**INTRODUCTION**

Much of the brain is organised in a series of layers, each receiving synaptic input from distinct neuronal subpopulations. To establish such stratified neuronal circuits, each ingrowing axon must be able to recognize its specific target layer. How is this layer-specific axon targeting achieved? This question has been partly answered from experiments demonstrating the layer-specific expression of growth cone attractants or repellents in multilayered structures such as the mammalian hippocampus (Del Río et al., 1997; Stein et al., 1999) and cerebral cortex (Castellani and Bolz, 1997; Castellani et al., 1998), and the chick optic tectum (Inoue and Sanes, 1997; Yamagata et al., 1995). If the various target tissue layers are labelled according to such a molecular code, then neurons innervating these structures must equip their axonal growth cones with the corresponding sets of receptors and signalling molecules needed to recognize the correct target layer and avoid non-target layers. While the target-derived cues have recently received much attention, relatively little is known of the intrinsic factors that program distinct neuronal subpopulations to seek their synaptic partners in specific target layers. To understand further how such intrinsic genetic programs determine target specificity in a multilayered neuronal network, we have recently embarked on a genetic analysis of axon targeting in the *Drosophila* visual system.

The *Drosophila* compound eye is composed of 800 ommatidia, each consisting of 8 different photoreceptor cells, R1-R8, that project their axons retinotopically into three distinct layers of the optic lobe: R1-R6 axons connect to targets in the first optic ganglion, the lamina, while R7 and R8 axons project through the lamina to terminate in distinct layers of the second ganglion, the medulla (for reviews, see Meinertzhagen and Hanson, 1993; Wolff et al., 1997). These connections are established during the late larval and pupal stages. Photoreceptors begin to differentiate in ommatidial clusters that are laid down in successive rows as a wave of morphogenesis sweeps from posterior to anterior across the eye imaginal disc. Axons of the eight newly formed photoreceptors in each ommatidial cluster project in a common bundle to the posterior margin of the eye disc and then through the optic stalk to the brain. Ommatidial fascicles first fan out retinotopically on the surface of the brain, before turning medially to penetrate the developing optic lobe. Within the optic lobe, each photoreceptor axon then seeks its appropriate target layer in either the lamina or medulla.

Photoreceptor axons arriving in the lamina enter into a complex dialogue with lamina precursor cells (Huang and Kunes, 1996; Huang et al., 1998; Selleck and Steller, 1991;
Winberg et al., 1992). This dialogue controls the differentiation of lamina neurons and glia, and also determines the final pattern of synaptic connections between R1-R6 axons and lamina neurons. Retinal axons deliver two signals that induce lamina precursor cells to produce lamina neurons. First, Hedgehog protein releases the precursor cells from G1 arrest (Huang and Kunes, 1996), and then Spitz induces their neuronal differentiation (Huang et al., 1998). Lamina glial cells migrate into the lamina from flanking ‘glial precursor zones’ (Perez and Steller, 1996). These cells migrate into position in advance of the incoming photoreceptor axons, but require as yet unidentified signals from these axons for their final differentiation (Perez and Steller, 1996; Winberg et al., 1992). Retinal input is also required for the continued migration of glia into the more anterior and still uninnervated regions of the lamina (Perez and Steller, 1996). As the lamina glia, but not the lamina neurons, are already in place as photoreceptor axons arrive in the lamina, Perez and Steller (1996) proposed that it is these glia that issue specific targeting signals instructing R1-R6 growth cones to terminate in the lamina. This idea is further supported by the fact that it is precisely between the two layers of lamina glial cells that R1-R6 growth cones stop their medial extension into the optic lobe. These growth cones then expand and, over a period of 4-5 days, undergo a stereotyped set of rearrangements before ultimately establishing their connections with lamina neurons (Meinertzhagen and Hanson, 1993). Currently, we do not know either the molecular nature of this putative glia-derived ‘stop’ signal, nor the intrinsic factors that specifically program R1-R6 axons to respond to it.

To identify such intrinsic factors, we have recently conducted a large scale genetic screen to isolate mutations that autonomously disrupt photoreceptor axon targeting (Newsome et al., 2000a). Several of the mutations identified in this screen specifically disrupt targeting of R1-R6 axons to the lamina, and complementation analysis has shown that these mutations affect three distinct genes (Newsome et al., 2000a). Here we present the phenotypic and molecular characterisation of the first of these genes, brakeless (bks). This gene encodes two nuclear protein isoforms that are necessary, but not sufficient, for targeting photoreceptor axons to the lamina. These studies form an important first step in our analysis of the genetic programs that determine target layer specificity in the Drosophila visual system.

