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A Role for the *deep orange* and *carnation* Eye Color Genes in Lysosomal Delivery in *Drosophila*

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Summary

***deep orange* and *carnation* are two of the classic eye color genes in *Drosophila*. Here, we demonstrate that Deep orange is part of a protein complex that localizes to endosomal compartments. A second component of this complex is Carnation, a homolog of Sec1p-like regulators of membrane fusion. Because complete loss of *deep orange* function is lethal, the role of this complex in intracellular trafficking was analyzed in *deep orange* mutant clones. Retinal cells devoid of *deep orange* function completely lacked pigmentation and exhibited exaggerated multivesicular structures. Furthermore, a defect in endocytic trafficking was visualized in developing photoreceptor cells. These results provide direct evidence that eye color mutations of the granule group also disrupt vesicular trafficking to lysosomes.**

Introduction

The typical red eye color of fruit flies results from the deposition in pigment granules of two types of pigments, the ommochromes and drosopterins, which are synthesized via independent biochemical pathways. Many of the classic *Drosophila* eye color genes encode components of either one of these two pathways (Phillips and Forrest, 1980; Lindsley and Zimm, 1992). A third group of mutations, however, affects both types of pigments and constitutes a distinct collection of eye color genes. The classification of these genes into the "granule group" is supported by a network of genetic interactions. For example, *carnation* (*car*) genetically interacts with *deep orange* (*dor*), *light*, and *garnet* (Lloyd et al., 1998).

Sequence similarities between proteins encoded by granule group genes and regulators of vesicular transport suggested that these classic *Drosophila* mutations might be a powerful resource for the genetic analysis of vesicular trafficking in Metazoa (Lloyd et al., 1998). For example, Garnet is similar to the δ subunit of the AP-3 adaptor complex in mammalian (Ooi et al., 1997; Simpson et al., 1997) and yeast cells (Cowles et al., 1997). The AP-3 complex functions in trafficking to lysosomes and other lysosome-related organelles, such as melanosomes and dense-cored granules (Kantheti et al., 1998; Dell'Angelica et al., 1999; Feng et al., 1999), and in synaptic vesicle formation (Faundez et al., 1998).

Two eye color genes are similar to *S. cerevisiae* vacuolar protein sorting (*VPS*) genes, which are required for transport of biosynthetic and endocytic cargo to the vacuole, a lysosome-like organelle in yeast (Wendland et al., 1998). First, the *Drosophila* eye color gene *light* is similar to *VPS41* (Warner et al., 1998). Second, the *Drosophila* Dor protein shares similarity with Vps18p: their C termini contain H2 variants of the RING finger Zn^{2+}-binding domain and are 23% identical (Rieder and Emr, 1997; Shestopal et al., 1997).

VPS18 belongs to the C class of *VPS* mutants (Banta et al., 1988; Raymond et al., 1992). The proteins encoded by these *VPS-C* genes, Vps11p, Vps16p, Vps18p, and Vps33p, form a complex acting late in vacuolar transport (Rieder and Emr, 1997). Mutations in these genes block delivery to the yeast vacuole and cause the accumulation of multivesicular bodies (MVBs), a prevacuolar compartment shared between endocytic and biosynthetic trafficking (Odorizzi et al., 1998). In vertebrate and invertebrate cells, MVBs are intermediates in endocytic trafficking between early endosomes and lysosomes (Gruenberg and Maxfield, 1995; Futter et al., 1996; Sunio et al., 1999).

Despite the sequence similarity of granule group genes to vacuolar trafficking genes, no direct evidence has linked any of these genes to lysosomes or other organelles that might be related to pigment granules. In fact, mutations in the *hook* gene (Krämer and Phistry, 1996) provide the only example of a defect in lysosomal delivery in *Drosophila*. In *hook* mutant tissue, MVBs are reduced in number and endocytosed ligands are prematurely degraded in lysosomes (Krämer and Phistry, 1999; Sunio et al., 1999).

In this paper, we demonstrate that *dor* mutations also alter trafficking to lysosomes. Null alleles of *dor* are lethal, and its pleiotropic effects indicate that the role of *dor* is not restricted to vesicular trafficking to pigment granules (Bischoff and Lucchesi, 1971; Shestopal et al., 1997). Dor, which is part of a protein complex that includes the Sec1p homolog Car, is required not only for the biogenesis of pigment granules but also for the normal delivery of proteins to lysosomes.

Results

The Conserved RING-H2 Domain of Dor Is Critical for Its Function

The importance of the C-terminal RING-H2 domain for Dor function is underscored by sequence analysis of two hypomorphic alleles; dor^1 and dor^4 are weak alleles that exhibit diminished fertility and an orange eye color (Lindsley and Zimm, 1992; Shestopal et al., 1997). In the dor^1 allele, a tyrosine replaced cysteine 979, which is one of the six cysteine residues that constitute the signature sequence of RING-H2 domains (Borden and Freemont, 1996; Shestopal et al., 1997). In the dor^4 allele, a

