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The *sidekick* gene, a member of the immunoglobulin superfamily, is required for pattern formation in the *Drosophila* eye

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SUMMARY

In the *Drosophila* eye imaginal disc the photoreceptor cells (R cells) differentiate according to a precise spatial and temporal order. The *sidekick (sdk)* gene is necessary to prevent extra R cells from differentiating during eye disc development. The extra cell appears between R3 and R4 early in R cell clusters and is most likely the result of the mystery cell inappropriately differentiating as an R cell. Mosaic analysis shows that *sdk* is required neither in the R cells nor in the extra cell, suggesting that *sdk* is necessary in the surrounding undifferentiated cells. The *sdk* gene codes for a protein that is a member of the immunoglobulin superfamily, having six immunoglobulin domains, thirteen fibronectin repeats and a transmembrane domain. The protein structure is consistent with its participation in cell-cell interaction during eye development.

Key words: *sidekick*, immunoglobulin superfamily, cell-cell interaction, *Drosophila*, eye development, photoreceptor cell

INTRODUCTION

The *Drosophila* eye comprises eight photoreceptor (R) cells, four cone cells, three types of pigment cell and the nerve-bristle group. All of the cells, with the exception of the nerve-bristle group are dependent on cell-cell interaction for their development in the eye-antennal imaginal disc (Lawrence and Green, 1979). The precise array of ommatidia begin development at the posterior end of the disc and progress to the anterior, allowing the examination of every stage of development of an ommatidium in a single disc (Ready et al., 1976; Tomlinson and Ready, 1987a; Wolf and Ready, 1991a). The R cells form a precluster just posterior to the morphogenetic furrow which demarcates the undifferentiated cells ahead of the furrow from the differentiating cells posterior to the furrow. Differentiation begins with R8, then the pairs R2 and R5, R3 and R4. The precluster also contains one or two mystery cells, so-called because their fate is unknown. They do not express neural antigens and either return to the pool of undifferentiated cells or undergo cell death (Tomlinson et al., 1987). All of the cells that are not a part of the precluster undergo mitosis after which R1 and R6, then R7 join the R cell cluster, making the final complement of 8 R cells. The remaining cells differentiate as either cone cells, three types of pigment cells, or as a member of the nerve-bristle group (Ready et al., 1976). They may also undergo cell death which eliminates any extra cells not needed for ommatidial assembly (Wolff and Ready, 1991b).

The R8 cell is established through a process of lateral inhibition (Jarman et al., 1994; Baker et al., 1990; Ellis et al., 1994; Baker et al., 1996) and presumably is instrumental in the induction of R2 and R5. The only established inductive cue by R8 is the exclusive expression in R8 of Bride of Sevenless (Boss), the ligand for the Sevenless tyrosine kinase, which is required in R7 for its development (reviewed by Zipursky and Rubin, 1994). Another tyrosine kinase, the *Drosophila* EGF receptor (EGFr) has been shown to be important for eye development (Baker and Rubin, 1989, 1992; Zak and Shilo, 1992). R1-6 cells (outer R cells) requires the EGFr (Xu and Rubin, 1993; Freeman, 1996), and expression of its ligand, Spitz, in R8, R2 and R5 induces the remaining R cells to join the cluster (Freeman, 1994b; Tio et al., 1994; Schweitzer et al., 1995a,b; Freeman, 1996; Tio and Moses, 1997). Spitz's activity is restricted by the competitive inhibitor, Argos, whose expression is dependent on EGFr activity, thus providing a regulatory feedback loop (Golembo et al., 1996). The cells that are most sensitive to the loss of the inhibitor Argos, are the mystery cells which differentiate as R cells in the absence of Argos (Freeman et al., 1992). It is unclear why the R cells are less sensitive to Argos than the mystery cells, but the effect of Argos on the R cells is dosage dependent since overproduction of *argos* causes a disruption of normal R cell development (Freeman, 1994a).

The mystery cells have been implicated as the source of extra R cells in other mutations which cause a rough eye phenotype. Of those, *fat facets (faf)* (Fischer-Vize et al., 1992a) and *groucho (gro)* (Fischer-Vize et al., 1992b) are not needed in the R cells nor in the mystery cells, suggesting a requirement in the surrounding undifferentiated cells. Faf is a deubiquinating enzyme whose expression is necessary early in eye disc development (Huang et al., 1995). Expression in the R cells will not rescue the phenotype (Huang and Fischer-Vize, 1996). This implies that inhibitory cues can emanate from cells outside the R cell cluster to restrict R cell development.

We describe the isolation and characterization of a mutation, *sidekick (sdk)*, that causes extra R cells to join the cluster. *sdk* codes for a member of the immunoglobulin (Ig) superfamily.

The superfamily is characterized by repeats that form a folded β sheet structure typically, but not necessarily, stabilized by cysteine bonds found at the ends of the repeats (Williams and Barclay, 1988). In *Drosophila*, members of this family function as tyrosine kinase receptors (Klambt et al., 1992), receptor-linked tyrosine phosphatases (Streuli et al., 1989), cell adhesion proteins (reviewed by Grenningloh et al., 1990), and regulators of cell death (Wolff and Ready, 1991b; Ramos et al., 1993). *Drosophila fasciclin II (fas II*) (Harrelson and Goodman, 1988), *fasciclin III (fas III*) (Patel et al., 1987; Snow et al., 1989), *neuroglian* (Bieber et al., 1989; Hall and Bieber, 1997), and *irregular chiasm C-roughest* (Ramos et al., 1993) are expressed on neurons and may function with other adhesion proteins to direct axon pathfinding; *fas II* is also required for proneural gene expression in the ocellar region of the eye-disc (Garcia-Alonso et al., 1995). Recently, another member of this family, *klingon*, was shown to be required for R7 cell development (Butler et al., 1997). Most of these proteins behave as homotypic adhesion proteins in cell adhesion assays in vitro. However, it is unclear whether they form functional heterotypic interactions with other adhesion proteins in vivo. The presence of Ig domains in *sdk* suggests that it functions in homotypic or heterotypic interactions in the eye during pattern formation to prevent extra cells from joining the precluster and differentiating as R cells.

