**shibire** mutations reveal distinct dynamin-independent and -dependent endocytic pathways in primary cultures of *Drosophila* hemocytes

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**Summary**

We have developed a primary cell culture system derived from embryonic and larval stages of *Drosophila*. This allows for high-resolution imaging and genetic analyses of endocytic processes. Here, we have investigated endocytic pathways of three types of molecules: an endogenous receptor that binds anionic ligands (ALs), glycosylphosphatidylinositol (GPI)-anchored protein (GPI-AP), and markers of the fluid phase in primary hemocytes. We find that the endogenous AL-binding receptor (ALBR) is internalized into Rab5-positive endosomes, whereas the major portion of the fluid phase is taken up into Rab5-negative endosomes; GPI-APs are endocytosed into both classes of endosomes. ALBR and fluid-phase-containing early endosomes subsequently fuse to yield a population of Rab7-positive late endosomes. In primary culture, the endocytic phenotype of ALBR internalization in cells carrying mutations in *Drosophila* Dynam (dDyn) at the *shibire* locus (*shi*ts) parallels the temperature-sensitive behavior of *shi*ts animals. At the restrictive temperature in *shi*ts cells, receptor-bound ALs remain completely surface accessible, localized to clathrin and α-adaptin-positive structures. On lowering the temperature, ALs are rapidly sequestered, suggesting a reversible block at a late step in dDyn-dependent endocytosis. By contrast, GPI-AP and fluid-phase endocytosis are quantitatively unaffected at the restrictive temperature in *shi*ts hemocytes, demonstrating a constitutive dDyn and Rab5-independent endocytic pathway in *Drosophila*.

Supplementary figures available online

Key words: Fluid phase, GPI-anchored proteins, Scavenger receptor, Endocytosis, Cell culture, shibire

**Introduction**

Metazoan systems like *Caenorhabditis* and *Drosophila* provide a unique opportunity to extract molecular details about the mechanisms of endocytosis as well as a broader idea about the role of endocytic trafficking in the development and physiology of multicellular animals (Narayanan and Ramaswami, 2001; Nurrish, 2002). For example, numerous genetic screens in *Drosophila* for mutations that result in reversible temperature-sensitive paralysis have led to the discovery of molecules affecting various aspects of neural transmission. Such behavioral screens, originally conceived as a means of isolating conditional mutations affecting muscle physiology, have largely identified molecules that affect axonal conduction (*para*) (Grigliatti et al., 1973) and synaptic vesicle recycling (*shibire, stoned, comatose, syntaxin*) (Grigliatti et al., 1973; Littleton et al., 1998; Siddiqui and Benzer, 1976). Complementary reverse-genetic approaches have also identified other molecules affecting nervous system function (*cysteine string protein, α-adaptin*) (Gonzalez-Gaitan and Jackle, 1997; Zinsmaier et al., 1994). Whereas specialized modes of trafficking such as synaptic vesicle recycling have been extensively investigated in *Caenorhabditis* and *Drosophila*, the diversity of endocytic pathways in these systems has yet to be characterized.

Not surprisingly, essential mediators of synaptic endocytosis like α-adaptin (Gonzalez-Gaitan and Jackle, 1997) and the *shibire* (*shi*) gene product *Drosophila* Dynam (dDyn), which is the only reported *Drosophila* homolog of vertebrate Dyn (Kosaka and Ikeda, 1983a; van der Bliek and Meyerowitz, 1991), are not restricted to the nervous system (Chen et al., 1992; Dorman et al., 1997). dDyn is known to regulate endocytosis in a variety of fly tissues (Kosaka and Ikeda, 1983a; Kosaka and Ikeda, 1983b; Tsuruhara et al., 1990). Zygotic null mutations at the *shi* locus are lethal and the mutants show neural hyperplasia (Poodry, 1990). Electronmicroscopy (EM) studies that addressed the phenotypes of temperature-sensitive alleles at the *shibire* locus (*shi*ts) in larval garland cells showed an accumulation of ‘coated pits’ at the plasma membrane and reduced uptake of fluid-phase tracers like horse radish peroxidase (HRP) (Kosaka and Ikeda, 1983b). These studies suggested that dDyn is required for all pathways of endocytosis in the cell types examined.

By contrast, over-expression of an analogous temperature-
sensitive mutant of Dyn (Dyn\textsuperscript{ts}) in HeLa cells led to an initial reduction of fluid-phase uptake at 39°C that subsequently recovered (Damke et al., 1995); the internalization of human transferrin was completely inhibited at this temperature. These results argued for pathways of fluid-phase uptake in mammalian cells that are induced or upregulated when Dyn function is perturbed. Glycosylphosphatidylinositol-anchored proteins (GPI-APs) (reviewed by Chatterjee and Mayor, 2001) and the D2 dopamine receptor (Vickery and von Zastrow, 1999) continue to be internalized into mammalian cells upon expression of dominant-negative Dyn isoforms. GPI-APs are endocytosed in a Dyn-independent manner into distinct endocytic compartments that contain a majority of the internalized fluid phase (Sabharanjak et al., 2002). These studies suggest that mammalian cells exhibit constitutive Dyn-independent pathways of endocytosis for both membrane and fluid-phase markers. However, mammals have three distinct genes encoding Dyn and as many as 25 splice variants (Cao et al., 1998), leaving open the possibility that alternate forms of Dyn might be involved in these endocytic events (McNiven et al., 2000). By contrast, in Drosophila dDyn, a multi-domain protein (see later) is encoded by a single locus that has six splice variants (Staples and Ramaswami, 1999; van der Biek and Meyerowitz, 1991). The availability of shibire\textsuperscript{ts} alleles that map to domains conserved across all the splice variants makes Drosophila an attractive system to dissect the involvement of dDyn in different endocytic processes.

Drosophila macrophages and hemocytes are a part of the innate immune system of the animal (Lanot et al., 2001; Tepass et al., 1994) and are capable of internalizing a variety of ligands by a scavenger receptor-mediated (dSR) pathway (Abrams et al., 1992). These cells are also phagocytic and have been shown to engulf both apoptotic cells and microbes (Franc et al., 1999); cells of this lineage in other metazoa are known to have multiple pathways of endocytosis (Gold et al., 1999; Racoosin and Swanson, 1992).

Here, we establish a methodology to reproducibly obtain primary cultures of macrophages and hemocytes, respectively from wild-type and mutant Drosophila embryos and larvae, respectively. The larval hemocytes have an endogenous anionic-ligand binding receptor (ALBR) with a similar ligand-binding specificity as dSR. In this system, we probed the existence of multiple endocytic pathways. Specifically, we have asked whether cells from embryonic and larval stages of wild-type and temperature-sensitive shibire animals are capable of dDyn-dependent and -independent endocytosis. We find that, first, the endocytic phenotype of ALBR-mediated endocytosis in cells from temperature-sensitive shibire mutants parallels the behavior of mutant flies; ALBR-mediated internalization is reversibly blocked by raising the temperature. Second, at the restrictive temperature, cell-surface, ALBR-bound ligands remain completely accessible to relatively large molecules (70 kDa proteins); upon shifting to low temperatures, the arrested structures are internalized in a surge, restoring ‘endocytic competence’. This is suggestive of a block in a late step of endocytosis in shibire mutants. By contrast, fluid-phase and GPI-AP endocytosis, which occurs via distinct endosomal structures, remains unaffected at the restrictive temperature in the shibire mutants, providing evidence for a constitutive dDyn-independent pathway.