**MATERIALS AND METHODS**

**Genetics**

The isolation of ethane methane sulfonate (EMS)-induced bks alleles, as well as the stocks used for analysis using the evFLP system, are described in Newsome et al. (2000a). We used the evFLP2 insertion on the X chromosome and the FRT42D P[w+;] c2R11 second chromosome to generate eye-specific mosaics. Additional markers used were a glass-lacZ insertion on the X chromosome, ro-tacZ on the third chromosome (provided by U. Gaul), Rh1-tacZ on the third chromosome (Newsome et al., 2000a), Rh4-tacZ on the second chromosome (Newsome et al., 2000a) and an om-b-tacZ insertion on the X chromosome (Newsome et al., 2000b). Mutant or mosaic larvae were identified by selecting against a P[w+] marker inserted on a CyO balancer chromosome. bks was mapped to the cytological division 55C1-2 based on its inclusion in the deficiencies Df(2R)Pc1115 and Df(2R)PC4, but not Df(2R)Pc111B and Df(2R)PC29. Transforms were generated as described by Rubin and Spradling (1982). For the rescue and overexpression experiments, we used two independent GMR-myc bksA insertions, three different GMR-myc bksB insertions and two GMR-bksB insertions (all on the third chromosome). Different insertions of the same transgene gave indistinguishable results in both rescue and overexpression assays, as did both the epitope-tagged and untagged versions of bksB. All flies were raised at 25°C.

**Isolation of bks cDNAs**

Genomic PCR products were used to probe the LD and LP cDNA libraries provided by the Berkeley Drosophila Genome Project (BDGP). Several clones from both libraries were analysed by restriction mapping, and partially sequenced. Full-length bksA and bksB cDNAs were isolated from both libraries. In parallel, additional bks cDNAs were identified from ESTs generated by BDGP. One of these, LD13770 was sequenced completely and found to represent a full-length bksA cDNA. Another EST clone, SD01229, was also sequenced completely and found to contain the 3’ 7.2 kb of bksB. A 3’ fragment of SD01229 was fused to a 5’ fragment of LD13770 at a unique and common NotI site to create a full-length bksB cDNA. This LD13770-SD01229 fusion differs from the longest bksB cDNAs isolated directly from the LD and LP libraries only in the length of the 5’ and 3’ untranslated regions. The LD13770 and LD13770-SD01229 cDNAs were used as bksA and bksB, respectively, for all experiments. The GenBank accession numbers for bksA and bksB are AF242193 and AF242194 respectively.

**Sequence comparisons**

Low-complexity regions of Bks were predicted using the SEG program (Wootton and Federhen, 1996). EST and genomic sequences showing homology to high-complexity regions of Bks were identified by TBLASTN searches. The predicted zinc finger domain in Bks was recognized by both the SMART (Schultz et al., 2000) and PFAM (Bateman et al., 2000) resources, albeit as a non-significant hit (P=0.50 and 0.24 respectively). However, the homologous region in SPAC16.05c was identified as a zinc finger domain by both programs with P values of 0.0051 and 0.0011, respectively. In addition, the predicted sequence domains from EST and genomic sequences with homology in this region were also reliably identified as zinc finger domains.

**GMR-bks transgenes**

Fragments of bksA (nucleotides 161-1081) and bksB (161-1712) encompassing the entire ORFs were subcloned into pGMR and pLD13770-SD01229 respectively into a pGMR transformation vector (Newsome et al., 2000a) to create GMR-bksA and GMR-bksB. GMR-myc bksA and GMR-myc bksB were then generated by modifying the 5’ end of the ORF to encode 8 tandem copies of the c-myc epitope. The predicted N-terminal sequence of these epitope-tagged Bks proteins is M(GSEQKLISEEDLN)GSE, where the last E corresponds to glutamate-2 of BksA or BksB. All flies were raised at 25°C.

**Generation of Bks antisera**

A GST-Bks fusion protein containing Bks amino acids 774-929 (a high-complexity region common to both Bks isoforms) was generated by amplifying the corresponding coding region from LD13770, inserting this product between the BamHI and EcoRI sites of pGEX-2T, and expressing the recombinant protein in bacteria according to
the manufacturer’s instructions (Pharmacia). Mice were immunized with the fusion protein and polyclonal antisera obtained according to standard protocols. Sera were first preabsorbed against wild-type embryos, and subsequently against dissected third instar imaginal discs homozygous for the l(2)k00702 allele. Specificity of the sera was confirmed by the strong specific staining patterns observed in engrailed-GAL4; UAS-myc bksA embryos and GMR-myc bksA eye imaginal discs, and by the failure to stain eye imaginal disc tissue homozygous for either bks1 or l(2)k00702.

