

# Mutations in the *Drosophila hook* Gene Inhibit Endocytosis of the Boss Transmembrane Ligand into Multivesicular Bodies

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**Abstract.** Transmembrane ligands can be internalized across cell boundaries into receptor-expressing cells. In the developing *Drosophila* eye imaginal disc, the bride of sevenless transmembrane protein (boss) is expressed on the surface of R8 cells. After internalization into neighboring R7 cells, the boss protein accumulates in multivesicular bodies. In a search for genes that affect this cell-type-specific pattern of boss endocytosis, we found that mutations in the *hook* gene inhibit the accumulation of boss in multivesicular bodies of R7 cells. In addition, *hook* flies exhibit pleiotropic phenotypes including abnormal bristle morphology and eye degeneration. The wild-type pattern of boss endocytosis was restored in *hook* mutants by a genomic rescue fragment containing the *hook* gene or by a hook cDNA ex-

pressed in R7 cells under control of a sevenless (*sev*) enhancer. The *hook* gene encodes a novel cytoplasmic protein of 679 amino acids with a central coiled-coil domain of some 200 amino acids. Truncated, epitope-tagged hook proteins coimmunoprecipitated the full-length protein, indicating dimerization mediated by the coiled-coil domain. The hook protein localizes to vesicular structures that are part of the endocytic compartment. The requirement of the hook protein in R7 cells for the accumulation of boss protein in multivesicular bodies, and the localization of the hook protein to endocytic vesicles indicate that the *hook* gene encodes a novel component of the endocytic compartment that plays an important role in the endocytosis of transmembrane ligands or their transport to multivesicular bodies.

**M**EMBRANE-bound ligands play important roles in local cell-cell communication. The term "juxtacrine signaling" has been proposed to describe the interaction between such transmembrane ligands and their receptors on adjacent cells (Bosenberg and Massague, 1993). The interaction between soluble ligands and their receptors is terminated by the internalization of the receptor/ligand complexes. In acidified endosomes, ligands are separated from their receptors and routed to lysosomes, where proteolysis terminates their life cycle (Gruenberg and Maxfield, 1995). The anchoring of complexes between transmembrane ligands and their receptors to both opposing plasma membranes raises the problem, how these complexes are removed from the cell surface. Analysis of the *Drosophila* bride of sevenless protein (boss)<sup>1</sup> indicates that internalization of transmembrane ligands across cell boundaries is one way by which cells terminate juxtacrine signaling (Cagan et al., 1992; Krämer, 1993).

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1. *Abbreviations used in this paper:* boss, bride of sevenless; HA, hemagglutinin; MVB, multivesicular bodies; sev, sevenless.

The boss protein functions as a neuronal inducer during the development of the compound eye in *Drosophila* (for review see Zipursky and Rubin, 1994). The ~800 ommatidia that compose the *Drosophila* eye initiate their development in the eye imaginal disc. Within this monolayer of polarized cells, the photoreceptor cells (R cells) of individual ommatidia emerge in a stereotyped pattern that is guided by cell-cell interactions (Tomlinson and Ready, 1987; Cagan, 1993). Neuronal development of the R7 cell is triggered by the activation of the sevenless receptor tyrosine kinase (*sev*) upon binding of its ligand, boss, a transmembrane protein containing seven membrane-spanning segments (Hart et al., 1990). The seven transmembrane domain of boss is required for its function. When the extracellular domain of boss was expressed as a soluble ligand, it antagonizes *sev* function instead of activating it (Hart et al., 1993).

In the eye disc, boss is specifically expressed on the apical surface of R8 cells. Upon binding to the *sev* receptor, the boss ligand is internalized into R7 cells in a developmentally regulated process (Krämer et al., 1991). In addition to R7, the R3 and R4 cells contact the boss-expressing R8 cell and present the *sev* receptor on their surface (Tomlinson et al., 1987). *Sev* receptor expressed on the R3

and R4 cells can bind to boss protein (Hart et al., 1993), but internalization of boss into the R3 or R4 cells has not been observed (Krämer et al., 1991; Cagan et al., 1992). *Split*, an allele of *Notch* and *rough* are two mutations that alter cell fates in the developing eye disc and cause the generation of additional R7 cells (Heberlein et al., 1991; Van Vactor et al., 1991). In these mutants, transformed cells in the positions of R3 and R4 cells internalize the boss protein (Van Vactor et al., 1991). It therefore appears that one consequence of commitment to an R3 or R4 cell fate is the inhibition of boss endocytosis.

Surprisingly, the entire boss protein, including its cytoplasmic tail, is transferred from the surface of the R8 cell into the R7 cell (Cagan et al., 1992). Antibodies against the extreme NH<sub>2</sub> as well as COOH terminus of boss were used to demonstrate that, within R7 cells, the entire transmembrane protein accumulates to high levels in endosomes that have the appearance of multivesicular bodies (MVBs) (Krämer et al., 1991; Cagan et al., 1992). Recently, internalization of transmembrane ligands has been observed for other receptor-ligand pairs (Bailey et al., 1992; Hu et al., 1993; Kooh et al., 1993). A striking example from *C. elegans* is the uptake of the Lag-2 transmembrane ligand into germline cells expressing its apparent receptor Glp-1 (Henderson et al., 1994). By expressing  $\beta$ -galactosidase fused to the cytoplasmic tail of Lag-2 in the distal tip cell, Kimble and coworkers could demonstrate that the entire Lag-2 fusion protein, including  $\beta$ -galactosidase, is internalized into vesicles in the germ line. Lag-2 exhibits similarity to the *Drosophila* delta and serrate ligands of the notch receptor (Henderson et al., 1994; Tax et al., 1994). These two transmembrane ligands appear to be internalized into notch-expressing cells (Kooh et al., 1993; Couso et al., 1995; Parks et al., 1995).

What mechanisms enable cells to internalize entire transmembrane ligands across cell boundaries? What is the molecular basis for the developmental regulation of boss endocytosis? From a genetic screen designed to identify genes involved in the cell-type-specific internalization of the boss ligand, we found that mutations in the *hook* gene inhibit the accumulation of the boss protein in MVBs in R7 cells. The *hook* mutation had originally been identified by Mohr (1927) based on a bristle phenotype. In this paper, we concentrate on the molecular characterization of *hook* and present evidence indicating that the hook protein is a novel component of the endocytic compartment.