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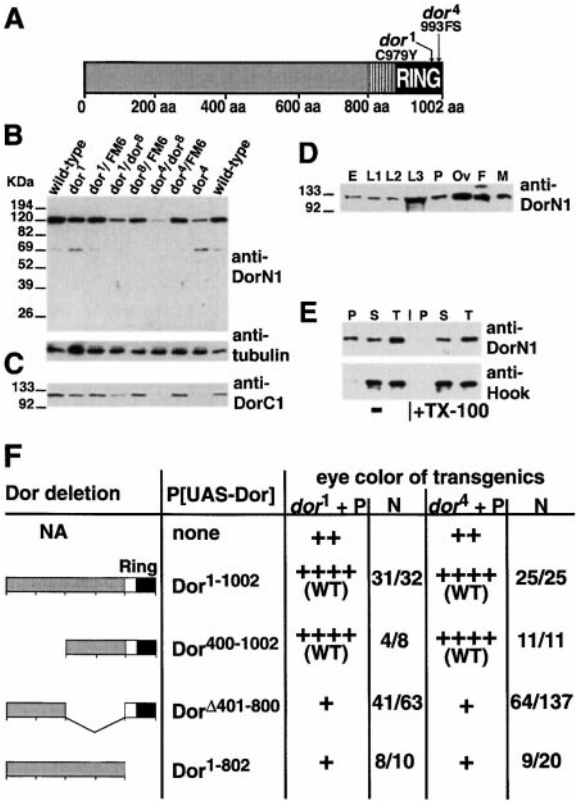

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Dor deletion	P[UAS-Dor]	eye color of transgenics dor^1 + P	N	dor^4 + P	N
NA	none	++		++	
Ring	Dor^{1-1002}	++++ (WT)	31/32	++++ (WT)	25/25
	$Dor^{400-1002}$	++++ (WT)	4/8	++++ (WT)	11/11
	$Dor^{\Delta 401-800}$	+	41/63	+	64/137
	Dor^{1-802}	+	8/10	+	9/20

Figure 1. The RING-H2 Domain Is Critical for Dor Function

(A) The Dor protein is a cytoplasmic protein of 1002 aa, with a coiled-coil domain (striped) and a RING-H2 domain (RING) near its C terminus (Shestopal et al., 1997). Mutations in the *dor*[1] and *dor*[4] alleles are indicated.

(B and C) Dor proteins in the depicted allelic combinations of the *dor* gene were detected by Western blotting using anti-DorN1 antibodies (B) or anti-DorC1 antibodies (C). The FM6 chromosome carries a wild-type *dor* gene. Anti-tubulin antibodies were used to control for loading.

(D) Expression of Dor during development was followed using the anti-DorN1 antibodies (E, embryo; L1, L2, and L3, first, second, and third instar larvae; P, pupae; Ov, ovaries; F, females; M, males).

(E) Dor protein is partially associated with the particulate fraction (P, post 100,000 g pellet) prepared from S2 cells. After solubilization of membranes with 1% Triton-X100 (+TX-100), Dor protein was found in the soluble fraction (S, post 100,000 g supernatant; T, total cell extract). The distribution of the soluble Hook protein is shown for comparison.

(F) Transgenic expression of full-length Dor or three Dor deletions using the UAS/Gal4 system altered eye color in *dor*[1] or *dor*[4] mutant backgrounds. Full-length Dor and $Dor^{400-1002}$ were sufficient to rescue the *dor* eye color defect. By contrast, expression of mutations without the central domain or the conserved C-terminal RING-H2 domain reduced eye pigmentation. The number of plus signs delineate the relative level of eye pigmentation. N refers to the fraction of flies that exhibited this level of pigmentation. The remaining flies expressed phenotypes more similar to *dor*[1] or *dor*[4].

frameshift after aa (amino acid) 993 substituted 30 new residues for the terminal nine amino acids (Figure 1A).

These mutations resulted in reduced levels of Dor protein. Antibodies directed against the N- and C-terminal domains of Dor recognized a protein close to the predicted size of 115 kDa on Western blots (Figures 1B and 1C). Identity of this band as the Dor protein was confirmed by the analysis of *dor* mutant alleles. In *dor*[1] and *dor*[4] homozygous flies, the Dor protein was detected at reduced levels, which were even further attenuated in flies heterozygous for the *dor*[8] null allele (Shestopal et al., 1997) and *dor*[1] or *dor*[4], respectively. Consistent with the pleiotropic effects of *dor* mutations and the ubiquitous expression of *dor* mRNA (Shestopal et al., 1997), the Dor protein was expressed during all stages of development (Figure 1D).

To confirm that expression of the Dor protein is sufficient to rescue the *dor* mutant phenotype, an epitope-tagged full-length Dor protein was expressed in transgenic flies using the Gal4/UAS system (Brand and Perrimon, 1993). When eye-specific expression was induced with the Gal4-GMR driver, wild-type eye color was fully restored in the *dor*[1] and *dor*[4] alleles (Figure 1F). Dor proteins with deletions of the central part of the protein ($Dor^{\Delta 401-800}$) or the conserved C-terminal domain (Dor^{1-802}) did not rescue the eye color phenotype. Instead, their expression further reduced the level of pigments in the *dor*[1] and *dor*[4] backgrounds, suggesting that these truncated proteins interfered with the remaining endogenous Dor function (Figure 1F). By contrast, a mutant Dor protein in which the N-terminal 399 aa was deleted ($Dor^{400-1002}$) still restored wild-type eye color when expressed in *dor*[1] and *dor*[4] mutant backgrounds. These results indicate that Dor's C-terminal RING-H2 domain is critical for Dor function.

