Genetic characterization of *Drosophila* RBP-J κ (*Suppressor of Hairless*) as a neurogenic gene in adult PNS development

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ABSTRACT

Drosophila RBP-J κ is a novel sequence-specific DNA binding protein encompassing the integrase motif which is highly conserved in various organisms. Its gene has been shown to be identical to *Suppressor of Hairless* which regulates adult peripheral nervous system (PNS) development. To elucidate the precise function of the RBP-J κ protein in adult PNS development, we analyzed transgenic flies that misexpress the RBP-J κ protein. Such studies have shown that RBP-J κ regulates PNS cell fate in at least two steps: commitment to sensory mother cell by lateral inhibition and terminal differentiation into the socket and shaft cells. Taken together with analysis of phenotypes of *Suppressor of Hairless* mutants, RBP-J κ shows the synergistic activity with neurogenic genes.

1. INTRODUCTION

Development of the *Drosophila* external sensory organ is regulated by a number of genes that specify cell fate at different steps of differentiation (for review, see Ghysen et al., 1993). During early neurogenesis a group of cells in the ectodermal layer acquire the potential to become neuronal precursors by activation of proneural genes such as *achaete-scute* complex genes and *daughterless*. Subsequently a single proneural cell is selected as sensory mother cell (SMC) through competitive cell-cell interaction mediated by the neurogenic genes such as *Notch*, *Delta*, *mastermind*, *neuralized*, *big brain*, *deltex* and *Enhancer of split* (*E(spl)*) (for reviews, see Simpson, 1990; Campos-Ortega and Haenlin, 1992; Fortini and Artavanis-Tsakonas, 1993). A neuronal selector gene *cut* is required to differentiate SMC into the external sensory organ precursor which undergoes two successive divisions, giving rise to four progeny cells: a neuron, a glia cell, a

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socket cell and a hair (shaft) cell. The final stage of SMC differentiation is regulated by cell lineage genes such as *numb*, *oversensitive*, *Bar H1*, *Bar H2*, *Hairless* and *Suppressor of Hairless (Su(H))* (for reviews, see Jan and Jan, 1993; Posakony, 1994).

Heterozygous *Hairless* flies show loss of many bristles on the head and thorax, and shortening of the L4-L5 wing veins, while homozygous *Hairless* mutation is lethal (Lindsley and Grell, 1968). Heterozygous *Hairless* flies frequently show conversion of the shaft cell to the socket cell, giving rise to the double-socket phenotype (Lees and Waddington, 1942; Bang et al., 1991). Recent molecular cloning of the *Hairless* gene shows that its encoded protein is a serine-rich nuclear protein without significant homology to other known proteins (Maier et al., 1992; Bang and Posakony, 1992). It has been reported that *Hairless* displays antagonistic interactions with members of neurogenic genes. *Notch* and *Delta* mutations are repressed by reduction of the *Hairless* activity (Lindsley and Grell, 1968; Lindsley and Zimm, 1985; Dietrich and Campos-Ortega, 1984). *Su (H)* appears to counteract the effect of *Hairless* because *Su (H)* mutations suppress *Hairless* mutations in heterozygous crosses although homozygous *Su (H)* is lethal (Nash, 1970; Ashburner, 1982). Although both *Hairless* and *Su (H)* are essential for normal development of the adult external sensory organ, the molecular mechanism of their regulation and interaction remains unknown.

The mouse RBP-J κ protein was initially purified from a mouse pre-B cell line as a DNA binding protein to the immunoglobulin recombination signal sequence and comprises the integrase motif but no other known DNA binding protein motifs such as zinc finger, basic region of helix loop helix protein and leucine zipper (Matsunami et al., 1989; Hamaguchi et al., 1989). Our recent studies have shown that mouse RBP-J κ is ubiquitously expressed (Hamaguchi et al., 1992) and binds to sequences containing CTGTGGGAA 15 times more strongly than to the recombination signal sequence (Tun et al., 1994), suggesting that an authentic binding target other than the recombination signal sequence may exist. More recently mouse RBP-J κ was shown to be involved in repression of the adenovirus pIX gene (Dou et al., 1994). Since homozygous RBP-J κ knock-out mice are embryonic lethal, mouse RBP-J κ is essential in the mammalian developmental process (C. Oka, T. Nakano, M. Kawaichi and T. Honjo, unpublished observation). The RBP-J κ gene is highly conserved among various species, and its human and *Drosophila* counterparts have been cloned (Furukawa et al., 1991; Amakawa et al., 1993). The *Drosophila* RBP-J κ gene has been shown to be identical with the *Su (H)* locus (Furukawa et al., 1992; Schweisguth and Posakony, 1992).

Understanding the precise role of this highly-conserved DNA binding protein is important to elucidation of the molecular mechanism of the nervous system development not only in *Drosophila* but also in vertebrates. We report that RBP-J κ is involved in at least two steps of PNS development by using transgenic

flies which are induced to express the RBP-J κ protein.

