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Expression and Function of Slit1α, a Novel Alternative Splicing Product for Slit1

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Slits are large molecular and extracellular glycoproteins that may function as chemorepellents in axon guidance and neural cell migration. The heterogeneity of the mRNA for *slit* has been described. Its variants indicate considerable potential for alternative splicing, resulting in the generation of multiple protein isoforms. We examined the regions in which these isoforms are expressed, and identified the highest expression of a splicing product for *slit1* in rat brain rather than in other organs. The splicing product, Slit1α, arises through alternative splicing at the C-terminus of Slit1, causing defects in the cysteine knot domain. We show that *slit1α* exists in the hippocampus and cerebral cortex in rat brain by *in situ* hybridization, and that it acts as a chemorepellent in olfactory bulb axon guidance *in vitro*. These findings suggest that Slit1α is an active Slit1 protein specific in the vertebrate nervous system.

Key words: alternative splicing, axon guidance, chemorepellent, Robo, Slit.

Abbreviations: LRR, leucine-rich repeats; EGF, epidermal growth factor; Robo, roundabout; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

Slits are extracellular ligands for Robo transmembrane receptors (*1*). Three slit genes, *slit1* to *slit3*, have been cloned in mammals (*2–5*). The Slit1 and Slit2 proteins act as chemorepellents and/or collapsing factors for olfactory (*5*, *6*), hippocampal (*6*), retinal (*6–9*), and motor axons (*10*). The *slit* genes encode large proteins found in the extracellular matrix. They contain four stretches of tandem LRR that mediate binding to Robo (*11–13*). The proteins also have nine EGF-like repeats similar to sequence in Notch EGF repeats. The EGF-like repeats are also involved in protein recognition and ligand binding (*14*, *15*). Slit contains a globular domain (ALPS-conserved domain) (*2*, *4*, *15*) found in other extracellular matrix proteins (agrin, laminin, and perlecan) and referred to as the ALPS (agrin, laminin, perlecan, and slit) motif (*16*). The motif in agrin is required for nerve-to-muscle signaling that triggers the formation of neuromuscular synapses (*17*). The C-terminal region of the Slit protein contains a cysteine knot that is a dimerization motif found in several secreted growth factors (*2*, *18*, *19*), and that mediates specific associations with other proteins (*15*). Except for the LRR domain, the functions of Slit structures are not known. In vertebrates, Slit2 can be proteolytically cleaved, and its amino-terminal fragment increases number of the sensory axon branches *in vitro* (*13*). The putative cleavage site is conserved among some but not all Slit family members, exceptions being vertebrate Slit1 and *Caenorhabditis elegans* Slit (*2*). Alternative spliced transcripts have been reported for *Drosophila slit* (*15*) and human *slit2* and *3* (*4*). Recently, Little et al. reported the structure of human and mouse *slit* genes, and found a conservation of exons and introns (*20*). There is the potential for alternative splicing resulting in the generation of multiple Slit isoforms, but their expressions and functions are not well known.

In this paper, we describe a novel alternative spliced mRNA product for *slit1* found specifically in vertebrate nervous system that acts as a chemorepellent factor for olfactory bulb axons.

MATERIALS AND METHODS

Animals—Sprague-Dawley rats (Kiwa Jikken Dobutsu Laboratory, Wakayama, Japan) were used for RNA extraction and culture experiments. The day on which a vaginal plug was detected was considered embryonic day 0, and the day of birth was considered neonatal day 0. Pregnant rats were anesthetized with chloral hydrate (40 mg/kg). All procedures were conducted in accordance with the Policy on the Use of Animals of Osaka Prefecture University.

Reverse Transcription and PCR for Adult Brains—RNA was isolated from neonatal and adult rat brains with Isogen (Nippon Gene, Toyama, Japan). The quantity of total RNA was measured by UV spectrophotometry. The mRNA was reverse-transcribed with an RT-PCR kit (AMV, ver. 2.1) that included an oligo-dT-adapter primer (Takara Bio, Otsu, Japan). Polymerase chain reaction (PCR) primers (Table 1) for *slit1* were designed on the basis of published cDNA sequence (Genbank accession number AF133730) (*2*). The cDNA was amplified on a thermal cycler (iCycler, Bio-Rad, Richmond, CA) with LA Taq or EX Taq DNA polymerase (Takara Bio). For amplification of the complete expression sequence from the cDNA, nested PCR was conducted with primer sets, resulting in the amplification of nucleotides (nt) 76–4755. Amplified products were separated by 1.5% agarose-gel electrophoresis, and stained with ethidium bromide.