Dor Is Present on Endosomal Compartments

Upon separation of cellular fractions of S2 tissue culture cells by differential centrifugation, about half of the Dor protein partitioned into the particulate fraction. This was likely a result of binding to membranes because all of the Dor protein is soluble in the presence of 1% Triton-X100 (Figure 1E). We visualized the subcellular distribution of the Dor protein in Garland cells harvested from third instar larva. In these cells, endocytic compartments were labeled by internalized, fluorescently labeled dextrans (Figure 2). At an early time point (5 min), Dor staining appeared closely associated with the internalized dextran molecules indicating that Dor is localized to endocytic compartments (Figure 2B). After a 30 min chase, the dextran was internalized further into the cells and separated from Dor immunoreactivity (Figure 2C). Preincubation of the Dor antibodies with excess Dor antigen abolished this staining (data not shown).

We next compared the localization of Dor to that of the Hook protein, which outlines large endosomes in S2:Sev cells (Krämer and Phistry, 1996). When these cells were double labeled for Hook and Dor, we found that a subset of the Dor immunoreactivity was closely associated with the Hook protein on the perimeter of such endosomes (Figures 2D–2G). Unlike the Hook protein, however, the Dor protein was not confined to the perimeter of these large endosomes, but was often found in their interior, where it colocalized with internalized dextran beads (arrowheads in Figure 2G). Similar colocalization between the internalized dextrans and Dor was also observed outside of Hook-positive endosomes (arrows in Figure 2G). Together, these experiments indicated that the Dor protein was closely associated with endocytic compartments identified by the internalized dextran and Hook immunoreactivity.

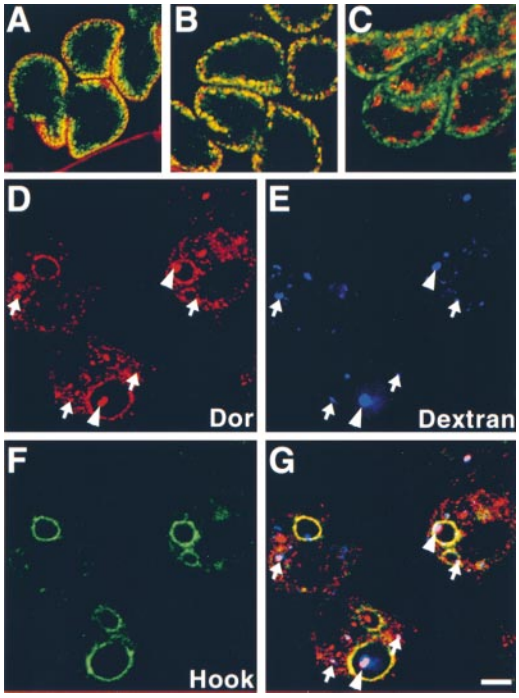

Figure 2. Dor Localizes to Endosomes

(A–C) Garland cells were isolated from early, third instar larvae and mixed with Texas red–labeled dextran beads (red). After 1 min (A), 5 min (B), and 30 min (C), cells were fixed and stained for Dor protein using the affinity-purified anti-DorN1 antibody (1:400) and FITC-labeled secondary antibodies (green). At an intermediate time point (B), dextran beads and Dor immunoreactivity appeared closely associated, whereas at a late time point (C), the dextran beads were separated from the Dor immunoreactivity.

(D–G) Dor partially colocalizes with internalized dextrans and Hook in S2:Sev cells. After internalization of dextran beads for 15 min, S2:Sev cells were fixed, and Dor (D) and Hook (F) immmunoreactivity and dextran beads (E) were visualized by confocal microscopy. A merged image of the three panels is shown in (G). Subsets of the Dor and Hook proteins colocalize to the large endosomes (G) formed in these cells (Krämer and Phistry, 1996). Dor protein also appeared closely associated with the dextran beads (arrows in [D], [E], and [G]), some of which were inside the Hook-positive endosomes (arrowheads). Specificity of staining was confirmed by staining in the absence of primary antibodies and by competition with an excess of antigens (Dor1–14 peptide and Hook–GST fusion protein; data not shown).

The scale bar represents 8 μm in (A)–(C) and 3 μm in (D)–(G).

dor Null Retinal Cells Have Altered MVBs

To test the consequences of complete loss of *dor* function, we generated clones of cells that were homozygous for the *dor*^{8} null allele (Shestopal et al., 1997) using the FLP/FRT method (Xu and Rubin, 1993). In adult compound eyes, clones were easily recognized by their loss of pigmentation. While partial loss-of-function alleles of *dor* caused intermediate eye color phenotypes (e.g., the *dor*^{4} allele in Figure 3C), the complete loss of *dor* function resulted in white patches (Figure 3B).