## Materials and Methods

### Fly Stocks and Transgenic Flies

All *hook* alleles used in this study (Table I) were generously provided by Ted Wright (University of Virginia, Charlottesville, VA) and have been previously described (Lindsley and Zimm, 1992; Stathakis et al., 1995). The deficiency Df(2L)TW130 deletes the entire *hook* gene as well as several neighboring essential genes (Stathakis et al., 1995). Standard procedures were used for scanning and transmission electron microscopy (Van Vactor et al., 1991). To generate the genomic rescue plasmid pCaSpeR-gHK, a 7-kb BamHI fragment from  $\lambda$ RS25 (Gilbert et al., 1984) was inserted into the transformation vector pCaSpeR (Thummel et al., 1988). To obtain cell-type-specific expression of the hook protein in the eye disc, a cDNA fragment encoding amino acids (aa) 2–679 of the hook protein with a NH<sub>2</sub>-terminal hemagglutinin (HA) epitope tag (MYPYDVPDYASS) was placed between the Asp718 and NotI sites of the transformation vec-

Table I. Summary of hook Alleles Used in This Study

Allele	Reference	Size of hook protein*
<i>hk</i> <sup>1</sup>	Mohr (1927)	truncated protein of ~60 kD
<i>hk</i> <sup>C1</sup>	(Stathakis et al., 1995)	truncated protein of ~65 kD
<i>hk</i> <sup>492</sup>	(Stathakis et al., 1995)	truncated protein of ~70 kD
Df(2L)TW130	(Wright et al., 1981)	complete deletion

\*See Fig. 6.

tor pBD365 (kindly provided by E. Hafen, University of Zürich, Switzerland). Expression from the hsp70 promoter in this vector is under control of a duplicated *sev* enhancer (Basler et al., 1991). Transgenic lines were established using standard techniques (Rubin and Spradling, 1982).

### DNA Manipulations

DNA manipulations were performed according to standard procedures (Ausubel et al., 1994). Four partial hook cDNAs were obtained from screening  $1.5 \times 10^6$  clones of an eye disc-specific phage library (kindly provided by Gerry Rubin, UC Berkeley Berkeley, CA). Two longer cDNAs that completely contained the single large open reading frame were obtained in a second screen of  $10^6$  clones of a disc-specific plasmid library (kindly provided by William Gelbart, Harvard University, Cambridge, MA). Deletion clones and specific oligonucleotides were used to sequence the six cDNAs and the corresponding genomic region from double-stranded DNA using Sequenase according to the manufacturer's protocols (USB, Cleveland, Ohio). The Blast and Coils2 programs were used to analyze the sequence (Altschul et al., 1990; Lupas et al., 1991).

### Antibodies

A hook-glutathione-S-transferase fusion protein encompassing the COOH-terminal 414 aa of the hook protein was expressed in bacteria. Inclusion bodies of the insoluble fusion protein were isolated and dissolved in 6M urea (Rio et al., 1986). After removal of urea by dialysis, insoluble material was pelleted at 20,000 g and the remaining soluble fusion protein was used for the production of antibodies in rabbits or coupled to Affinica-gel (Schleicher and Schuell, Keene, NH) for affinity purification of antisera (Harlow and Lane, 1988).

### Cell Culture

S2 cells were grown and transfected as previously described (Krämer et al., 1991). Constructs encoding truncated hook proteins in which either the first 163 aa were replaced by an NH<sub>2</sub>-terminal HA epitope tag (MYPYDVPDYASS) or the COOH-terminal 118 aa were replaced by a Myc epitope tag (DPGEEQKLISEEDLL) were placed under control of the metallothionein promoter (Bunch et al., 1988). Using lipofectin (GIBCO-BRL, Gaithersburg, MD),  $3 \times 10^6$  S2 cells were cotransfected with 20  $\mu$ g of the respective hook-expression construct and 5  $\mu$ g of the plasmid pPC4 (Joker et al., 1989) which confers resistance to  $\alpha$ -amanitin. Stable cell lines expressing the hook deletion proteins were established after selecting with 5  $\mu$ g/ml  $\alpha$ -amanitin and limited-dilution cloning.

### Immunoprecipitation and Western Analysis

To express truncated hook proteins at levels similar to the endogenous hook protein, transfected cells were induced by a 1-h pulse of 0.7 mM CuSO<sub>4</sub>, washed twice and then incubated in Schneider's medium (GIBCO-BRL) supplemented with 10% FCS for 16 h. After the incubation period,  $10^7$  cells were collected by centrifugation (400 g). Cell pellets were solubilized in isotonic lysis buffer (0.2% Triton X-100; 142.5 mM KCl; 5 mM MgCl<sub>2</sub>; 10 mM Hepes, pH 7.2; 1 mM EGTA; 0.5 mM PMSF; 0.1% aprotinin; 0.7  $\mu$ g/ml pepstatin; 1  $\mu$ g/ml leupeptin) for 15 min at 4°C. The insoluble fraction was removed by a centrifugation for 15 min at 20,000 g. The supernatant was added to 20  $\mu$ l of Sepharose-resins which had been coupled to the mAb 12CA5 (Wilson et al., 1984) or mAb 9E10 (Evan et al., 1985). After 1 h at 4°C, the resins were pelleted and washed three times with lysis buffer. Bound proteins were eluted by incubation in 10% SDS for 5 min at 37°C. Eluted proteins were separated by an 8% polyacrylamide gel, transferred to nitrocellulose, and detected by anti-hook antibodies (1:4,000) using ECL (Amersham Corp., Arlington Heights, IL).

## Immunohistochemistry

To assess boss endocytosis, eye imaginal discs were dissected, fixed, and stained using mAb  $\alpha$ boss1 and HRP-labeled secondary antibodies as previously described (Krämer et al., 1991; Van Vactor et al., 1991). Boss endocytosis was tested in the allelic combinations  $hk^1/Df(2L)TW130$ ,  $hk^{C1}/Df(2L)TW130$ ,  $hk^{492}/Df(2L)TW130$ , and  $hk^{492}/hk^{C1}$ , and found indistinguishable from the  $hk^1$  phenotype shown in Fig. 1 A. Hook protein was visualized using anti-hook antibodies at a concentration of 1:1,000 and Cy3- or Cy5-labeled secondary antibodies. Tissue derived from  $hk^{492}/Df(2L)TW130$ , which expresses dramatically reduced levels of hook protein (see Fig. 6), was used as negative control. Ovaries were stained as previously described (McKearin and Ohlstein, 1995).

Fluorescence-labeled secondary antibodies were detected by confocal microscopy with an MRC1000 (Bio-Rad Labs, Hercules, CA). For double staining of eye discs, anti-boss NNI rabbit antibodies (Krämer et al., 1991) and anti-delta mAb 202 (kindly provided by Marc Muskavitch, Indiana University, Bloomington, IN) were used at dilutions of 1:2,000 and 1:5, respectively. FITC-conjugated goat anti-mouse and Cy3-conjugated goat anti-rabbit antibodies (Jackson Laboratories, West Grove, PA) were used at dilutions of 1:500. Images were acquired as a Z-series with a stepsize of 0.72  $\mu$ m. The 6–8 most apical optical sections contained all the large vesicles stained with delta or boss antibodies; these images were projected into one image which was stored as a Tiff image. The images were opened in Photoshop, montaged into one figure containing images obtained from wild-type and mutant eye discs, adjusted for contrast, and printed. To quantify the difference in delta endocytosis between wild-type and mutant eye discs, we counted the number of vesicles stained with anti-delta antibodies that appeared larger than 0.3  $\mu$ m.

