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Characterization of a novel member of murine semaphorin family☆

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Abstract

Semaphorin gene family contains a large number of secreted and transmembrane proteins, and some of them are functioning as the repulsive and attractive cues of the axon guidance during development. Here we report murine orthologues of a novel member of class 6 semaphorin gene, semaphorin 6D (Sema6D), mapped on the chromosome 2. Sema6D is mainly expressed in the brain and lung, and the ubiquitous expression in the brain continues from embryonic late stage to adulthood, as determined by Northern blot and in situ hybridization. We also found that Sema6D has five different splicing variants, and the expression patterns of individual isoforms differ depending on the tissues. Thus, Sema6D may play important roles in various functions including the axon guidance during development and neuronal plasticity.

Keywords: Semaphorin; Axon guidance; Brain; Alternative splicing

During embryogenesis, axons reach their specific targets correctly to form the complex neural network found in the mature functional nervous system. Several groups of axon guidance molecules such as semaphorins, ephrins, netrins, and slits have been reported to repel or attract the growing axons that express their cognate receptors [1].

Semaphorins are secreted and transmembrane proteins containing a conserved domain (semaphorin domain) of about 500 amino acids are found in both vertebrates and invertebrates [2,3]. So far, more than 20 kinds of semaphorin genes have been identified and classified into seven classes and a virus semaphorin [4]. Among them, semaphorin 3A (Sema3A) is the first identified semaphorin in vertebrates on the basis of its ability to induce the collapse of axonal growth cones of dorsal root ganglion (DRG) [5]. Sema3A-deficient mice showed a severe abnormality in the axonal projection pattern in the peripheral nervous system during embryogenesis [6]. Neuropilins are functional receptors for class 3 semaphorins and plexins are also known as receptors for other types of semaphorins [7–15]. Moreover, semaphorin 4D/CD100 functions in the immune system and semaphorin 3C (Sema3C) functions during cardiac development [16,17]. Thus, semaphorin family genes perform many important biological functions besides the axon guidance.

To identify a novel semaphorin gene, we searched human expressed sequence tag (EST) databases and identified a novel human semaphorin cDNA, KIAA1479. By using KIAA1479 as a probe, here, we cloned a novel murine transmembrane semaphorin gene, semaphorin 6D (Sema6D), and its four splicing variants. Northern blot and in situ hybridization analyses suggest their important roles in the brain development and functions.

Materials and methods

Identification and cloning of semaphorin 6D. We searched human EST databases and identified a novel human semaphorin cDNA,

☆ *Abbreviations:* aa, amino acid; bp, base pair; DRG, dorsal root ganglion; E, embryonic day; EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ORF, open reading frame; RT, reverse transcriptase; Sema6D, semaphorin 6D; SSC, standard saline citrate; VEGF, vascular endothelial growth factor.

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KIAA1479, which is a member of class 6 semaphorin and thus was named semaphorin 6D (SEMA6D) according to the rule of Semaphorin Nomenclature Committee [4]. To clone the murine semaphorin 6D (Sema6D) cDNA, adult mouse (C57BL/6) brain cDNA library was screened with a 1.5 kb *Hin*cII fragment of human SEMA6D cDNA as a probe. Hybridization was performed in 6× SSC, 5× Denhardt's solution, 0.2% SDS, and 100 μg/ml herring sperm DNA at 55 °C overnight. The filters were washed with 2× SSC–0.2% SDS four times at 55 °C. The positive clones were sequenced by use of an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, CA, USA). Thus, a 6330 bp Sema6D-1 cDNA containing the open reading frame and four isoforms were obtained. The accession numbers of Sema6D-1, Sema6D-2, Sema6D-5, Sema6D-6, and Sema6D-4 are deposited as AB091532, AB091533, AB091534, AB091535, and AB091536, respectively.

RT-PCR. Reverse transcriptase (RT) reaction was performed by ThermoScript RT (Invitrogen, CA, USA) with 2 μg of total RNA. PCR was performed by TaKaRa Ex Taq (TaKaRa, Japan) with an exon 15 primer TTAAGCCAGGGAGTTTGTGAGAGA and an exon 19 primer TTCATGTGGACCATCTGATTGGAT. PCR products were electrophoresed on 4% agarose gel.