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Table 1. **Nucleotide sequences of the oligonucleotides used in the amplification of *slit1* by PCR.**

Product (nt)	5′ Primer sequence	3′ Primer sequence
76–4755	5′-GGATAGCACCTGGAGAGTAGC-3′	5′-TTACGCACACGGGGCACAGC-3′
182– 669	5′-GGTCCTCTTCGGGGTTGA-3′	5′-GCTGATCTGGTTCTTGTC-3′
499–1300	5′-CTACGACTGAACCGCAACC-′′	5′-ACAGGCCTCCAAATACACC-3′
1274–1879	5′-TGCCCCGAGGTGTATTTG-3′	5′-TGCTCAGGTTTATTTTCT-3′
1827–2416	5′-GGCCACTGGGTTGTTTA-3′	5′-GGAGGTGCTTGTTGCTA-3′
2292–2871	5′-TGTGGCCTTCCCTGACTTCA-3′	5′-CAACAGTTTGCCCTCCATCT-3′
2864–3704	5′-AACTGTTGCTCACCACACCT-3′	5′-CAGTTCTGCAGGTCAGTGAA-3′
3662–4334	5′-TGGACCGGGACACTTACC-3′	5′-TCGAGGGGCACGCATTTC-3′
4175–4725	5′-GCCGAAAACTCTACTGTCT-3′	5′-GGGCTTCTCCACCTCCTCA-3′

Amplification by PCR with different primer pairs, resulted in the amplification of nt 182–669, 499–1300, 1274–1879, 1827–2416, 2292–2871, 2864–3704, 3662–4334 and 4175–4725 (Table 1). The amplified products were separated by 5% poly-acrylamide-gel electrophoresis. The regions nt 4175–4725 from the cDNA and genomic DNA were amplified by PCR. PCR products were separated by 1% agarose-gel electrophoresis. A molecular weight standard (Nippon Gene) was used. The nucleotide sequences of the PCR products were determined with a Thermo Sequenase primer cycle sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ) and an SQ-5500 sequencer (Hitachi, Tokyo, Japan).

RNase Protection Assay—The 1.7-kbp product from the amplification of nt 4175–4725 was cloned into pT7Blue T-vector (EMD Bioscience, Darmstadt, Germany). A fragment containing nt 4442–4954 and partial sequences of pT7Blue about 40-bp long was obtained by digestion with the restriction enzymes *Hind*III and *Rsa*I. The isolated fragment was cloned into pGEM-4Z (Promega, Madison, WI). The vector containing the insert was linearized on the 5′-side of the insert. A probe for *slit1* was generated with SP6 RNA polymerase (Invitrogen, Carlsbad, CA). An RNase protection assay was performed with a kit (RPAIII; Ambion, Austin, TX). In brief, total RNA was hybridized with ^{32}P-labeled cRNA overnight, and digested with ribonucleases A and T1. The hybridized RNA was then separated in a 5% poly-acrylamide-gel containing 8 M urea. After electrophoresis, the portions of RNA protected by the probe were visualized by autoradiography.

RT-PCR for Aadult Tissues—RNA from the brains, hearts and kidneys of adult rats was used. The fragment nt 4175–4725 of *slit1* was amplified as described above for adult brain. Electrophoresis was performed using a 1.0% agarose-gel. The amount of cDNA was standardized according to the results of amplification of the housekeeping gene for G3PDH, carried out at the same time.

In Situ Hybridization—An alternative splicing product, *slit1α*, cDNA fragment was obtained from the 1.7-kbp genomic cDNA by digestion with the restriction enzymes *Rsa*I and *Sma*I. The fragment was subcloned into pGEM-4Z vector and used to generate digoxigenin (DIG)-labeled cRNA probes for SP6 or T7 RNA polymerase. The cRNA probes were partially digested with 0.1 M Na_2CO_3 (pH 10) at 60°C for 45 min to enhance permeability. Whole brains of 7 weeks old Sprague-Dawley rat were fixed in 4% paraformaldehyde in PBS, embedded in paraffin and sectioned to a thickness of 8 μm. The samples were deparaffined, acetylated with 0.1 M triethanolamine (pH 8.0) and hybridized with 50 μl of hybridization buffer (50% formamide, 2× SSC, 1 μg/μl salmon testis DNA, 1 μg/μl yeast tRNA, 1 μg/μl BSA, 10% dextran sulfate, 0.1 M dithiothreitol) containing 400 μg/ml DIG labeled cRNA probe per sample at 55°C. After hybridization, the samples were treated with 20 μg/ml RNaseA, and dehydrated. The hybridization signals were detected with anti-DIG-AP Fab fragments (Roche, Basel, Switzerland) and NBT/BCIP solution.

