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Mutational analysis of the *eyeless* gene and phenotypic rescue reveal that an intact Eyeless protein is necessary for normal eye and brain development in *Drosophila*

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ABSTRACT

Pax6 genes encode evolutionarily highly conserved transcription factors that are required for eye and brain development. Despite the characterization of mutations in *Pax6* homologs in a range of organisms, and despite functional studies, it remains unclear what the relative importance is of the various parts of the Pax6 protein. To address this, we have studied the *Drosophila* Pax6 homolog *eyeless*. Specifically, we have generated new *eyeless* alleles, each with single missense mutations in one of the four domains of the protein. We show that these alleles result in abnormal eye and brain development while maintaining the OK107 *eyeless* GAL4 activity from which they were derived. We performed *in vivo* functional rescue experiments by expressing in an *eyeless*-specific pattern Eyeless proteins in which either the paired domain, the homeodomain, or the C-terminal domain was deleted. Rescue of the eye and brain phenotypes was only observed when full-length Eyeless was expressed, while all deletion constructs failed to rescue. These data, along with the phenotypes observed in the four newly characterized *eyeless* alleles, demonstrate the requirement for an intact Eyeless protein for normal *Drosophila* eye and brain development. They also suggest that some endogenous functions may be obscured in ectopic expression experiments.

Introduction

Pax6/*eyeless* is involved in the development of the eye and the brain of both invertebrates and vertebrates. In *Drosophila melanogaster*, the *Pax6* homolog *eyeless* is initially expressed throughout the undifferentiated eye disc, where it persists until the cells differentiate with the progression of the morphogenetic furrow (Halder et al., 1998; Quiring et al., 1994). *eyeless* is required for normal development of the *Drosophila* eye, as mutations in the *eyeless* gene result in irregular facets as well as in a reduction of eye size (Lindsley and Zimm, 1992). Misexpression of *eyeless* in antennal, leg, or wing imaginal discs is able to initiate the formation of ectopic eyes (Halder et al., 1995), establishing *eyeless* as a key regulator of *D. melanogaster* eye development. In the vertebrate eye, *Pax6* is expressed in the optic vesicle/optic cup, lens, and cornea (Walther and Gruss, 1991) and is required for normal development of each of these eye structures (Ton et al., 1991; Hill et al., 1991; Grindley et al., 1995; Quinn et al., 1996). *Pax6* also has a role in retinogenesis, where it is required to maintain the retinogenic potential of retinal progenitor cells (Marquardt et al., 2001). Loss of *Pax6* function results in aniridia or Peter's anomaly in humans, and the *small eye* phenotype in the mouse and rat. Ectopic expression of *Pax6* in *Xenopus laevis* results in the formation of differentiated ectopic eyes (Chow et al., 1999), highlighting the conserved role of Pax6 during eye formation.

In the *Drosophila* embryonic brain, *eyeless* is expressed in all three neuromeres of the brain (Kammermeier et al., 2001) while in later stages of brain development, *eyeless* is primarily expressed in the protocerebrum in e.g. the optic lobes, the mushroom bodies, and the pars intercerebralis. Loss of *eyeless* activity leads to defects in these structures (Callaerts et al., 2001; Clements et al., 2008; Kurusu et al., 2000; Noveen et al., 2000). Vertebrate *Pax6* is involved in the development of the forebrain (Mastick et al., 1997; Mastick and Andrews, 2001; Warren and Price, 1997), midbrain (Matsunaga et al., 2000), and hindbrain (Osumi et al., 1997;

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Stoykova et al., 2000; Takahashi and Osumi, 2002), where it has roles in the patterning of neuromeres, neuronal differentiation, and axon guidance.

The Pax6/Eyeless protein contains two DNA-binding domains, the 128-amino acid (aa)-long paired domain containing two helix–turn–helix (HTH) motifs (Bopp et al., 1986; Treisman et al., 1991) and a 60-aa-long paired-like homeodomain (Frigerio et al., 1986; Ton et al., 1991; Walther and Gruss, 1991). In addition, Pax6/Eyeless contains a proline, serine, and threonine-rich domain at its carboxy-terminus and a glycine-rich region of variable length that links the amino-terminal paired domain with the more carboxy-terminal homeodomain. A comparison of *Pax6* genes from many species revealed a high degree of structural conservation, particularly within the two DNA-binding domains (Callaerts et al., 1997, 2006). The high degree of sequence conservation observed in Pax6 is suggestive of a high degree of conservation of macromolecular interactions as well, despite the vast differences in both structure and development of brain and eye in diverse phyla. Indeed, not only are all *Pax6* genes studied so far expressed in the brain/nerve ring and the eye/photoreceptor, several of the target genes are conserved as well. These include the lens crystallins, the *sine oculis*/*Six* gene family, the *eyes absent*/*Eya* gene family, and the *dachshund*/*Dach* gene family (Callaerts et al., 1997, 2006; Wawersik and Maas, 2000; see Gehring and Ikeo, 1999; van Heyningen and Williamson, 2002; Kozmik, 2008 for further reviews on Pax6).