When sections through such *dor*^{8} clones were examined by electron microscopy, all retinal cell types were present in most ommatidia (Figure 3D), indicating that *dor* is not directly required for cell fate decisions during development of the compound eye. Within the *dor*^{8} mutant clones, only few ommatidia exhibited minor morphological defects (e.g., arrow in Figure 3D). Because

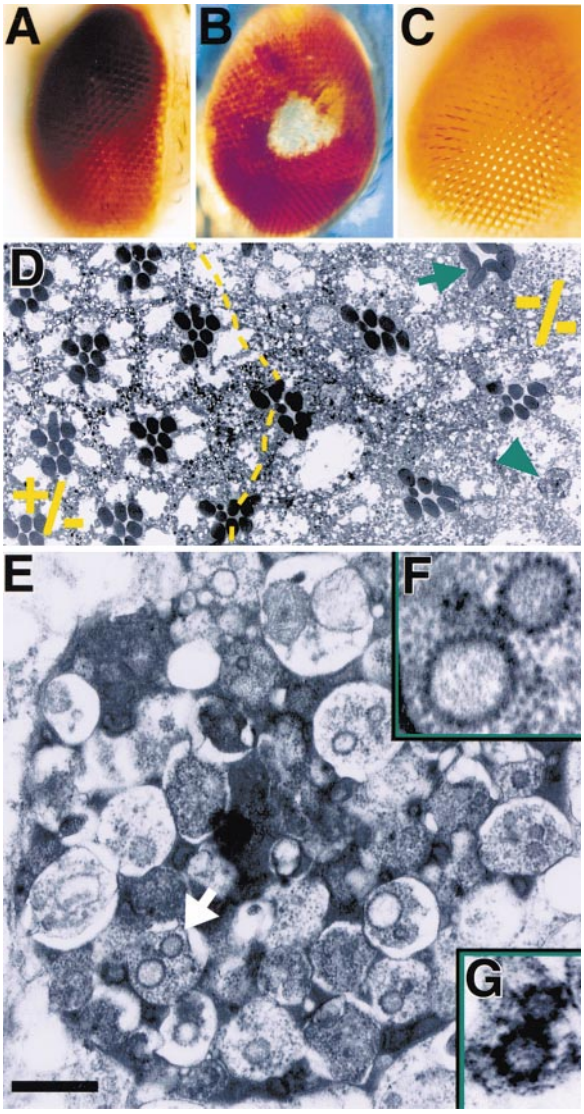

Figure 3. Clones Null for *dor* Lack Pigmentation

Micrographs of adult, compound eyes demonstrate the red pigmentation in wild-type eyes (A) and the complete lack of pigmentation in *dor*^{8} mutant clones (B) induced by FLP-induced mitotic recombination (Xu and Rubin, 1993). Partial loss of *dor* function in the *dor*^{4} allele results in the orange eye color for which the gene was named (C). A section through the border of a clone of *dor*^{8} cells (D) revealed only minor defects in the cellular composition of ommatidia (e.g., arrow in [D]), indicating that *dor* function is not required for cell fate decisions in the developing eye. The heterozygous part of the eye is labeled +/−, while the −/− labeling denotes the *dor*^{8} homozygous clone, which can be recognized by the lack of pigment granules. (E) In all three *dor*^{8} clones examined by electron microscopy, we observed rare, large multivesicular structures with diameters of up to 3 μm (see arrowhead in [D]), which were not observed in wild-type ommatidia (see left part of [D]). Many of the internal vesicles in these multivesicular structures appeared coated (e.g., [F], a magnification of the vesicles labeled with the arrow in [E]). Such internal coated vesicles were also seen in smaller MVBs in *dor*^{8} mutant photoreceptor cells (G) but were not observed in wild-type cells. The scale bar corresponds to 10 μm in (D), 500 nm in (E), and 150 nm in (F) and (G).

the majority of rhabdomeres were normal, the function of the Golgi and the secretory apparatus appeared to be unaffected by the *dor*^{8} mutation. Mutations known

to alter synthesis and transport of Rhodopsin to the cell surface of photoreceptor cells have established the morphology of rhabdomeres as a sensitive indicator for defects in the secretory pathway (Ranganathan et al., 1995).

Strikingly, we observed giant multivesicular structures in *dor*^{8} mutant pigment cells. These MVBs reached diameters of more than 3 μm (Figure 3E), about ten times the size of normal MVBs in insect cells (Sunio et al., 1999). A second unusual feature of these MVBs in *dor*^{8} cells was the appearance of internal vesicles decorated by a coat (Figures 3E–3G). Closer examination revealed their presence in small MVBs in *dor*^{8} photoreceptor cells as well (Figure 3G). Such internal coated vesicles were not observed in wild-type *Drosophila* cells and have not previously been described in the MVBs of yeast or vertebrate cells. Our observation of these coated internal vesicles in MVBs of *dor*^{8} mutant cells is consistent with a role of *dor* in biosynthetic or endocytic trafficking.

Mutations in *dor* Inhibit Lysosomal Delivery In Vivo

To address directly the role of *dor* in endocytic trafficking, we used the Boss ligand as a marker for the endocytic pathway in vivo. During early development of the compound eye, Boss is specifically expressed by R8 photoreceptor cells and internalized into neighboring R7 cells (Krämer et al., 1991). After internalization into R7, Boss is degraded in less than 2 hr. Therefore, Boss detected in R7 cells by immunofluorescence represents the steady-state level of Boss protein in the endocytic compartments of R7 cells (Sunio et al., 1999).

Clones of cells that lacked *dor* function were induced and stained in third instar eye disks (Figure 4). Expression of the Boss protein on the surface of R8 cells appeared normal in *dor* mutant R8 cells, further indicating that the *dor* mutation does not alter Golgi function or secretion (examples are outlined in Figure 4A). In wild-type ommatidia, internalized Boss was detected in R7 cells at low levels (white arrowheads in Figures 4A and 4B). By contrast, in *dor*^{8} mutant R7 cells, internalized Boss accumulated far above the normal steady-state level (orange arrows in Figures 4A and 4B). A straightforward explanation for the accumulation of internalized Boss ligand in *dor* mutant R7 cells is the inhibition of lysosomal delivery by *dor* mutations.

