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Interaction between Ataxin-2 Binding Protein 1 and Cubitus-interruptus during wing development in *Drosophila*

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ABSTRACT

Animal growth and development is dependent on reiterative use of key signaling pathways such as Hedgehog (Hh) pathway. It is widely believed that Cubitus-interruptus (Ci) mediates all functions of Hh pathway. Here we report that CG32062, the *Drosophila* homologue of Ataxin-2 Binding Protein 1 (dA2BP1), functions as a cofactor of Ci to specify intervein region between L3 and L4 veins of the adult wing. Specifically, Ci-mediated transactivation of *knot/collier* (*kn*) in this region of the developing wing imaginal disc is dependent on dA2BP1 function. Protein interaction studies and chromatin-immunoprecipiation experiments suggest that Ci helps dA2BP1 to bind *kn* promoter, which in turn may help Ci to activate *kn* expression. These results suggest a mechanism by which Ci may activate targets such as *kn*, which do not have classical Ci/Gli-binding sites.

Introduction

Growth and patterning during fly wing development are mediated by signaling from its anterior-posterior (A/P) and dorsal-ventral (D/V) organizers. The A/P compartmentalization is controlled by the expression of *engrailed* (*en*; Lawrence and Morata, 1976), a selector gene that specifies posterior identity (Kornberg et al., 1985). En activates *hedgehog* (*Hh*), which is a short-range morphogen that diffuses to the anterior compartment, and activates Smoothened (Smo) by releasing it from Patched (Ptc). In the presumptive A/P boundary, Smo activates Cubitus-interruptus (Ci) by stabilizing its full-length isoform, which in turn activates targets of Hh, particularly Decapentaplegic (Dpp) and Knot/Collier (Kn). Ci also activates Ptc and thus, a higher concentration of Ptc receptor is maintained at the AP boundary (reviewed by Aza-Blanc et al., 1997).

A combination of repressor activity of 75 kDa form and activator activity of 155-kDa form of Ci regulate the expression of different sets of target genes (Methot and Basler, 2001; Wang and Holmgren, 1999). This, however, is thought to be mediated by Ci binding to similar binding sites (Muller and Basler, 2000). Targets of Ci such as *ptc, dpp, wg* possess well characterized binding sites on their promoters (Alexandre et al., 1996; Hidalgo and Ingham, 1990; Muller and Basler, 2000; Von Ohlen et al., 1997). However, classical Gli/Ci-binding sites are not present on *kn* promoter (Hersh and Carroll, 2005). This suggests that the mechanism of Ci-mediated activation of *kn* may be different from the mechanism activating *ptc* or *dpp* expression.

Previously, we reported the identification of *CG32062* in an enhancer-trap screen to identify genes that show differential expression between wing and haltere discs (Bajpai et al., 2004). Here we report functional characterization of *CG32062*. Sequence analyses suggest that protein encoded by *CG32062* (hereafter referred to as *dA2BP1*) is the closest homologue of human Ataxin-2 Binding Protein 1 (A2BP1). In mammals including human, expansion of polyglutamine repeats in Ataxin-2 causes Spinocerebellar ataxia type 2 (SCA2), a neuro-degenerative disease. The Ataxin-2 activity is modulated by A2BP1, which binds to the C-terminus of the former (Shibata et al., 2000).

dA2BP1 shows a dynamic expression pattern; both spatially and temporally. The protein is predominantly nuclear with traces of the protein in the cytoplasm. Removal of *dA2BP1* function causes formation of ectopic veins and loss of intervein region, a phenotype similar to those caused by loss-of-function alleles of *kn*. Epistasis experiments suggested *dA2BP1* interacts with Ci to activate the expression of *kn*. Protein interaction studies and chromatin-immunoprecipiation experiments suggest that Ci helps dA2BP1 to bind *kn* promoter, which in turn may facilitate Ci to activate *kn* expression. Results presented here suggest that dA2BP1 may facilitate activation of those targets of Ci such as *kn*, which do not have classical Ci/Gli-binding sites.