MATERIALS AND METHODS

Fly stocks

Stocks used for P-element mutagenesis, *Birmingham 2*; *ry*; *y* and *CyO/Sc*; *ry Sb* P[$ry^+\Delta 2$-3]*/TM6*, have been described by Robertson et al. (1988). Flies that were used to generate mosaic eyes were P[ry^+; *hs-neo; FRT*]*18A*; ry^{506} and *MKRS*, P{ry^+, *Hsp70: FLP=hsFLP*}*3/TM6B*. The deficiencies and duplications used to map *sdk* were *Dp(1;f)101*, *Dp(1:f)135*, *Df(1)A94*, *Df(1)S39*, *Df(1)sc8*, *Df(1)260-1*, *Df(1)svr*, *Df(1)su(s)83*. All of the above stocks were obtained from the Bloomington Stock Center. The *seven-up (svp)* enhancer-trap line *AE127* was obtained from the Rubin laboratory.

Isolation of *sdk* alleles

The P-element allele, sdk^{P1}, was isolated in a F_1 screen for the X chromosome using P-elements from the *Birmingham 2; ry* line as follows. *Birm2; ry*; *y* virgin females were crossed to *CyO/Sp*; *ry Sb* P[*Δ2-3* ry^+]*/TM6* males and maintained at 16°C. The *Sb* male progeny were crossed to *C(1)DX, y w f* virgin females at 25°C and, approximately, 1700 male progeny were screened for abnormal eye morphology and for an irregular deep pseudopupil (Francheschini and Kirschfeld, 1971). Additional alleles were obtained using gamma ray mutagenesis by irradiating *y* males with 4000 rads and mating to sdk^{P1} females.

Reversion analysis

Reversion of the sdk^{P1} phenotype was carried out by mobilizing the P-element. sdk^{P1} females were mated to *ry Sb* P[*Δ2-3* ry^+]*/TM6b* male flies. The *Sb* male progeny were crossed to *C(1)DX, y w f* females and the male progeny were scored for a wild-type eye.

Mosaic analysis

Mosaic analysis was carried out using $sdk^{P1}w$ flies. Mitotic recombination was induced using the FRT/FLP system as described by Golic and Lindquist (1989); Xu and Rubin (1993). The FRT from strain P[ry^+; *hs-neo*; *FRT*]*18A*; ry^{506} was recombined onto the *w* sdk^{P1} chromosome. To generate larvae with the appropriate genotype for inducing mitotic recombination, P[ry^+; *hs-neo*; *FRT*]*18A*; ry^{506} females were crossed to males *MKRS* P{ry^+, *Hsp70: FLP=hsFLP*}*3/TM6B*. *w* sdk^{P1} *FRT18A* females were crossed to male progeny carrying *MKRS* from the first cross above. The progeny from the second cross were subjected to 38°C heat shock for 1 hour during late embryo to early 1st instar stages. Adult mosaic patches were analyzed by sectioning and microscopy.

In situ hybridization to polytene chromosomes

The P-element in sdk^{P1} was mapped by in situ hybridization to polytene chromosomes as described by Engels et al. (1986). The probe was a 588 bp *Hin*dIII-*Eco*RI P-element fragment derived from the pW8 vector (Klemenz et al., 1987) that was labeled with biotin-14-dATP by nick translation (BRL BioNick Labeling System). The signal was detected using the BRL DNA Detection System.

Histology and microscopy

Fixation and sectioning of adult eyes were carried out as described by Reinke and Zipursky (1988) with the following modifications. Eyes were fixed in 2% glutaraldehyde in PBS and subsequently, subjected to the following steps: a standard ethanol dehydration series, incubation in propylene oxide for 10 minutes, two times; 4 hours to overnight incubation in 1:1 epon araldite:propylene oxide solution, and incubation in 100% epon araldite for 4 hours.

For scanning electron microscopy, adult heads were dehydrated through a graded series of ethanol over 2 days followed by critical point drying using liquid CO_2 in a Tousimis Samdri 790 Critical Point Drier (Rockville, MD). Samples are sputter coated with gold-palladium in a Denton Vacuum Desk-1 Sputter Coater (Cherry Hill, NJ) and examined in a JEOL JSM6400 SEM using an accelerating voltage of 10 kV.

Immunocytochemistry and cobalt sulfide staining

Antibody staining was performed according to the method of Kimmel et al. (1990). Third instar larvae eye discs were dissected in 1× PBS and incubated in PEMP (0.1 M Pipes pH 7.0, 2 mM EGTA, 1 mM $MgSO_4$) for 45 minutes on ice. In some cases, the peripodial membrane was removed during this incubation to aid antibody accessibility. Discs were washed 4 times for 10 minutes in PBT (1× PBS, 0.2% BSA, 0.1% Triton X-100) and then incubated in a 1/20 dilution of an anti-β-galactosidase monoclonal antibody (40-1a from Developmental Studies Hybridoma Bank) in PBTS (PBT + 10% normal goat serum) overnight at 4°C, followed by four 10-minute washes in PBT. Subsequent incubation with horseradish peroxidase-labeled goat anti-mouse IgG (H&L) antibody (Jackson Laboratory) was performed at 1/50 dilution for 4 hours at 4°C in PBTS. Discs were washed 4× 10 minutes with PBT, stained with diaminobenzidine (0.5 mg/ml DAB, 1× PBS, 0.015% H_2O_2) and mounted in 80% glycerol in PBS.

Cobalt sulfide staining of pupal eye discs was carried out according to the method of Melamed and Trujillo-Cenoz (1975).

Isolation of the *sdk* genomic region and cDNA

Inverse PCR (Ochman et al., 1988) was performed to clone the genomic sequences flanking the P-element insertion in sdk^{P1}. sdk^{P1} genomic DNA (4 μg) was digested with *Nco*I to completion and, subsequently, ligated in 1.2 ml using T4 DNA ligase (Promega) at 0.02 U/ml for 12 hours at 15°C, followed by an ethanol precipitation and resuspension in 60 μl of H_2O. A 100 μl standard PCR reaction was performed using 1 μg of the ligated DNA for 30 cycles at 94°C (2 minutes), 55°C (2 minutes), 72°C (2 minutes, 30 seconds) and a final extension of 10 minutes at 72°C. The primer was a 31 bp oligo made to the P-element 31 bp direct repeat sequence, inverted to read outward toward the flanking genomic DNA. The 2.7 kb PCR product obtained was subcloned into pCRII T/A cloning vector (Invitrogen).