**Histology**

Larval eye-brain whole mounts and adult head sections were prepared and stained as described in Newsome et al. (2000a). Anti-Bks antisera was used at a dilution of 1:50. For in situ hybridization, riboprobes were prepared from pBluescript plasmids containing either the full-length LD13770 clone (for the common probe), or a 1.6 kb PsI fragment of SD01229 (unique to bksB). Eye imaginal discs were prepared essentially as described in Cubas et al. (1991), and hybridization and detection performed as described in Tear et al. (1996).

**RESULTS**

**bks acts autonomously to control photoreceptor axon targeting**

We recovered two EMS-induced alleles of bks, bks1 and bks2, in a genetic screen for mutations that autonomously disrupt the projection patterns of photoreceptor axons from the eye imaginal disc into the optic lobe of the brain (Newsome et al., 2000a). In this screen, we analysed photoreceptor projections in genetic mosaics in which the developing eye, but not the optic lobe, was genetically homozygous for a newly induced mutation. Eye-specific mosaics were generated by using the FLP/FRT system for site-specific recombination (Golic, 1991; Xu and Rubin, 1993) together with an eyFLP transgene to provide FLP recombinase activity exclusively in proliferating eye imaginal disc cells (Newsome et al., 2000a). In animals of the genotype eyFLP; FRT bks / FRT cl2R11, for example, FLP recombinase activity in a dividing cell in the developing eye induces recombination at the FRT sites to create two daughter cells, one homozygous for the bks mutation and the other homozygous for both bks+ and the cl2R11 mutation. The latter is a recessive cell lethal mutation, introduced to eliminate these homozygous bks+ cells. Thus, a heterozygous bks cell is effectively replaced by its homozygous bks daughter. The continuous high levels of FLP recombinase activity in the developing eye ensure that this occurs with almost 100% efficiency. As a result, almost all heterozygous cells in the developing eye are replaced by homozygous bks cells during the proliferative phase of eye development, which precedes photoreceptor cell fate specification and axon targeting. In the visual system of such animals, axonal connections are therefore established between homozygous bks mutant photoreceptors in the eye and wild-type (bks+) target cells in the brain. For simplicity, we refer to these animals here as bks mosaics.

*bks* mosaics show a striking defect in their photoreceptor axon projections, as visualized in third instar larvae using MAb24B10 to label all photoreceptors and their axons (Fig. 1). In wild type (Fig. 1A,B), photoreceptor axon fascicles project through the optic stalk to their appropriate topographic locations in the optic lobe. R1-R6 axons terminate in the lamina, where their growth cones expand to form a dense layer of staining, the lamina plexus, that lies between two glial cell layers. R7 and R8 axons continue beyond this layer to terminate in the medulla. In *bks* mosaics (Fig. 1C-E), photoreceptor axons project normally through the optic stalk to the brain, but fail to segregate into their distinct target layers. Only a vestigial lamina plexus is formed, while thick bundles of photoreceptor axons project through to the medulla. These features suggest that the primary defect in these mutants is the failure of ingrowing R1-R6 axons to stop in the lamina, which inspired the name *brakeless* (Y. Rao and S. L. Zipursky, personal communication). The projection defects observed in *bks1* and *bks2* mosaics, as well as *bks1 / bks2* transheterozygotes, are indistinguishable.

**bks is specifically required for R1-R6 axon targeting**

To examine the projection errors of *bks* mutant photoreceptors in further detail, we introduced into *bks* mosaics a series of markers to label the axons of specific photoreceptor subclasses (Fig. 2). We first sought to confirm that R1-R6 axons extend through the lamina to terminate in the medulla in *bks* mosaics.

Fig. 1. *brakeless* disrupts lamina targeting. (A,C) Schematic diagram of photoreceptor axon projections in wild-type (A) and *bks* mosaic (C) third instar larvae. (B,D,E) Whole-mount eye-brain complexes of wild-type (B) and *bks1/m* mosaic (D,E) larvae, stained with MAb24B10 to visualize all photoreceptor axons. Photoreceptors in the developing eye imaginal disc (ed in A and C; not shown in B, D and E) project axons through the optic stalk (os) into the optic lobe of the brain. (A,B) In wild-type animals, R1-R6 axons stop in the lamina (1a), where their growth cones expand to form a continuous band of staining, the lamina plexus, indicated by arrows in B. R7 and R8 axons continue through to lamina to terminate in the medulla (me). At this early stage, few R7 axons stain with MAb24B10. (C-E) In *bks* mosaics, fewer axons terminate in the lamina, while the medulla is massively hyperinnervated. (D) A relatively mild *bks* mosaic phenotype with a discontinuous and broad lamina plexus. (E) A slightly younger example showing a more extreme *bks* phenotype in which the lamina plexus is almost entirely absent. Scale bars, 40 μm.
To label specifically these axons, we used a ro-\(\beta\)-Galactosidase marker to visualize R2-R5 axons in larvae and an Rh1-\(\beta\)-Galactosidase marker to label all R1-R6 axons in adults. In wild-type animals, all photoreceptor axons expressing these markers terminate in the lamina (Fig. 2A,C), whereas in bks mosaics most of these axons project through the lamina and terminate in the medulla (Fig. 2B,D). This observation confirms that many R1-R6 axons project through the lamina in bks mosaic larvae, and further demonstrates that these inappropriate projections are maintained in the adult. Within the medulla, mistargeted R1-R6 axons all appear to make R7-like projections (compare Fig. 2D and E).