Antibody Production—A polyclonal antibody was raised in New Zealand White rabbits according to standard procedures using the histidine tag fusion protein. A His-tag fusion protein encompassing a portion of the 1st–6th EGF-like repeats of Slit1 was constracted from the *Rattus norvegicus* Slit1 sequence (Genbank accession number AF133730) (*2*), subcloned into pET-16b (Takara Bio). The fusion protein was purified using a nickel column (Qiagen, Tokyo, Japan). Affinity-purified His-Slit1 was emulsified with Freund's complete adjuvant and injected intramusculary into rabbits. Four booster doses of fusion protein emulsified in Freund's incomplete adjuvant were administered biweekly. A final booster dose was administered 1 week later, and the blood was collected by cardiac puncture. The serum IgG fraction containing the anti-Slit1 antibody was purified with a MAb Trap kit (Amersham Pharmacia Biotech).

Plasmid Construction and Transfection—The coding region for full-length *slit1* or *slit1α* was inserted in frame into p3XFLAG-CMV13 (Sigma, St. Louis, MO) to allow expression of the proteins with FLAG tags (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C) at their C-termini. The inserted sequence and its orientations were confirmed by DNA sequencing. COS-7 cells were transfected with this plasmid by means of Geneporter transfection reagent (Gene Therapy Systems, San Diego, CA).

Immunoblot Analysis—To investigate the expression of Slit1 protein *in vivo*, rat brains were homogenized with a polytronhomogenizer in PBS containing 2.5mM EDTA, 0.1mM PMSF, protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The crude homogenate was centrifuged at 1,000 × *g* for 5 min at 4°C. The supernatant was precipitated with acetone, solubilized in SDS-PAGE sample buffer, subjected to SDS-PAGE in a 7% gel, and then transferred to a PVDF membrane (Millipore, Billerica, MA). The membrane was stained with the anti-Slit1 antibody. To examine the expression and secretion of Slit1 and Slit1α proteins from COS-7 cells to the medium, conditioned media and high salt (1M NaCl) extracts from cells transfected with truncation constructs containing Slit1 or Slit1α fragments tagged with FLAG, were proved