2. MATERIALS AND METHODS

P-element transformation of Drosophila

RBP-J κ cDNA used to generate the transgenic *Drosophila* lines of hs1-RBP-J κ contains the full-length open reading frame (ORF) (Schweisguth and Posakony, 1992). The RBP-J κ cDNA for hs2-RBP-J κ is as described (Furukawa et al., 1991). The phenotype using hs2-RBP-J κ is slightly stronger than that of hs1-RBP-J κ . A DNA fragment encoding human *c-myc* tag consisting of 13 amino acids was attached to the position just before the stop codon of RBP-J κ ORF. The *Eco*RI sites at both ends of these cDNAs are blunt-ended and cloned into the blunt-ended *Kpn*I site of pWH1 vector (Schneuwly et al., 1987). Transgenic flies were generated by standard P element-mediated germ-line transformation techniques (Rubin and Spradling, 1982) using P Δ 2-3 as the recipient strain. Independent 4 transformant lines were analyzed. Western blot analysis was used to verify that all transformants express *Drosophila* RBP-J κ proteins of the expected sizes upon heat shock.

Generation of anti-Drosophila RBP-J κ monoclonal antibody (mAb)

The conserved region (nucleotide 729–1122) of *Drosophila* RBP-J κ cDNA (Furukawa et al., 1991) was expressed in *E. coli* using pET-3 vector (Rosenberg et al., 1987). Expressed protein of 17 kDa was isolated by gel electrophoresis. mAb was generated by injections of the isolated protein with complete adjuvant into BALB/c mouse foot pad. The detailed procedure and characterization of mAb will be described elsewhere.

Fly stocks and immunohistochemistry

Flies were reared on cornmeal-yeast medium at 25°C under constant illumination. Males and females from the strain Canton-Special were used as wild-type. The enhancer-trap line *A101* was used to identify SMCs. Immunohistochemistry was done according to Usui and Kimura (1993) using mAb against *lacZ* (Promega), neuronal antigen (22C10, Bellen et al., 1989) and neuroglian (BP-104, Hortsch et al., 1990).

3. RESULTS

Effect of RBP-J κ misexpression on microchaete formation

To determine the precise function of *Drosophila* RBP-J κ in the adult PNS development, we expressed the *Drosophila* RBP-J κ protein under the control of the heat-shock promoter in transgenic flies. We examined appearance of mechanosensory bristles on the adult notum as their developmental process has been

analyzed in great detail; most SMCs of macrochaetes appear by 6 hr after puparium formation (APF) (Huang et al., 1991) and those of microchaetes emerge between 8 and 12 hr APF (Usui and Kimura, 1993). Expression of the *Drosophila* RBP-J κ protein was induced in transgenic flies by heat shock of 37°C for 1 hr at 6 hr APF when most SMCs of microchaetes are believed to be singled out initially in proneural clusters (Usui and Kimura, 1993). Most of microchaetes disappeared on nota in transgenic flies (Figs 1C, D), although a normal pattern of microchaetes was formed in wild type flies that received the same treatment (Figs 1A, B). Only a small number of microchaetes survived whereas macrochaetes were not lost except for one macrochaete that transformed into the double socket phenotype. The SMCs of macrochaetes begin to be formed from late larvae up to early pupae and we confirmed that the heat shock treatment at this stage led to loss of macrochaetes (data not shown). Induced expression of the RBP-J κ protein was confirmed by Western blotting with an anti-RBP-J κ mAb that we have developed (data not shown).

These results suggested that loss of microchaetes might be caused by disorder of SMC formation. To investigate this hypothesis, we used the enhancer trap line *A101*, in which the *lacZ* protein is produced specifically in SMC (Huang et al., 1991; Bellen et al., 1989). We made *A101;hs-RBP-J κ* flies by genetic cross. Effect of the heat shock treatment at 6 hr APF on the SMC formation was examined with the anti-*lacZ* antibody. Nota of heat-treated and untreated flies were stained with this antibody at 14 hr and 24 hr APF. In control animals all the SMCs corresponding to macrochaetes and microchaetes were detected (Figs 2A, C, E and G). On the other hand, nota of heat shocked flies at 14 hr APF showed loss of many SMCs (Figs 2B and F), although the SMCs of macrochaetes remained intact. These heat shocked flies showed loss of many SMCs also at 24 hr APF indicating that SMC did not appear even at later stages (Figs 2D and H). This clearly indicates that loss of bristles is caused from defect of SMC formation. These results show that the number of SMCs is regulated by the amount of the RBP-J κ protein.

Socket cell commitment is enhanced by RBP-J κ

Hairless is known to be involved in the cell fate determination of the socket and shaft cells (Bang and Posakony, 1992). We assayed the biological role of the RBP-J κ protein in this terminal differentiation stage of SMC by using the transformants described above. The differentiation into socket and shaft cells is considered to begin around 26 hr APF (Usui and Kimura, 1993). The heat shock treatment at this stage induced the double-socket phenotype of microchaete of transformant flies (Figs 3A, B). Almost all shaft cells of microchaetes disappeared and transformed into socket cells on the notum. The shafts of macrochaetes are formed but they are slightly short and deformed. Some macrochaetes were transformed into double sockets (Fig. 3B). We also examined the

Fig. 1. Loss of bristles by overexpression of *Drosophila* RBP-J χ . Binocular images of nota of heat shock treated flies at 6 hr APF are shown. (A), notum bristles of wild type fly; (B), magnification of (A); (C), notum bristles (loss of most of microchaetes) in hs1-RBP-J χ fly; (D), magnification of (C) showing loss of both shaft and socket cells.