5′ RACE analysis

First strand cDNA synthesis was performed using 1 μg of third instar larvae total RNA primed with a 20 bp oligo, 5′GTAAACCGAGGAGCCTGAAG3′, using the Gibco-BRL 5′ RACE kit. The subsequent PCR procedure was followed as recommended using the adapter

primer provided and the oligo above. To ensure specificity, 2 μl of the PCR reaction was subjected to a second round of PCR with the adapter primer and a primer, 5′GCAGCTGTCGCAGTAGCAGAG3′, which is 60 bp 5′ to the first oligo. The PCR products were electro-eluted from an agarose gel and subcloned into the pCRII T/A cloning vector (Invitrogen).

DNA sequencing and sequence analysis

Double strand DNA sequencing was done by the chain termination method (Sanger et al., 1977) using Sequenase (United States Biochemical Corp.) or by the Perkin Elmer ABI 373 automated DNA sequencing system using either Sequenase or *Taq* DNA polymerase. The cDNA clone was sequenced completely on both strands. The sequence homology search was carried out using FASTA (Wisconsin GCG) and NCBI BLAST programs.

RESULTS

Isolation of *sdk*

P-element mutagenesis was carried out employing the *Birmingham* line to isolate mutations on the X chromosome which affect eye development. F_1 male flies were screened and *sdk* was identified by its rough eye phenotype. Examination of *sdk/+* females indicated that it is a recessive gene. A multiply marked X chromosome was used to map *sdk* to the tip of the X chromosome, distal to the *w* gene. In situ hybridization to polytene chromosomes using a P-element probe showed that *sdk* is located at 1B14-1C1. Deficiencies [*Df(1)svr* and *Df(1)su(s)83*] and a duplication [*Dp (1;f)101*] that cover this region were used to confirm the P-element localization.

To establish that the P-element is responsible for the mutation, reversion analysis was carried out to obtain wild-type revertants. *sdk* reverted to wild type approximately 2% of the time in hemizygous males. Pcr experiments were performed on *sdk* revertants to establish whether the P-element had been removed. Two revertant lines were examined and sequence analysis revealed that both retained some P-element sequence. sdk^{rev104} retained 28 bp of the P-element and 8 bp of the direct repeat that was generated when the P-element inserted. sdk^{rev104} retained 46 bp of the P-element and the 8 bp repeat. Those that reverted to wild type had multiples of three nucleotides left, maintaining the open reading frame. Three lines were examined that initially were thought to have a rougher phenotype than sdk^{P1}. When established as lines these flies displayed the same variability in roughness as sdk^{P1}. Those that did not revert retained P element sequence also, but did not maintain an open reading frame.

We performed a gamma ray mutagenesis to obtain additional alleles of *sdk*, and isolated four new alleles, sdk^{7AX}, sdk^{N23}, sdk^{N6} and sdk^{D14}. One allele, sdk^{N23}, shows a cytological rearrangement in the 1C region and sdk^{D14} has a 5 bp deletion in the coding region. sdk^{N23}, sdk^{7AX} and sdk^{D14} will be discussed in more detail below. None of the new alleles has a more severe phenotype than sdk^{P1}, and no alleles of *sdk* over deficiencies which uncover *sdk* are more severe. This and the molecular analysis of *sdk* alleles presented below indicate that sdk^{P1} most likely represents the null phenotype.

sdk causes abnormalities in R cell development

The R cells were examined by light microscopy which shows that *sdk* causes extra R cells to develop in approximately 10% of the ommatidia. The extra R cell can be either an outer, R1-6 type, or an inner, R7 type (Fig. 1). In more proximal sections R8 is located in its normal position and is present in every ommatidium (not shown). Although the position and type of the extra outer R cell cannot be determined by examining the adult R cells, the extra inner R cell is always located between R3 and R4, the position the mystery cell occupies early in ommatidial assembly. This suggests that the extra R cells that occur in *sdk* are derived from an inappropriately recruited mystery cell.

sdk affects early ommatidial assembly

We were interested in finding the earliest stage of ommatidial development that is affected by *sdk*. Since pigment cells are missing, the extra R cells could be caused by misdirection of the pigment cell fate to an R cell fate late in ommatidial assembly, signifying that *sdk* is primarily involved in specifying cell fates at late stages of eye development. To address this question we employed a *seven-up (svp)* enhancer trap line, *AE127*, which expresses *lacZ* in R1,6,3, and 4 early in eye disc development (Mlodzik et al., 1990a). *sdk;AE127/ +* discs were examined for *lacZ* expression. There is an extra staining cell between R3 and R4 in *sdk*; *AE127/ +* discs just posterior to the morphogenetic furrow (Fig. 2). No extra cells were observed near R1 or R6. We also examined a line that stains only in R8, *A2-6* (Mlodzik et al.,

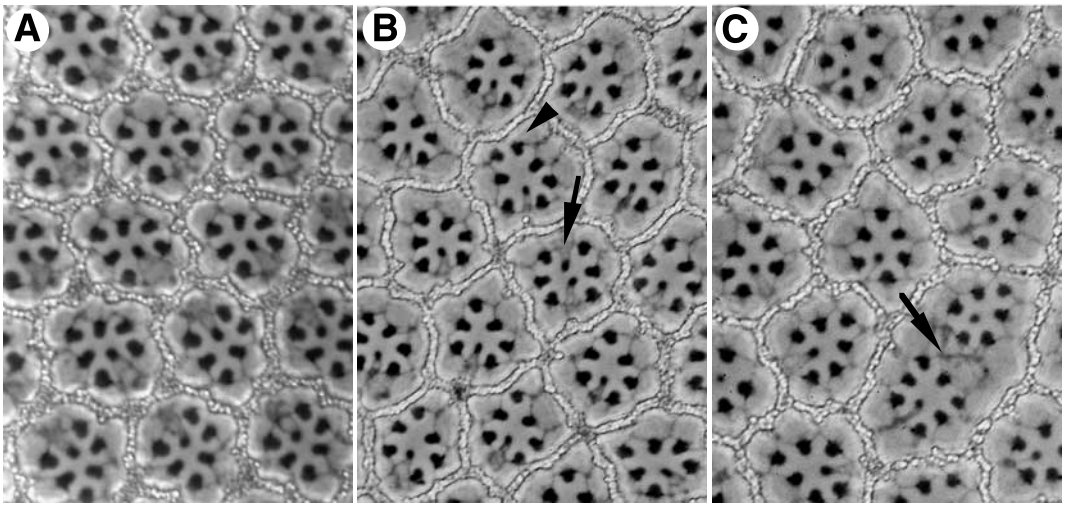


Fig. 1. Extra R cells differentiate in *sdk* eyes. Sections of adult wild-type (A) and *sdk* (B and C) eyes show that *sdk* has extra R cells in some ommatidia. The arrow in B indicates an extra inner R cell and the arrowhead indicates an extra outer R cell type. In C an arrow indicates a missing pigment cell between ommatidia. In both A and B some ommatidia are misoriented.