## Internalization Experiments

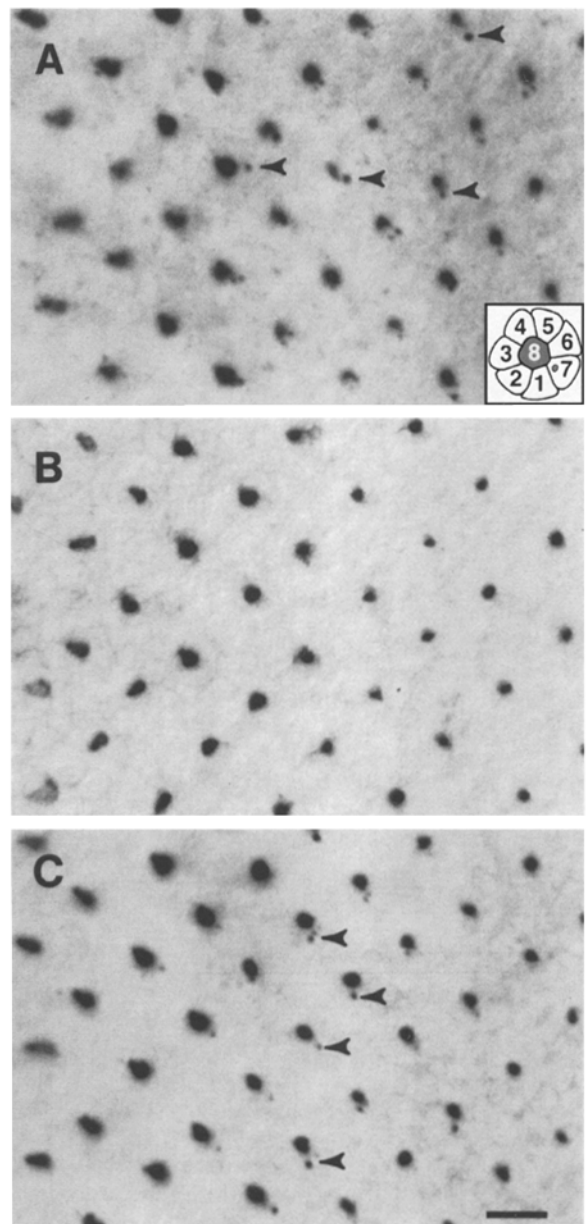
To follow internalization of boss into sev-expressing S2 cells,  $2 \times 10^6$  S2: boss and SL2-sev cells were mixed and pelleted at 400 g. After 15 min of internalization at 25°C, cells were fixed at 4°C and stained as previously described (Krämer et al., 1991; Cagan et al., 1992). Dilutions were 1:3,000 for mAb  $\alpha$ boss1, 1:1,000 for rabbit anti-hook antibodies, and 1:500 for FITC- and Cy3-labeled secondary antibodies from goat (Jackson Laboratories).

To visualize internalization of anti-sev antibodies, SL2-sev cells were incubated with a 1:500 dilution of goat anti-sev antiserum G14 (kindly provided by Ernst Hafen, University of Zürich) for 30 min at 4°C. Unbound antibodies were removed by a wash in ice-cold PBS and internalization was initiated by warming cells to 25°C. At various time points internalization was stopped by fixing the cells in a 100-fold excess of ice-cold fixative and staining as described above with anti-hook antibodies (1:1,000) and secondary antibodies from donkey labeled with FITC or Cy3, respectively (1:500). To follow fluid phase internalization, Texas red-labeled dextrans (mol wt 10,000, lysine fixable; Molecular Probes, Eugene, OR) were incubated at 1 mg/ml with SL2-sev cells. After 20 min of internalization at 25°C, cells were fixed and stained for hook protein as described above, but secondary antibodies were Cy5-labeled (1:500).

## Results

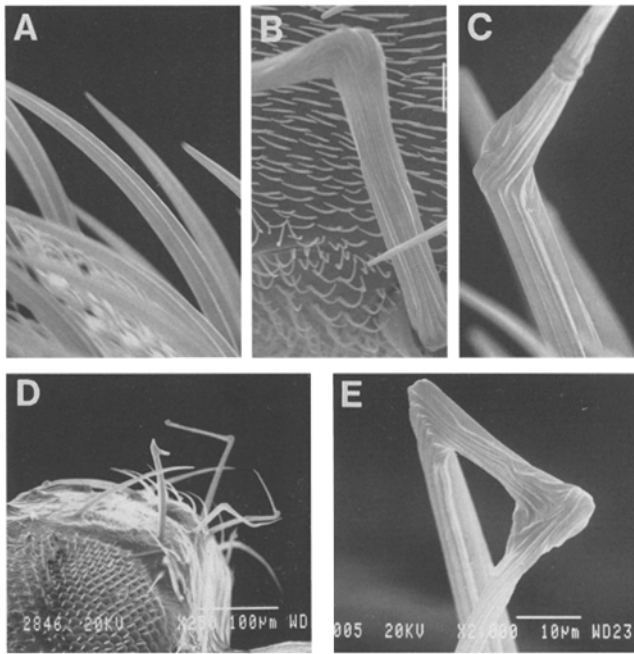
### Mutations in the hook Gene Inhibit Boss Endocytosis into MVBs in R7 Cells

Accumulation of the boss transmembrane ligand in MVBs of R7 cells can be visualized by staining eye discs with mAb  $\alpha$ boss1 (Krämer et al., 1991; Cagan et al., 1992). The specific expression of boss in a single cell in each ommatidium, the R8 cell, combined with the specificity of internalization only into R7 cells results in an easily discernible pattern of boss staining in wild-type eye imaginal discs that can be used as an assay for boss endocytosis (Fig. 1 A). Some 60 mutations, which had previously been reported to affect the *Drosophila* compound eye (Lindsley and Zimm, 1992), were analyzed for changes in the pattern of boss endocytosis (Cagan, R., and H. Krämer, unpublished observations). We discovered that mutations in the *hook* gene



**Figure 1.** Requirement of the *hook* gene for endocytosis of the boss protein into MVBs in R7 cells. Localization of the boss protein in eye imaginal discs was visualized using mAb  $\alpha$ boss1 and HRP-coupled secondary antibodies (Krämer et al., 1991). In wild-type eye discs (A) the boss protein was detected on the apical surface of R8 cells (large dots) and in MVBs in R7 cells (arrowheads). Boss staining in MVBs of R7 cells is no longer visible in  $hk^1$  mutant eye discs (B). Expression of the hook protein in R7 cells under control of the sev enhancer restores boss endocytosis in R7 cells in  $hk^1$  mutant eye discs (C). The inset in A indicates the position of the photoreceptor cells in the ommatidia. The bar in C corresponds to 3  $\mu$ m in A, B, and C. Posterior is to the right. The left edge of the images corresponds to the third row posterior to the morphogenetic furrow, the first row of boss expression.

