped with the 3′ end of HFBCB09. One clone, 39286, which had the longest insert (1.8 kb), was selected for DNA sequencing. To identify the cDNA sequence of the region connecting the two clones HFBCB09 and 39286, we performed RNA PCR on human placenta poly(A)$^+$ RNA (Clontech, Palo Alto, CA), using primers derived from the 3′ end of HFBCB09 and the 5′ end of 39286 (see Figure 1A). The product (PCR1) had at its 5′ end the last 111 nt of HFBCB09 and at its 3′ end the first 144 nt of 39286. The intervening sequence of 309 nt translated into an amino acid sequence that showed a high degree of similarity to the corresponding region in the bovine cDNA sequence. This sequence was confirmed by sequencing another RNA PCR product derived from total RNA of SK-N-SH neuroblastoma cell line and a different set of primers. 5′ RACE was performed using a human pancreas Marathon cDNA library (Clontech), and primers containing sequences complementary to nt 1095–1115 and to nt 771–800 of the δ-adaptin cDNA. DNA sequencing of HFBCB09, 39286, the 5′ RACE product and PCR1 was performed on both strands. That the two non-overlapping ESTs (HFBCB09 and 39286) are derived from a single cDNA was further confirmed by multiple tissue Northern blot analyses which showed mRNA of identical size and tissue distribution when probed with sequences derived from these two ESTs (see Figure 2).

Identification of the cDNA sequence of the Drosophila ortholog of δ-adaptin

Initial appraisal of the reported *Drosophila garnet* cDNA sequence in the GenBank database suggested that it was incomplete at both the 5′ and 3′ ends, and contained intronic sequences. To obtain the missing sequences, we constructed a cDNA library for 5′ and 3′ RACE from *Drosophila* strain Canton S poly(A)$^+$ RNA, using the Marathon cDNA Amplification kit (Clontech). 5′ and 3′ RACE (Advantage cDNA PCR kit; Clontech) were performed using oligonucleotides corresponding to coding sequences near the 5′ and 3′ missing ends [codons 254–263 (complement) and codons 786–796, respectively, of the *Drosophila garnet* cDNA ORF]. The RACE products yielded additional DNA sequences corresponding to nt 1–921 and nt 2704–3526 of the *garnet* cDNA. To obtain the intervening sequence, we used PCR (Advantage cDNA PCR) to amplify the region encompassing nt 201–3226, from the Marathon cDNA library we had constructed (see above). DNA sequencing of the RACE and PCR products was performed on both strands. As further confirmation of the cDNA sequence, we sequenced another PCR product encompassing nt 170–3406.

Cloning and DNA sequencing

RNA PCR was performed using the GeneAmp XL RNA PCR kit (Perkin Elmer, Foster City, CA) and PCR from DNA template was performed using either the UlTma or AmpliTaq polymerases (Perkin Elmer). PCR products were cloned into either the pNoTA/T7 vector using the Prime PCR cloner system (5′→3′, Boulder, CO) or the pCR2.1 vector using the TA cloning system (Invitrogen, San Diego, CA). The ABI Prism Dye terminator cycle sequencing kit (Perkin Elmer) was used for DNA sequencing reactions, and sequencing was performed on an automated ABI sequencer (model 377).

Northern blot analysis

Human multiple tissue Northern blots were purchased from Clontech. *Drosophila* total RNA was prepared from homogenized whole flies using the Trizol reagent (Gibco-BRL, Gaithersburg, MD), according to the instructions of the manufacturer. RNA samples were electrophoresed on formaldehyde–agarose gels and transferred to GeneScreen Plus membrane (Dupont NEN, Boston, MA). ^{32}P-labeled DNA probes were prepared using the Megaprime DNA labeling system (Amersham, Arlington Heights, IL) and hybridization was carried out in Hybrisol (Oncor, Gaithersburg, MD) at 42°C for 16–20 h. Blots were washed twice in 2× SSC/0.05% SDS for 5 min at room temperature, and twice in 0.1× SSC/0.1% SDS for 20 min at 50°C, and then exposed to X-ray film.

Cells

The following human cell lines were used: M1 fibroblasts (gift of E.Long, NIH); RD4 rhabdomyosarcoma (gift of J.Harford, NIH); HeLa epitheloid, SK-N-MC neuroblastoma and SK-N-SH neuroblastoma (American Type Culture Collection, Rockville, MD). All cell lines were maintained in DMEM supplemented with 9% FBS, 100 U/ml penicillin, 100 U/ml streptomycin and 2 mM L-glutamine.

Antibodies

Antibodies to the σ3, μ3 and β3 subunits of the AP-3 complex were described previously (Dell'Angelica *et al*., 1997a). Rabbit antibody to BSA was from Cappel (Cochranville, PA). Sequences corresponding to residues 29–48 and 783–800 of human δ-adaptin were used to generate anti-peptide antibodies in rabbits (antibodies named GA1 and GA2, respectively) (service purchased from Genemed Biotechnologies, San Francisco, CA). Affinity purification of GA1 was carried out using peptide immobilized on SulfoLink gel (Pierce, Rockford, IL).

