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Research paper

BmRobo1a and BmRobo1b control axon repulsion in the silkworm *Bombyx mori*

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ABSTRACT

The development of the nervous system is based on the growth and connection of axons, and axon guidance molecules are the dominant regulators during this course. Robo, as the receptor of axon guidance molecule Slit, plays a key role as a conserved repellent cue for axon guidance during the development of the central nervous system. However, the function of Robo in the silkworm *Bombyx mori* is unknown. In this study, we cloned two novel *robo* genes in *B. mori* (*Bmrobo1a* and *Bmrobo1b*). BmRobo1a and BmRobo1b lack an Ig and a FNIII domain in the extracellular region and the CC0 and CC2 motifs in the intracellular region. BmRobo1a and BmRobo1b were colocalized with BmSlit in the neuropil. Knock-down of *Bmrobo1a* and *Bmrobo1b* by RNA interference (RNAi) resulted in abnormal development of axons. Our results suggest that BmRobo1a and BmRobo1b have repulsive function in axon guidance, even though their structures are different from Robo1 of other species.

1. Introduction

In bilaterally symmetric animals, the midline is a critical control center during the development of the central nervous system (CNS). Midline crossing is an important regulating mode to affect the normal development of the nervous system, and the molecular mechanisms mediating midline crossing and axon path-finding are research focuses in many species (Goodman, 1994; Evans and Bashaw, 2010). Longitudinal axons normally do not cross the midline, while commissural axons are guided toward the midline and cross it only once to form a characteristic ladder-like structure, which is mediated by attractant and repellent signals (Pappu and Zipursky, 2010). As a potent midline repellent signal for axons, Robo was initially identified in a genetic screen for axon guidance mutants in the ventral nerve cord of the embryonic nervous system (Kidd et al., 1998). The structure of *Drosophila* Robo possesses an extracellular region of 5 Ig domains followed by 3 fibronectin type III domains (FNIII), a transmembrane region and a long intracellular tail containing four conserved cytoplasmic (CC0, CC1, CC2 and CC3) domains. Later, the other two Robo family members have been discovered in *Drosophila*, named Robo2 and Robo3. Four Robo family members have been identified in vertebrates, which are Robo1/Dutt1, Robo2, Robo3/Rig1 and Robo4/Magic Robo (Rajagopalan et al., 2000a; Lee et al., 2001; Huminiecki et al., 2002).

In order to exert its function in neural development, Robo needs to interact with Slit ligand. The Robo-Slit signaling axis was first established as a pathway to guide axon pathfinding, controlling neuronal migration and promoting axon branching. In *Drosophila*, three Robo receptors show distinct roles during the axon guidance. Robo1 and Robo2 coordinate to promote the repulsive functions in the midline, while Robo3, together with Robo2, controls the lateral position of axons in the developing CNS (Rajagopalan et al., 2000b; Simpson et al., 2000). Further researches reveal that Robo proteins are extensively involved in the developmental processes of various organs, such as the lung, liver, kidney, eye and reproductive systems (Piper et al., 2000; Greenberg et al., 2004; Thompson et al., 2006; Dickinson and Duncan, 2010; Zhou et al., 2011).

The silkworm, *B. mori*, owns a type of nervous system of ventral nerve cord, which controls numerous life activities, such as motion, hormone secretion and organs development. The nervous system of the silkworm has a simpler structure than that of vertebrates and is easy to operate in molecular biological research. Moreover, compared with *Drosophila*, its size and shape are larger. Thus the silkworm is an excellent model organism for studying nervous system development. In this paper, we cloned two new *robo* genes in the silkworm, named *Bmrobo1a* and *Bmrobo1b*. BmRobo1a and BmRobo1b were located in the CNS and colocalized with Slit ligand. The result of RNAi experiment showed that BmRobo1a and BmRobo1b had the function of midline repellent in the silkworm.