inhibited the accumulation of boss protein in the MVBs of R7 cells (Fig. 1 B). The first *hook* mutation was recognized by its effect on bristle morphology (Mohr, 1927): macrochaetes form with sharply bent or truncated ends (Fig. 2). Based on this phenotype, several *hook* alleles have previ-

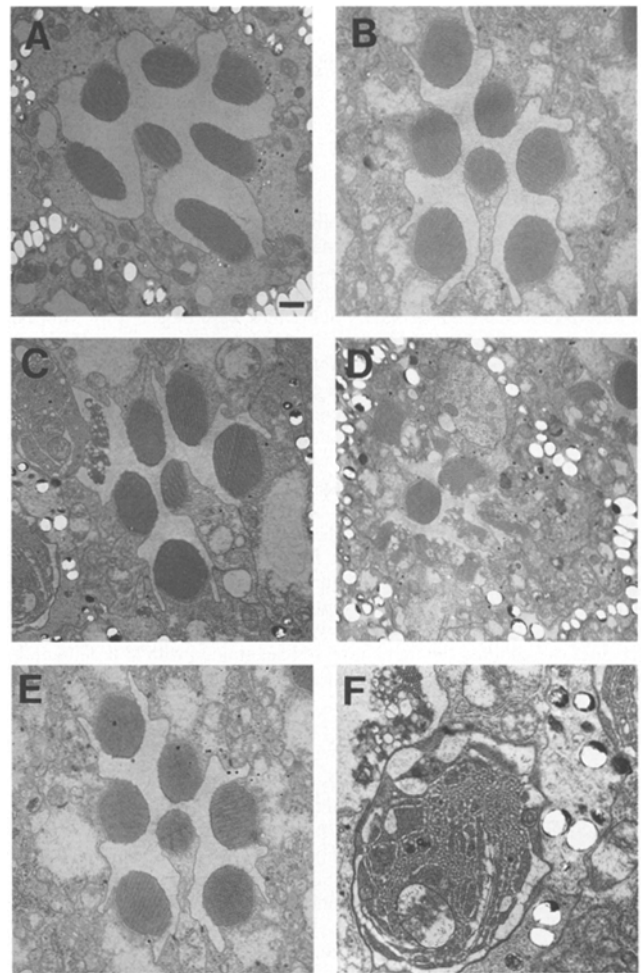


**Figure 2.** Effect of the *hook* mutation on bristle development. Macrochaetes of wild-type flies are straight and without sharp bends (A). Macrochaetes of *hk*<sup>1</sup> mutant flies, especially on the head and the thorax, exhibit frequently sharp bends (B–E). Alternatively, the tips of bristles might be shorter or blunt-ended instead of the evenly tapered end of wild-type flies. The phenotype of other *hook* alleles is indistinguishable from that of the *hk*<sup>1</sup> flies. Note that the microchaete do not appear to be affected (B). Bars: The bar in B represents 10  $\mu$ m (B) or 15  $\mu$ m (A and C); (D) 100  $\mu$ m; (E) 10  $\mu$ m.

ously been identified (Stathakis et al., 1995). We tested boss endocytosis in several combinations of *hook* alleles (see Materials and Methods) and found that boss staining of MVBs in R7 cells was reduced in all alleles indistinguishable from *hk*<sup>1</sup>.

Two different mechanisms have previously been shown to change the pattern of boss endocytosis. First, mutations in the genes *rough* and *Notch* cause cell fate changes in the developing eye disc (Tomlinson et al., 1988; Cagan and Ready, 1989; Heberlein et al., 1991). As a consequence, cells in the positions of R3 and R4 cells can adopt an R7-like fate and internalize the boss protein (Van Vactor et al., 1991). Second, the *shibire* gene encodes the *Drosophila* homologue of the dynamin protein which is directly required for the early stages in endocytosis (Kosaka and Ikeda, 1983). In *shibire*<sup>ts1</sup> mutant eye discs endocytosis was blocked at the nonpermissive temperature and boss immunoreactivity in R7 cells was greatly reduced (Krämer et al., 1991).

The *hook* mutations could affect boss endocytosis indirectly due to a switch in R7 cell fate to a cell that no longer internalizes boss (e.g., a R3 or R4 cell) or they could directly affect boss endocytosis. To distinguish between these two possibilities, we analyzed several *hook* alleles for the presence of R7 cells. In adult compound eyes, R7 cells can be recognized due to their unique morphology and position in ommatidia (Fig. 3 A). Adult eyes of *hk*<sup>492</sup>/Df(2L)TW130 mutant flies exhibited a full complement of photoreceptor



**Figure 3.** Presence of the R7 cell in *hook* mutant eyes. In transmission electron micrographs of ommatidia from adult wild-type flies (A) the R7 cell can be recognized by the small rhabdomere located in the center of the ommatidium in distal sections. In *hook* mutant flies of the genotype *hk*<sup>492</sup>/Df(2L)TW130 (B); or *hk*<sup>1</sup>/Df(2L)TW130 (E) the number of R7 cells is not affected. The homozygous *hk*<sup>1</sup> mutation (D and F) or the *hk*<sup>1</sup>/*hk*<sup>C1</sup> allelic combination (data not shown) cause strong ommatidial degeneration when the flies are raised under normal room light conditions. Degeneration is reduced, but not completely suppressed when *hk*<sup>1</sup> flies are raised in the dark (C). After 10 d in the dark, fewer than 7% of ommatidia of *hk*<sup>1</sup> flies exhibited more than two degenerated photoreceptor cells. F shows a higher magnification of a degenerated photoreceptor cell in a *hk*<sup>1</sup> mutant fly. Bars: A corresponds to 1  $\mu$ m (A–E); (F) 2  $\mu$ m.