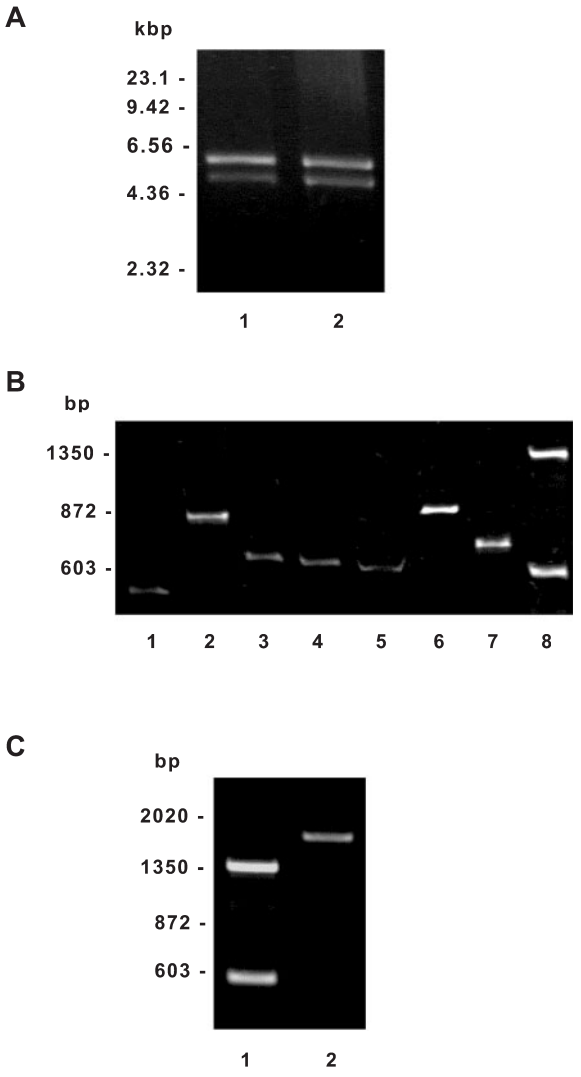

Fig. 1. **Electrophoretic patterns of cDNAs containing the coding sequence for rat *slit1*.** (A) The electrophoretic pattern of cDNAs containing the complete coding sequence for rat-brain *slit1*. Neonatal- and adult-brain mRNAs were reverse-transcribed with an oligo-dT-adapter primer, and the cDNA was amplified by PCR. The amplified products from neonatal (lane 1) and adult (lane 2) rat brains were separated by 1.5% agarose-gel electrophoresis. (B) The electrophoretic pattern of cDNAs containing parts of the coding sequence for newborn rat-brain *slit1*. cDNA was amplified by PCR with different primer pairs, resulting in the amplification of nt 182–669 (lane 1), 499–1300 (lane 2), 1274–1879 (lane 3), 1827–2416 (lane 4), 2292–2871 (lane 5), 2864–3704 (lane 6), 3662–4334 (lane 7) and 4175–4725 (lane 8). The amplified products were separated by 5% poly-acrylamide-gel electrophoresis. (C) Amplification of *slit1* in cDNA and genomic DNA. cDNA was isolated from newborn rat brain. Genomic DNA was isolated from rat liver. The regions nt 4175–4725 from the cDNA (lane 1) and genomic DNA (lane 2) were amplified by PCR. PCR products were separated by 1% agarose-gel electrophoresis. Molecular masses are shown at the left.

with the anti-Slit1 antibody and anti-FLAG M2 monoclonal antibody (Sigma). An Immunostar kit (Wako, Osaka, Japan) was used for detection.

Collagen-Gel Assay—Olfactory bulbs were extracted from embryos on day 15, and then cultured (*21*). The explants were cultured for 48 h with aggregates of COS-7 cells transfected with *slit1* or *slit1*α or control vector. The samples were cultured at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 100 ng/ml heparin, in a humidified atmosphere containing 5% CO_2. Explants were fixed in ice-cold 4% paraformaldehyde and immunolabeled (*6*). The neurite lengths of the explants were measured as described by Chédotal *et al.* (*22*).

RESULTS

Identification of Splice Variants by RT-PCR—To detect splice variants of *slit1*, we applied a combination of RT-PCR. Initially, nt 76–4725 of *slit1* were amplified from the cDNA of neonate rat brain. Two amplified cDNA, approximately 4.7 and 5.5 kbp in size, were obtained. The same result was obtained in the case of adult rat brain cDNA (Fig. 1A). Nested PCR was performed with the primer pairs listed in Table 1, resulting in the amplification of cDNA products for covering the whole coding sequence of *slit1*. Separation of the amplified PCR products with the primer pairs nt 182–669, 499–1300, 1274–1879, 1827–2416, 2292–2871, 2864–3704 and 3662–4334 (Table 1) by agarose-gel electrophoresis showed that for each primer pair one cDNA fragment was predominantly amplified (Fig. 1B, lanes 1–7). Sequencing of these PCR products indicated that the nucleotide sequence of the amplified cDNA was identical with that reported for *slit1* (*2*). In contrast with the PCR products from the region nt 182–4334 of *slit1*, amplification of the cDNA in the region nt 4175–4725 resulted in the detection of two PCR products (Fig. 1B; lane 8). Sequencing of these PCR products revealed that the smaller fragment was part of the *slit1* cDNA sequence while the larger one had a 792-bp insert (Fig. 1C; lane 1). PCR of the *slit1* nt 4175–4725 from genomic DNA produced a 1.7-kbp product (Fig. 1C, lane 2). Sequencing of the amplified genomic cDNA showed it to be identical to that of the *slit1* cDNA except for 383-bp and 792-bp insertions. The ends of these insertion sequences had donor and acceptor consensus sequence sites, AG and GT (*23*, *24*). These results suggest that the 383-bp sequence is an intron, and is cut off by splicing in both mRNA species. They also suggest that the 792-bp sequence is an exon, and that it is removed by alternative splicing to produce a *slit1* variant (Fig. 2A). Insertion of the 792-bp nucleotide by alternative splicing results in the expression of a 180-kDa Slit1 variant protein that lacks the cysteine knot domain in the C-terminal region owing to the presence of a termination codon in the inserted exon (Fig. 2B). We named the product containing the 792-bp insert "*slit1*α" (accession number AB073215).