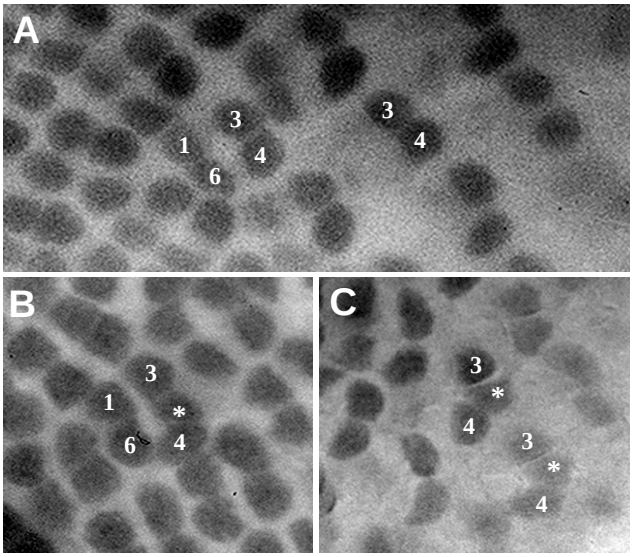


Fig. 2. Stained eye imaginal disc demonstrates early *sdk* phenotype. A *svp* enhancer trap line, *AE127*, was stained with anti-β-galactosidase. *AE127*/+ (A) shows the wild-type *svp* staining pattern. *svp* stains R3 and R4 near the furrow, and R3, R4, R1 and R6 in older ommatidia more posterior to the furrow. *AE127*/+; *sdk* discs (B,C) show an extra β-galactosidase staining cell between R3 and R4 indicated by an asterisk. This is the position normally occupied by the mystery cell. The ommatidium marked in B is mature, with staining in R1, R6, R3, R4 and the mystery cell. The ommatidium in C is closer to the morphogenetic furrow so only R3, R4 and the mystery cell stain for *svp*. Anterior is to the left.

1990b), and found no extra cells staining with this marker (data not shown). These results indicate that *sdk* affects early R cell development and that the extra outer R cell is most likely a mystery cell that has remained with the R cell cluster, differentiating as an R3 or R4.

sdk affects multiple stages of eye development

Scanning EM analysis shows that *sdk* causes pattern defects at multiple stages of eye development. Ommatidia are often fused and the bristle pattern is aberrant (Fig. 3). Some bristles are missing and at other positions two bristles are observed (not shown). This phenotype is very similar to the dominant phenotype of *Delta (Dl)* /+, a neurogenic mutation also affecting multiple stages of eye development (Dietrich and Campos-Ortega, 1984; Parks et al., 1995). The scanning EM also revealed defects that appear as holes in the lens (not shown); this is most likely the result of cone cell defects.

We also observed missing secondary pigment cells between ommatidia (Fig. 1); approximately o.4% of the pigment cells are missing. Pigment cells are quite often affected when there is a large overproduction of R cells and cone cells. However, in *sdk* the number of extra R cells observed is modest and the loss of pigment cells is not necessarily associated with the same ommatidium that has an extra R cell. The effect on the pigment cells is most likely caused by an independent requirement for *sdk* in later eye development.

We also examined the cone cells in *sdk* since the results from the scanning EM indicated that there might be problems either in their function or development. Cobalt sulfide staining of pupal cone cells revealed that 2.9% of the ommatidia have extra cone cells and 0.74% have too few cone cells (Fig. 4). It is not clear whether this is an indirect effect caused by the R cell defect, or whether this represents a separate function for *sdk*. We have not determined if an ommatidium that has an extra R7 cell is the same ommatidium that is missing a cone cell. The cone cell fate is closely related to the R7 cell fate (Tomlinson and Ready, 1987b). However, the percentage of extra R cells is much greater than the percentage of missing cone cells.

sdk behaves nonautonomously in R cell development

R cell development is dependent on both inductive and inhibitory mechanisms that result in the invariant number of eight R cells per ommatidium. It is crucial to know which cells require *sdk* to begin to understand the mechanism by which this gene acts. Mosaic analysis of a null allele of *sdk*, sdk^{P1}, was carried out using the FLP/FRT method of generating somatic clones in the eye (Xu and Rubin, 1993). Only mutant ommatidia could be scored because *sdk* does not affect all ommatidia. The results from 55 mosaic eyes are shown in Table 1. The extra R7 cells can be mutant or wild type for *sdk*, indicating that *sdk* is non-autonomous. Although we cannot definitively identify and score the extra outer R cell type, two mutant ommatidia were found in which all of the R cells, including the extra cell were wild type. Thus, *sdk* is non-autonomous and is not required in the R cells or in the extra R cell. We have also noted that a phenotypically mutant ommatidium is quite often surrounded by wild-type ommatidia. This suggests that

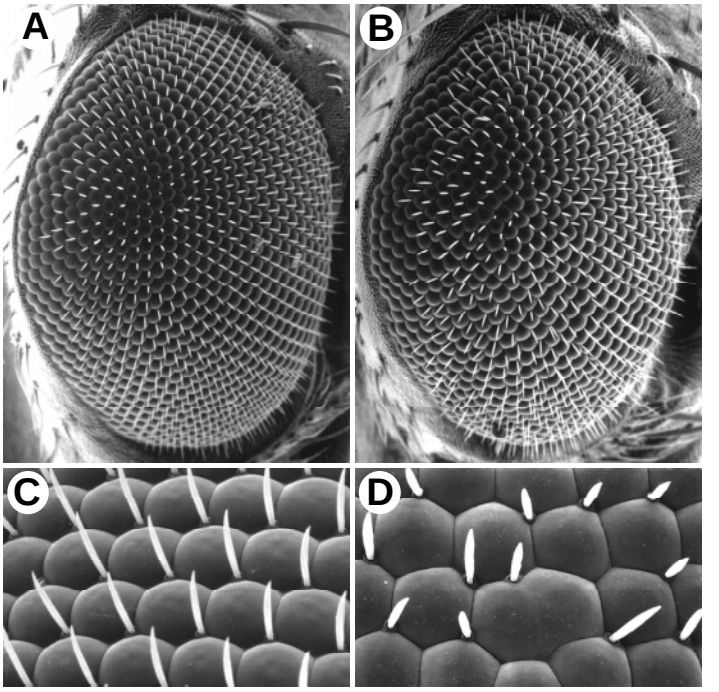


Fig. 3. Scanning electron micrograph of adult eyes. (A,C) Wild type, (B,D) *sdk*. C and D show an enlargement of A and B respectively. In B the *sdk* eye is mildly rough, and in D *sdk* is missing some bristles; some facets are fused and malformed.