Materials and Methods

Materials

All chemicals, apart from the fluorochromes Cy3, Cy5 (AP Biotech, UK), Alexa488, FITC (Molecular Probes, Eugene, OR), and Biotin-XX-SSE (Molecular Probes), were obtained from Sigma-Aldrich (St Louis, MO). Antibodies to mammalian Rab7 and Rab5, and anti-Drosophila α-adaptin, were obtained from M. Zerial (Dresden, Germany) and N. J. Gay (Cambridge, UK), respectively. Anti-GFP monoclonal (1B3AB) was obtained from S. Sundaresan and labeled with fluorophores according to manufacturers’ instructions. All secondary antibodies were from Jackson Laboratories. PI-PLC was purified in the laboratory from PI-PLC-expressing bacterial strains (Koke et al., 1991).

Fly stocks

UAS-GFP-Rab5 (Entchev et al., 2000), UAS-GFP-GPI (Greco et al., 2001), UAS-GFP-Clc (Chang et al., 2002) and Collagen-Gal4 (Asha et al., 2003) were obtained from M. Gonzalez-Gaitan, S. Eaton, I. Mellman and C. Dearolf, respectively. Other fly strains were obtained as previously described (Krishnan et al., 1996).

Cell culture

Stage 11 and 12 embryos were collected from flies freshly transferred to sucrose-agar bottles. Cell cultures were derived by homogenizing 40-80 embryos in a Potter-Elvehjem Tissue Grinder (2 ml; Wheaton, Millville, NJ) using a loose-fitting pestle in complete medium [Schneider’s Incomplete Medium (Gibco-BRL, Gaithersberg, MD) supplemented with 10% non-heat-inactivated FBS (Gibco-BRL), 1 μg/ml bovine pancreatic insulin, penicillin, streptomycin and L-glutamine]. The resulting cell suspension was transferred to 35 mm cover-slip bottom dishes (Sabharanjak et al., 2002), and maintained in an incubator at 21°C. Macrophages were identified by their characteristic morphology (Eschalier, 1997) in culture after a period of 2-4 days. Complete medium was ‘aged’ for a period of 24-36 hours at 4°C prior to use.

Hemocytes from the third larval instar were obtained as described previously (Lanot et al., 2001) with modifications (Sriram et al., 2003). Male flies carrying UAS-GFP-protein transgenes were crossed with virgin shibire\textsuperscript{ts}-CollagenGal4 flies and the progeny male larvae expressing GFP-tagged proteins were used to obtain hemocytes expressing the appropriate transgene in the mutant background. The cells were used for experiments 2 hours after dissection, unless otherwise mentioned.

Probes for endocytosis and immunodetection

mBSA was prepared as described earlier (Haberland and Fogelman, 1985). Fluorescent conjugates were made according to the instructions provided by the manufacturer with minor modifications. Biotinylated-Cy3-mBSA (B-Cy3mBSA) was made by conjugation of BSA with BiotinXXSSE at a molar ratio of 1:5 according to the instructions provided by the manufacturer, prior to conjugation to Cy3 and subsequent maleylation. FITC or Lissamine Rhodamine conjugated to Dextran (10 kDa; F-Dex, LR-Dex, respectively) was used as a fluid-phase tracer. Immunofluorescence detection of antibodies was carried out as described (Sriram et al., 2003). To enhance contrast in detecting membrane-bound forms of ectopically expressed GFP-Rab5 or GFP-Clc, cells were fixed for shorter time periods and permeabilized to remove cytosolic fluorescence.

Uptake assays

For uptake experiments, cells were incubated with endocytic probes in Schneider’s Incomplete Medium supplemented with BSA (1.5
images were processed using MetaMorph software. Images were as described previously (Sabharanjak et al., 2002). Fluorescence quantitative digital imaging and confocal microscopy was carried out in a given experiment. Fluorescence imaging, quantification and processing in a given experiment.

For uptake assays at 31°C, the dishes were secured onto an aluminum block immersed in a water bath. The dishes were then covered and probes were added through a hole in the dish lid. The water bath and dishes inside were kept covered except when the probe was added or the cells were washed. In all uptake experiments at 31°C, cells were first incubated at this temperature for a period of 5 minutes prior to being labeled.

To visualize the specific endocytic uptake of GFP-GPI, cells were pre-incubated with Fl-anti-GFP (2-4 μg/ml, 0°C) for 20 minutes followed by incubation of cells at room temperature for 45 seconds or 5 minutes. PI-PLC treatment was carried out (0.3 mg/ml; 30 seconds, room temperature) to quantitatively remove cell-surface GFP-GPI and bound Fl-anti-GFP prior to fixation and imaging. Cells were fixed with 2.5% paraformaldehyde in medium 1 (150 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, pH 6.9). Prior to imaging (even in fixed cells), endosomal pH was neutralized by the addition of 10 μM Nigericin in high-potassium buffer (120 mM KCl, 5 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, pH 7.4) or with freshly prepared ammonium chloride (20 mM in medium 1), and finally imaged in medium 1.

**Assays for surface accessibility of mBSA**

**Quantitative assay:** After endocytosis of B-Cy3mBSA by ALBRs, to detect surface-accessible receptors, cells were washed and cooled rapidly on ice and further incubated with Cy5-SA for an additional 15 minutes to probe for surface accessibility of B-Cy3-mBSA, and then fixed and imaged on a wide-field microscope. Cy5-SA-labeled cells were imaged and the Cy5-fluorescence was completely photobleached prior to imaging Cy3 to eliminate the possibility of quenching B-Cy3mBSA fluorescence by bound Cy5-SA.

Quantitative assay: to quantify the total accessible pool of receptors, cells were labeled with B-Cy3mBSA on ice for 15 minutes, washed and then labeled with Cy5-SA for 15 minutes, and taken for imaging. These assays used medium 1 as incubation buffer since Schneider’s medium contains free biotin that competed for the binding of Cy5-SA to B-Cy3mBSA. To measure the amount accessible at any other temperature, cells were labeled with B-Cy3mBSA for a period of 5 minutes at the given temperature, washed rapidly and labeled with Cy5-SA for an additional 15 minutes at the same temperature. In experiments designed to assay the reversibility of the temperature-sensitive endocytic defect, after incubation with B-Cy3mBSA at high temperatures, cells were rapidly transferred to ice prior to the addition of Cy5-SA at the same temperature. The ratio of Cy5 to Cy3 fluorescence represents the fraction of the B-Cy3mBSA probe accessible to Cy5-SA. The ratios of Cy5 to Cy3 fluorescence at a given temperature were normalized to the ratio obtained for total accessible pool. To estimate the extent of nonspecific binding of Cy5-SA, in each experiment cells were labeled with B-Cy3mBSA for 15 minutes on ice and subsequently incubated with biocytin-treated (2 μM) Cy5-SA. The nonspecific value never exceeded 10% of the fraction of the total accessible pool in a given experiment.