The high degree of sequence conservation argues for conserved functions for the DNA binding paired and homeodomains, as well as the transactivating C-terminal domain. Pax6 has effectively three DNA-binding HTH domains: the amino-terminal (PAI) and carboxy-terminal (RED) subdomains of the paired domain and the homeodomain. By using multiple combinations of DNA binding domains, Pax proteins potentially regulate a multitude of different functions (Jun and Desplan, 1996). This hypothesis is supported by data in which a missense mutation in the paired domain can either abolish or increase the DNA binding and transcription dependent on homeodomain binding sites (Singh et al., 2000). Furthermore, point mutations in the transactivation domain can reduce or abolish the DNA-binding activity of the paired domain or the homeodomain (Singh et al., 2001). The activity of the transactivation domain requires the entire domain (152-aa in human) for maximal activity (Tang et al., 1998). These data suggest a requirement of the paired domain, homeodomain, and transactivation domain for Pax6 function. However, given the high degree of conservation and its known DNA binding roles, surprisingly few homeodomain mutations have been found in comparison to the many paired domain and transactivation domain mutations, suggesting that the homeodomain may not be necessary for Pax6 function. Indeed, in *Drosophila*, the *eyeless* phenotype in the eye was rescued in 79% of cases by an Eyeless protein lacking the homeodomain, which was also able to induce the formation of ectopic eyes (Punzo et al., 2001). In contrast, the characterization of missense mutations affecting only the homeodomain of Pax6 (Azuma et al., 2003; Favor et al., 2001; Morrison et al., 2002; Redeker et al., 2008; Thaung et al., 2002) suggests that the homeodomain is required for normal function.

Here we describe a requirement for the paired domain, the linker region, the homeodomain, and the C-terminal domain of the *D. melanogaster Pax6* homolog *eyeless* during eye and brain development. Furthermore, we demonstrate that the C-terminal domain of Eyeless functions as a transactivation domain as in Pax6. We describe four new *eyeless* alleles created by mutagenesis of the *eyeless* Gal4 driver OK107. This Gal4 driver expresses the yeast transcriptional activator Gal4 in an *eyeless* pattern in the *Drosophila* eye and brain (Adachi et al., 2003), allowing the targeted expression of any gene or construct that is preceded by the Gal4 upstream activating sequence (UAS) in cells that normally express *eyeless* (Brand and Perrimon, 1993). The four new alleles of *eyeless* also maintained the Gal4 activity of OK107, and thus allowed us to attempt rescue of the eye and brain phenotypes of homozygous mutant animals by driving the expression of Eyeless deletion constructs in an *eyeless* expression pattern. We describe, for the first time, complete rescue of the *eyeless* phenotype with full-length Eyeless protein. On the other hand, deletion of the paired domain, the homeodomain, or the C-terminal domain of the Eyeless protein resulted in the failure of these proteins to rescue either the eye or the brain phenotype of homozygous *eyeless* mutants. The new alleles are each caused by a single missense mutation in one of the four major domains of the Eyeless protein, resulting in abnormal eye and brain development. These data, along with the rescue experiments, strongly suggest that an Eyeless protein with each domain intact is necessary for normal Eyeless function. In addition, in one of the alleles, $ey^{OK107/6}$, Eyeless protein failed to localize to the nucleus, revealing a nuclear localization signal (NLS) in the recognition helix of the homeodomain.

Materials and methods

Mutagenesis screen

In order to generate *eyeless* alleles on the OK107 chromosome, we mutagenized 1500 homozygous OK107 males with 35 mM EMS as described (Lewis and Bacher, 1968; see Supplemental Fig. 1 for mutagenesis scheme). These males were then crossed *en masse* to ci^D females in order to obtain OK107*/ci^D males (asterisk indicates mutagenized chromosome). 3314 males of this genotype were crossed individually to ey^{D1Da}/ci^{lacZ} females. The progeny of this cross consisted of four possible genotypes (OK107*/ey^{D1Da}, OK107*/ci^{lacZ}, ey^{D1Da}/ci^D, and ci^{lacZ}/ci^D, which is lethal). Flies without the ci^D phenotype were then screened for the eyeless phenotype (eyes reduced or lost), identifying multiple independent putative alleles. Of these, non-ci^D flies without an *eyeless* phenotype (presumed genotype: OK107*/ci^{lacZ}) were crossed to ey^{D1Da}/ci^D flies in order to (1) verify the phenotype and (2) establish the $ey^{OK107/X}/ci^D$ stocks (the name $ey^{OK107/X}$ refers to the entire series of alleles generated in this screen).

Analysis of phenotypes

The eye was examined by comparing the size of each mutant eye to the eye of a wildtype (Oregon-R) animal. Mutant eyes were then assigned to one of the following categories: 75–100% (eye relatively normal in size), 50–75%, 25–50%, 1–25% (eye present but extremely small, usually less than 50 ommatidia), or no eye (eye completely absent). Brain phenotypes were gauged on the basis of the morphology of the mushroom bodies and the central complex, both of which are dependent on *eyeless* for normal development. This was determined by whole-mount staining of adult brains with the monoclonal antibody 1D4. Brain defects were assigned to one of the following categories: no defects (structure phenotypically wildtype), mild (a few, subtle defects observed), severe (significant disorganization of neuropil structure), or very severe (structure completely or nearly completely absent).

Fly stocks

Flies were maintained at 25°C on standard agar-cornmeal-molasses medium. Fly stocks or alleles used were OK107 (Connolly et al., 1996), ey^{D1Da} and ey^{JD} (Callaerts et al., 2001), ey^{EH} (Benassayag et al., 2003), ey^2 (available from the Bloomington Stock Center), $Pabp2^{CC00380}$ (Buszczak et al., 2007), and ci^{lacZ} (from Thomas Kornberg). Fly stocks used for rescue of the *eyeless* phenotype were UAS-*ey* (Halder *et al*., 1995) and UAS-*ey*ΔPD, UAS-*ey*ΔHD, and UAS-*ey*ΔCTD (Punzo et al., 2001).