Affinity purification of the AP-3 complex

Cytosol from 1.5×10^{10} Jurkat cells (kindly provided by Weiguo Zhang and Larry Samelson, NIH) was passed through a protein A–Sepharose column (3 ml) and then loaded onto a 1.4 ml column containing anti-σ3 antibody covalently coupled to protein A–Sepharose. Bound material was eluted with 0.1 M glycine, pH 2.5, and analyzed by immunoblotting.

Immunoblot analysis

Samples were subjected to SDS–PAGE on 4–20% Tris–glycine gels (Novex, San Diego, CA) and transferred to nitrocellulose. Blots were incubated in antibody diluted in blocking buffer (5% non-fat dry milk/0.2% Tween-20/PBS), for 1 h at room temperature. Horseradish peroxidase-conjugated antibodies were detected by an ECL system (Amersham).

Preparation and fractionation of cell lysates

Confluent (or near-confluent) cells were labeled for 14–18 h at 37°C, with 0.2–0.8 mCi/ml [^{35}S]methionine (Easy Tag Protein Label, Dupont NEN) in a mixture of methionine-free and regular DMEM (9:1, v/v) supplemented with 9% fetal bovine serum and penicillin/streptomycin/L-glutamine as mentioned above. For preparation of whole-cell lysates, cells were washed twice in ice-cold PBS, and then lysed in ice-cold IP lysis buffer (1% Triton X-100, 50 mM Tris–HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA) supplemented with 0.25 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride, 10 μM leupeptin, 7 μM pepstatin and 10 mM iodoacetic acid. The lysate was centrifuged at 14 000 *g* for 15 min at 4°C, and BSA (0.1%, w/v final) added to the supernatant fraction. This fraction was then filtered through a 0.45 μm Millex-HA membrane (Millipore, Bedford, MA) and used for immunoprecipitation. To prepare a cytosolic fraction, cells were washed twice with ice-cold PBS, recovered by scraping in the presence of homogenization buffer (10 mM HEPES, pH 7.0, 150 mM KCl, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM DTT) and lysed by 15 passages through a 25-gauge syringe. The lysate was centrifuged at 120 000 *g* for 90 min at 4°C and the supernatant was considered the cytosolic fraction. Fractionation of whole-cell lysates on a linear 5–20% sucrose gradient (in 1% Triton X-100, 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.02% NaN_3) was carried out by centrifugation in a SW41Ti rotor at 39 000 r.p.m. for 16 h.

Immunoprecipitation

Protein A–Sepharose beads with pre-adsorbed antibodies (prepared by tumbling at 4°C for 1 h in lysis buffer) were added to cell lysates or fractions, and the mixture tumbled at 4°C for 1 h. The beads were washed four times with cold IP wash buffer (same as lysis buffer, except with 0.1% Triton X-100), once with PBS, and then resuspended in elution buffer (1% SDS, 0.1 M Tris–HCl, pH 7.4, 10 mM DTT) and heated at 95°C for 5 min to elute bound proteins. The eluate was diluted 20-fold with IP lysis buffer supplemented with 0.1% (w/v) BSA and 10 mM iodoacetic acid, and then used for a second immunoprecipitation. After the final PBS wash, the beads were resuspended in SDS buffer, heated at 95°C for 5 min, then loaded onto a 4–20% SDS–polyacrylamide gel (Novex). After electrophoresis, the gel was soaked in 2% glycerol/1 M sodium salicylate, dried and exposed to X-ray film for fluorography.

Flies

Drosophila melanogaster garnet mutants (g^3 and g^{50e} alleles) were obtained from the Mid-American *Drosophila* Stock Center (Bowling Green, OH).

Microscopy

Whole *Drosophila* eyes dissected from wild-type and mutants g^3 and g^{50e} were fixed by immersion in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, overnight at 4°C. After rinsing in buffer, the eyes were post-fixed in 1% osmium tetroxide in the same buffer for 1 h

at 4°C, and in-block stained with 2% uranyl acetate in 0.1 M sodium acetate buffer, pH 5.0, overnight on ice. After dehydration in ethanol and substitution with propylene oxide, the samples were embedded in Araldite resin (CY212). Cross-sections 0.5 μm thick at the R7 rhabdomere level were mounted on glass slides and photographed unstained with a Leica photomicroscope. Thin sections from the same areas were collected on single slot, Pyoloform and carbon-coated grids, contrasted with uranyl acetate and lead citrate and photographed with a Philips CM-10 electron microscope.

Analysis of eye pigments of Drosophila

Extraction of the red (pteridine) and brown (ommachrome) pigments from the eyes of *Drosophila* for quantitative estimation was carried out following the method of Real *et al*. (1985). For the red pigments, eight fly heads were placed in 0.4 ml of AEA (30% ethanol acidified to pH 2.0 with HCl), tumbled for 22 h at room temperature, and the solution used for spectrometry. For the extraction of brown pigments, 10 fly heads were first homogenized in 0.15 ml 2 N HCl, and then 1 mg sodium metabisulfite and 0.2 ml 1-butanol were added. The mixture was tumbled at room temperature for 30 min and then centrifuged at 4000 *g* for 5 min. The organic layer was removed and washed twice with 0.15 ml 0.66% sodium metabisulfite by tumbling for 30 min each time followed by centrifugation. The organic layer was used for spectrometry. Absorption spectra in the visible range were obtained using a Beckman DU 640 spectrophotometer.

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