Identification of mRNA Species by RNase Protection Assay—To confirm the presence of the variant containing the insertion of 792-bp sequence by alternative splicing, we performed RNase protection assay with a 552-bp probe consists of part of the 383-bp intron, the 212-bp exon, and part of the 792-bp exon (Fig. 2A). An unprotected band was detected around 550-bp (Fig. 3, lane 1). Two bands were found to migrate slightly faster than the unprotected band, confirming the presence of mRNAs for the two splicing variants (Fig. 3, lanes 2 and 3). The upper band, included the 212-bp exon and part of the 792-bp exon, was *slit1*α. The lower band, included the

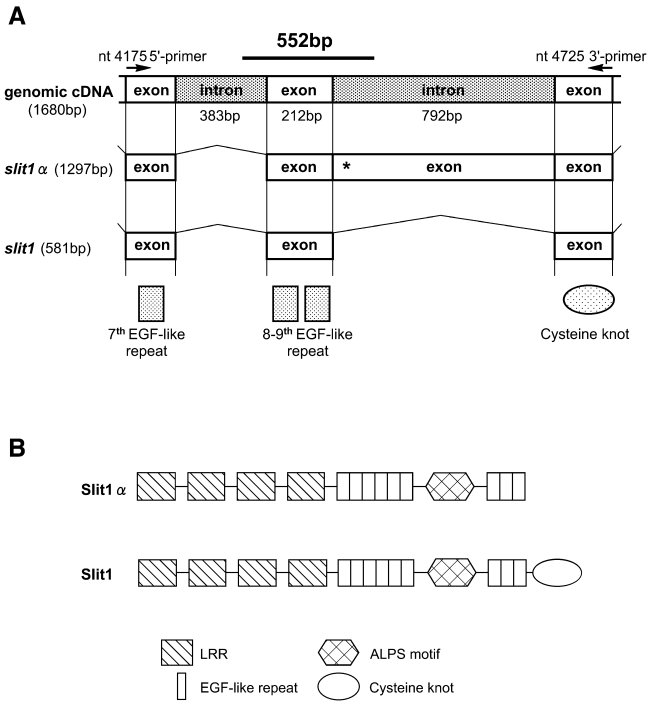

Fig. 2. **Proposed structure of the 3′-region of rat *slit1*.** (A) The major mRNA species contain some exons, but the 383-bp intron has been deleted by splicing. The numbers at the left are the sizes of the RT-PCR products with primers spanning nt 4175–4725. The 1297-bp product, *slit1α*, comprised four exons, one of which is inserted the 792-bp exon. The 581-bp product, *slit1*, comprises three exons with sequences that have been previously reported. The bar labeled 552-bp shows the length and position of the undigested probe used in the RNase protection assay. The asterisk (*) shows the position of termination codon. Diagrams at the bottom show the protein domains of Slit1. (B) Diagrams depicting the functional domains of Slit1 and Slit1α proteins. Slit1α lacks the cysteine knot domain in the C-terminal region owing to the presence of a termination codon in the inserted 792-bp exon.

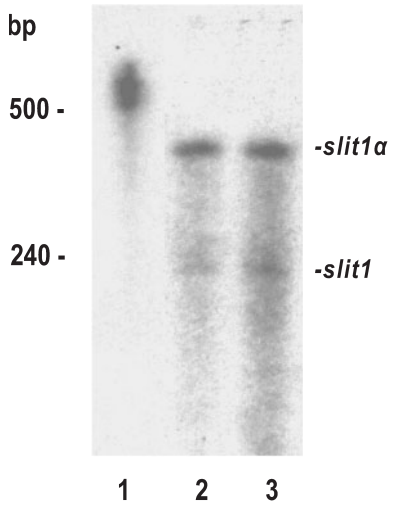

Fig. 3. **Identification of *slit1* mRNA species by RNase protection assay.** An RNase protection assay was performed with RNA from neonatal rat brain (lane 2) and adult rat brain (lane 3). The undigested probe (552-bp of *slit1*) is in lane 1. Twenty micrograms each of total RNA from neonatal or adult brain was hybridized with the probe, and then digested with ribonucleases A and T1.

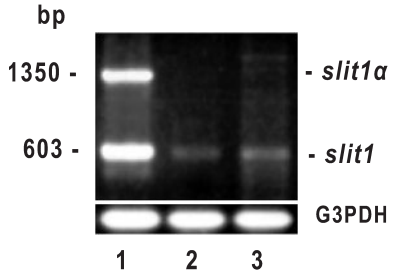

Fig. 4. **Expression of *slit1* and *slit1α* mRNA in adult tissues.** cDNAs from brain (lane 1), heart (lane 2) and kidney (lane 3) of adult rats were amplified by PCR with a primer set, resulting in the amplification of nucleotide sequence (nt) 4175–4755 of *slit1*. The PCR products were separated by 1% agarose-gel electrophoresis and stained with ethidium bromide. The mRNA for the housekeeping gene G3PDH, used to standardize the mRNA amounts, is shown at the bottom.

212-bp exon, was *slit1* (Fig. 2A, Fig. 3, lanes 2 and 3). These results suggest that the 383-bp intron was removed from both mRNA species, and that the 792-bp sequence was inserted as an exon for *slit1α* by alternative splicing (Fig. 2A).