We next used an Rh4-\(\beta\)-Galactosidase marker to examine R7 projections and found that these axons are correctly targeted to the medulla in bks mosaics (Fig. 2E,F). We also noticed that both mistargeted R1-R6 axons and correctly targeted R7 axons appear to project in topographically correct fashion along the anteroposterior axis (Fig. 2D,F). To also examine retinotopic mapping along the dorsoventral axis, we used an omb-\(\beta\)-Galactosidase reporter, which is expressed in the dorsalmost and ventralmost photoreceptors in the eye imaginal disc. These photoreceptors project their axons retinotopically to the dorsoventral extremes of the optic lobe. Most of the photoreceptors expressing the early omb-\(\beta\)-Galactosidase marker do not yet label with mAb24B10, and are therefore only singly labelled. Note also that the \(\beta\)-Galactosidase fusion protein is localized to microtubules, and so does not fill the entire growth cone. (H) bks mutations do not disrupt the retinotopic mapping of these axons. Scale bars, 40 \(\mu\)m.

Retina and lamina cell fates are correctly specified in bks mosaics

The specific failure of R1-R6 axons to terminate in the lamina in bks mosaics might be due to either (a) a transformation of...
in bks mosaics. Thus, by both morphological and molecular criteria, R1-R6 cells are not transformed towards an R7 cell fate in bks mosaics. Indeed, the most common abnormality in bks mutant ommatidia is a cell fate transformation in the opposite direction: in 40 of the 63 abnormal bks ommatidia observed, the R7 cell appeared to be transformed to an R1-R6 cell, as judged by the size and position of its rhabdomere (arrows in Fig. 3A).

Lamina precursor cells require retinal innervation for their final differentiation, including, most likely, their ability to instruct R1-R6 growth cones to seek targets within the lamina. We therefore examined lamina cell fates in bks mosaics using the neuronal marker Elav (Robinson et al., 1988) and the glial marker Repo (Campbell et al., 1994). Lamina neurons were found to differentiate normally in bks mosaics (data not shown). More importantly, the glia that are thought to provide targeting signals for R1-R6 growth cones (Perez and Steller, 1996) migrate into the developing lamina and express Repo in bks mosaics (Fig. 3C) just as they do in wild type (Fig. 3B).

The ordered arrangement of these glia into rows is however slightly disrupted in bks mosaics, but we suspect that this minor irregularity is a consequence rather than cause of the continued growth of R1-R6 axons through this layer. Thus, the lamina glial cells are in place and appear to be differentiating normally in bks mosaics. As bks function is not disrupted in these cells, they presumably still issue their normal targeting instructions to incoming retinal axons.

We therefore conclude that the R1-R6 targeting errors in bks mosaics are not due to the transformation of these cells towards an R7 fate, nor the failure of lamina cells to provide targeting instructions to R1-R6 growth cones. Rather, our data strongly suggest that, in bks mosaics, R1-R6 growth cones are unable to sense or respond to a specific stop signal produced in the lamina.

