Evolutionarily Conserved Repulsive Guidance Role of Slit in the Silkworm Bombyx mori

Qi Yu1, Xiao-Tong Li1, Chun Liu2, Wei-Zheng Cui1, Zhi-Mei Mu1, Xiao Zhao1, Qing-Xin Liu1*

1 Laboratory of Developmental Genetics, Shandong Agricultural University, Ta’ian, Shandong, China, 2 State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing, China

Abstract

Axon guidance molecule Slit is critical for the axon repulsion in neural tissues, which is evolutionarily conserved from planarians to humans. However, the function of Slit in the silkworm Bombyx mori was unknown. Here we showed that the structure of Bombyx mori Slit (BmSlit) was different from that in most other species in its C-terminal sequence. BmSlit was localized in the midline glial cell, the neuropil, the tendon cell, the muscle and the silk gland and colocalized with BmRobo1 in the neuropil, the muscle and the silk gland. Knock-down of BmSlit by RNA interference (RNAi) resulted in abnormal development of axons and muscles. Our results suggest that BmSlit has a repulsive role in axon guidance and muscle migration. Moreover, the localization of BmSlit in the silk gland argues for its important function in the development of the silk gland.

Introduction

The Slit protein plays an important role in a variety of physiological and pathological processes, such as nervous system development, heart morphogenesis, muscle migration and tumor metastasis [1–6]. The slit gene was first identified in Drosophila and its homologues have been found in many species. There is a single slit in invertebrates. Three slit genes, slit1, slit2, and slit3 are found in tetrapods and there are four slit genes in teleosts [7–11]. It is generally considered that during animal evolution there is a global trend towards increases in gene size, complexity, and diversity [12]. Therefore, it is necessary to understand to what extent the evolutionary diversification of the slit gene contributes to the increase in the complexity of animals.

In insects, much of our knowledge about functions of the slit gene has come from studies in the dipteran Drosophila melanogaster [5,13,14]. Recently, the investigation of slit-mediated axon guidance has been extended to the coleopteran Tribolium castaneum [15]. The silkworm Bombyx mori has served as a lepidopteran model animal with many experimental advantages, such as large body size, short lifecycle, ease of rearing and rich genetic resources [16]. In addition, the completion of silkworm genome sequencing facilitates studies on molecular biology [17]. Separated by more than 240 million years from D. melanogaster, B. mori provides an important window on certain evolutionary changes of the lepidoptera relative to the diptera [16]. However, as a crucial guidance molecule for diverse cell types, the slit gene in the silkworm has not been described.

Here we report the characterization of Bmslit, a slit orthologue from B. mori. Multiple sequence alignment and domain analysis indicate that BmSlit has a relatively conserved structure but with a different form of C-terminal sequence. BmSlit localizes to the midline glial cell, the neuropil, the tendon cell, the muscle, and the silk gland and plays an important role in axon guidance and muscle migration in B. mori.

Materials and Methods

Experimental Animals

The silkworm strains Zhg×Chun54, 9202×La7 and Dazao were used in this study. Eggs were incubated at 25°C. Larvae were reared on fresh mulberry leaves or artificial diet at 25°C.

Identification and Cloning of Bmslit

To identify the slit orthologue in the silkworm, the fruit fly Slit protein sequence was used as the query sequence to perform BLAST search against the silkworm genome database (http://silkworm.genomics.org.cn/). Total RNA was extracted from the brain of day 3 fifth instar larvae. The first-stranded cDNA was synthesized using reverse transcriptase AMV (Roche) and an initial fragment of Bmslit was amplified by PCR using Primer 1F and Primer 1R (Table S1). Rapid amplification of cDNA ends (RACE) was performed using primers 5’-RACE: Primer 2-1 and Primer 2-2; 3’-RACE: Primer 3-1 and Primer 3-2 (Table S1) according to the manufacturer’s instructions of the SMART PCR cDNA Amplification kit (Clontech).
Sequence Analysis

Protein sequence alignment was performed by DNASTAR MAX Version 3.0 (MiraiBio, San Francisco, CA). Domain architectures for BmSlit were determined by SMART [18]. To investigate the evolutionary relationships between Slit of *B. mori* and other organisms, the neighbor joining (NJ) tree with Poisson model was constructed using MEGA5 [19].

Generation of Anti-BmSlit Antibody

The nucleotide sequence encoding 107 amino acids at the C-termini of BmSlit was amplified by PCR using Primer 4-F and Primer 4-R (Table S1). The PCR product was cloned into the expression vector pET28a and transformed into *Escherichia coli* BL21 (DE3) cells. The fusion protein was purified by HisTrap HP column (GE Healthcare) and used to generate polyclonal antibodies in mice (AbMax Biotechnology, Beijing, China).