We have shown previously that the depletion of MVBs in *hook* mutants caused a reduced level of Boss immunoreactivity in R7 cells (Sunio et al., 1999). Because this *hook* phenotype is opposite that of *dor*, we wished to establish the phenotype of double mutant cells. We generated such *dor*^{8}, *hook*^{11} double mutant clones within eye disks that were mutant for the *hook*^{11} null allele (Figure 4C). In the *hook*^{11} mutant ommatidia surrounding the clones, Boss was not detected in R7 cells due to the premature degradation of Boss. By contrast, this phenotype was suppressed in the *dor*^{8}, *hook*^{11} cells; internalized Boss protein accumulated in such double mutant R7 cells. These results indicate a requirement of Dor for lysosomal delivery even in cells in which MVBs are destabilized by the lack of Hook function. Together, these experiments indicated that the function of *dor* in lysosomal delivery in *Drosophila* parallels that of its homolog *VPS18* in vacuolar delivery in yeast.

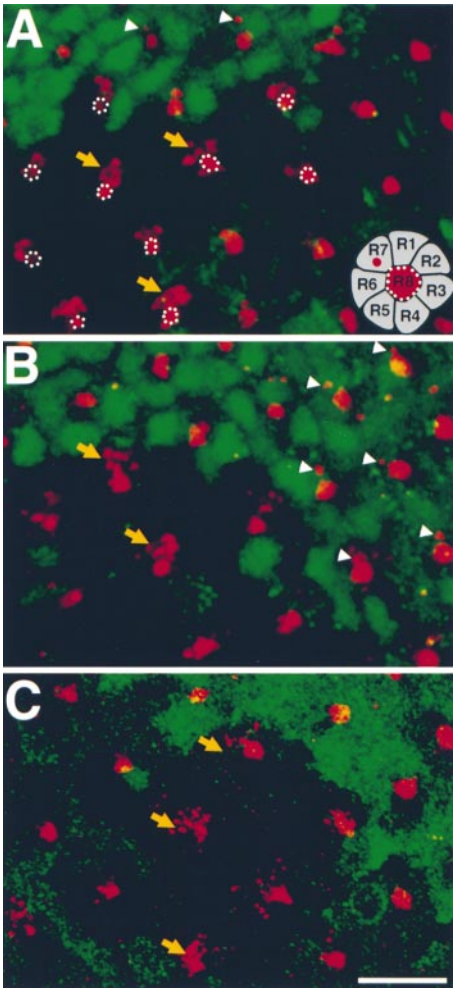

Figure 4. Cells Mutant for *dor*^{8} Are Deficient in Lysosomal Delivery of the Boss Ligand

Clones of *dor*^{8} mutant cells in otherwise wild-type (A and B) or *hook*^{11} mutant eye discs (C) were detected by the absence of the myc epitope tag (green). Boss immunoreactivity on the surface of R8 cells and internalized into R7 cells is visualized in red. In wild-type R7 cells (green area in [A] and [B]), Boss immunoreactivity (red) is marked by the white arrowheads. Boss accumulation in *dor*^{8} mutant R7 cells is labeled with orange arrows. The inset in (A) displays the stereotypic positioning of the photoreceptor cells relative to each other. To facilitate orientation, R8 cells in mutant ommatidia are outlined by punctate lines in (A). (C) Double mutant *dor*^{8}, *hook*^{11} cells were generated in *hook*^{11} mutant eye discs. Boss is not detected in *hook*^{11} R7 cells (marked in green), unless they are also mutant for *dor* (orange arrow). Posterior is to the left in all images. Scale bar, 4 μm.

Dor Is a Component of a Multiprotein Complex

Vps18p is a component of a large protein complex including three other proteins encoded by C class *VPS* genes (Rieder and Emr, 1997). To test whether the similarity between Vps18p and Dor extends to this level, the native size of the soluble Dor protein was analyzed by gel filtration and sucrose density gradient centrifugation. These methods indicated that Dor in its native state is part of a protein complex whose estimated molecular mass is 370 kDa (Table 1). As a control, we determined the molecular mass of the Hook protein to be 185 kDa,

Table 1. Native Molecular Mass of Protein Complexes Containing Dor and Hook

Protein	Dor	Hook
Sedimentation coefficient ($S_{20,w}$)[a]	8.6	9.3
Stoke's radius (nm)[b]	10.0	4.8
Calculated native molecular mass (kDa)[c]	370	185
Molecular mass by SDS-PAGE (kDa)[d]	120	85

[a]Sedimentation coefficients were determined by centrifugation on 5%–20% sucrose density gradients.
[b]Stoke's radii were determined by gel filtration (Sevrioukov et al., 1998).
[c]Native molecular mass was calculated from the sedimentation coefficient and the Stoke's radii using the Svedberg equation (Tanford, 1961).
[d]Molecular mass was determined by SDS-PAGE and Western blotting.

in close agreement with our previous demonstration that Hook dimerizes (Krämer and Phistry, 1996).

car Encodes the *Drosophila* Homolog of Vps33p

To identify additional constituents of the Dor complex, we searched the *Drosophila* EST database for homologs of C class *VPS* genes. This search identified a homolog of *VPS33* (Figure 5), which is a member of the *SEC1* family (Thompson et al., 1997). A nearest neighbor analysis of a representative set of these proteins revealed that Dm-Vps33 is most closely related to the Vps33 subset of this family (Figure 5B).