**Results**

A system for the study of endocytosis

Primary cultures derived from Canton S (CS wild-type strain) embryos of stage 11/12 (Materials and Methods) yield neurons, different types of muscle, and fat cells, as identified by both morphology and specific markers (e.g. antibodies and GAL4-mediated cell-type-specific expression of reporters (Brand and Perrimon, 1993; Eschalier, 1997; Seecof and Unanue, 1968) (A. Guha and S. Mayor, unpublished). In these cultures, macrophages are morphologically distinct and can be visually discriminated from other cells after 2-4 days (Fig. 1A; A. Guha and S. Mayor, unpublished). Embryonic macrophages in vivo internalize polyanionic ligands (ALR) such as acetylated LDL and maleylated BSA (mBSA) via a unique class of dSRs (Abrams et al., 1992). Embryonic cultures incubated with Cy3-labeled mBSA (Cy3mBSA) revealed a population of cells that specifically internalized this anionic ligand into endosomes via ALBRs (Fig. 1B). None of the other cell types in culture internalized Cy3mBSA at detectable levels at the concentrations used (data not shown). The hemolymph of third instar larva is also a rich source for cells of the hemocyte lineage (Lanot et al., 2001) that are endocytically active and show ALBR-mediated Cy3mBSA uptake (Fig. 1C, D). Moreover, the binding and internalization of Cy3mBSA by cells in both types of cultures could be competed by the addition of excess unlabeled mBSA (Fig. 1D, inset; and 1E). The binding of Cy3-BSA could also be competed by the anionic ligands, fucoidan or LPS (Fig. 1E), but not by BSA, poly IC or Dextran (data not shown). This is similar to the binding specificity of type C1 dSRs (Pearson et al., 1995; Ramet et al., 2001). These results show that internalization of Cy3mBSA via ALBRs is likely to be mediated by an endogenous dSR.

In this study, we have mainly used larval hemocytes since they are better suited for the study of endocytic processes because large numbers of ALBR-expressing cells can be obtained more reproducibly. They also exhibit considerably less auto-fluorescence than their culture-derived embryonic counterparts. Nevertheless, qualitatively similar results have been obtained in both types of cell cultures from different genetic backgrounds (Supplementary figure S2).

Pathways of receptor-mediated and fluid-phase endocytosis in hemocytes

To study the pathways of receptor-mediated and fluid-phase endocytosis in larval hemocytes from wild-type (CS) animals, Cy3mBSA was used to label the ALBR-mediated pathway whereas FITC or Lissamine Rhodamine conjugated to Dextran (10 kDa; F-Dex, LR-Dex, respectively) were used as fluid-phase tracers. When cells are pulsed with Cy3mBSA and F-Dex for 5 minutes and chased for 2 minutes both probes extensively colocalize in endosomes (Fig. 2A-C). These endosomes are positive for the endosomal marker Rab7 (Fig. 2D-F) and are multivesicular (Siriram et al., 2003), indicating that F-Dex and Cy3-mBSA are rapidly delivered to late endosomes in these hemocytes. These rates are similar to those
observed in mammalian macrophages in culture wherein multivesicular endosomes containing fluid-phase cargo are visualized as early as 5-8 minutes after a pulse of BSA-gold in mammalian macrophages (Rabinowitz et al., 1992). Consistent with the passage through an early endosomal compartment, endosomal intermediates formed in a short pulse of 45 seconds of Cy3mBSA and F-Dex do not colocalize with Rab7 (Fig. 2G,H).

To confirm that the punctate structures formed by Cy3mBSA in short internalization times are endosomal, we incubated cells with biotinylated Cy3mBSA (B-Cy3mBSA) for 45 seconds and assayed for the surface accessibility of the biotin-tag by incubation with Cy5-labeled Streptavidin (Cy5-SA) on ice. As a control, we ascertained that, if cells were labeled at 0°C, almost all punctate Cy3mBSA structures are colocalized with Cy5-SA (data not shown). After 45 seconds, a fraction of peripherally distributed B-Cy3mBSA structures remains accessible to Cy5-SA, indicating cell-surface localization (compare Fig. 3A and B, small arrowheads). At the same time, endocytosed B-Cy3mBSA (defined as Cy5-SA inaccessible structures) is distributed in numerous small endosomal structures (Fig. 3A, small arrows) distinct from endosomes formed by F-Dex present in the incubation medium (Fig. 3C, arrowheads; merge in Fig. 3D). After the 45 seconds pulse, when cells were chased for 5 minutes in the absence of fluorescent probes, both Cy3mBSA and F-Dex colocalize in late endosomes (Supplementary figure S1A-C). Following a 2 minutes pulse, the majority of Cy3mBSA and F-Dex were found to colocalize in late endosomal structures, but compartments containing only F-Dex or Cy3-mBSA are also seen (Supplementary figure S1D-F).

The difference in distribution of B-Cy3mBSA- and F-Dex-containing endosomes after a 45 seconds pulse suggested two possibilities. First, that F-Dex and B-Cy3mBSA are internalized via independent pathways. Second, that F-Dex and Cy3mBSA are internalized via a common intermediate wherein the observed segregation reflected differences in their relative rates of internalization. To discriminate between these alternatives, we incubated cells with B-Cy3mBSA alone for 3 minutes to label all early endosomal structures and then pulsed with F-Dex and B-Cy3mBSA together for an additional 45 seconds. Under these conditions, we find that the majority of F-Dex-containing structures remain devoid of B-Cy3mBSA (Fig. 3E-H). After a 5 minutes chase, F-Dex and B-Cy3mBSA are once again extensively colocalized (data not shown). These experiments suggest that the difference in distribution of Cy3mBSA and F-Dex after brief pulses could be due to the involvement of different endocytic pathways, and that the ALBR-mediated pathway has a poor capacity for fluid-phase uptake. Consistent with this, if cells were pulsed with tenfold higher concentrations of F-Dex (5-8 mg/ml), the fluid-phase marker was detected in the distinct endosomes as well as in Cy3mBSA-labeled endosomes under the same conditions of imaging (data not shown). These results confirm that Cy3mBSA (or B-Cy3mBSA)-labeled early endosomes do not account for a major fraction of the fluid-phase uptake in hemocytes.

The Cy3mBSA structures accessed in 45 seconds are positive for Rab5 (Fig. 4A-C, small arrows) consistent with their designation as early endosomes (Zerial and McBride, 2001). At the same time, early (~45 seconds) endosomes of the fluid-phase pathway do not stain for Rab5 (Fig. 4D-F, arrowheads), suggesting that these endosomal compartments are part of a Rab5-independent pathway.