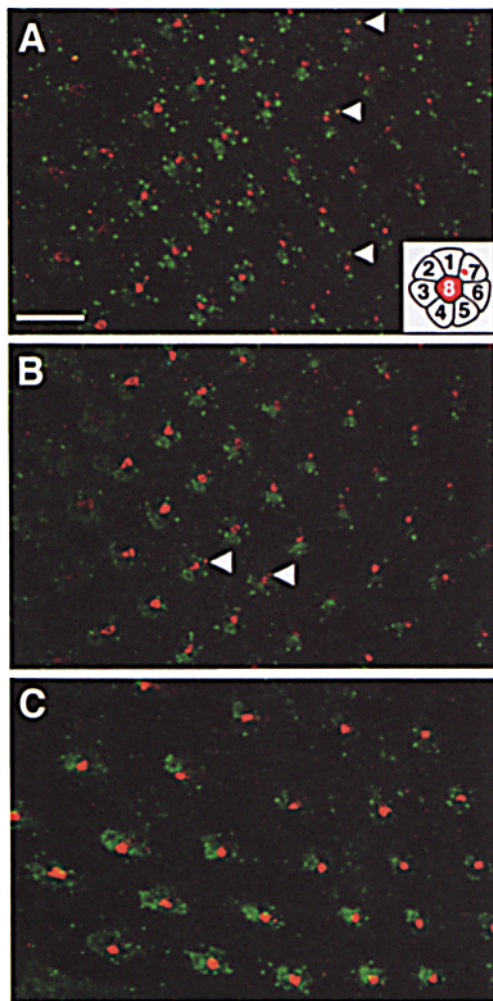
cells, indicating that the fate of *hook* mutant R7 cells was not changed (Fig. 3 B).

In *hk*<sup>1</sup> and *hk*<sup>1</sup>/*hk*<sup>C1</sup> flies, we were initially unable to score for the presence of R7 cells due to a strong degeneration of photoreceptor cells (Fig. 3 D). Light-dependent eye degeneration has previously been described for mutations in many genes that function in phototransduction in *Drosophila* (Pak, 1991; Colley et al., 1995; Ranganathan et al., 1995). We tested whether *hook*-induced eye degeneration is also light-dependent. Eye degeneration was suppressed in *hk*<sup>1</sup> or *hk*<sup>1</sup>/*hk*<sup>C1</sup> mutant flies raised in the dark even 10 d after eclosion (Fig. 3). Under these conditions

57% of ommatidia ( $n = 496$ ; four eyes) did not exhibit signs of degeneration in the *hk<sup>1</sup>* allele. In 37% of ommatidia only one or two of the rhabdomeres showed indications of degeneration as shown in Fig. 3 C. R7 cells were present in all these ommatidia. Thus, inhibition of boss endocytosis in *hook* mutants was not due to a change in R7 cell fate; instead our findings were consistent with a direct role of the *hook* gene in boss endocytosis.

### The Effect of *hook* Mutations Is Not Specific for boss Endocytosis

To address whether the effect of the *hook* mutation on en-



**Figure 4.** The *hook* mutation affects endocytosis of the delta transmembrane ligand. Eye imaginal discs were double-stained to visualize the boss (red) and delta (green) proteins. (A) In wild-type eye imaginal discs, the delta protein was found predominantly in vesicular structures in the apical region of photoreceptor cells as previously described (Kooh et al., 1993; Parks et al., 1995). Vesicles in R7 cells are frequently labeled by anti-boss and anti-delta antibodies (arrowheads) indicating that both proteins are internalized into the same MVBs. (B) In *hk<sup>1</sup>* mutant eye discs, the number of vesicles stained for boss or delta was reduced. The most prominent staining with anti-delta antibodies corresponds to the surface of cells that express delta. Vesicles stained with anti-delta antibodies are still visible but appear smaller and less intensely stained relative to wild type. In some

instances, vesicles in R7 cells appear to be stained with anti-boss antibodies indicating that boss endocytosis may be not completely blocked by *hook* mutations (arrowheads). (C) Similarly, in *shi<sup>151</sup>* mutant eye discs that were kept at the nonpermissive temperature (35°C for 1 h), endocytosis of boss and delta proteins was reduced resulting in the absence of the brightly stained vesicles. Strong staining can be observed associated with the surface of cells that express the boss and delta proteins, as previously reported (Krämer et al., 1991; Parks et al., 1995). In all images posterior is to the right and the furrow is towards the left side. The inset in A indicates the position of the photoreceptor cells in the ommatidia. Bars: (A and B) 5  $\mu$ m; (C) 3.5  $\mu$ m.

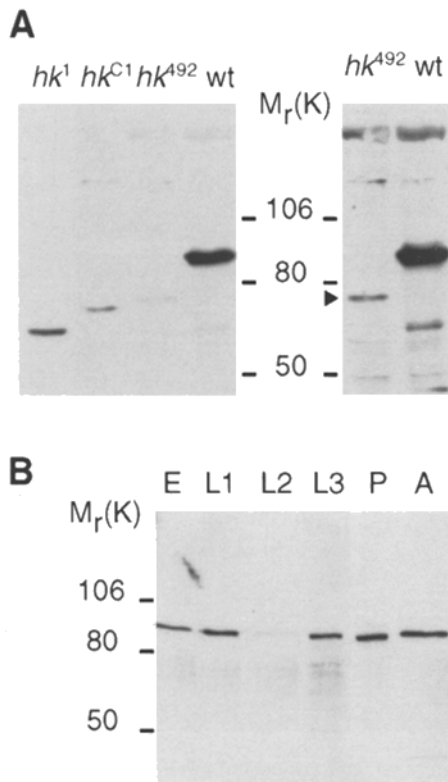
docytosis is specific for boss or also affects other ligands, we analyzed the distribution of the delta protein in eye imaginal discs. The delta transmembrane ligand is required for cell fate decisions in many tissues including the developing eye (Campos-Ortega, 1993; Muskvavitch, 1994; Parks et al., 1995). In eye imaginal discs, the delta protein has been found in MVBs in photoreceptor precursor cells (Parks et al., 1995). In R7 cells, delta protein colocalized in MVBs with internalized boss protein (Fig. 4 A) indicating that the punctate delta staining at least partially corresponded to endocytosed delta protein as previously described (Kooh et al., 1993; Parks et al., 1995). Similarly, the colocalization of delta in MVBs with the notch protein, the receptor of the delta ligand, was previously interpreted as an indication that delta might be internalized after binding to the notch receptor. However, the dynamic changes in delta expression in many cell types in the developing eye disc made it very difficult to distinguish whether the delta protein in MVBs had been expressed by the same cell, or whether it was internalized from the surface of neighboring cells (Parks et al., 1995). In *hook* mutant eye discs, the distribution of delta was changed; the most prominent staining localized to the surface of delta expressing cells (Fig. 4 B). In *hook* mutant eye discs the number of stained MVBs was reduced to 67 per 100 ommatidia ( $n = 270$ ) compared to 213 stained MVBs in 100 wild-type ommatidia ( $n = 158$ ). These numbers were likely to underestimate the effect of the *hook* mutation as they did not take into account the reduced intensity of labeling (Fig. 4 B). The change in distribution of the delta protein was similar, although less pronounced, to that observed in *shibire<sup>ts1</sup>* mutant eye discs that were placed at the nonpermissive temperature (35°C) for 60 min before dissection and fixation (Fig. 4 C). We concluded that the effect of *hook* mutations was not specific for boss endocytosis.