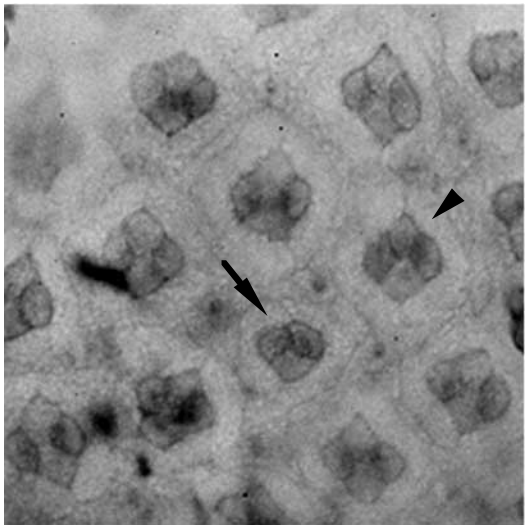


Fig. 4. Cone cell phenotype of *sdk*. Cobalt sulfide staining of a *sdk* pupal eye shows that *sdk* is missing a cone cell in some ommatidia (arrow). Arrowhead points to a typical wild-type facet with four cone cells. Some facets have an additional cone cell (not shown).

Table 1. Mosaic analysis of *sdk*

Cell	w^+ sdk^+	w^- sdk^-
R1	37	32
R2	17	52
R3	18	51
R4	32	37
R5	27	42
R6	35	34
R7*	35	32
R8*	27	27
Extra†		
outer R	35	32
inner R	2	0

*69 mutant ommatidia were scored, but because of the difficulty in scoring R7 and R8 for the presence of pigment granules, the genotypes of these cells in some ommatidia are not reported.

†The extra R7 can be identified as opposite that of the endogenous R7. The position of the extra outer R cells is assumbed to be between R3 and R4, based on the eye disc staining pattern of extra R cells in *sdk* mutants.

sdk cannot act over long distances, and if *sdk* is secreted, it cannot diffuse over a distance of more than a few cells.

Genetic interactions with *sdk*

Three other genes, *faf, gro* and *argos* affect the same stage of development and have a phenotype similar to *sdk*. Since *sdk* is not fully penetrant, we tested these genes for dominant genetic interactions with *sdk* to observe whether they would increase the penetrance of the *sdk* phenotype. These genes were tested for dominant genetic interactions with *sdk* as heterozygotes in *sdk* males. No change in the *sdk* phenotype was observed. We also analyzed members of the *sev* pathway for interactions with *sdk*. *sos*, *gap*, *ras*, *raf*, and *yan* showed no effect on *sdk* when heterozygous in *sdk* males. Both *Ellipse* and *torpedo*, alleles of the *Drosophila* EGF receptor, were examined as heterozygotes in *sdk* males, and were found to have no effect on *sdk*.

Since extra R7s appear in *sdk*, we generated flies that are *boss*;*sdk* to test the dependence of the extra R7s on the *sev-boss* interaction. The extra R7s formed in *sdk* are independent of *boss* (data not shown), indicating that *sdk* does not act by increasing the activity of the Ras pathway through the Boss-Sevenless interaction.

Isolation of *sdk* genomic region and cDNA

sdk^{P1} genomic DNA flanking the P-element insertion site was isolated by inverse PCR (see materials and methods) using oligos complementary to the ends of the P-element. A 2.7 kb genomic fragment was subcloned and used as a probe to isolate phage and cosmid clones from *Drosophila* genomic DNA libraries. Overlapping phage DNA fragments were isolated that cover 80 kb of genomic DNA and a cosmid clone was isolated that includes much of the region proximal to the P-element (Fig. 5A).

The cosmid clone was used as a probe to screen an eye disc cDNA library and four cDNAs were obtained from 1×10^6 plaques. The full-length cDNA was isolated by walking 3′ from the original cDNAs, using both eye disc and pupal cDNA libraries. RACE analysis of the 5′ end of the cDNA revealed the presence of three species of mRNA. Sequence analysis of these mRNAs indicate that there is alternate splicing between exon 1 and exon 2. There are three donor sites in exon 1 and one acceptor site in exon 2 (Fig. 5B). This occurs in the 5′ untranslated region and does not change the amino acid sequence of the open reading frame.

The P-element insertion is at the 3′ border of exon 2, just preceding the intron. Molecular analysis of sdk^{N23} revealed a