In Chinese Hamster Ovary (CHO) cells, fluid-phase uptake takes place predominantly via a pathway that selectively incorporates GPI-APs. This pathway is also Rab5 independent, and independent of clathrin, dynamin and caveolin (Sabharanjak et al., 2002). To examine if fluid-phase uptake occurs via a similar pathway in Drosophila hemocytes, we have followed the endocytosis of exogenously added fluorescently labeled antibodies (Fl-anti-GFP) against GFP-
GPI transgenically expressed in hemocytes using the UAS-Gal4 system (Brand and Perrimon, 1993). Visualization of endocytosed GFP-GPI was facilitated by the quantitative removal (data not shown) of cell-surface GFP-GPI (and FI-anti-GFP) by treatment with PI-PLC (Materials and Methods). Observation at 45 seconds post-internalization shows that the peripheral small early endosomes containing Cy5mBSA as well as the large fluid-filled early endosomes are both labeled with PI-PLC-resistant FI-anti-GFP specifically internalized by GFP-GPI (Fig. 5A,B); cells that do not express GFP-GPI do not show uptake of detectable amounts of FI-anti-GFP (data not shown). These results suggest that, similar to CHO cells, the fluid-phase uptake is also mediated by a Rab5-negative, GPI-AP-enriched endosomal pathway. At later times, FI-anti-GFP (endocytosed via GFP-GPI) is delivered to late endosomes (Fig. 5C,D). The delivery of GPI-APs to late endosomes in *Drosophila* hemocytes is in contrast to the eventual delivery of GPI-APs to endosomal recycling compartments in CHO cells. However, it is similar to the route followed by GPI-APs in Baby Hamster Kidney cells, consistent with the differential sorting and fate of endocytosed GPI-APs observed in multiple cell types (Fivaz et al., 2002; Sabharanjak et al., 2002).

Together, these results suggest that hemocytes from *Drosophila* internalize ALBR-bound ligands and fluid-phase markers into two distinct classes of early endosomes, Rab5-positive and negative, respectively. These later fuse to form Rab7-positive late endosomes.

**Role of dDyn in endocytosis**

We next asked if dDyn plays any role in endocytosis of the two types of probes. For this purpose we obtained cells from *shi* alleles, *shi*<sup>ts1</sup> and *shi*<sup>ts2</sup>. Both these alleles contain missense mutations (van der Bliek and Meyerowitz, 1991) conserved across all splice variants (Staples and Ramaswami, 1999). The *shi*<sup>ts2</sup> mutation (G141S) lies in the GTP binding and hydrolysis domain (G domain) and the *shi*<sup>ts1</sup> mutation (G267D) lies at the interface of the G domain and the ‘middle’ domain (Fig. 6A). *shi*<sup>ts1</sup> and *shi*<sup>ts2</sup> flies paralyze within 3 minutes at 27°C and 27.5°C (the restrictive temperature), respectively, and recover just as rapidly when returned to 21°C (the permissive temperature) (Krishnan et al., 1996).

Hemocytes from CS, *shi*<sup>ts1</sup> and *shi*<sup>ts2</sup> animals were pulsed with Cy3mBSA and F-Dex for 5 minutes at 21°C. At this temperature, the uptake of both probes in *shi*<sup>ts1</sup> and *shi*<sup>ts2</sup> cells is indistinguishable from that of CS cells; both Cy3mBSA and F-Dex colocalize in large centrally located endosomes (Fig. 6B-D). In CS cells, following a 5 minutes pulse at 31°C, Cy3mBSA and F-Dex are seen in endosomes indistinguishable from those formed at 21°C (compare Fig. 6B with 6E). However, the distribution of Cy3mBSA is dramatically altered in both mutant *shi* alleles; Cy3mBSA has a punctate peripheral distribution (Fig. 6F,G; small arrowheads). The distribution of Cy3mBSA in *shi*<sup>ts1</sup> and *shi*<sup>ts2</sup> cells at 31°C is comparable to cells labeled on ice (Fig. 6H-J), a condition in which all endocytic activity is inhibited, suggesting an arrest in the internalization of ALBR-ligands.
In contrast to Cy3mBSA, fluid-phase endocytosis appears unaffected; F-Dex is internalized into similar endosomes in cells from the mutant shi animals (Fig. 6F,G; green) when compared with the endocytic structures observed in cells from CS animals (Fig. 6E, green) at the non-permissive temperature (see below for a detailed analysis of fluid-phase and GPI-AP endocytosis in shi\textsuperscript{ts} mutants). These observations confirm that the inhibition of ALBR internalization at the restrictive temperature is not result of any nonspecific cellular toxicity.

Nature of endocytic defect in shi\textsuperscript{ts} mutants at restrictive temperature

The process of formation of the endocytic vesicle minimally comprises three distinct stages: invagination, closure and fission (Sever et al., 2000a). At the synapse, shi\textsuperscript{ts} mutants exhibit a block at a late stage in this process (Ramaswami et al., 1994). To understand the nature of the endocytic defect in hemocytes, and to quantify the extent of Cy3mBSA internalization at 31°C, we devised a quantitative surface-accessibility assay (Fig. 7A). In this assay, we determined the accessibility of Biotinylated-Cy3mBSA (B-Cy3mBSA) to exogenously added Cy5-labeled Streptavidin (Cy5-SA). Thus, the ratio of Cy5-SA to B-Cy3mBSA fluorescence is a read out of the extent of the accessibility of B-Cy3mBSA to Cy5-SA added at a given temperature (Materials and Methods). Maximum accessibility was determined from cells incubated with B-Cy3mBSA on ice followed by Cy5-SA incubation at the same temperature (Fig. 7B, shi\textsuperscript{ts1}ice). In shi\textsuperscript{ts1} cells at 31°C, most of the B-Cy3mBSA was surface accessible; Cy5 to Cy3 fluorescence ratio in a population of shi\textsuperscript{ts1} cells labeled on ice is indistinguishable from that observed when the cells are labeled at 31°C (Fig. 7B, shi\textsuperscript{ts1} 31°C). In sharp contrast, only a small fraction of the ALBR ligand is accessible to Cy5-SA at the permissive temperature of 21°C; Cy5 to Cy3 fluorescence ratio in a population of shi\textsuperscript{ts1} cells at 21°C (Fig. 7B, shi\textsuperscript{ts1} 21°C) resembles that obtained for nonspecific binding of the Cy5-SA to cells (Fig. 7B, shi\textsuperscript{ts1} Noise). Greater than 90% of total ligand-bound ALBR remained arrested at the cell surface in both shi\textsuperscript{ts1} and shi\textsuperscript{ts2} cells at 31°C. At the same temperature, ~50% of ligand-bound ALBR remains at the cell surface in CS cells (Fig. 7C), compared with the ~25% detected at 21°C. These experiments show that shi\textsuperscript{ts1} and shi\textsuperscript{ts2} cells quantitatively arrested ALBR sequestration at 31°C but not at 21°C, confirming that ligand-bound ALBR is internalized by a dDyn-dependent mechanism. These results are consistent with studies conducted in mammalian cells overexpressing Dyn\textsuperscript{ts} (analogous to shi\textsuperscript{ts1}) on the internalization of biotinylated transferrin endocytosed via the transferrin receptor wherein the coated pits with wide openings also appear to accumulate at the restrictive temperature (Damke et al., 1995).