To identify mutants in the gene encoding Dm-Vps33, we determined its chromosomal location. Dm-Vps33 mapped to a P1 clone (DS00611) derived from the chromosomal location 18C8-D4 (Hartl et al., 1994). Within this genomic region, the eye color gene *car* was a strong candidate gene to encode Dm-Vps33; *car* genetically interacts with *dor* and maps to 18D1,2 (Lindsley and Zimm, 1992). Sequencing of the only available allele, car^1, revealed that two amino acids differed between the car^1 and wild-type sequences. The first was the change to a valine of leucine 26, a residue not conserved between Vps33p homologs. The second alteration, however, substituted a valine for glycine 249, a residue conserved in all Vps33p homologs analyzed (Figure 5C).

To confirm the identity of the *car* gene, we rescued the mutation by introduction of a transgene. Expression of the Dm-Vps33 cDNA under control of the GMR promoter/enhancer cassette (Figure 5D) restored eye color of the car^1 mutation to wild type (n = 5 transgenic lines). Together, these data indicated that the *car* gene encodes the *Drosophila* homolog of Vps33p, which is necessary for production of normal pigment granules.

The *car* Gene Product Is a Constituent of the Dor Complex

A physical interaction between the Dor and Car proteins was suggested by sucrose density gradient centrifugation (Figure 6). Car protein was found in fractions 15–17, consistent with its being in a monomeric form (Figure 6A). Significantly, a second peak of Car protein was detected in the same fractions (8–10) as the Dor complex, which had only a single peak at 8.6 S_{20w}.

Coimmunoprecipitation experiments confirmed the interaction between Car and Dor. Car protein endogenously expressed in *Drosophila* S2 cells was immunoprecipitated using two different antibodies prepared against a GST–Car fusion protein (Figure 6B, lanes 5 and 6). Car could also be precipitated with antibodies against

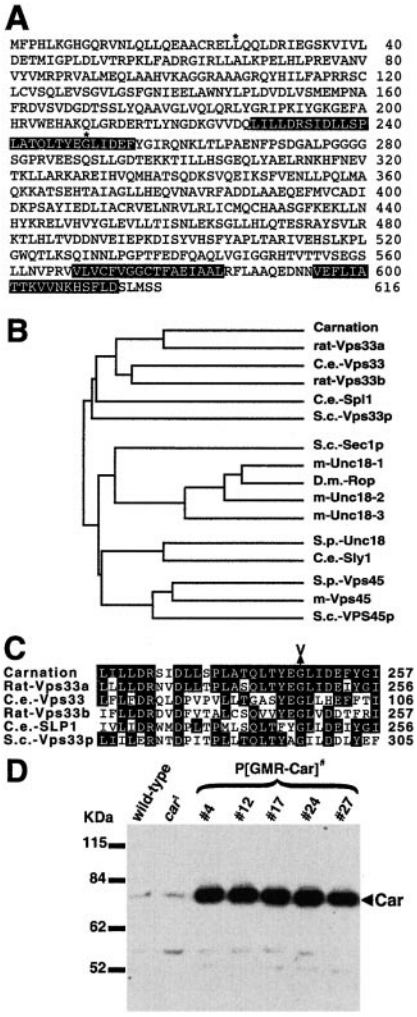

Figure 5. The Car Protein Is a Member of the Sec1p Family of Proteins

(A) Sequence of Car, a *Drosophila* homolog of Vps33p. Dark shading indicates domains with the strongest similarity to other members of the Sec1p protein family. Stars mark the two amino acids changed in the car^1 allele.

(B) Nearest neighbor analysis (Thompson et al., 1997) of representative members of the Sec1p protein family demonstrates that the Vps33p-like proteins form a distinct subfamily. Accession numbers of the protein sequences used: Vps33p, #173185; r-Vps33a, #1477468; r-Vps33b, #1477470; C.e.-Vps33, #868239; C.e.-Slp1, #1353066; Sec1p, #266998; m-unc18-1, #435433; m-unc18-2, #A57022; m-unc18-3, #2501517; D.m.-Rop, #639709; S.p.-Unc-18, #3581893; C.e.-Sly1 #3877054; S.p.-VPS45, #1175476; Vps45p, #468233; m-VPS45, #1703494.

(C) Lineup of the highly conserved domain I of the VPS33 proteins. Glycine residue 249, which is conserved in all Vps33p homologs, is mutated in the car^1 allele.

(D) Western analysis of Car expression in heads of wild-type and car^1 flies, and five transgenic lines expressing Car using the GMR vector (Hay et al., 1994), all of which rescued the car^1 eye color phenotype.