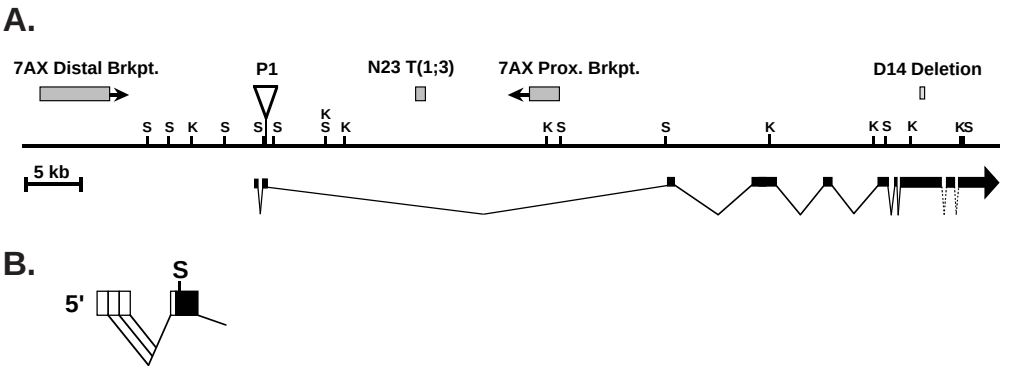
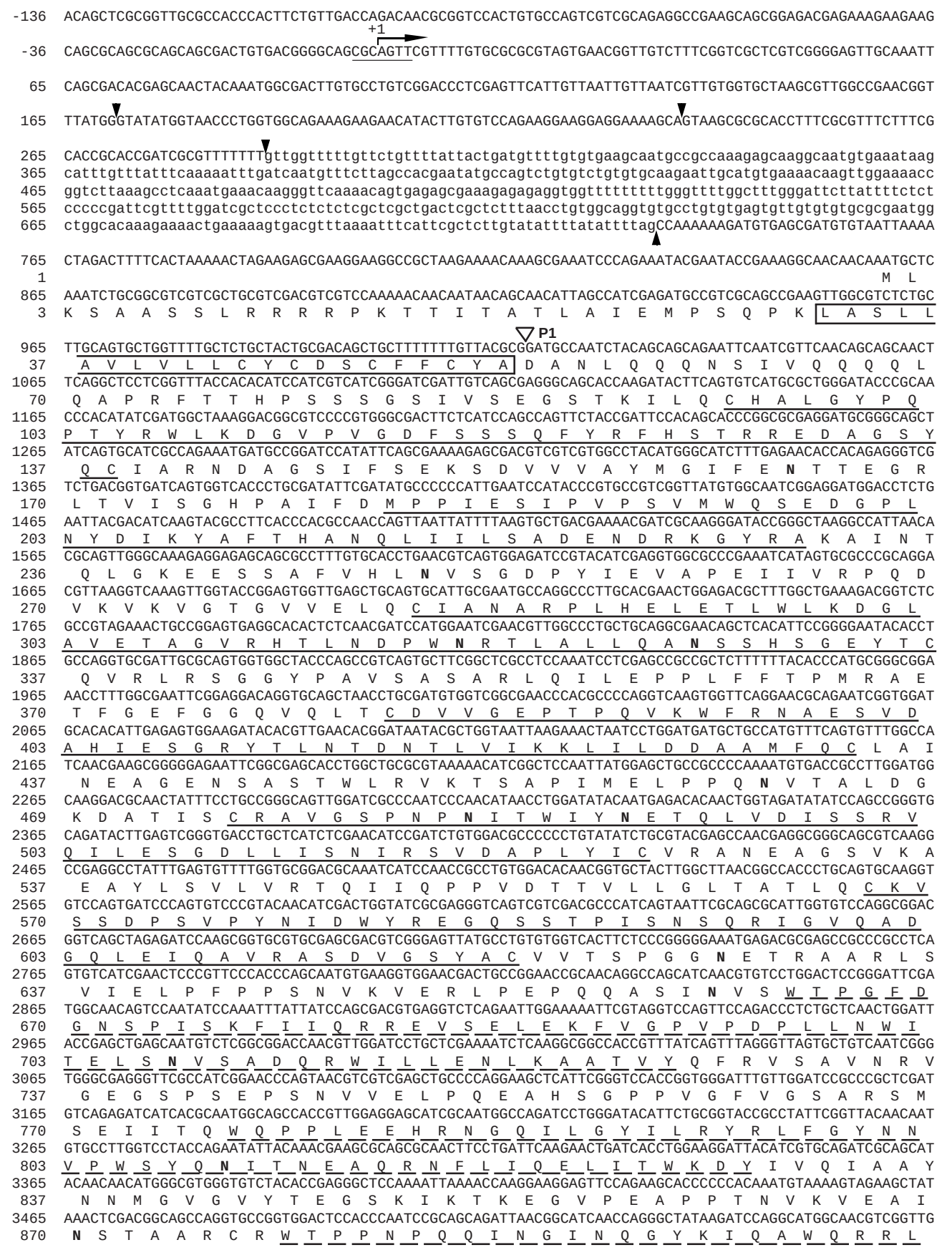


Fig. 5. Structure of the *sdk* gene and cDNA. (A) A restriction enzyme map of the *sdk* genomic locus is shown, spanning 62 kb. The site of the P-element insertion that was used to isolate flanking genomic DNA is marked. The position of the breakpoints for two alleles, sdk^{N23} and sdk^{7AX} is indicated as well as the position of the 5 bp deficiency in exon eight found in sdk^{D14}. The structure of the *sdk* cDNA is shown below the restriction enzyme map of the genomic locus. The positions of the eighth intron is approximate and is based on restriction map differences between genomic DNA and cDNAs. All other exon-intron boundaries were determined by DNA sequence analysis. S, *Sal* I; K, *Kpn* I. (B) Enlargement of exon 1 and exon 2 showing alternate splicing in exon 1.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P1