To test whether the reversible nature of the temperature-sensitive shi mutation (Koenig et al., 1983; Kosaka and Ikeda, 1983b) is reproduced in the primary culture cells, we asked if Cy3mBSA arrested at the cell surface at 31°C in shi\textsuperscript{ts} cells (Fig. 7C) is capable of...
being internalized into subsequently formed Cy5mBSA-containing endosomes at the permissive temperature (see scheme in Fig. 8A). The extensive colocalization of Cy3mBSA in Cy5mBSA-containing endosomes formed at the permissive temperature in hemocytes from \textit{shi}ts animals provides evidence for the reversible nature of the arrest of dDyn-mediated internalization in these cells (Fig. 8B).

To gain further insight into the nature of structures containing surface-accessible receptors that accumulate at the restrictive temperature we followed the scheme outlined in Fig. 8C. This protocol addresses the rapidity with which surface-accessible structures that accumulate at the restrictive temperature in \textit{shi} mutant cells become inaccessible to large-molecular-weight molecules (Cy5-SA). After rapid transfer to ice (<5 seconds to equilibrate to 0°C), we find that a surprisingly significant fraction (~65%) of surface-accessible B-Cy3mBSA pre-bound at 31°C (Fig. 7C) becomes inaccessible to Cy5-SA (Fig. 8D). In comparison with the quantitative accessibility of B-Cy3mBSA at 32°C, transfer to ice results in a significant reduction in accessible receptors. This suggests a rapid sequestration of the B-Cy3mBSA in this experimental paradigm. As observed in a representative figure (right panel in Fig. 8C), although the majority (87%) of the B-Cy3mBSA fluorescence remains inaccessible to Cy5-SA, the B-Cy3mBSA is predominantly peripherally distributed (Fig. 8C, 32°C, arrowheads), which is indistinguishable from cells labeled on ice (Fig. 6H-J), or cells from \textit{shi}ts animals held at the high temperature (Fig. 6F,G). The distribution of Cy5-SA-inaccessible ligands in this paradigm is quite different from the distribution of internalized B-Cy3mBSA in cells incubated at the permissive temperature (21°C) post to transfer to ice (Fig. 8C, 21°C). These results suggest that the temperature-sensitive step in dDyn mutants is at a late step in the sequestration process mediated by dDyn.

**Surface-accessible ligands accumulate in clathrin- and adaptin-positive structures**

Since various Dyn-dependent pathways described in mammalian cells (McNiven et al., 2000) are clathrin dependent or independent, we ascertained whether internalization of ALBR occurs by a clathrin-mediated pathway. For this purpose, we examined the colocalization of ectopically
expressed GFP-clathrin light chain (GFP-Clc) (Chang et al., 2002) with Cy3mBSA-labeled ALBR incubated at 0°C. We find that most of the surface-localized (and quantitatively surface-accessible; Fig. 7B) receptors are present in GFP-Clc-positive punctate structures (Fig. 9A-C, small arrowheads). At the restrictive temperature in shi^ts2 cells, the surface-accessible receptors are also localized in GFP-Clc-positive structures (Fig. 9D-F, small arrowheads). Furthermore α-adaptin, a component of the clathrin-recruiting AP-2 complex (Chang et al., 1993), also colocalizes with the peripheral, surface-accessible Cy3mBSA puncta in both shi^ts1 and shi^ts2 cells (Supplementary figure S3). These data support the conclusion that ALBR ligands are internalized into primary larval hemocytes by a clathrin- and dDyn-mediated pathway.

Fluid-phase and GPI-AP endocytosis in shi^ts cells

Unlike the internalization of Cy3mBSA, uptake of F-Dex into shi^ts1 and shi^ts2 cells at the restrictive temperature does not appear to be perturbed (Fig. 6E-G). A quantification of the amount of F-Dex internalized (Fig. 10A) shows that fluid-phase uptake in shi^ts1 and shi^ts2 cells at 21°C, 31°C and 33°C is comparable with CS cells at the same temperatures. A common pathway of endocytosis for both Cy3mBSA and F-Dex would predict that a decrease in the rate of mBSA internalization should be accompanied by a decrease in the rate of F-Dex uptake. This does not take place, thus ruling out the possibility of a common pathway. It is unlikely that the conditions of primary culture medium components upregulate alternative endocytic pathways in hemocytes, since cells directly extracted into buffered saline also show dDyn-independent F-Dex internalization (Supplementary figure S2A-F).

To determine whether F-Dex-containing endosomes formed at the higher temperature in shi^ts cells are bona fide endocytic structures, we examined if these endosomes are capable of merging with ALBR-ligand-containing endosomes formed during the recovery of shi^ts cells at 21°C. For this purpose, we first incubated cells with F-Dex (and Cy3mBSA) at 31°C and then shifted the temperature to 21°C in the presence of Cy5mBSA (see schematic, Fig. 8A). After shifting to 21°C, Cy3mBSA, Cy5mBSA and F-Dex colocalize in both shi^ts1 and
shi^ts2 cells (Fig. 8B). These results show that the fluid-filled structures formed at restrictive temperatures are capable of merging with ligand-bound ALBR subsequently internalized from the cell surface in shi^ts cells at the permissible temperature.

The endocytosis of GPI-APs via the fluid-filled endosomes in mammalian cells has been shown to be Dyn independent (Sabharanjak et al., 2002). To ascertain the requirement for dDyn in GPI-AP internalization, we examined GPI-AP endocytosis in cells from shi^ts animals. At the restrictive temperature, Fl-anti-GFP is taken up into cells from shi^ts2 in fluid-filled endosomes similar in morphology (Fig. 10B) and extent as in cells from wild-type animals at the same temperature (data not shown). Furthermore, in the same cells, Cy5-mBSA is excluded from the endocytic pathway at the restrictive temperature (Fig. 10C). In cells expressing comparable levels of GFP-GPI, the ratio of internalized Fl-anti-GFP to the total amount of GFP fluorescence is 0.57±0.37 (15 cells) in wild type and 0.79±0.27 (20 cells) in the shi^ts2 background. Thus, the shi^ts2 mutation does not prevent the internalization of GPI-APs. This further reiterates the existence of a distinct dDyn-independent pathway involved in GPI-AP endocytosis in Drosophila hemocytes. Taken together, these data show that mBSA internalization and F-Dex (and GPI-AP) uptake occur by distinct mechanisms; ALBR-bound mBSA internalization is dDyn-dependent.