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Materials and Methods

Genetics

Recombinant chromosomes and combinations of GAL4 drivers and UAS lines, generation of different mutations and markers, molecular cloning and associated nucleic acid and protein techniques (expression, purification, immunoprecipitation etc) were as per the standard genetic and molecular procedures. The LD15974 (Berkeley *Drosophila* Genome Project), the cDNA representing full-length CG32062-RE isoform, was used to express dA2BP1 in *E. coli* (bacterially expressed protein was used to raise polyclonal antibodies against dA2BP1) and to generate transgenic UAS-dA2BP1. Cloning strategies, sequence of PCR primers, details of GST-pull down assay and immunoprecipitation experiments are described in the supplement text.

Several flystocks were obtained from various sources: GAL4 strains used are Actin5c>CD2>GAL4 (Pignoni and Zipursky, 1997), *C96*-GAL4 (Gustafson and Boulianne, 1996), *ap*-GAL4 (Calleja et al., 1996), *EN403*-GAL4 (Bajpai et al., 2004), *MS1096*-GAL4 (Capdevila et al., 1994), *omb*-GAL4 (personal communication to Flybase, *Calleja, 1996.10.16*), *ptc*-GAL4 (Brand and Perrimon, 1993) and *vg*-GAL4 (Simmonds et al., 1995). Other fly-strains used: UAS-Ci (Aza-Blanc et al., 1997), UAS-Ci-HA (Wang and Holmgren, 1999), $dpp^{BS3.0}$-lacz (Blackman et al., 1991), UAS-Dsh (Neumann and Cohen, 1996), *EN403*-lacZ (Bajpai et al., 2004), UAS-mCD8::GFP (Lee and Luo, 1999), *hs*-FLP (Xu and Rubin, 1993), UAS-Kn (Mohler et al., 2000), *kn*-lacZ (Hersh and Carroll, 2005)and UAS-$Notch^{intra}$ (Fortini et al., 1993). The P-insertion $y^1\ w^{67c23}$; *P{EPgy2}EY01049/TM3,* $Sb^1\ Ser^1$ (Bloomington Stock # 15489) in dA2BP1 is described in Flybase.

Histology

RNA in situ and immunochemical staining on embryos and imaginal discs were performed as described earlier Patel et al. (1989) and Tautz and Pfeifle (1989). LD15974 and RE20611 were used as templates to generate DIG-labelled probes for RNA in situ for *dA2BP1* and *dpp*, respectively. Antibodies used were, anti-dA2BP1 (1:1), monoclonal anti-Cut (1:10; Blochlinger et al., 1993), anti-β-Galactosidase and anti-GFP (Molecular Probes), anti-HA (1:1000; Roche), anti-DSRF (1:100; Montagne et al., 1996), anti-dpERK1/2 (1:50; Sigma, USA), anti-Ci (raised in rat; 1:5; Motzny and Holmgren, 1995), anti-Caspase 3 (1:300; Cell Signaling), monoclonal anti-Wg (1:500; Brook and Cohen, 1996), anti-Delta (1:100; Qi et al., 1999)) anti-Ubx (1:30; White and Wilcox, 1984); anti-Ptc (1:50; Capdevila et al., 1994), anti-Hh (1:1000; Tabata and Kornberg, 1994), anti-En (1;200; Patel et al., 1989). Antibodies against Wg and Dl were obtained from Developmental Studies Hybridoma Bank (DSHB), Iowa, USA. All secondary antibodies conjugated to Alkaline Phosphatase or Horse Radish Peroxidase were purchased from Jackson Immuno Research Laboratories, USA and those conjugated to fluorophores were purchased from Molecular Probes, USA. Protein A Agarose (Amersham) or Protein G Agarose (Upstate) were used for immunoprecipitation experiments.