bks encodes two novel protein isoforms
bks was mapped to the cytological region 55C1-2 on chromosome arm 2R. Two lethal P-element insertions at this site, l(2)04440 and l(2)k00702, failed to complement the lethality associated with both the bks1 and bks2 alleles. Furthermore, in larvae transheterozygous for either P insertion and bks1 or bks2, R1-R6 axons overshoot their lamina targets just as they do in bks mosaics. l(2)04440 and l(2)k00702 are therefore bks alleles. These two P elements belong to a larger set of 11 non-complementing P elements identified by the Berkeley Drosophila Genome Project (BDGP), including the insertion l(2)04525. The insertion site of the l(2)04525 P element has been determined by BDGP (Fig. 4A). Analysis of flanking genomic sequences predicted the existence of a coding region spanning approximately 10 kb, including coding exons on both sides of the P-element insertion site. We used genomic PCR fragments corresponding to this predicted gene to isolate cDNAs from both embryonic and larval-pupal libraries, and also identified several additional cDNAs from ESTs generated by BDGP. A total of 13 cDNA clones were analysed in detail and found to fall into two classes. The longest clones in each class were 3.1 kb and 8.1 kb in length, with complete open reading frames encoding proteins of 929 and 2302 amino acids respectively. We refer to these two cDNA classes as bksA and bksB respectively. Comparison of the cDNA and genomic sequences indicated that the longer bksB transcript arises from...
Fig. 4. Molecular characterisation of the \textit{bks} gene. (A) Genomic organisation of the \textit{bks} locus at 55C1-2. The structure of the two transcripts is shown, with black indicating the coding regions. Grey boxes show genomic fragments used to screen cDNA libraries. The approximate insertion site of the \textit{l(2)04525} P element was determined by BDGP. (B,C) RNA in situ hybridization of \textit{bksB}-specific antisense (B) and sense (C) riboprobes to eye imaginal discs. An identical staining pattern was obtained with probes derived from the region common to both \textit{bks} transcripts. (D) Predicted BksB protein sequence, with the region common to BksA underlined. Low-complexity regions are shown in lower case. Grey shading indicates the conserved regions shown in E-G. The putative zinc finger motif (residues 1112-1137) is boxed. (E-G) Predicted protein sequences showing homology to high-complexity regions of Bks. These are not multiple alignments, but rather pairwise alignments with the Bks sequence. Sequences were compared using the PAM120 matrix. Protein sequences have been derived from \textit{Homo sapiens} (Hs), \textit{Mus musculus} (Mm), \textit{Xenopus laevis} (Xl) and \textit{Danio rerio} (Dr) expressed sequence tags (ESTs), and from \textit{Fugu rubripes} (Fr) genome sequence survey (GSS) entries. The Fugu genome sequence was assembled from the entries FR0036427, 34, 36, 54, 57, 61 and 71. Mm_ESTs include the entries AI644380 and AI644389.
alternative splicing at a donor site that overlaps the stop codon of \textit{bksA}, leading to the addition of further 3’ coding exons in \textit{bksB} (Fig. 4A). In situ hybridization experiments performed with probes prepared from either the common region of \textit{bksA} and \textit{bksB}, or the unique region of \textit{bksB}, showed that \textit{bks} transcripts are uniformly and ubiquitously expressed in the eye imaginal disc (Fig. 4B,C; data not shown).

The two Bks proteins have a most unusual structure, with long stretches of low-complexity sequence (Fig. 4D). Both are also unusually hydrophilic. BksA has a particularly high content of serine (24.0%) and glycine (14.0%) residues, and polar residues in general (DEGKQRST, 67.3%). Hydrophobic residues (LVIFM) make up only 10.8% of BksA, whereas 99% of the proteins in SWISS-PROT have a hydrophobic amino acid content in excess of 13.0% (Brendel et al., 1992). The unique region of BksB has a somewhat different composition, but also of low complexity, with proline (14.3%), glutamine (13.5%) and glycine (11.4%) as the most abundant residues.

Scattered amongst these low-complexity regions are several small islands of high complexity. One of these, unique to BksB (residues 1112-1137) could be reliably identified as a classical C\textsubscript{2}H\textsubscript{2} zinc finger domain (Parraga et al., 1988). This region also shows high homology to predicted proteins from human, mouse, zebrafish and frog EST sequences and yeast and pufferfish genomic sequences (Fig. 4F). For the pufferfish genomic sequence, homology extends beyond the predicted zinc finger domain to include additional high-complexity sequences in the common region at the C terminus of BksA and continuing into the unique region of BksB (residues 869-968, Fig. 4E). Other ESTs also show homology to this region, which includes an almost perfectly conserved 63 amino acid motif in predicted pufferfish and mouse proteins. Remarkably, this motif is not found in any other \textit{Drosophila} protein, and is not encoded at all within the \textit{C. elegans} genome. In addition to these highly conserved motifs, another high-complexity region near the C terminus of BksB (residues 1986-2094) also shows significant but somewhat lower homology to a predicted human protein (Fig. 4G). These conserved high-complexity regions are likely to represent important functional domains present in Bks proteins.

**Bks proteins are localized in the nucleus**

To examine the cellular localization of Bks proteins, we generated antisera recognizing a region of high sequence complexity at the C terminus of BksA, corresponding to the central region of BksB. These sera detect a widely expressed nuclear antigen in eye-antennal imaginal discs (Fig. 5A-D). This antigen is not restricted to R1-R6 cells, or even to photoreceptors, but appears to be present in all cells of the imaginal disc. To verify that we have correctly identified the \textit{bks} gene, and that these antisera specifically recognize Bks proteins, we also stained discs carrying small patches of homozygous \textit{bks\textsuperscript{1}} cells. No staining was detected within these mutant cells (Fig. 5E-H), confirming the identity of the \textit{bks} gene and the specificity of the antisera. We also observed greatly reduced Bks levels in cells homozygous for either of the two \textit{bks} P-element alleles \textit{l(2)04440} or \textit{l(2)k00702}, while \textit{bks\textsuperscript{2}} cells were found to express approximately normal levels of Bks proteins (data not shown). As the Bks antisera do not distinguish the two Bks isoforms, we also generated transgenic animals expressing c-myc epitope-tagged versions of either BksA or BksB under the control of the eye-specific promoter \textit{GMR}. Using anti-c-myc antibodies to stain the eye discs of animals carrying either of these \textit{GMR-myc\textsuperscript{bksA}} and \textit{GMR-myc\textsuperscript{bksB}} transgenes, we confirmed that indeed both Bks isoforms are predominantly nuclear proteins (data not shown).