Fig. 7. Cells from shi^ts animals arrest mBSA at the plasma membrane in cell-surface-accessible structures. (A) Cartoon depicting the scheme adopted for detecting surface accessibility of mBSA bound to ALBR using Cy5-SA and B-Cy3mBSA. (B) Histogram shows the ratio of Cy5 to Cy3 fluorescence obtained for shi^ts1 cells at the temperatures and conditions indicated. The cartoons indicate the expected surface-accessible or -inaccessible status of ALBR ligand (B-Cy3mBSA) as probed using Cy5-SA. (C) Histogram shows the average extent of B-Cy3mBSA bound to the cells that remains accessible to Cy5-SA (Cy5/Cy3 ratio) after a pulse for 5 minutes at 21°C and 31°C in the indicated alleles, relative to the total accessible pool of receptors (Cy5/Cy3 ratio determined for Ice condition; arrow). Bars represent means±s.e. obtained from three experiments, each with at least 75 cells from two dishes in each condition. Note that both shi^ts alleles quantitatively arrest Cy3mBSA internalization at this temperature.
Discussion

The results presented in this report provide evidence that primary cultures of Drosophila hemocytes are capable of at least two distinct constitutive pathways of endocytosis (see model in Fig. 10D): fluid-phase and ALBR-mediated endocytic pathways are genetically distinguishable in cells from the \textit{shi}^{ts} mutant animals. These observations are not restricted to larval hemocytes since we obtain qualitatively similar results regarding distinct pathways of endocytosis in embryonic macrophages derived from \textit{shi}^{ts1} embryos (Supplementary...
In terms of the molecular identity of the ALBR, the competition studies carried out in larval hemocytes suggest that the endogenous ALBR has a binding specificity similar to that of type C1 dSRs (Pearson et al., 1995; Ramet et al., 2001). Furthermore, our results suggest that ALBR is internalized via a clathrin- and adaptin-mediated pathway and that type C1 dSRs have an adaptin-binding motif (YXXφ) at 549-552. However, these results do not unequivocally establish that the ALBR is identical to a specific dSR.

At the restrictive temperature, the receptors are trapped at the cell surface in clathrin- and adaptin-decorated sites, accessible to large molecules (Cy5-SA). This accessibility is quantitatively similar to that observed when cells from both wild type and mutants are directly labeled on ice, indicating a complete blockage of uptake and sequestration. These results are analogous to observations in mammalian cells overexpressing Dynš, a homologue of shiš1, wherein coated pits with wide openings accumulated at the restrictive temperature (Damke et al., 1995). In contrast to studies where, in mammalian cells overexpressing Dynš, the receptors retained avidin accessibility even after transfer to ice (Damke et al., 1995), the receptors in shiš cells pre-treated at restrictive temperature become completely sequestered upon transfer to ice. This difference is likely to reflect the intrinsic nature of mutations in dDyn in the absence of interference from copolymerization with endogenous wild-type Dyn molecules. The rapidity with which receptor sequestration takes place (within 5 seconds), suggests that the block in dDyn function in the shiš mutants is at a stage where commitment to endocytic sequestration has already occurred. The sharp transition in the extent of sequestration induced by shifting to ice after incubation at the restrictive temperature is suggestive of a structural role for dDyn in sequestration. Consistent with these results is a previous report where synaptic endocytosis is blocked in shi mutants at a late stage (Ramaswami et al., 1994). Current models for the role of dynamin in receptor-mediated endocytosis via clathrin-coated pits fall into two broad classes (reviewed in Hinshaw, 2000; Sever et al., 2000a), one in which the GTPase activity of the enzyme is directly involved in a mechano-chemical scission of the invaginated pit (Marks et al., 2001), and the other where Dyn is a regulatory GTPase involved in recruiting molecular players responsible for the fission process (Sever et al., 2000b). Although our results do not distinguish between these two models, they support a structural role for dDyn at late stages in the sequestration process without precluding a regulatory role in the earlier stages of the formation of the endocytic invagination. Understanding of the stage in the GTP-GDP cycle at which the shiš mutants are blocked will further discriminate between the two models (Damke et al., 2001).

These results reported here also demonstrate that the process of dDyn-dependent internalization is intrinsically temperature sensitive; >85% of the receptors are sequestered at permissive temperatures compared with ~50-60% at the restrictive
temperature in the same time interval in wild-type flies. Interestingly, this also has a correlate in the temperature sensitivity of the paralysis of wild-type (CS) flies in our hands; CS flies paralyze in a manner very similar to \textit{shi}2 flies but at temperatures >40°C. Mutations at the \textit{shi} locus, \textit{shi}1 and \textit{shi}2, can thus be seen as enhancers of the intrinsic temperature sensitivity of this process.

By contrast, fluid-phase uptake does not exhibit any temperature sensitivity in wild-type and \textit{shi} animals, arguing strongly for independent regulation. Cells completely lacking any \textit{shibire} gene product are likely to be autonomously cell-lethal (Grant et al., 1998), precluding an analysis of fluid-phase uptake in cells without any dDyn expression. Therefore, these data do not completely rule out a role for dDyn in fluid-phase uptake but they certainly rule out a role similar to the function of dDyn in receptor-mediated uptake in this endocytic process. Uptake of fluid occurs by various means (Lamaze and Schmid, 1995); however, except for macropinocytosis and the pathway for the internalization of GPI-APs (Sabharanjak et al., 2002), all other pathways require Dyn function in mammalian cells. Live imaging with cultured larval hemocytes expressing either GFP-actin or other actin-binding proteins fused to GFP have revealed that these cells undergo extensive membrane ruffling (A. Guha and S. Mayor, unpublished). Whereas macropinocytosis correlates with membrane ruffling (Racoosin and Swanson, 1992), the earliest F-Dex-filled compartments are unlike typical macropinosomes. Macropinocytosis is usually a triggered process resulting in large, phase-lucent organelles at the time of formation (Racoosin and Swanson, 1992). Although endosomes labeled in a 5 minutes pulse in larval hemocytes develop into phase-bright structures, the majority of the earliest F-Dex-filled endosomes formed in a 45 seconds pulse are not, arguing against the dDyn-independent pathway resembling macropinocytosis.

Another marker that has been extensively used to study fluid-phase endocytosis in \textit{Drosophila} and other cell culture systems is HRP. The reduced uptake of HRP seen in garland cells from \textit{shi}1 animals at the restrictive temperature (Kosaka and Ikeda, 1983b) would, therefore, appear contrary to the results presented here. However, at comparable molar concentrations, it is likely that HRP may be taken up via multiple means, including receptor-mediated pathways. We find that HRP is internalized via a yeast-mannan-competable pathway in hemocytes (Sriram et al., 2003), consistent with the possibility that HRP uptake is not restricted to the fluid phase. Furthermore, similar to the distinct cdc42-regulated, dynamin-independent pinocytic pathway recently described in mammalian cells (Sabharanjak et al., 2002), our results show that the \textit{Drosophila} hemocytes are also capable of dDyn-independent GPI-AP endocytosis via the fluid-phase pathway. Another point of similarity with the fluid-phase pathway recently described in mammalian cells is the absence of Rab5 on fluid-phase-containing endosomes (Sabharanjak et al., 2002). This suggests that Rab5 defines only a subset of early endosomal

![Fig. 10](image-url)