### The *hook* Gene Encodes a Novel Protein

The *hook* gene has previously been mapped to position 37B10 of the second chromosome next to the *DOPA decarboxylase* gene cluster which has been cloned in a genomic walk covering some 100 kb (Wright et al., 1981; Gilbert et al., 1984; Stathakis et al., 1995). Within this walk, the *hook* gene was localized to a 7-kb EcoRI fragment by restriction fragment length polymorphisms (data not shown). Germline transformation using a 6.5-kb genomic fragment containing the *hook* transcription unit yielded seven transgenic lines. Three lines with insertions on the third chro-

instances, vesicles in R7 cells appear to be stained with anti-boss antibodies indicating that boss endocytosis may be not completely blocked by *hook* mutations (arrowheads). (C) Similarly, in *shi<sup>151</sup>* mutant eye discs that were kept at the nonpermissive temperature (35°C for 1 h), endocytosis of boss and delta proteins was reduced resulting in the absence of the brightly stained vesicles. Strong staining can be observed associated with the surface of cells that express the boss and delta proteins, as previously reported (Krämer et al., 1991; Parks et al., 1995). In all images posterior is to the right and the furrow is towards the left side. The inset in A indicates the position of the photoreceptor cells in the ommatidia. Bars: (A and B) 5  $\mu$ m; (C) 3.5  $\mu$ m.





**Figure 6.** Western analysis of hook expression. (A) Extracts from wild-type (*wt*), *hk<sup>1</sup>* (*hk<sup>1</sup>*), *hk<sup>C1</sup>*/Df(2L)TW130 (*hk<sup>C1</sup>*) or *hk<sup>492</sup>*/Df(2L)TW130 (*hk<sup>492</sup>*) flies were resolved by SDS-PAGE and analyzed on Western blots. Anti-hook antibodies raised against a hook-GST fusion protein recognized a ~85-kD protein in wild-type flies. This band was absent in all *hook* alleles. Instead, truncated proteins of apparent molecular mass of 72–62 kD were detected. The two right lanes were overexposed to show more clearly the low amount of truncated protein specific for the *hk<sup>492</sup>* allele (arrowhead). Sequence analysis predicted a molecular weight of 77 kD for the hook protein, slightly smaller than observed. We do not know whether posttranslational modifications or structural features of the hook protein are responsible for the anomalous migration. (B) Western analysis of the hook protein in extracts from different stages of *Drosophila* development. Loading in the different lanes corresponds to two embryos (E), two first instar larvae (L1), one second instar larvae (L2), 0.3 third instar larvae (L3), 0.1 pupae (P), or 0.1 adult fly (A). The apparent shift in molecular weight in the lane derived from pupae was caused by the highly abundant p83 cuticular protein (Mitchell and Petersen, 1989); this protein, with similar migration, displaced the hook protein during the SDS-PAGE.

tent with the well-established function of coiled-coil domains in the dimerization of proteins.

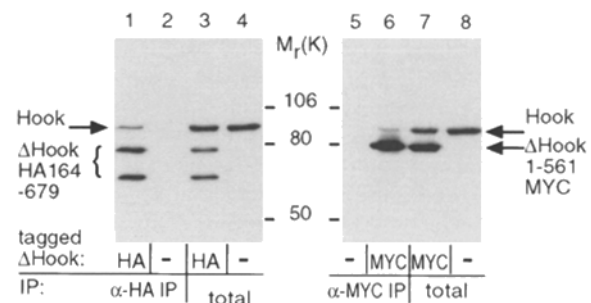
### The hook Protein Is Required in R7 Cells for boss Endocytosis into MVBs

The effect of *hook* mutations on boss endocytosis was consistent with a role of the hook protein in endocytosis within R7 cells. Alternatively, the hook protein could be required in R8 cells for the transfer of boss across the two cell boundaries. To distinguish between these two possibilities we expressed a cDNA encoding the full-length hook protein under control of a *sev* expression cassette, in which a

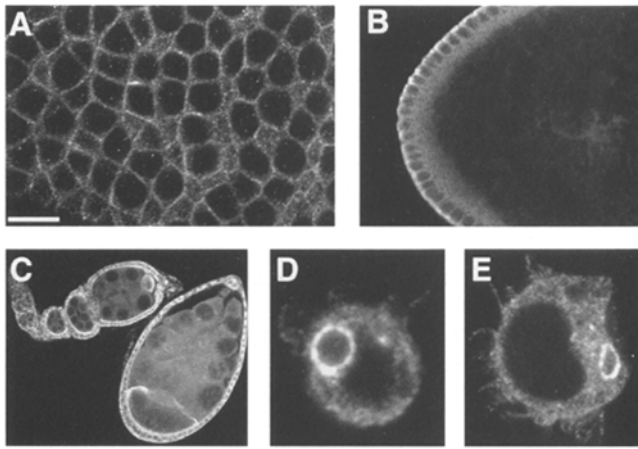
duplicated *sev* enhancer was placed in front of the *hsp70* promoter. This cassette directed expression in several cells in the eye disc including R7 but excluding R8 (Tomlinson et al., 1987; Basler et al., 1991). A similar approach had previously been used to confirm the cell-autonomous requirement of *Star* in R7 development (Kolodkin et al., 1994). P-element-mediated germ line transformation yielded nine transformed lines, four of which carried insertions on the third chromosome. These four lines were crossed into a *hk<sup>1</sup>* background and found to restore boss endocytosis (Fig. 1 C). Consistent with the expected tissue-specific expression under control of the *sev* enhancer, the bristle phenotype of the *hk<sup>1</sup>* allele was not rescued by any of the four lines. We cannot exclude the possibility that low-level expression from the *sev* expression cassette in the R8 cells provided the rescue activity. However, we consider this possibility unlikely given that all four lines tested restored boss endocytosis into MVBs in R7 cells and none of them exhibited leaky expression outside the eye disc as indicated by the lack of rescue of the *hook* bristle phenotype. In summary, these results were most consistent with a requirement for the hook protein within R7 cells for boss endocytosis into MVBs.

### The hook Protein Localizes to Endosomes

Western blots were used to follow expression of the hook protein during *Drosophila* development. The hook protein is expressed at all stages from embryos to adult flies (Fig. 6 B). Immunofluorescence was used to assess the subcellular distribution of the hook protein. In *Drosophila* embryos, shortly after cellularization, the hook protein appeared in a punctate pattern consistent with a localization to small vesicular structures in these cells (Fig. 8, A and B). The majority of the punctate staining appeared close to the plasma membrane similar to distributions that had previously been observed for other proteins localized to the



**Figure 7.** The hook protein dimerizes. Hook protein is endogenously expressed in S2 cells (lanes 4 and 8). In addition, hook proteins either NH<sub>2</sub> terminally truncated by 163 aa and tagged by an HA epitope (lanes 1 and 3) or COOH terminally truncated by 118 aa and tagged by a Myc epitope (lanes 6 and 7) were expressed under control of the metallothionein promoter (Bunch et al., 1988). Total cell extracts (lanes 3, 4, 7, and 8) or eluates from immunoprecipitates with mAb 12CA5 (lanes 1 and 2) or mAb 9E10 (lanes 5 and 6) were separated by SDS-PAGE. Hook protein was detected by Western blots using anti-hook antibodies. The hook protein endogenously expressed in S2 cells is not precipitated by either anti-HA or anti-MYC antibodies (lanes 2 and 5). However, in the presence of epitope-tagged hook proteins the full-length protein is coimmunoprecipitated (lanes 1 and 6).