**Bks proteins are necessary but not sufficient for lamina targeting**

The nuclear localization of Bks proteins excludes them from playing a direct role in axon targeting, but raised the interesting possibility that they might control the choice of target layer by regulating the expression of specific guidance receptors. Target layer specificity might even be determined by the specific Bks isoform(s) expressed in each of the different photoreceptor classes. To test such a model, we asked whether the expression

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**Fig. 5.** Bks proteins are localized in the nucleus. Eye imaginal discs were stained with antisera against Bks proteins (red) and the neuronal nuclear protein Elav (green). (A-D) Wild-type discs, showing the ubiquitous expression of Bks in the antennal (ad) and eye (ed) imaginal discs (A) and, at higher magnification, its nuclear localization (B). Differentiating photoreceptors express the nuclear marker Elav (C). The merged image (D) shows that Bks proteins colocalize with the nuclear Elav protein, but is not restricted to differentiating photoreceptors. (E-H) An eye disc from a larva of the genotype \textit{ey\textsubscript{FLP1}; FRT42D arm-lacZ/FRT bks\textsuperscript{1}} contains large patches of \textit{bks\textsuperscript{1}} homozygous mutant cells. These cells can be detected by the lack of staining with anti-\textit{\beta}-galactosidase (E), and also do not stain with the Bks antisera (F), confirming that these sera specifically recognize Bks proteins. Note that in this case no cell lethal mutation is present on the \textit{bks\textsuperscript{1}} arm, and so the disc is a mosaic of approximately equal numbers of both mutant and wild-type cells. (G) Elav staining shows that \textit{bks} mutant cells undergo normal photoreceptor differentiation, although the levels of Elav protein in \textit{bks} mutant cells appear to be slightly reduced. (H) The three images are merged. Scale bars, A, 100 \textmu m; B-D, 20 \textmu m; E-H, 40 \textmu m.
either BksA or BksB alone would restore lamina targeting of R1-R6 axons in bks mosaics, and possibly even retarget R7 or R8 axons to the lamina.

We first introduced the GMR-myc bksA and GMR-myc bksB transgenes into bks mosaics and assayed R1-R6 projections using the Rh1-tlacZ marker (Fig. 6; Table 1). The GMR-myc bksA transgene partially rescues the R1-R6 targeting defects in bks mosaics (Fig. 6C; Table 1). Most R1-R6 axons now correctly target the lamina. However, a small number of R1-R6 axons in each hemisphere, predominantly those in the posterior regions of the eye, still project through the lamina to terminate in the medulla. Complete rescue of the bks targeting defect was only obtained with the GMR-myc bksB transgene (Fig. 6D; Table 1). Identical results were obtained for both the

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Serial sections were prepared through the heads of animals of the indicated genotypes. Animals additionally carried an Rh1-tlacZ reporter, allowing R1-R6 projections to be visualized with anti-β-galactosidase staining. Hemispheres were then scored for the severity of R1-R6 mistargeting: –, no R1-R6 axons targeting the medulla; +, 1-2 R1-R6 axon bundles target the medulla; ++, several R1-R6 axon bundles target the medulla (as shown for example in Fig. 6B); +++, a strong bks phenotype, in which many R1-R6 axons target the medulla (Fig. 6B). Only hemispheres that could be entirely reconstructed from the serial sections were scored. Numbers in parentheses after the GMR transgene indicate the number of different insertions assayed. No significant differences were observed between different insertions of the same transgene, and the results have therefore been pooled. n indicates the total number of hemispheres scored for each genotype.

Table 1. Transgenic rescue of R1-R6 targeting defects in bks mosaics

Fig. 6. Either Bks isoform can rescue the R1-R6 targeting defect in bks mosaics. Adult head sections of flies carrying an Rh1-tlacZ marker were stained with anti-β-galactosidase antibodies to visualize R1-R6 axons. (A) In wild type, R1-R6 axons project from the retina (re) and terminate in the lamina (la, arrows). No R1-R6 axons extend through to the medulla (me). (B) In bks2 mosaics, the majority of R1-R6 axons continue through the lamina to terminate in the medulla (arrows). (C) In bks2 mosaics additionally carrying a GMR-myc bksA transgene, most R1-R6 axons now correctly target the lamina. Only small bundles of R1-R6 axons, usually from the posterior retina, still project through to the medulla (arrows). (D) A GMR-myc bksB transgene restores lamina targeting to all R1-R6 axons (arrows) in bks2 mosaics. Scale bar, 50 μm.