**Fig. 10.** Fluid-phase and GPI-AP uptake is unperturbed at restrictive temperatures in cells from mutant alleles of \textit{shi}. Histogram shows the average amount of F-Dex (FDx; ±s.d.) internalized per cell in the indicated alleles and temperatures in an incubation period of 5 minutes. The similarity in uptake across all alleles, even at restrictive temperatures, demonstrates that mutations at the \textit{shi} locus do not perturb the uptake of the fluid phase. Data shown are from three experiments each with at least 75 cells. (B,C) Hemocytes from \textit{shi}2 COLG-Gal4;UAS-GFP-GPI animals were incubated with Fl-anti-GFP (green) at 31°C for 15 minutes in the presence of LR-Dex (B, red) or Cy5mBSA (C, red) prior to fixation and imaging on a wide-field microscope. Endocytosis of Fl-anti-GFP into LR-Dex (B, arrows)-containing endosomes is unaffected, whereas Cy5mBSA is blocked at the cell surface (C, small arrowheads). Insets (red, top; green, middle; merge, bottom) show the area marked by the asterisk. Bar, 5 μm; inset, 1 μm. (D) Model depicts the existence of dDyn-independent endocytic pathways for the fluid phase (yellow) and GPI-APs (green) in larval hemocytes. ALBR ligands (red) are endocytosed via clathrin and dDyn-dependent pathways into Rab5-positive early endosomes (EE), whereas the fluid phase (yellow) marks a separate Rab5-negative EE, before finally coming together in Rab7-positive late endosomes (LE).
compartments, strengthening the notion that there may be multiple early endosomal systems that are connected to each other via cell-type-dependent trafficking pathways (Fivaz et al., 2002; Sabharanjak et al., 2002). This primary cell culture system will provide an opportunity for analysis of the molecular players involved in this pathway in a genetically amenable system, and will allow an exploration of the trafficking consequences of alterations in this pathway in the whole animal.

In conclusion, we show that Drosophila cells exhibit Dyn (dDyn)-dependent and -independent pathways of endocytosis. These pathways converge in a population of Rab7-positive endosomes and subsequently target endocytosed cargo for degradation in lysosomes (Sriram et al., 2003). It is conceivable that several GPI-AP-containing membranes in Drosophila, including 'argosomes' (Greco et al., 2001) and modulators of signaling such as heparan sulfate proteoglycans (Selleck, 2000) are endocytosed via the dDyn-independent pathway, distinct from dDyn-dependent pathways utilized to regulate signaling receptors (Ceresa and Schmid, 2000; Pierce and Leftkowitz, 2001). This could provide a mechanism by which cells can independently modulate their responses to the extracellular environment. At least in the case of Notch and Delta, the clearance of inactive Notch at the cell surface appears to be facilitated by a dDyn-independent pathway (Parks et al., 2000). The primary culture system described in this report makes it possible to examine, at high resolution, endocytic trafficking facilitated by a dDyn-independent pathway (Parks et al., 2000). Clathrin-independent pinocytosis is induced in cells overexpressing a shibire temperature-sensitive mutant of dynamin. J. Cell Biol. 131, 69-80.

Danne, H., Babu, T., van der Bliek, A. M. and Schmid, S. L. (1995). Clathrin-independent pinocytosis is induced in cells overexpressing a temperature-sensitive mutant of dynamin. J. Cell Biol. 131, 69-80.

References

Abroms, J. M., Lux, A., Steller, H. and Krieger, M. (1992). Macrophages in Drosophila embryos and L2 cells exhibit scavenger receptor-mediated endocytosis. Proc. Natl. Acad. Sci. USA 89, 10375-10379.

Asha, H., Nagy, I., Kovacs, G., Stetson, D., Ando, I. and Deraolt, C. R. (2003). Analysis of Ras-induced overproliferation in Drosophila hemocytes. Genetics 163, 203-215.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401-415.

Cao, H., Garcia, F. and McNiven, M. A. (1998). Differential distribution of dynamin isoforms in mammalian cells. Mol. Biol. Cell 9, 2595-2609.

Ceresa, B. P. and Schmid, S. L. (2000). Regulation of signal transduction by endocytosis. Curr. Opin. Cell Biol. 12, 204-210.

Chang, H. C., Newmyer, S. L., Hull, M. J., Ebersold, M., Schmid, S. L. and Mellman, I. (2002). Hsc70 is required for endocytosis and clathrin function in Drosophila. J. Cell Biol. 159, 477-487.

Chang, M. P., Mallet, W. G., Mostov, K. E. and Brodsky, F. M. (1993). Adamin self-aggregation, adaptor-receptor recognition and binding of alpha-adaptin subunits to the plasma membrane contribute to recruitment of adaptor (AP2) components of clathrin-coated pits. EMBO J. 12, 2169-2180.

Chatterjee, S. and Mayor, S. (2001). The GPI-anchor and protein sorting. Cell. Mol. Life Sci. 58, 1969-1987.

Chen, M. S., Burgess, C. C., Vallee, R. B. and Wadsworth, S. C. (1992). Developmental stage- and tissue-specific expression of shibire, a Drosophila gene involved in endocytosis. J. Cell Sci. 103, 619-628.

Danne, H., Babu, T., van der Bliek, A. M. and Schmid, S. L. (1995). Clathrin-independent pinocytosis is induced in cells overexpressing a temperature-sensitive mutant of dynamin. J. Cell Biol. 131, 69-80.

Danne, H., Binns, D. D., Ueda, H., Schmid, S. L. and Baba, T. (2001). Dynamin GTPase domain mutants block endocytic vesicle formation at morphologically distinct stages. Mol. Biol. Cell 12, 2578-2589.

Dornan, S., Jackson, A. P. and Gay, N. J. (1997). Alpha-adaptin, a marker for endocytosis, is exported in complex patterns during Drosophila development. Mol. Biol. Cell 8, 1391-1403.

Entchev, E. V., Schwabedissen, A. and Gonzalez-Gaitan, M. (2000). Gradient formation of the TGF-beta homolog Dpp. Cell 103, 981-991.

Echaller, G. (1997). Drosophila Cells in Culture. New York: Academic Press.

Fivaz, M., Vilbois, F., Thurnheer, S., Pasquali, C., Abrami, L., Bickel, P. E., Parton, R. G. and van der Goot, F. G. (2002). Differential sorting and fate of endocytosed GPI-anchored proteins. EMBO J. 21, 3989-4000.

Franke, N. C., Heitzler, P., Ezekowitz, R. A. and White, K. (1999). Requirement for cromeront in phagocytosis of apoptotic cells in Drosophila. Science 284, 1991-1994.

Gold, E. S., Underhill, D. M., Morrissette, N. S., Guo, J., McNiven, M. A. and Aderem, A. (1999). Dynamin 2 is required for phagocytosis in macrophages. J. Exp. Med. 190, 1849-1856.

Gonzalez-Gaitan, M. and Jackie, H. (1997). Role of Drosophila alpha-adaptin in presynaptic vesicle recycling. Cell 88, 767-776.

Grant, D., Unadkat, S., Katzen, A., Krishnan, K. S. and Ramaswami, M. (1998). Probable mechanisms underlying interallelic complementation and temperature-sensitivity of mutations at the shibire locus of Drosophila melanogaster. Genetics 149, 1019-1030.

Greco, V., Hanus, M. and Eaton, S. (2001). Argosomes: a potential vehicle for the spread of morphogens through epithelia. Cell 106, 633-645.

Grigliatti, T. A., Hall, L., Rosenbluth, R. and Suzuki, D. T. (1973). Temperature-sensitive mutations in Drosophila melanogaster. XIV. A selection of immobile adults. Mol. Gen. Genet. 120, 107-114.