Chromatin immunoprecipitation (ChIP) experiments

The ChIP protocol used is modified from Papp and Muller (2006) and Sanchez-Elsner et al. (2006). Chromatin of 500 bp-2 kb was isolated from wing discs of larvae of desired genotype and subjected ChIP using goat anti-Ci (Santacruz, USA) or anti-dA2BP1 antibodies. The immunoprecipitated DNA was subjected to PCR amplification with primers designed to amplify those regions of *kn* and *dpp* genes, which harbour Ci-binding motifs (Hersh and Carroll, 2005; Muller and Basler, 2000). Primers against a region of *kn* with no known binding sites for Ci, which is more than 20 kb away from the region with Ci binding site, was also included in all experiments. ChIP experiments for a region of 3rd exon of *Rpl32* served as negative control and the same was used to normalize the differences between mock and test of a given experiment. Details of the procedure and sequences of primers used are given in the Supplement Text.

Electromobility shift assays

The ability of purified dA2BP1 (bacterially expressed) to bind *kn* promoter was examined using electromobility shift assays as described earlier (Pallavi et al., 2006). The protein + DNA mix was electrophoretically resolved on 5% polyacrylamide gels. Probes used: (1) PCR amplified *kn* and *dpp* cis-regulatory regions using the same primers as used for ChIP. (2) a 60-mer oligo comprising Ci-binding sites on *kn* and its mutated version. Sequences of the two oligomers used are shown in the Supplement Table 1.

Results

Loss of dA2BP1 causes loss of intervein tissue in the adult wing

The *dA2BP1* gene in *Drosophila*, is a complex locus spanning more than 80 kb of the genomic region located at 67E1-2 polytene position (Bajpai et al., 2004). Based on the analyses of ESTs for this gene, *dA2BP1* is predicted to have 17 exons coding for 5 isoforms (http://www.flybase.org). In addition to a predicted RNA-recognition motif, all isoforms of *dA2BP1* have 2 long poly-Q domains (Suppl. Fig. 1).

To address the role of this gene in the growth and patterning, we started by examining the expression pattern of this gene in developing tissues. First, we raised antibodies against a variant of dA2BP1 protein containing all the predicted functional domains of dA2BP1 (Suppl. Fig. 1). We examined the expression of dA2BP1 during embryonic development and in the larval imaginal discs. In embryos, expression of dA2BP1 was primarily in the central and peripheral nervous systems (Suppl. Fig. 2A, B). Expression was also noted in embryonic pericaridial cells (Suppl. Fig. 2B).

In the 3rd instar larva, expression of dA2BP1 was observed in all imaginal discs. In wing imaginal discs, expression was seen in the pouch region as well as the notum. Within the pouch region, cells away from the dorsal-ventral (D/V) compartment boundary showed relatively higher levels of expression as compared to the cells at the D/V boundary (Fig. 1A). This is similar to the RNA in situ pattern reported for this gene (Bajpai et al., 2004). The expression of dA2BP1 in the notum appeared to be in myoblasts; this was confirmed by its co-localization with the transcription factor Cut, which marks these cells (Fig. 1A). In addition to the pouch and notum regions, dA2BP1 expression was also observed in peripodial cells of the wing disc (data not shown).

As mentioned earlier, *dA2BP1* was identified in an enhancer trap screen. The enhancer trap line *EN403-lacZ* is inserted within the 49 kb long intron of the *dA2BP1* gene (Bajpai et al., 2004). To determine if the anti-dA2BP1 staining pattern in the wing discs is in the same cells that express *EN403-lacZ*, we carried out a double staining with anti-dA2BP1 and anti-βgal antibodies on *EN403-lacZ* wing discs. dA2BP1 and β-gal were found colocalized in the wing pouch (Fig. 1B), suggesting that the anti-A2BP1 antibodies recognize the gene product trapped by *EN403*-lacZ.

In addition to the wing disc, expression of dA2BP1 was also detected in other imaginal discs. In the leg and antennal discs, staining was observed in the central region (data not shown); in the eye imaginal discs, the protein was detected predominantly in cells posterior to the morphogenetic furrow (Suppl. Fig. 2E). In all cases, the protein appeared to be nuclear (Fig. 1A; Suppl. Fig. 2).

We had previously shown that expression of dA2BP1 is dependent on Notch signaling at the D/V boundary (Bajpai et al., 2004). However, the functional role of dA2BP1 in wing patterning was not explored. To address this question, we attempted to generate loss-of-function