Fig. 7. Overexpression of Bks does not retarget R7 and R8 axons to the lamina. (A,B) Eye-brain complexes from GMR-myc bksA (A) and GMR-myc bksB (B) larvae were stained with mAb24B10 to visualize all photoreceptor axons. R8 axons extend normally through the lamina to terminate in the medulla. At this early stage of development, few if any R7 axons stain with mAb24B10. (C,D) R7 projections were assessed in adults using the Rh4-tlacZ marker. Horizontal head sections of adults carrying the Rh4-tlacZ marker and either GMR-myc bksA (C) or GMR-myc bksB (D) were stained with anti-β-galactosidase (green) to visualize R7 axon projections, and counterstained with MAb22C10 (red) to reveal the structure of the optic lobe. In both cases, R7 axons project to the medulla just as they do in wild type (Fig. 2E), os, optic stalk; re, retina; la, lamina; me, medulla. Scale bars, 40 μm.
protein null bks¹ allele and the protein-positive bks² allele (Table 1). Thus, R1-R6 cells expressing only one of the two Bks isoforms still target their axons to the lamina, arguing against models in which the different isoforms specify different target layers. Evidently, the unique regions of BksB, including the putative zinc finger domain, are also largely, though not entirely, dispensable for Bks function in photoreceptor axon targeting.

Finally, we examined R8 projections (using MAb24B10 to stain larval eye-brain complexes) and R7 projections (using the adult marker Rh4-talacZ) in both wild-type and bks mosaic animals carrying either the GMR-movbksA or GMR-movbksB transgene. Despite the high levels of expression provided by the GMR promoter, neither BksA nor BksB is sufficient to retarget R7 or R8 axons to the lamina (Fig. 7 and data not shown). Thus, Bks proteins are necessary, but not sufficient, for lamina targeting.

DISCUSSION

We have identified a novel gene, brakeless (bks), and shown that its function is required during visual system development specifically for the correct targeting of R1-R6 axons to the lamina. In eye-specific bks mosaics, mutant R1-R6 cells instead make R7-like projections through the lamina to the medulla. Other aspects of visual system development appear completely normal. Photoreceptor and lamina cell fates are properly specified, the projections of R7 axons to the medulla are undisturbed, and retinal axons, including the mistargeted R1-R6 axons, maintain their retinotopic order with respect to both the anteroposterior and dorsoventral axes.

The specific requirement for lamina targeting of R1-R6 axons distinguishes bks from most other genes presently known to function in photoreceptor axon guidance. Mutations in several other genes, such as dock, Pak and trio, result in highly disorganised projection patterns in which the mistargeting of R1-R6 axons is only a very minor component (Garrity et al., 1996; Hing et al., 1999; Newsome et al., 2000b). These genes are believed to encode components of a common signal transduction pathway likely to mediate diverse guidance decisions. However, as most R1-R6 axons still terminate correctly in the lamina in these mutants, this signal transduction pathway is unlikely to directly participate in the response of R1-R6 growth cones to ‘stop’ signals in the lamina.

More specific targeting errors have been reported for mutations in the genes Ptp69D, nonstop and limbo (Garrity et al., 1999; Martin et al., 1995; Newsome et al., 2000a). Of these, only in Ptp69D mutants have the projection defects of photoreceptor axons been analysed in sufficient detail to allow a direct comparison with bks (Garrity et al., 1999; Newsome et al., 2000a). Like bks, Ptp69D function is also required in the eye for the correct targeting of photoreceptor axons, and, as in bks mutants, photoreceptor and lamina cell fates are correctly specified in Ptp69D mutants. However, the targeting errors made by Ptp69D mutant photoreceptors are both qualitatively and qualitatively different from those observed in bks mosaics. In Ptp69D mosaics, only 5-20% of R1-R6 axons fail to terminate in the lamina, whereas in bks mosaics nearly all R1-R6 axons continue beyond the lamina. Also, in Ptp69D mutants, approximately half of the R1-R6 axons that extend beyond the lamina make R8-like projections in the medulla (Garrity et al., 1999; Newsome et al., 2000a), as do many R7 axons (Newsome et al., 2000a). In contrast, neither R1-R6 nor R7 axons appear to make R8-like projections in bks mosaics. In view of these differences, we conclude that Ptp69D and bks most likely control distinct aspects of photoreceptor axon targeting.