Abbreviations: *Bmrobo1a*, *roundabout1a* gene from *Bombyx mori*; *Bmrobo1b*, *roundabout1b* gene from *Bombyx mori*; *Bmslit*, *slit* gene from *Bombyx mori*; CNS, central nervous system; HSPGs, heparan sulfate proteoglycans; GAPs, GTPase-activating proteins; Ig, Immunoglobulin; FNIII, fibronectin type III; RNAi, RNA interference; CC, conserved cytoplasmic motifs; TM, transmembrane domain; RACE, Rapid amplification of cDNA ends; ORF, opening reading frame; UTR, untranslated region; egfp, enhanced green fluorescent protein; dsRNA, double-stranded RNA.

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2. Materials and methods

2.1. Silkworm strain

The silkworm strain *Dazao*, provided by the State Key Laboratory of Silkworm Genome Biology, Southwest University, was used in this study. The silkworms were cultured at standard temperature of 25 °C with fresh mulberry leaves under a photoperiod of 12 h light and 12 h dark.

2.2. Molecular cloning of Bmrobo1a and Bmrobo1b

In order to identify *robo1* orthologues in the silkworm, the amino acid sequence of *Drosophila* Robo1 was used as the query sequence to conduct BLAST search against the silkworm genome database (http://www.silkdb.org/silkdb). Total RNA from the brains of day 3 fifth instar larvae was isolated with TRIzol reagent (TaKaRa). The first-stranded cDNA was synthesized using reverse transcriptase AMV (Roche). Initial fragments of *Bmrobo1a* and *Bmrobo1b* were amplified by PCR with Primer 1F, Primer 1R and Primer 2F, Primer 2R, respectively (Table S1). Rapid amplification of cDNA ends (RACE) of *Bmrobo1a* and *Bmrobo1b* were performed using primers (5′-RACE: Primer 3-1, Primer 3-2 and Primer 4-1, Primer 4-2; 3′-RACE: Primer 5-1, Primer 5-2 and Primer 6-1, Primer 6-2) (Table S1) according to the manufacturer's instructions of the SMART PCR cDNA Amplification kit (Clontech).

2.3. Sequence analyses

The Robo sequences of other species were obtained from the NCBI database by the BLAST algorithm. The GenBank accession numbers of protein sequences are given in Table S2. Alignments of the deduced amino acid sequences were performed by MUSCLE program of MEGA6 and a phylogenetic tree was constructed by the neighbor-joining (NJ) method of MEGA6 (Tamura et al., 2013). Immunoglobulin (Ig), fibronectin type III (FNIII) domains and transmembrane region were predicted by simple modular architecture research tool (SMART, http://smart.embl.de).

2.4. Antibody generation

The nucleotide sequences encoding 60 and 86 amino acids of BmRobo1a and BmRobo1b were amplified by PCR with the Primer pair of 7F and 7R, Primer pair of 8F and 8R, respectively (Table S1). The PCR products were cloned into the pET-28a expression vector, and transformed into *Escherichia coli* BL21 (DE3) cells. HisTrap HP column (GE Healthcare) was used to purify the fusion protein, which was injected into rabbits to generate polyclonal antibodies (AbMax Biotechnology).

2.5. Antibody staining

The process of antibody staining was performed as described (Liu et al., 2000). Anti-BmRobo1a and BmRobo1b antibodies were used at 1:100 and 1:50 dilutions, respectively. Other antibodies used were: mouse anti-BmSlit polyclonal antibody (1:100, Yu et al., 2014), mouse MAb 22C10 (1:20; Developmental Studies Hybridoma Bank), Alexa 488-conjugated goat anti-rabbit IgG (1:1000; Jackson ImmunoResearch), Cy3-conjugated donkey anti-mouse IgG (1:1000; Jackson ImmunoResearch).

2.6. Construction of the dsRNA

Partial sequences of *Bmrobo1a* (526 bp) and *Bmrobo1b* (521 bp) in the intracellular region were selected as templates to synthesize the dsRNAs of the two genes, with the Primer pair of 9F and 9R, Primer pair of 10F and 10R, respectively (Table S1). The dsRNAs were synthesized by the RiboMAX Large Scale RNA production Systems-T7 and SP6 (Promega) according to the manufacturer's instructions.