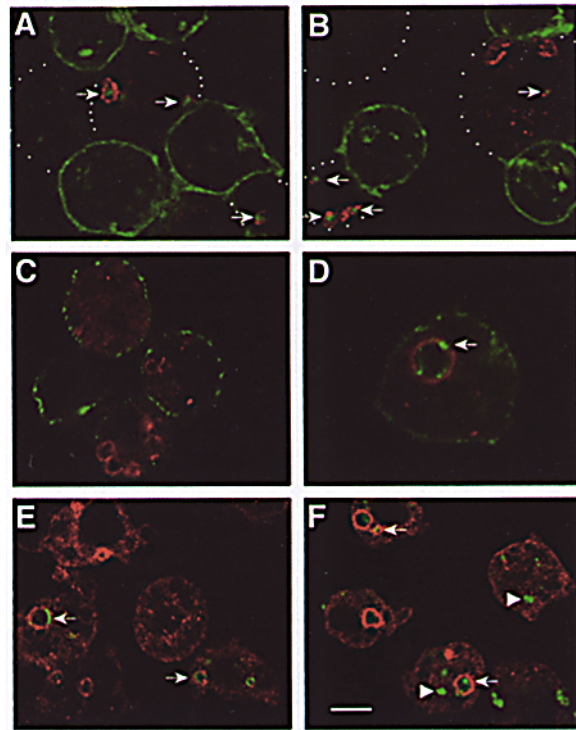
**Figure 8.** Subcellular localization of the hook protein. The hook protein was visualized using affinity-purified anti-hook antibodies, Cy3-labeled secondary goat anti-rabbit antibodies (Jackson Laboratories) and confocal microscopy. In tangential sections (A) and cross sections (B) of stained embryos the localization of the punctate hook staining to the cortical areas of cells is visible. In ovaries (C) strong expression of hook can be observed in follicle cells surrounding the germ cells. In oocytes the hook protein is most abundant in the cortical area. In cultured Schneider S2 cells (D and E), the hook protein is detected in a punctate pattern that presumably corresponds to small vesicular structures. However, the most prominent structures stained are large vesicles outlined by the anti-hook antibodies. Bars: (A) 4  $\mu\text{m}$ ; (B and C) 10  $\mu\text{m}$ ; (D and E) 5  $\mu\text{m}$ .

cortical area of cells in the *Drosophila* embryo (Schejter and Wieschaus, 1993). A similar cortical localization of hook was detected in oocytes, while the nurse cells exhibited a lower level of expression and an even distribution in the cytoplasm (Fig. 8 C). Hook protein was endogenously expressed in cultured Schneider SL2-sev cells (Fig. 7). In these cells, hook protein was detected in a punctate pattern that we interpreted as staining of small vesicular structures. In addition, large vesicles were highlighted by the hook-antibodies (Fig. 8, D and E).

Similar large vesicular structures had previously been observed when endocytosis of boss into sev-expressing S2 cells was followed (Krämer et al., 1991; Cagan et al., 1992). To test whether the hook-positive vesicular structures are the MVBs into which boss is internalized, we mixed S2 cells that either expressed boss or sev protein. After endocytosis into sev-expressing cells, boss protein was detected within hook-positive MVBs (Fig. 9, A and B).

To test whether these structures were specifically associated with endocytosis of transmembrane ligands or whether they were part of the general endocytic compartment in SL2-Sev cells, we used antibodies against the sev receptor as soluble ligands. Antibodies were bound to the SL2-Sev cells in the cold (Fig. 9 C). Internalization was initiated by warming the cells to 25°C. After 10 min of internalization, anti-sev antibodies were detected in hook-positive MVBs (Fig. 9, D and E). In these endosomes, the internalized antibodies were always unevenly distributed (arrows in Fig. 9, D and E) indicating that they only occupied a small subcompartment of the MVBs.

To follow fluid phase endocytosis, we used Texas red-



**Figure 9.** Hook-positive vesicles are part of the endocytic compartment. Internalization of the boss transmembrane ligand (A and B) or anti-sev antibodies (C–E) into hook-positive vesicles. Fluorescence-labeled antibodies were used to detect the boss protein (green in A and B), anti-sev antibodies (green in C–E), and the hook protein (red in A–F) by confocal microscopy. (A and B) Boss-expressing cells were aggregated with sev-expressing cells as described (Krämer et al., 1991). Upon binding to the sev-receptor, the boss protein is internalized into sev-expressing cells and can be detected in large vesicles outlined by the hook protein. Boss-expressing cells are outlined by the surface boss-staining. The outlines of sev-expressing cells are indicated by the dotted lines. Boss protein can be detected in vesicles in the sev-expressing cells. In many cases these vesicles are clearly outlined by the hook antibodies (arrows). (C–E) Anti-sev antibodies were bound to sev-expressing cells at 4°C to prevent internalization. After 1 h of incubation antibodies are found clustered on the surface of the sev-expressing cells (C). No binding to S2 cells not expressing sev was detected (data not shown). Internalization was started by warming the cells to 25°C. After 10 min at 25°C internalized antibodies could be detected in hook-positive endosomes (arrows in D and E). Staining frequently appears restricted to small compartments within those vesicles. F shows dextran particles (mol wt 10,000; green) internalized into SL2-sev cells. After 20 min at 25°C, dextran particles can be observed in hook-positive endosomes (arrows), but in addition, intense labeling of structures not stained with the hook-antibodies can be observed (arrowheads). Bars: F represents 3  $\mu\text{m}$  (A, B, and D); (C and E) 5  $\mu\text{m}$ ; (F) 7.5  $\mu\text{m}$ .

labeled dextrans of a molecular weight of 10,000. After 20 min of endocytosis, we could frequently localize Texas red-labeled dextrans in hook-positive MVBs (arrows in Fig. 9 F). However, many of the more densely labeled clusters of dextrans appeared not associated with hook-positive MVBs (arrowheads in Fig. 9 F). We assume that these clusters label late endosomes or lysosomes as they could be observed more frequently after 30 min of internalization than at earlier time points. We conclude that the large



vesicular structures stained by the anti-hook antibodies are not specialized compartments for the uptake of transmembrane ligands across cell boundaries. Rather they appear to be a component of the general endocytic compartment. The multivesicular morphology of vesicles into which the boss protein is internalized (Cagan et al., 1992) and the time course of internalization into the large hook-positive vesicular structures suggested that they constitute early endosomes in these *Drosophila* cells.