Distribution of Splice Variants of slit1 in Sdult Tissues—To investigate the proportions of splicing products of *slit1* in adult tissues, we examined the expression of each type of mRNA in brain, heart, and kidney (Fig. 4). Each *slit1* splicing product had a distinct expression pattern: *slit1* was expressed in the brain, heart and kidney, while *slit1α* was specific to the brain. The expression level of *slit1* was highest in the brain, although it was detectable in heart and kidney.

Localization of slitla in Adult Brain—To clarify in more detail of expression pattern of *slit1α* mRNA in adult rat brain, we performed *in situ* hybridization using a probe specific for *slit1α*. The *slit1α* mRNA was found to be expressed in the hippocampus and cerebral cortex (Fig. 5, arrow), with the strongest signals detected in dentate gyrus and CA3 regions.

Detection of Slit1α Protein In Vivo—To confirm the presence of Slit1 proteins *in vivo*, we performed Western blotting analysis using an anti-Slit1 antibody. Slit1 and Slit1α proteins expressed in COS-7 cells were detected with the anti-Slit1 antibody (Fig. 6, lanes 2–4) and an anti-FLAG antibody directed against the FLAG tag present within Slit1 and Slit1α constructs (Fig. 6, lanes 5–7). Two bands were detected in rat brain extract (Fig. 6, lane 1) corresponding to Slit1 and Slit1α. The presence of the anti-Slit1 antibody was also demonstrated by recognizing recombinant Slit1 and Slit1α expressed in COS-7 cells. These results indicate that both Slit proteins are present in rat brain.

Slit1α Repels Olfactory Bulb Axons—To investigate the repelling activity of Slit1α, we compared the effects of these recombinant Slit1 proteins on the axons of olfactory bulb neurons (in particular, mitral cells) from 15-day embryos. After constructs encoding the *slit1*, *slit1α*, or control vector were separately transfected into COS-7 cells, the conditioned media were collected. Western blot

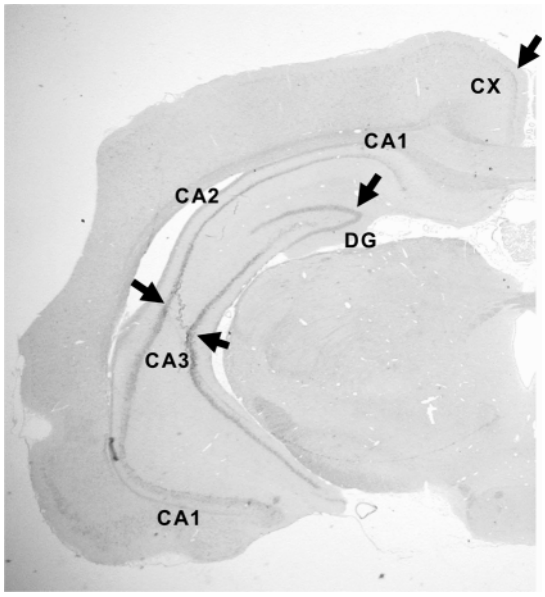

Fig. 5. **Localization of *slit1α* mRNA in a rat adult brain transverse section.** The sample was hybridized with a *slit1α*-specific cRNA probe. *Slit1α* mRNA was stained in the hippocampus and cerebral cortex. The strongest signals were detected in dentate gyrus and CA3 regions. CA1–CA3: CA fields of Ammon's horn; CX: cerebral cortex; DG: dentate gyrus.

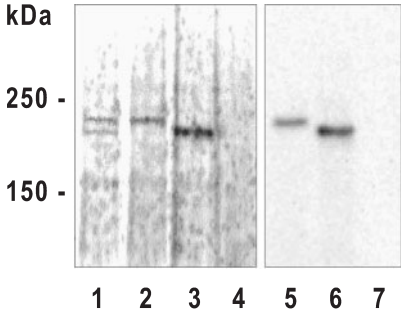

Fig. 6. **Immunoblotting of Slit1 and Slit1α proteins.** Two bands were detected in rat brain by the anti-Slit1 antibody (lane 1). Constructs encoding *slit1* (lanes 2 and 5), *slit1α* (lanes 3 and 6), or the control vector (lanes 4 and 7) were transfected into COS-7 cells. Conditioned media from the cells were subjected to immunodetection with the anti-Slit1 antibody (lanes 2–4) and the antibody recognizing the FLAG tag at the C-terminus (lanes 5–7).