Fig. 6 continued overleaf

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7065  TGTAGGCACACTGCGAAGCGGTACCTTGGGGACTCTGGGCAGAAAGTCTACCAGTCGACCGCCGCCGGGTGTCCATCTCGGCAAGAGTCCGCCACGCCCA
2070   V  G  T  L  R  S  G  T  L  G  T  L  G  R  K  S  T  S  R  P  P  P  G  V  H  L  G  K  S  P  P  R  P
7165  TCGCCCGCTTCGGTGGCGTATCACAGCGACGAGGAGAGCTTGAAGTGCTACGACGAAAATCCAGACGACAGCAGCGTGACCGAGAAGCCCTCGGAGGTCA
2103  S  P  A  S  V  A  Y  H  S  D  E  E  S  L  K  C  Y  D  E  N  P  D  D  S  S  V  T  E  K  P  S  E  V  S
7265  GCAGCTCGGAGGCATCGCAGCATTCGGAGAGCGAAAACGAGAGCGTGCGCAGCGACCCCCACTCCTTTGTGAATCACTATGCCAACGTGAATGACTCACT
2137    S  S  E  A  S  Q  H  S  E  S  E  N  E  S  V  R  S  D  P  H  S  F  V  N  H  Y  A  N  V  N  D  S  L
7365  GCGACAGTCGTGGAAGAAGACCAAGCCGGTGCGAAACTACTCCAGCTACACCGATTCGGAGCCGGAGGGCAGTGCCGTGATGAGCCTCAATGGTGGCCAG
2170   R  Q  S  W  K  K  T  K  P  V  R  N  Y  S  S  Y  T  D  S  E  P  E  G  S  A  V  M  S  L  N  G  G  Q
7465  ATTATCGTCAATAATATGGCCAGGTCGAGGGCACCACTGCCCGGCTTCTCGTCATTCGTCTGACAATCAACTGAGTTCTAAGATCTAAGCACCAGTAGCA
2203  I  I  V  N  N  M  A  R  S  R  A  P  L  P  G  F  S  S  F  V  *
7565  ACGGCACCGTCATCCGCGAGACCTTCGTCTAGGCTGAGAACGAGGAGGGTCCGAAGAAGGAACCCGGAGCTGGGGGGTTGGCTGGGGCCACTCGAACCCG
7665  TTGGAGGACGACACACACACCCACATATCAGACAGGCCCTCCTGCCCTCAGCCCCTCCACCTTCACGACGATACGCTGCTACAGGGATATACATATTACT
7765  TAATACCAGACATTCAGTAAGATTATCTTGTATGCATTTAGTTTGAGTTCTTAATTATGTGCTTTTTACGTTGGGTTGTGCACTTCCGCGTCTTAACGTT
7865  ATCCTTTTAACTAAAAATGTTTGTATGCCCAGTACTTCCCATACTCGTAAAACGTATAAATTTGACCAACAGGCATTAATTGAAGTAATATTTTTAAATA
7965  TGCATAACCTAGTGTAAGGAGCACATTATTTTCAGCGTCAGTTTCCCAGCATGACTCTGCTCCTATTTTATAGTATTTATTTTTGGATGTACAGTGTGAG
8065  TGCGATGCTGCCTGCCACGCCTCCAGATGTGGCATTTATGGAATAGGGCGCGACAAATCGAATCCGAATCCTGTGATAAAACGATTTCCTTGTGCCCTAA
8165  TAAGTTTTTCCAATGATTTCCAAGTGTGTTCAGCGCGTGGCAGGTAGTCTGGTCTTTTAAGTGACTGTGAGTGTGAGTGACGTACGCGCTTCGTCTGCGC
8265  AATCTCATTATATATAAAATGACAAAAGGTTATGTTTTATGTTTAGTTTTTTGTGCGAGTAAATCTAGAAAATCTACAAGTACGAAGACAAGGACAAAAC
8365  ACCGATGCAATCGGTGGCCTAATAAGAAATGAAGAAAACGAGTTCATGAAACACGCGAAAGAAATCATACGCAGTAAACGAAAGTCTTTCTCTTATAAGA
8465  AAGGATAAGTGTATTTTTTTGGAAAGGCATCGAAGAAAGGCTATAGCCATGAGCATGAGAAGAATATCTAAGAAAGATGGCTTAGGTAATATTGATAGGC
8565  CATATTATTGCAATTTCCCTACCACATAAGTTCACAAACGCAAGCAAAAGTAACACAAAGCAAACAAACAAACAAACGTAGCAAACATGCAAGCATTCAA
8665  AAGCTGCATAGCACACAAACAATCGAAGAATCGATTCGTTCAGAGCGCATGCGTAGCAGATTTAGCATTAAGTACGATCTTCTAGAGGTAAGATGCATTT
8765  TTTTTTGTTAGTTGGGGGCCAGTCTCTACGGGGAGACTTTGCCCTGATACTCTAGATACGTAATTTTCGATTGTTTAATCCCCGAAGCTTAGAGATAATT
8865  GTACTCGTGTTTGTATTTGAATGCGGTTTATATTTATGACTATATTTATATATCGATGTATACATGTATATCTAAAAACTTAAACGACTGATTATATATT
8965  TTCCCCTGATTAGTTGTATACTTAACTCCTTTTTTGTAATACCTATGCATTAACTATGCTAAGACCACTTTGGCACTTTGGCGACCGTTGAAATGAAAAC
9065  GGAGTGGATAACTTTCTGCGTTTTCCCCAAATCGCAAGCTAATTTCAACTTCAAAGACTCAAAGCACAAGTTCATCTAGTTAAGTAAATGTATTTGTAAT
9165  TGTTGAATTGATGATTAAAGGATAGTTCTCCACTAATTGAGGAGGCACGTTTGATGTGGAGGAACTATGGAGATGAAAAAGGTGGAGCTTAGGGATCTTT
9265  CGCTCGTTGCTAGTTGAAATTCGTGCAACAAGCGCAAAGTATTAAAATATTGATAAATACAATTTAAATGTGTAAATGTAATTGATCGAGCATCCGAATA
9365  TGAATCAATAAAAGAACATGAAATCAATGGAAAAAAAAAAAAAAAAAA   9412
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Fig. 6. DNA sequence and predicted amino acid sequence of *sdk* cDNA. The sites for alternate splicing in the 5′ non-coding region are indicated with solid arrowheads; downward pointing for the three 5′ sites and pointing up for the single 3′ site. The hydrophobic potential internal signal sequence and transmembrane domains are boxed. The P-element insertion site is indicated with an open arrowhead. The six Ig repeats are underlined with solid lines and the thirteen fibronectin repeats with broken lines. The five bp deficiency in sdk^{D14} is indicated by a shaded box. In the last fibronectin repeat a mini exon is inserted between amino acids 1923 and 1925. Instead of reading RDDS (shown in bold), the second D is replaced by EKGEPSFVY. Potential glycosylation sites are indicated by bold type. The putative polyadenylation site is doubly underlined. The GenBank accession number is BankIt 95112 U88578.

breakpoint in the second intron, and cytological analysis indicates that this breakpoint is caused by a translocation of the tip of the X chromosome to the third chromosome. sdk^{7AX} is caused by a small inversion from the second intron into the 5′ flanking region of the gene. The sdk^{D14} allele has a 5bp deletion in exon eight that interrupts the open reading frame (Fig. 5A).

sdk is a member of the Immunoglobulin superfamily

The sequence and conceptual open reading frame of *sdk* is shown in Fig. 6. *sdk* is a 240 kDa member of the immunoglobulin superfamily. It contains six Ig repeats, thirteen fibronectin type III repeats, a transmembrane domain and a 200 amino acid intracellular domain. The intracellular domain contains no homology to any known proteins. There is a hydrophobic region near the N terminus which may function as an internal signal sequence. Alternate splicing in intron 9 results in the insertion of nine amino acid in the thirteenth fibronectin repeat. We do not know what percentage of the mRNAs have the micro exon. Of the 3 cDNAs isolated that cover this region only one has it.