2.7. Injection of dsRNA into silkworm embryos

The injection of dsRNA was performed as described (Yu et al., 2014). About 3 nL of 1 μg/μL *Bmrobo1a* and *Bmrobo1b* dsRNAs were microinjected into the silkworm embryos (*Dazao*) by the TransferMan NK2 micromanipulator and Femto Jet 5247 microinjector (Eppendorf) under an SZX16 microscope (Olympus). The injection opening was sealed with instant glue (Konishi Co.), and the injected embryos were incubated at 25 °C and 90% relative humidity until embryonic stage 22. Embryos for control were injected with the same amount of enhanced green fluorescent protein (*egfp*) dsRNA.

2.8. Microscopy and image treatment

The fluorescence microscope (Olympus BX53) and laser confocal scanning microscope (Leica SD AF) were used to obtain microscopic images. For the confocal microscopy, the step size of stacks was 1 μm. The images were processed with Adobe Photoshop CC image programs.

3. Results

3.1. Cloning and characterization of Bmrobo1a and Bmrobo1b

Partial sequences of *Bmrobo1a* (1324 bp) and *Bmrobo1b* (1269 bp) were cloned by PCR reactions. The full length sequences of *Bmrobo1a* (3896 bp) and *Bmrobo1b* (3324 bp) were obtained by the RACE method, and deposited in GenBank (Accession Nos. KF831574 and KF831575). *Bmrobo1a* consists of an opening reading frame (ORF) of 2931 bp, a 5′-untranslated region (UTR) of 249 bp and a 3′-UTR of 716 bp, while *Bmrobo1b* possesses an ORF of 3018 bp, a 5′-UTR of 165 bp and a 3′-UTR of 141 bp. The ORFs of *Bmrobo1a* and *Bmrobo1b* encoded polypeptides of 976 amino acids and 1005 amino acids, respectively. The two genes are both on silkworm chromosome 6. *Bmrobo1a* consists of 7

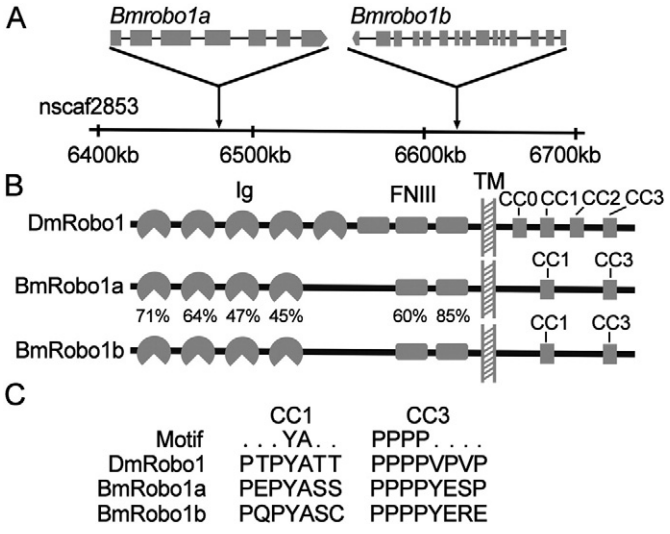

Fig. 1. Genomic localization and molecular characterization of *Bmrobo1a* and *Bmrobo1b*. (A) The genomic localization of *Bmrobo1a* and *Bmrobo1b* and their gene structures. The two genes are on the nscaf2853 of the 6 chromosome, exons and introns of the two genes are also sketchily represented. (B) Protein structure comparison of BmRobo1a, BmRobo1b and DmRobo1. Numbers show the percent amino acid identity between individual Ig and FNIII domains. Species are abbreviated as: Bm, *Bombyx mori*; Dm, *Drosophila melanogaster*. Lengths of cytodomains are roughly to scale. (C) Comparison of conserved cytoplasmic (CC) motifs for BmRobo1a, BmRobo1b and DmRobo1. CC1 and CC3 motifs are fairly well conserved between BmRobo1a, BmRobo1b, while CC0 and CC2 are not conserved in these two new Robo homologs of silkworm.