Haberland, M. E. and Fogelman, A. M. (1985). Scavenger receptor-mediated recognition of maleyl bovine plasma albumin and the demaleylated protein in human monocyte macrophages. Proc. Natl. Acad. Sci. USA 82, 2693-2697.

Hinshaw, J. E. (2000). Dynamin and its role in membrane fission. Annu. Rev. Cell Dev. Biol. 16, 483-519.

Koenig, J. H., Saito, K. and Iedea, K. (1983). Reversible control of synaptic transmission in a single gene mutant of Drosophila melanogaster. J. Cell Biol. 96, 1517-1522.

Koke, J. A., Yang, M., Henner, D. J., Volwerk, J. J. and Griffith, O. H. (1991). High level expression in Escherichia coli and rapid purification of phosphatidylinositol-specific phospholipase C from Bacillus cereus and Bacillus thuringiensis. Protein Expr. Purif. 2, 51-58.

Kosaka, T. and Iedea, K. (1983a). Possible temperature-dependent blockage of synaptic vesicle recycling induced by a single gene mutation in Drosophila. J. Neurobiol. 14, 207-225.

Kosaka, T. and Iedea, K. (1983b). Reversible blockage of membrane retrieval and endocytosis in the garland cell of the temperature-sensitive mutant of Drosophila melanogaster, shibirets1. J. Cell Biol. 97, 499-507.

Krishnan, K. S., Chakravarty, S., Rao, S., Ragharum, V. and Ramaswami, M. (1996). Allleviation of the temperature-sensitive paralytic phenotype of shibire(ts) mutants in Drosophila by sub-anesthetic concentrations of carbon dioxide. J. Neurogenet. 10, 221-238.

Lamaze, C. and Schmid, S. L. (1995). The emergence of clathrin-independent pinocytotic pathways. Curr. Opin. Cell Biol. 7, 573-580.

Lanot, R., Zachary, D., Holder, F. and Meister, M. (2001). Postembryonic hematopoiesis in Drosophila. Dev. Biol. 230, 243-257.

Littleton, J. T., Chapman, E. R., Kreber, R., Garment, M. B., Carlson, S. J., Fran, E. S., Underhill, D. M., Morrissette, N. S., Guo, J., McNiven, M. A. and Aderem, A. (1999). Dynamin 2 is required for phagocytosis in macrophages. J. Exp. Med. 190, 1849-1856.

McNiven, M. A., Cao, H., Pitts, K. R. and Yoon, Y. (2000). The dynamin family of mechanoenzymes: pinching in new places. Trends Biochem. Sci. 25, 115-120.

Narayan, R. and Ramaswami, M. (2001). Endocytosis in Drosophila: progress, possibilities, prognostications. Exp. Cell Res. 271, 28-35.

Nurshad, S. J. (2002). An overview of C. elegans trafficking mutants. Traffic 3, 2-10.
Parks, A. L., Klueg, K. M., Stout, J. R. and Muskavitch, M. A. (2000). Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. Development 127, 1373-1385.

Pearson, A., Lux, A. and Krieger, M. (1995). Expression cloning of dSR-CI, a class C macrophage-specific scavenger receptor from Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 92, 4056-4060.

Pierce, K. L. and Lefkowitz, R. J. (2001). Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors. Nat. Rev. Neurosci. 2, 727-733.

Poodry, C. A. (1990). shibire, a neurogenic mutant of Drosophila. Dev. Biol. 138, 464-472.

Rabinowitz, S., Horstmann, H., Gordon, S. and Griffiths, G. (1992). Immunocytochemical characterization of the endocytic and phagolysosomal compartments in peritoneal macrophages. J. Cell Biol. 116, 95-112.

Racoosin, E. L. and Swanson, J. A. (1992). M-CSF-induced macropinocytosis increases solute endocytosis but not receptor-mediated endocytosis in mouse macrophages. J. Cell Sci. 102, 867-880.

Ramawat, M., Krishnan, K. S. and Kelly, R. B. (1994). Intermediates in synaptic vesicle recycling revealed by optical imaging of Drosophila neuromuscular junctions. Neuron 13, 363-375.

Ramel, M., Pearson, A., Manfruelli, P., Li, X., Koziel, H., Gobel, V., Chung, E., Krieger, M. and Ezekowitz, R. A. (2001). Drosophila scavenger receptor CI is a pattern recognition receptor for bacteria. Immunity 15, 1027-1038.

Saharanja, S., Sharma, P., Parton, R. and Mayor, S. (2002). GPI-anchored proteins are delivered to the endosomal recycling compartment via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. Dev. Cell 2, 411-423.

Seeof, R. L. and Unanue, R. L. (1968). Differentiation of embryonic Drosophila cells in vitro. Exp. Cell Res. 50, 654-660.

Selleck, S. B. (2000). Proteoglycans and pattern formation: sugar biochemistry meets developmental genetics. Trends Genet. 16, 206-212.

Sever, S., Damke, H. and Schmid, S. L. (2000a). Garrotes, springs, ratchets, and whips: putting dynamin models to the test. Traffic 1, 385-392.

Sever, S., Damke, H. and Schmid, S. L. (2000b). Dynamin-GTP controls the formation of constricted coated pits, the rate limiting step in clathrin-mediated endocytosis. J. Cell Biol. 150, 1137-1148.

Sharma, P., Sabharanjak, S. and Mayor, S. (2002). Endocytosis of lipid rafts: an identity crisis. Semin. Cell Dev. Biol. 13, 205-214.

Siddiqi, O. and Benzer, S. (1976). Neurophysiological defects in temperature-sensitive paralytic mutants of Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 73, 3253-3257.

Sriram, V., Krishnan, K. S. and Mayor, S. (2003). deep-orange and carnation define distinct stages in late endosomal biogenesis in Drosophila melanogaster. J. Cell Biol. 161, 593-607.

Staples, R. R. and Ramaswami, M. (1999). Functional analysis of dynamin isoforms in Drosophila melanogaster. J. Neurogenet. 13, 119-143.

Tepass, U., Fessler, L. L., Aziz. A. and Hartenstein, V. (1994). Embryonic origin of hemocytes and their relationship to cell death in Drosophila. Development 120, 1829-1837.

Tsuruhara, T., Koenig, J. H. and Ikeda, K. (1990). Synchronized endocytosis studied in the oocyte of a temperature-sensitive mutant of Drosophila melanogaster. Cell Tissue Res. 259, 199-207.

van der Bliek, A. M. and Meyerowitz, E. M. (1991). Dynamin-like protein encoded by the Drosophila shibire gene associated with vesicular traffic. Nature 351, 411-414.

Vickery, R. G. and von Zastrow, M. (1999). Distinct dynamin-dependent and -independent mechanisms target structurally homologous dopamine receptors to different endocytic membranes. J. Cell Biol. 144, 31-43.

Zerial, M. and McBride, H. (2001). Rab proteins as membrane organizers. Nat. Rev. Mol. Cell. Biol. 2, 107-117.

Zinsmaier, K. E., Eberle, K. K., Buchner, E., Walter, N. and Benzer, S. (1994). Paralysis and early death in cysteine string protein mutants of Drosophila. Science 263, 977-980.