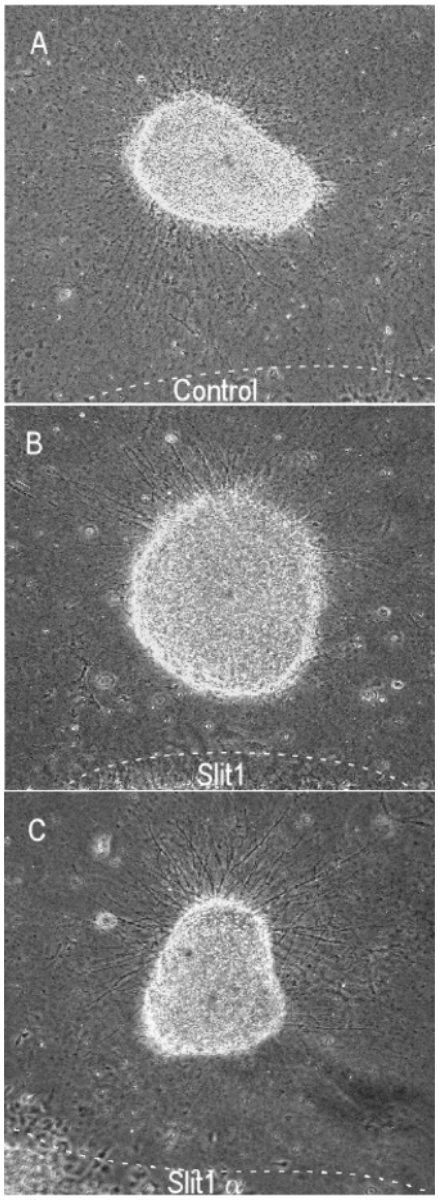

Fig. 7. **Effects of Slit1 and Slit1α proteins on rat olfactory bulb axons.** Olfactory bulb explants of tissues obtained from 15-day embryos were cocultured with COS-7 cells transfected with control vector (A), Slit1 (B), or Slit1α (C). Axons growing next to control cells grew radially, but axons cocultured with cells expressing Slit1 or Slitα grew away from the cells.

analysis showed that the proteins were secreted extracellularly into the culture medium (Fig. 6; lanes 2–7). We used a three-dimensional collagen-gel assay to test the ability of the proteins to function as diffusible chemorepellent with respect to axons. Olfactory bulb axons were repelled both by cells that expressed Slit1 (Fig. 7B, Table 2) and those that expressed Slit1α (Fig. 7C, Table 2). The Slit1α protein was similar to Slit1 protein in that it functioned as a chemorepellent.

DISCUSSION

Slit is a large extracellular matrix protein that is thought to function in axonal guidance in *Drosophila* and mammals. Heterogeneity of the mRNA for *slit* has been described (*4*, *15*, *20*). Although it has been suggested that the considerable potential for alternative splicing results in the generation of multiple Slit isoforms, their expression patterns and functions have not been clarified. The *slit1* mRNA is mainly expressed in vertebrate brain, as compared with other organs (*4*, *25*). Alternative splicing products for *slit1* may involve axonal pathfinding, or contribute to refinement of the connectivity within neural networks.

We searched for alternative splicing regions in *slit1*, and identified the alternative splicing product containing a 792-bp insertion in the 3′-region between the 9th EGF-like repeat and the cysteine knot (Figs. 1B and 2A). We

Table 2. **Patterns of axonal growth in olfactory bulb explants cocultured with COS-7 cells transfected to express one of three proteins.**

Protein	Number of explants	Pattern of axonal growth		
		No repulsion	Moderate repulsion	Strong repulsion
Slit1	23	1	16	6
Slit1α	27	2	15	10
Control	26	25	1	0

Results are classified as follows: no repulsion (length of axons growing toward and growing away from COS-7 cells differed by less than twofold in length); moderate axonal repulsion (axons growing away were two to three times longer than those growing toward the COS-7 cells); strong axonal repulsion (axons growing away were more than three times longer than those growing toward the COS-7 cells). Attraction of axons (toward COS-7 cells) was not observed.