Ptp69D encodes a receptor tyrosine phosphatase, the activation of which is thought to reduce adhesion between follower R1-R6 and R7 growth cones and the pioneer R8 axon (Newsome et al., 2000a). The signal mediated by PTP69D would thus facilitate the independent targeting of follower axons, but not provide specific targeting instructions. For their final choice of target layer, photoreceptor growth cones would therefore rely on additional signals. Our data suggest that bks function is specifically required for the signalling events that instruct R1-R6 growth cones to target the lamina. These two signals do not necessarily act sequentially. Rather, we envisage that R1-R6 growth cones are induced to stop in the lamina by the simultaneous and synergistic action of both PTP69D and such a specific targeting signal.

Bks proteins are localized in the nucleus, and so cannot directly participate in the growth cone response to targeting signals. Two types of models can therefore be proposed for Bks function, positioning Bks either upstream or downstream of the signalling events in the growth cone. In an ‘upstream’ model, Bks might be needed to provide R1-R6 growth cones with the molecular machinery required to receive their specific targeting signals. Alternatively, in a ‘downstream’ model, Bks might act in nuclear processes required to stop further axon extension in response to these signals.

How might Bks act in a ‘downstream’ model? Stop signals for R1-R6 growth cones are thought to be provided by the lamina glia (Perez and Steller, 1996). These glial cells are therefore only intermediate targets, or ‘guideposts’, that R1-R6 growth cones use for navigational purposes before ultimately forming synapses with the lamina neurons. Another well-characterised guidepost for navigating growth cones is the midline of the central nervous system. Commisural axons that project across the midline encounter a mirror-image set of guidance cues on each side of the midline, but respond quite differently to these cues before and after their passage across the midline. Studies in both vertebrates (Dodd et al., 1988) and invertebrates (Condron, 1999) have documented changes in the expression of growth cone receptors as commissural axons cross the midline. Furthermore, blocking gene transcription interferes with the correct targeting of commissural axons once they have crossed the midline (Von Bernhardi and Bastiani, 1995). These observations suggest that, at least at the midline, signals provided by an intermediate target might induce transcriptional responses that determine subsequent growth cone behaviour. In a similar scenario, stop signals issued to R1-R6 growth cones by lamina glial cells might act via Bks either to induce the expression of genes required for these growth cones to remain within the lamina, or to downregulate genes required for their further extension into the optic lobe. However, if this were the case, one might expect R1-R6 axons to at least temporarily stall in the lamina before continuing through to the medulla in bks mosaics. We see no evidence of this, as even in young bks eye-brain complexes most R1-R6
axons already extend fully through to the medulla (see, for example, Fig. 1E).

In view of this, we favour models in which Bks acts ‘upstream’ of targeting events in the growth cone, perhaps by regulating the expression of specific growth cone receptors or signalling molecules. The biochemical functions of the two Bks proteins are at present unknown, but the presence of a zinc finger domain in BksB suggests that it may be a DNA-binding protein. The shorter BksA isoform lacks this motif but retains another highly conserved domain of unknown function. BksA might therefore antagonise BksB function, perhaps by forming inactive BksA:BksB heterodimers or by sequestering common binding partners into inactive complexes. Such mechanisms have been documented for the inhibition of several well-characterised transcriptional activators by closely related proteins that lack the DNA binding domain (Norton et al., 1998; Ron and Habener, 1992; Treacy et al., 1991). In such a model, different expression patterns of BksA and/or BksB might in turn determine the expression of specific targeting receptors in each of the different photoreceptor classes. In this way, Bks proteins might control photoreceptor axon targeting in a manner analogous to control of motoraxon targeting by LIM homeobox proteins (Sharma et al., 1998; Thor et al., 1999). However, as attractive as such a model may be, the evidence speaks strongly against it. Expression of either BksA or BksB alone is sufficient for the lamina targeting of most R1-R6 axons, but neither isoform is sufficient to retarget R7 or R8 axons to the lamina. Thus, if Bks proteins act upstream of targeting events in the growth cone, they cannot be the sole determinant of targeting specificity. We therefore propose that Bks proteins act in concert with other as yet unknown transcription factors to regulate the expression of specific targeting receptors.

In conclusion, our analysis of the role of bks in the lamina targeting of R1-R6 axons has clearly demonstrated the existence of a specific targeting mechanism for these axons and provided a first entry point in the molecular characterisation of this process. Several other mutations with similar targeting defects have also been isolated (Martin et al., 1995; Newcombe et al., 2000a; T. P. Newsome, B. Asling, B. J. D., unpublished data) and the molecular characterisation of some of these genes is currently in progress (S. L. Zipursky, personal communication, K.-A. S. and B. J. D., unpublished data). We anticipate that the detailed investigation of these genes, together with further analysis of Bks function, will lead to rapid progress in our understanding of the mechanisms that control layer-specific targeting decisions in the Drosophila visual system.

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