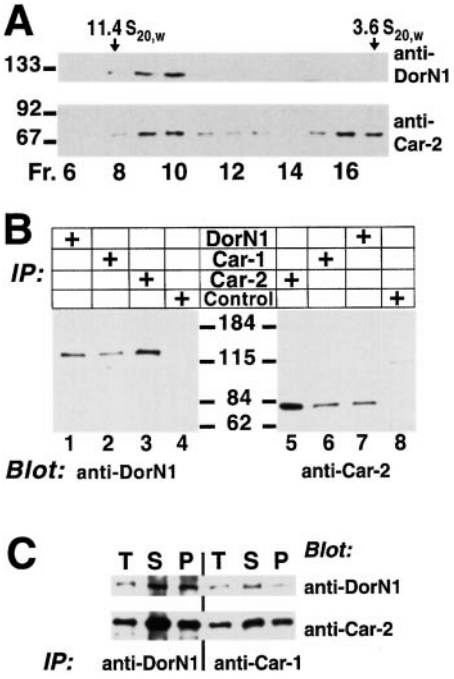

Figure 6. The Dor and Car Proteins Are Part of a Protein Complex

(A) The Car protein cosediments with the Dor complex in sucrose density gradients (10%–20%). Proteins in the different fractions were detected by Western analysis. $S_{20,w}$ values of two markers are indicated.

(B) Endogenous Dor and Car proteins were coimmunoprecipitated from extracts of S2 cells using the indicated antibodies (*IP*). Control antibody was the anti-hemagglutinin antibody 12CA5.

(C) Car and Dor were coimmunoprecipitated from cytosol (S) and solubilized particulate fraction (P) from S2 cells. Total extract (T) is shown for comparison. Proteins were detected by Western blotting with the designated antibodies (*Blot*).

Dor (lanes 7) but not with control antibodies (lane 8). Similarly, Dor was precipitated with antibodies directed against Dor (lane 1) or Car (lane 2 and 3), but not with control antibodies (lane 4). This association of the Dor and Car proteins was independent of their membrane association, because the two proteins could be coimmunoprecipitated from cytosol as well as from the solubilized particulate fraction (Figure 6C). These experiments indicated that the Dor and Car proteins are components of the same high molecular weight protein complex regulating endocytic trafficking.

Discussion

car and *dor* belong to the classic set of eye color genes in *Drosophila* (Beadle and Ephrussi, 1936; Merrell, 1947). Here, we provide genetic, histological, and biochemical evidence that they encode components of the multiprotein Dor complex, which is necessary for the delivery of proteins to lysosomes and pigment granules. This is a direct demonstration that members of the granule group of eye color mutations in *Drosophila* function in lysosomal delivery.

Such a role for the Dor/Car complex is suggested by our analysis of the null phenotype of dor^8 clones. First, these clones lack pigment granules, indicating defects in granule biogenesis. Second, the accumulation of the Boss ligand in R7 photoreceptors within the dor^8 clones revealed the inhibition of lysosomal delivery of either the Boss ligand or, alternatively, the lysosomal enzymes necessary for Boss degradation. The partial localization of Dor to the limiting membranes of endosomes in S2:Sev cells further supports the involvement of the Dor/Car complex in trafficking to lysosomes.

Our data provide direct evidence for a role in lysosomal delivery for metazoan homologs of C class *VPS* genes. The proteins encoded by these genes constitute a complex required for the docking or fusion between a prevacuolar, multivesicular compartment and the lysosome-like vacuole (Rieder and Emr, 1997). Although we have not yet identified all the components of this complex in *Drosophila*, strong support for the homologous nature of the Dor/Car and *VPS-C* complexes is provided by the resemblance of their mutant phenotypes and the conservation of two of their components. In addition to the similarity between Dor and Vps18p (Shestopal et al., 1997), we demonstrate here that the Car protein is similar to Vps33p (Banta et al., 1990; Wada et al., 1990).

Car and Vps33p belong to the large family of Sec1p-related proteins (Halachmi and Lev, 1996), which appear to play a crucial role in vesicle docking or fusion. This view is supported by the tight binding of family members to Syntaxin isoforms (Hata et al., 1993; Garcia et al., 1994; Pevsner et al., 1994) and the genetic requirement of various family members for membrane fusion events (e.g., Banta et al., 1990; Cowles et al., 1994; Harrison et al., 1994; Piper et al., 1994; Schulze et al., 1994; Wu et al., 1998; Peterson et al., 1999; Tall et al., 1999). Together, these data point to a role of Sec1p homologs in regulating the formation of the core complex of t-SNAREs and v-SNAREs (Pfeffer, 1999).

A model that restricts the function of the Dor/Car complex to vesicle docking or fusion cannot easily explain the striking finding of coated vesicles within MVBs of dor^8 mutant cells (Figure 3). Interestingly, a form of the COP1 coatomer complex is required for vesicular transport from early to late endosomes (Whitney et al., 1995; Aniento et al., 1996; Gu et al., 1997). It is tempting to speculate that one of the consequences of *dor* mutations is a defect in the uncoating of such COP1-dependent vesicular intermediates in endosomal trafficking. Although we have no data supporting a direct role of the Dor complex in such a function, the localization of the Dor complex to distinct structures internal to endosomes in S2:Sev cells (Figure 2G) suggests that Dor might function in the dramatic morphological changes of internal membrane structures observed in late endosomes (van Deurs et al., 1993; Futter et al., 1996; Sunio et al., 1999).

In summary, our analysis of the *dor* and *car* eye color genes revealed their role in vesicular transport to lysosomes and pigment granules. Since the turn of this century, more than 85 eye color genes have been described in *Drosophila*. Our data suggest that a large subset of these genes, the granule group, is of general importance in regulating transport to lysosome-like organelles. Only now are we beginning to appreciate the potential of these genes in the analysis of molecular mechanisms regulating vesicular trafficking.