## Discussion

Endocytosis in *Drosophila* is required for viability as indicated by the phenotype of mutations in the *shibire* and *clathrin heavy chain* genes. Mutations in the *clathrin heavy chain* gene are lethal (Bazin et al., 1993). However, clathrin's dual role in endocytosis at the plasma membrane and in vesicle budding at the *trans*-Golgi network (Robinson, 1994) complicates the interpretation of the mutant phenotype. A more specific requirement for endocytosis has been observed for the *shibire* gene which encodes the *Drosophila* homologue of the dynamin protein (Chen et al., 1991; van der Blik and Meyerowitz, 1991). Dynamin is required for coated vesicles to pinch off the plasma membrane (Kosaka and Ikeda, 1983; Herskovits et al., 1993; van der Blik et al., 1993; Damke et al., 1994; Takel et al., 1995). Mutations in the *shibire* gene inhibit the uptake of the boss, delta, and serrate transmembrane ligands into neighboring cells (Krämer et al., 1991; Couso et al., 1995; Parks et al., 1995) similar to their effect on the secreted ligands wingless and hedgehog (Tabata and Kornberg, 1994; Bejsovec and Wieschaus, 1995). Interestingly, in preliminary experiments we could not detect a change in the internalization of wingless or hedgehog in *hook* mutant embryos (data not shown), indicating differences in the mechanism of uptake of transmembrane ligands compared to these soluble proteins.

The *hook* gene appears to be dispensable for viability. Wright and coworkers performed several screens over deficiencies that uncover the *hook* gene. They identified 16 lethal complementation groups in the *Ddc* gene cluster but all four *hook* alleles recovered were viable (Stathakis et al., 1995). In addition, the combination of two overlapping deficiencies has been reported to be viable and to display a *hook* phenotype (Wright et al., 1981). Consistent with the viability of *hook* mutations is the finding that they inhibit endocytosis of transmembrane ligands into MVBs but do not completely block it (Fig. 4).

We consider two possible explanations for these findings. First, redundant pathways might be available to bypass the specific step in endocytosis in which the hook protein functions. Redundant pathways in endocytosis have previously been observed. In HeLa cells, overexpression of dominant-negative mutants of dynamin results in an initial reduction of the level of fluid phase pinocytosis, but within 30 min the induction of a dynamin- and clathrin-independent pinocytotic pathway restores fluid phase uptake to wild-type levels (Damke et al., 1995). The observation that all *hook* alleles still express truncated proteins (Fig. 6 A) points towards the second possible explanation, that none of the *hook* alleles on hand completely eliminate *hook* function. A phenotype that allows us to assess the strength

of different alleles is the light-dependent eye degeneration (Fig. 2). When compared in *trans* to the *hk<sup>1</sup>* allele, *hk<sup>1</sup>* and *hk<sup>C1</sup>* alleles exhibit a stronger eye degeneration phenotype than the Df(2L)TW130 which completely removes the *hook* gene. These findings indicate that it is unlikely that the *hk<sup>1</sup>* and *hk<sup>C1</sup>* alleles retain *hook* function in regard to the eye-degeneration phenotype. However, the relationship between the eye degeneration and the endocytosis phenotypes is currently not well understood. Therefore, we cannot rule out that the *hook* alleles available are hypomorphic alleles.

In this paper, we have provided evidence that mutations in the *hook* gene disrupt endocytosis of transmembrane ligands across cell boundaries. It must be stressed, however, that our experiments monitor the accumulation of transmembrane ligands in vesicles at an advanced stage of endocytosis. Hook function could be required at multiple steps along the pathway leading to the endosomal accumulation of these ligands. While the pathway of receptor-mediated endocytosis has been well described for soluble ligands (recently reviewed in Gruenberg and Maxfield, 1995), such a detailed description has not yet been obtained for the internalization of transmembrane ligands across cell boundaries. Both phagocytosis and receptor-mediated endocytosis have been suggested as possible mechanisms (Cagan et al., 1992).

The phagocytosis model proposes that a large portion of one cell would be engulfed by the adjacent cell. This type of phagocytosis has been observed in vertebrate retinas where the retinal pigment epithelial cells engulf parts of the underlying rod outer segments (Dickson and Harvey, 1992). A second model proposes that uptake of the boss transmembrane ligand into the adjacent R7 cells proceeds along a pathway similar to receptor-mediated endocytosis of soluble ligands. Uptake of the transmembrane boss ligand via clathrin-coated pits would result in coated vesicles containing boss and internal membranes derived from the R8 cell surface. Such coated vesicles containing internal membranes have not yet been observed in R7 cells (Cagan et al., 1992), but immunoelectron micrographs visualizing the serotonin-induced internalization of adhesion molecules in *Aplysia* suggest that this pathway does exist (Bailey et al., 1992; Hu et al., 1993).

The actin cytoskeleton plays an active role in the engulfment of phagocytosed material (Allen and Aderem, 1995), and actin has also been implicated in receptor-mediated endocytosis from apical microvilli of polarized epithelial cells (Gottlieb et al., 1993; Jackman et al., 1994). An intriguing connection between the function of *hook* and the actin cytoskeleton is provided by the visible bristle phenotype of *hook* mutations (Fig. 2). Mutations in the *forked*, *chickadee*, and *singed* genes cause malformations of bristles similar to *hook* mutations (Cant et al., 1994; Petersen et al., 1994; Verheyen and Cooley, 1994; Tilney et al., 1995). The *chickadee* and *singed* genes encode *Drosophila* homologues of the actin-binding proteins profilin and fascin. These proteins are required for the formation of actin bundles in the early stages of extension of the bristle shaft but the function of these actin-binding proteins is not restricted to the formation of bristles, as the pleiotropic effects of *chickadee* and *singed* mutations indicate (Cant et al., 1994; Verheyen and Cooley, 1994). To date, roles of these proteins

in phagocytosis or the endocytosis of transmembrane ligands have not been tested.

In summary, we have identified a novel component of the endocytic compartment encoded by the *hook* gene. The hook protein localizes to endosomes that are common to fluid phase pinocytosis and receptor-mediated endocytosis of soluble and transmembrane ligands. Mutations in the *hook* gene revealed that the hook protein is a necessary component of the pathway that functions in the uptake of transmembrane ligands into MVBs. Only a very limited set of mutations is currently available that affects endocytosis in multicellular organisms. The addition of the *hook* mutations to this collection opens a new avenue to the genetic analysis of endocytosis.

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