In Situ Hybridization

In situ hybridization was carried out as previously described [20]. A 570 bp DNA fragment of *Bmslit*, residing in the fifth EGF domain to the LamG domain, was amplified by PCR using Primer 5-F and Primer 5-R (Table S1) and cloned into pGEM-T Easy Vector (Promega). *Bmslit* probe was generated by digesting the recombinant plasmid with NeoI and transcribing with DIG RNA Labeling Kit (Roche).

Western Blot Analyses

Western blot was carried out as previously described [21]. The primary antibody was anti-BmSlit antibody (1:100) and the secondary antibody was horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:20000, Jackson ImmunoResearch).

Immunofluorescence Staining

Antibody staining was carried out as previously described [22]. Whole embryos or silk glands were dissected in PBS and fixed in 4% formaldehyde in PEM buffer (PEM: 100 mM Pipes-KOH at pH 7.0, 2 mM EGTA, 1 mM MgSO4) for 40 minutes on ice, then permeabilized for 15 minutes at room temperature in PBS containing 0.5% NP40, and blocked for 2 hours in PBS containing 0.1% BSA and 5% goat serum. The samples were stained with the primary antibodies for 2 hours, followed by incubation with secondary antibodies for 1.5 hours at room temperature. The primary antibodies we used were as follows: anti-BmSlit antibody (1:100), anti-BmRobo1 antibody (1:100, developed by our lab) and

---

Figure 1. Sequence analysis of BmSlit. (A) Protein structure comparison of *Bombbyx mori* Slit (BmSlit), *Drosophila melanogaster* Slit (dSlit) and *Homo sapiens* Slits (hSlit1, hSlit2, hSlit3). (B) Phylogenetic tree of Slit. Numbers next to the branches indicate bootstrap values with 1000 replicates. The scale bar represents a distance of 0.1 amino acid substitutions per site. doi:10.1371/journal.pone.0109377.g001
mouse monoclonal antibody 22C10 (1:20, Developmental Studies Hybridoma Bank). The secondary antibodies Alexa 488-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) and Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) were used at a dilution of 1:500. The anti-HRP-FITC antibody (1:200, Jackson Immunoresearch) was also used.

**RNA Interference**

The same sequence with the in situ hybridization probe was used for the Bmslit RNA interference. Double-stranded RNA (dsRNA) of Bmslit and enhanced green fluorescent protein (EGFP) gene was synthesized in vitro by RiboMAX Large Scale RNA Production Systems (Promega). About 3 nl of 1 μg/μl Bmslit dsRNA solution was injected into silkworm embryos, which were collected within 3 hours of oviposition. The same amount of egfp dsRNA was used as a control.

**Microscopy and Image Treatment**

Images were acquired by the laser scanning confocal microscope (Leica SD AF) and fluorescence microscope (Olympus BX53) and treated with Adobe Photoshop CS6 image programs. For confocal microscopy, the step size of stacks was 1 μm.
Repulsive Guidance Role of BmSlit

Results

Isolation and Sequence Analysis of Bmslit

Bmslit was located on chromosome 24 of B. mori according to the silkworm genome database. The full length cDNA of Bmslit was isolated from the brain of silkworm, which has been submitted to GenBank (accession number KF739412). The ORF (open reading frame) sequence of Bmslit consisted of 4002 bp, encoding 1333 amino acid residues. The predicted BmSlit protein contained 1333 amino acid residues. The predicted BmSlit protein contained 1333 amino acid residues. The predicted BmSlit protein contained 1333 amino acid residues. The predicted BmSlit protein contained 1333 amino acid residues. The predicted BmSlit protein contained 1333 amino acid residues. The predicted BmSlit protein contained 1333 amino acid residues.

Expression Pattern of Bmslit

Figure 4. Knock-down phenotypes of Bmslit. (A) The embryos injected with egfp dsRNA were stained with anti-BmSlit antibody. The expression of BmSlit in midline glial cells was not affected (arrow). (B) The embryos injected with Bmslit dsRNA were stained with anti-BmSlit antibody. The expression of BmSlit in midline glial cells was significantly reduced (arrowhead). (C) In embryos injected with egfp dsRNA (as a control for the RNAi method) and stained with 22C10 antibody, the axons were visible on each side of the midline (arrow) (100%, n = 78). (D) The axon phenotype of embryos injected with Bmslit dsRNA (82.5%, n = 80). The axons were closer to the midline than in the control animal (arrowhead). (E) In embryos injected with egfp dsRNA and stained with anti-BmSlit antibody, the muscles were visible on each side of the midline (arrow) (100%, n = 76). (F) The muscle phenotype of embryos injected with Bmslit dsRNA (80%, n = 75). Some muscles crossed the midline (arrowhead). Scale bars represent 100 μm.