Fig. 2. Pattern of SMC appearance on the notum of adult flies. Whole mount preparations or dissected notum were labeled with the anti-lacZ antibody. The *hs2-RBP-J α* adults, either untreated (A, C, E and G) or heat-treated (B, D, F and H) at 6 hr APF were sacrificed at 14 hr APF (A, B, E and F) and 24 hr APF (C, D, G and H). (A) and (C), images of SMCs of the whole notum at 14 hr and 24 hr APF, respectively. (E) and (G), small areas of (A) and (C), respectively: note that four daughter cells of SMC appear at 24 hr APF. (B) and (D), loss of many SMCs at 14 hr and 24 hr APF,

Fig. 3. Double-socket phenotype caused by overexpression of *Drosophila* RBP-J κ . (A), notum of hsl-RBP-J κ fly with the heat shock treatment at 26 hr APF. Most of bristles lost shafts and remaining shafts are relatively short. (B), enlargement of (A) showing double socket-type bristles.

effect of overexpression of the RBP-J κ protein on bristle formation of other organs. The heat shock treatment at 16 hr APF caused transformation of many bristles into the double socket form on abdomen and legs (Figs 4B, C). The heat shock treatment at 6 hr APF resulted in loss of bristles on the legs (Fig. 4D).

4. DISCUSSION

Genetic studies using a number of mutants have clearly shown that *Su (H)* is required for the formation of adult sensory organs. The experiments described here showed that *Drosophila* RBP-J κ is required for at least two steps during development of adult sensilla; SMC formation and terminal differentiation of socket and shaft cells. Loss-of-function *Su (H)* mutants showed a remarkable increase in number of SMCs in imaginal discs (Schweisguth and Posakony, 1992). In agreement with this report, present studies on transformants with heat shock-induced high dosages of *Su (H)* display loss of SMCs on the notum, abdomen

respectively, in heat-treated transformant. (F), enlargement of (B) showing loss of many SMCs. (H), enlargement of (D) showing loss of many SMC and appearance of some of differentiated four daughter cells.

Fig. 4. Phenotype of overexpression of *Drosophila* RBP-J κ . (A), hs1-RBP-J κ fly without the heat shock treatment. (B), loss of bristles in heat shock treated hs1-RBP-J κ fly. (C), double-socket bristles on abdomen of (B). (D), loss of bristles on the legs of (B).

and leg. These results demonstrate that RBP-J κ participates in regulation of the number of SMCs in a manner similar to neurogenic genes, indicating that *Drosophila* RBP-J κ can be classified as a neurogenic gene. In this respect, it is interesting to note that loss of SMCs has been observed in Notch overexpression flies (Rebay et al., 1993). This suggests that RBP-J κ may be involved in Notch signal transduction pathway. A previous study concerning the phenotype of flies with duplicated *Su(H)*⁺ loci reported that up to four copies of *Su(H)*⁺ did not show a significant loss of bristles in a wild type background (Ashburner, 1982). The difference could be due to the fact that chromosomal gene duplication does not always correlate with a dose of the gene product.

We have also found that a high dosage of RBP-J κ results in the reduction of shaft cells. Some loss-of-function *Hairless* mutants show the double-socket phenotype whereas overexpression of *Hairless* shows the double-shaft phenotype (Bang and Posakony, 1992). These phenotypes seem to result from the transformation of the shaft cell into a socket cell, or the converse. Although no double-shaft phenotype of *Su(H)* mutants was found, the present results demonstrate that higher amounts of the RBP-J κ protein can induce differentiation of shaft/socket precursor cells into the socket cell. Thus the balance between the *Hairless* and RBP-J κ products is considered to determine the fate of the socket

and shaft cells. In fact, we have obtained the evidence that *RBP-J κ* product acts as a transcriptional activator of the *E (spl) m8* promoter and *Hairless* product represses it in a dose dependent manner (T. Furukawa, K. Tamura, K. Kimura, Y. Kobayakawa, T. Tanimura, M. Kawaichi and T. Honjo, submitted). To ensure the balanced differentiation between the shaft and socket cell fates, it is plausible that flies adopt a strategy using the competitive cell-cell interaction that has been demonstrated in SMC determination (Simpson, 1990). Various studies proposed that neurogenic genes participate in cell-cell interaction in tissues other than the nervous system (for review, Artavanis-Tsakonas and Simpson, 1991). In fact, it has been reported that a loss-of-function mutant of *Notch* results in the double shaft phenotype (de Celis et al., 1991).

During preparation of this manuscript a somewhat similar but independent result was reported by Schweisguth and Posakony (1994).

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