DISCUSSION

It is unclear how the mystery cells are excluded from becoming R cells. They are a part of the precluster that forms in the morphogenetic furrow, and although they do not express neuronal antigens, they express Sevenless which is also expressed in a subset of R cells and the cone cells (Tomlinson et al., 1987). The expression of Sevenless in the mystery cells is transitory, but indicates that the association with the precluster is more than just a physical association and that expression of this marker may suggest a propensity for the mystery cells to differentiate as R cells. No genes have been identified that are required in the mystery cells to cause their exclusion. An inhibitory cue from R3 and R4, the two R cells in closest contact with the mystery cells, has been implicated via the *svp* gene (Mlodzik et al., 1990a). However, *svp* is a transcription factor required for the differentiation of R3 and R4 and is unlikely to be directly involved with a signal from R3 and R4 to the mystery cells.

The mystery cells are also different from other cells in the precluster with respect to their sensitivity to Argos. Argos is a secreted protein expressed in all cells posterior to the morphogenetic furrow and is required to prevent the mystery cells from differentiating as R cells (Freeman et al., 1992). Argos acts by interfering with the EGFr pathway which is required for R cell differentiation (Xu and Rubin, 1993; Tio et al., 1994; Schweitzer et al., 1995a; Freeman, 1996; Tio and Moses, 1997). Although all the cells in the precluster are exposed to Argos, the mystery cells must be more sensitive to Argos action, possibly because of lower EGFr activity in these cells. It is possible that the position of the mystery cells causes a lowered initial induction of this pathway because of a restricted access to Spitz (Freeman, 1996) or that its position makes it more susceptible to cues from the surrounding pool of undif-

ferentiated cells. Thus, the undifferentiated cells may play a more active role than previously assumed in determining the fate of cells in the precluster.

sdk, a member of the Ig superfamily, affects the fate of the mystery cell during early pattern formation in the eye disc. It is also needed for cone cell and accessory cell differentiation at later stages of development. Sdk contains six C2-type Ig repeats and thirteen fibronectin type III repeats. A potential transmembrane domain separates the extracellular region from a 200 amino acid intracellular domain. The intracellular domain has no homology to any known proteins. *sdk* is not required in the R cells nor in the mystery cell and is, therefore, presumably necessary in the surrounding undifferentiated cells to prevent the conversion of the mystery cell to an R cell.

We have shown that *sdk* is needed at the precluster stage of development when R2, 3, 4 and 5 are determined. At this time one or two mystery cells are present in the precluster between R3 and R4. The mystery cells do not express neural antigens and normally leave the precluster. In *sdk* eye discs an extra cell stains with *svp*, an R1, R3, R4, and R6 marker, and most likely represents the conversion of a mystery cell into an extra R cell. We never saw more than one Sca-expressing cell (R8 marker) nor an extra cell appearing later near R1 and R6.

Two other mutations, *faf* (Fischer-Vize et al., 1992a) and *groucho* (Fischer-Vize et al., 1992b), also behave non-autonomously and are also not required in the R cells nor the mystery cells. It is unclear how Faf, a deubiquinating enzyme (Huang et al., 1995), acts in preventing the mystery cells from joining the R cell cluster. Groucho, a nuclear protein that negatively regulates hairy-related bHLH proteins (Paroush et al., 1994), may act by interfering with neuronal differentiation, but it is not required in the mystery cells so its action must be indirect. Thus, no obvious common pathway emerges from an examination of the potential functions of these three proteins.

The Ig superfamily contains functionally diverse members ranging from putative cell adhesion proteins such as Neuroglian, Fas II, and Fas III in *Drosophila* and NCAM and L1 in vertebrates to tyrosine kinase receptors and receptor-linked tyrosine phosphatases (Grenningloh et al., 1990). One member of this family in *Drosophila*, Amalgam, is secreted and has no known function (Seeger et al., 1988). Members of the so-called cell adhesion family function as homophilic adhesion molecules in cell aggregation assays in vitro, suggesting a role in cell-cell interaction and recognition. Many members of this family, including Fas II, Fas III, Neuroglian, and NCAM express two forms of protein, one form with an intracellular domain and another that is linked to the membrane with a PI-anchor or has a minimal intracellular domain (Grenningloh et al., 1990). It has been shown that *neuroglian*, can function as an adhesion molecule in vitro with or without its intracellular domain (Hortsch et al., 1995). It is not clear how these proteins function during axon pathfinding and generally mutations in the genes that code for them cause subtle phenotypes (Bieber et al., 1989; Elkins et al., 1990; Grenningloh et al., 1991). The subfamily of Ig adhesion proteins may be redundant, with many members contributing to the same activity. *sdk* does not affect all ommatidia and this may reflect a similar redundancy of function in the eye.

Fas II had previously been shown to be needed in axon pathfinding in the MP1 pathway (Grenningloh et al., 1991) and recently was demonstrated to be required for proneural gene expression in the developing ocelli (Garcia-Alonso et al., 1995). It is unclear whether Fas II is functioning in signal transduction in both situations or only during proneural gene induction. A striking example of the separation of function between the extracellular domain and the intracellular domain is the *irregular chiasm C-roughest* locus. It is needed for correct axon pathfinding and for the control of cell death in the eye. Deletion of the intracellular domain affects its role in apoptosis without affecting its role in axon pathfinding (Ramos et al., 1993; Reiter et al., 1996).

We have found no evidence that *sdk* expresses different forms of the protein similar to that observed with other members of this family that act as adhesion proteins, since we have not seen any evidence of alternate splicing in the region that includes the transmembrane domain. Also, if there is a secreted form, it acts over a very short range since neighboring wild-type cells at the edge of a mutant patch cannot rescue an ommatidium with a *sdk* phenotype in mosaic eyes. The presence of a long intracellular domain is intriguing and may indicate a potential role in signal transduction. However, the lack of homology with any known functional domain will require further investigation into the role of the intracellular amino acids. Sdk may function as an adhesion molecule either through heterotypic interactions with another adhesion molecule expressed on the mystery cell, or indirectly through homotypic interactions with the undifferentiated cells.

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