doi:10.1371/journal.pone.0109377.g004

Figure 4. Knock-down phenotypes of Bmslit. (A) The embryos injected with egfp dsRNA were stained with anti-BmSlit antibody. The expression of BmSlit in midline glial cells was not affected (arrow). (B) The embryos injected with Bmslit dsRNA were stained with anti-BmSlit antibody. The expression of BmSlit in midline glial cells was significantly reduced (arrowhead). (C) In embryos injected with egfp dsRNA (as a control for the RNAi method) and stained with 22C10 antibody, the axons were visible on each side of the midline (arrow) (100%, n = 78). (D) The axon phenotype of embryos injected with Bmslit dsRNA (82.5%, n = 80). The axons were closer to the midline than in the control animal (arrowhead). (E) In embryos injected with egfp dsRNA and stained with anti-BmSlit antibody, the muscles were visible on each side of the midline (arrow) (100%, n = 76). (F) The muscle phenotype of embryos injected with Bmslit dsRNA (80%, n = 75). Some muscles crossed the midline (arrowhead). Scale bars represent 100 μm.

doi:10.1371/journal.pone.0109377.g004

Discussion

Slit is an evolutionarily conserved multifunctional protein and its guidance role has been studied in many organisms, such as planarians, nematodes, flies, and vertebrates [2,3,8,9]. In this study, we identified and characterized an orthologue of the slit gene from the silkworm B. mori. It has been known that the interaction between Slit and its receptor Robo is mediated through the second LRR domain of Slit and the first Ig domain of Robo [23]. Comparing with the Drosophila counterpart, BmSlit lacked the seventh EGF domain and the CT domain, but it was similar to the protein structure of Dugesia japonica Slit. Multiple sequence alignment showed that the LRR2 domain of BmSlit is clearly conserved. Therefore, although BmSlit lacked the partial C-terminal sequence, the interaction between BmSlit and its receptor may not be affected. We also found the colocalization of BmSlit and BmRobo1, which further supported the ligand-receptor relationship between BmSlit and BmRobo1.
As a multifunctional molecule, the Slit protein controls the development of diverse tissues [24]. We showed that BmSlit was localized in the midline glial cell, the neuropil, the tendon cell, the muscle and the silk gland. RNAi-mediated knockdown of BmSlit produced abnormal arrangements of axons and muscles. These results indicate that BmSlit contributes to the development of axons and muscles. In addition, surprisingly, we found that BmSlit colocalized with BmRobo1 in the silk gland. The silk gland is derived from the labial segment and then migrates dorsally and posteriorly [25]. So far, the molecular mechanism underlying the migration of silk gland is unclear. However, our finding raises an intriguing possibility that BmSlit is involved in the migration of the silk gland.

Supporting Information

Figure S1  Sequence alignment of Bombyx mori Slit with Drosophila melanogaster Slit (NP_476727.1) and Homo sapiens Slits (Slit1, NP_003052.2; Slit2, NP_004778.1; Slit3, NP_001258875.1). Species are abbreviated as: Bm, Bombyx mori; d, Drosophila melanogaster; h, Homo sapiens.

References

1. Brose K, Bland KS, Wang KH, Arnott D, Henzel W, et al. (1999) Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. Cell 96: 795–806.
2. Hu H (1999) Chemorepulsion of neuronal migration by Slit2 in the developing mammalian forebrain. Neuron 23: 703–711.
3. MacMullin A, Jacobs JR (2006) Slit coordinates cardiac morphogenesis in Drosophila. Dev Biol 295: 134–164.
4. Medioni C, Bertrand N, Mesbah K, Hudry B, Dupays L, et al. (2010) Expression of Slit and Robo genes in the developing mouse heart. Dev Dyn 239: 3303–3311.
5. Kramer SG, Kidd T, Simpson JH, Goodman CS (2001) Switching repulsion to attraction: changing responses to Slit during transition in mesoderm migration. Science 292: 737–740.
6. Bauer K, Dowjok A, Bouserhoff AK, Reichert TE, Bauer R (2011) Slit-2 derived from the labial segment and then migrates dorsally and posteriorly [25]. So far, the molecular mechanism underlying the migration of silk gland is unclear. However, our finding raises an intriguing possibility that BmSlit is involved in the migration of the silk gland.

Acknowledgements

We are grateful to Susumu Hirose for critical comments on the manuscript. We thank Qing-You Xia for assistance with microinjection and Hua Tang for help with the confocal microscopy.

Author Contributions

Conceived and designed the experiments: QY QXL. Performed the experiments: QY XTL. Analyzed the data: QY XTL WZC XZ. Contributed reagents/materials/analysis tools: CL ZMM. Wrote the paper: QY QXL.

Table S1  Primer sequences used in this study.

(TIF)

(DOC)