designated this variant *slit1α*. Some splicing products were also found in other regions of *slit1* (Fig. 1B, lanes 1–7), but their expressions were quantitatively lower than that of *slit1α* (Fig. 1B, lane 8). Little *et al.* predicted the occurrence of multiple mRNA splicing patterns for *slit1* (*20*). Splicing products found in the RT-PCR experiment partially correspond to their predicted patterns (Fig. 1B). Our investigation of the genomic structure of rat *slit1* in the 3′-region corresponding to the sequence between the 9th EGF-like repeat and the cysteine knot (Fig. 1C) showed that the 792-bp in the *slit1α* mRNA are inserted as an exon, which is deleted as an intron in the *slit1* mRNA (Fig. 1C; lane 1, Fig. 2A). Between the 7th EGF-like repeat and 8th EGF-like repeat of Slit1, we found a portion of the previously published *slit1* cDNA sequence containing the 383-bp intron (Figs. 1C and 2A). The intron was deleted from the 3′-untranslated region of prespliced *slit1* and *slit1α* mRNAs. These exon and intron gene structures of rat *slit1* in the 3′-region appear similar to those in humans (*20*). The 212-bp exon in the rat *slit1* cDNA (Fig. 2A) correspond to exon 36 in the human *slit1* cDNA (20; Table 1). The 383-bp and 792-bp introns in rat *slit1* (Fig. 2A) correspond to the 335-bp and 1019-bp introns in human *slit1* (20; Table 1). These gene structures are conserved in vertebrates, suggesting the expression of *slit1α* by alternative splicing may also be conserved in vertebrates.

We confirmed the presence of *slit1α* mRNA by an RNase protection assay (Fig. 3). The 383-bp intron located in the 7th EGF-like and 8th EGF-like repeats was not detected, and we, therefore, concluded that the *slit1α* mRNA is an alternative splicing product, not a prespliced mRNA. These data indicate that in addition to *slit1*, *slit1α* is also expressed in neonatal and adult rat brain. To investigate the distribution of the *slit1* and *slit1α* mRNAs in other tissues, we examined the expression of the *slit1* and the *slit1α* mRNAs in brain, heart, and kidney. The *slit1α* was expressed specifically in rat brain, but not in heart or kidney (Fig. 4), suggesting that Slit1α plays an exclusive role in the nervous system. To confirm the localization of the *slit1α* mRNA in rat brain, we performed *in situ* hybridization. The *slit1α* mRNA was found to be expressed in the cerebral cortex and hippocampus (Fig. 5). Itoh *et al.* revealed that the *slit1* mRNA is expressed in the cerebral cortex and hippocampus by *in situ* hybridization (*4*). These results suggest that *slit1* and *slit1α* are coexpressed in rat brain.

Two bands were detected around 200 kDa in rat brain extract by immunoblot analysis using an anti-Slit1 antibody (Fig. 6, lane 1). Based on a comparison with the molecular weights of the Slit1 and Slit1α proteins expressed in COS-7 cells (Fig. 6, lane 2–7), the bands were identified as the Slit1 and Slit1α proteins. It has been reported that 40-kDa C-terminal fragment and 160-kDa N-terminal fragment are detected in 293T cells expressing the *slit1* construct, although Slit1 was not processed in rat primary neurons expressing the *slit1* construct (*26*). In our immunoblot analysis, the cleaved form of Slit1 was not detected in either Slit1-expressing COS-7 cells or rat brain extract. These results suggest that in addition to the alternative splicing of *slit1*, the proteolytic processing of Slit1 also regulates the function of the Slit1 protein *in vivo*.

It has been reported that the cysteine knot functions as a dimerizing motif in several secreted growth factors, or mediates specific associations with other proteins (*2*, *15*, *18*, *19*). Slit1α lacks the cysteine knot owing to a terminal codon in the inserted 792-bp exon (Fig. 2). To investigate whether Slit1 is able to promote dimerization via the cysteine knot domain in the C-terminus, western blotting analysis was performed under non-reducing conditions. The dimeric form was not detected (data not shown), indicateing that cysteine knot motif, conserved in all of the slit family, may not function as a dimerization motif, but rather may mediate specific associations with other proteins (*15*).

Slit proteins repel axons in a process mediated by binding to Robo, identified as a Slit receptor. Battye *et al.* demonstrated that the LRR domain in the N-terminal region of Slit2 mediates binding to Robo (*11*, *12*). Nguyen *et al.* reported that the N-terminal portion from LRR to the 5th EGF-like repeat of Slit1 is enough to repel olfactory bulb axons (*27*). We found that Slit1 and Slit1α had similar activities in repelling olfactory bulb axons in a collagen-gel repulsion assay (Fig. 7, Table 2). Our data also show that the N-terminal region of Slit1 mediates the repelling of olfactory bulb axons. Slit1α can function as a chemorepellent in neonate and adult rat.

The present results indicate that a Slit1 isoform, Slit1α, is an active Slit1 protein. It is specifically expressed in rat brain during the developmental stage and in the neuronal system after the developmental stage, and is functional as a chemorepellent for olfactory bulb axon guidance *in vitro*. However, its functions *in vivo* are not fully known. It is necessary to explore the functions of Slit1α in each part of brain in order to understand the role of Slit proteins in nervous system.

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