Experimental Procedures

Molecular Biology

Clones encoding Dor (AA391097) or Car (Dm-VPS33; AA264748) were identified in the collection of *Drosophila* EST sequences (Berkeley *Drosophila* Genome Project/HHMI EST Project, unpublished). Full-length sequence of the *car* cDNA was obtained using an automated ABI sequencer and submitted to Genbank, under the accession number AF133260. P1 filter panels were used for chromosomal mapping of Dm-VPS33 according to the manufacturer's procedures (Clontech). Alleles of *dor* and *car* were sequenced by PCR-amplifying genomic DNA. Sequences were directly determined without subcloning. Differences between mutant alleles and wild-type sequences were confirmed from independent PCR reactions. Dor cDNAs encoding either the full-length Dor protein or three truncation mutations eliminating aa 1–399 ($Dor^{400-1002}$), aa 402–799 ($Dor^{\Delta 401-800}$), or aa 803–1002 (Dor^{1-802}) were generated using PCR.

Fly Work

The $hook^{11}$ null allele has been described (Krämer and Phistry, 1999). Other fly stocks (Lindsley and Zimm, 1992; Shestopal et al., 1997) were obtained from the Bloomington Stock Center (Bloomington, Indiana). For the generation of transgenic flies, DNA fragments encoding Dor proteins were cloned into the pUAS transformation vector (Brand and Perrimon, 1993) and the Car cDNA was cloned into the pGMR vector (Hay et al., 1994). Expression of Dor proteins in compound eyes of flies carrying the various P[UAS-Dor] transgenes was induced using P[GMR-Gal4] (Freeman, 1996). Clones homozygous for dor^{8} were generated from dor^{8} heterozygous flies that were otherwise wild type or $hook^{11}$ using the FRT/FLP technique (Xu and Rubin, 1993).

Antibodies

Bacterial GST fusion proteins containing aa 510–1002 of Dor or aa 191–616 of Car were expressed, purified from inclusion bodies, and used for the production of antibodies in chicken (anti-DorC1) or rabbits (anti-Car1 and anti-Car2), respectively. Additional antibodies were raised in rabbits against an N-terminal peptide (aa 2–16) of the Dor protein (anti-DorN1). Specific antibodies were affinity purified from the sera using the fusion proteins or peptides coupled to agarose beads (Affigel 10, Bio-Rad) as described (Harlow and Lane, 1988).

Biochemistry

For cell fractionation experiments, approximately 10^8 S2 cells were lysed in 0.4 ml of ice-cold hypotonic buffer (5 mM Tris/HCl [pH 7.5], 1.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM KCl, and a protease inhibitor mix). Unbroken cells and nuclei were removed by a 10 min spin at 1,000 × g. The supernatant was adjusted to 150 mM NaCl, 50 mM Tris (pH 7.5) and spun at 95,000 × g for 2 hr (TLA 120.1 rotor). Supernatant was collected, and the pellet was adjusted to equal volume using 150 mM NaCl, 50 mM Tris (pH 7.5), 2% Triton X-100. Both were analyzed by Western blotting. Native molecular weights were determined by a combination of gel filtration (200HR FPLC Superdex) and sucrose density gradient centrifugation (5%–20%) of supernatants.

For coimmunoprecipitation experiments, approximately 2×10^7 S2 cells were disrupted in 0.5 ml lysis buffer (10 mM HEPES, 142 mM KCl, 5 mM $MgCl_2$, 1% Triton X-100 [pH 7.2]) for 15 min at 4°C. Lysates were cleared at 20,000 × g for 15 min. Either 0.2 μg anti-DorN1 or 1 μg anti-Dm-VPS33–1 were incubated with 250 μl of lysate for 1 hr at 4°C. Antibodies were collected using 10 μl protein A beads at 4°C for 1 hr and a 1 min 15,000 × g spin. Beads were washed three times with lysis buffer, and proteins were eluted with 100 μl 1× SDS loading buffer for 10 min at 37°C. Before separation on SDS-PAGE, DTT was added to 50 mM. On Western blots, Dor or Car proteins were detected with the respective affinity-purified antibodies (1:2000). Primary antibodies were visualized using HRP-coupled secondary antibodies and enhanced chemiluminescence (Super-Signal; Pierce).

Histology

Double immunofluorescence detection of Boss (anti-Boss NN1; 1:2500) (Krämer et al., 1991) and the MYC epitope (9E10; 1:500) in eye discs was performed using a Bio-Rad MRC1024 confocal microscope (Sevrioukov et al., 1998). Garland cells, dissected from third instar larvae, or S2 cells were incubated with Texas red or FITC-labeled dextran beads (MW 70,000; 1 mg/ml) for 1 or 5 min at RT, and excess beads were removed. Either directly or after an additional 30 min, cells were fixed in 4% paraformaldehyde for 30 min. Fixed Garland cells were stained using rabbit anti-DorN1 (1:400) as described (Krämer and Phistry, 1996). For double labeling in S2 cells, Hook protein was detected using Dig-labeled anti-Hook antibodies and Cy5-labeled anti-Dig antibodies. For electron microscopic analysis, adult eyes were fixed, embedded in plastic, and sectioned as previously described (Van Vactor et al., 1991).

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