Northern blot hybridization. Northern blot membranes purchased from Seegene, (Seoul, Korea) were used. Hybridization was performed in 50% formamide, 6× SSC, 5× Denhardt's solution, 0.2% SDS, and 100 μg/ml herring sperm DNA at 42 °C overnight. The filters were washed with 2× SSC–0.2% SDS three times and 0.2× SSC–0.2% SDS once at 65 °C. A 2.5 kb *Ban*II–*Bgl*II fragment of Sema6D-1 cDNA was used as a probe.

In situ hybridization. In situ hybridization was performed as described [18]. Anesthetized ICR mice were perfusion-fixed with 4% paraformaldehyde and dissected organs were sectioned after paraffin embedding. After in situ hybridization staining, the sections were counterstained with Kernechtrot stain solution (Muto Pure Chemicals, Tokyo, Japan). To generate probes, Sema6D-1 fragment (2317–2886, 570 bp) was cloned. Digoxigenin-labeled riboprobes were prepared by transcription of linearized plasmids using T7 or T3 RNA polymerase and DIG RNA labeling kit (Roche Molecular Biochemicals, Mannheim, Germany). Sense probes were used as controls.

Results

Identification and cloning of human and murine semaphorin 6D genes

We identified one novel transmembrane semaphorin cDNA, KIAA1479. Sequence analysis revealed that KIAA1479 contained a predictable open reading frame (ORF) and was a novel member of class 6 semaphorin (Fig. 1B). KIAA1479 was named semaphorin 6D (SEMA6D) according to the rule of Semaphorin Nomenclature Committee [4]. SEMA6D encodes 1011 amino acid (aa) protein with a predicted molecular weight of about 113 kDa. Then, murine semaphorin 6D (Sema6D) was cloned by use of human SEMA6D cDNA as a probe (Fig. 1A). The length of Sema6D cDNA was 6330 bp and the ORF of Sema6D are 3033 bp (1011 aa). Murine Sema6D protein showed a 95.3% identity with human SEMA6D protein (Fig. 1B), and similarity to other class 6 semaphorins was: Sema6A1 (45% identity), Sema6B (44% identity), and Sema6C (38% identity) [19–22].

During the Sema6D cloning, we obtained four kinds of Sema6D isoforms. Because human SEMA6D was recently reported by others independently [23], these Sema6D and isoforms were renamed Sema6D-1 (1011 aa predictable protein), Sema6D-2 (998 aa), Sema6D-5 (1030 aa), Sema6D-6 (1054 aa), and Sema6D-4 (1073 aa) according to human SEMA6D nomenclature. These isoforms were generated by alternative splicing (Fig. 2 and see below).

Genomic structure and chromosomal localization

Sema6D gene was mapped on chromosome 2 by the use of Celera mouse genomic database. Comparison between Sema6D cDNA and mouse genomic DNA sequences revealed that Sema6D gene is composed of at least 19 exons. Sema6D gene spans more than 56 kb of the genomic DNA. The first exon including 5′ noncoding region was about 42 kb distant from the second exon that includes the initiation codon ATG. Thus, the length of other 18 exons and 17 introns of Sema6D gene is about 14 kb. In Sema6D isoforms, exon 16a (39 bp) was deleted in the ORF of Sema6D-2 cDNA, while exon 17 (57 bp) was added to Sema6D-5. Sema6D-6 lacked exon 16a and attached exon 18 (168 bp). Sema6D-4 did not have exon 16a but had exons 17 and 18 (Fig. 2).

Expression pattern

To clarify the temporal and regional expression of Sema6D, Northern blot hybridization was performed (Fig. 3). Sema6D transcript was about 6.5 kb in size, and the length was comparable to the size of the cloned Sema6D cDNA. Its expression during embryogenesis was analyzed (Fig. 3A). Sema6D was detected on embryonic day 10.5 (E10.5) and its expression continued until birth. It was expressed predominantly in adult brain and lung, moderately in heart, small intestine, skeletal muscle, uterus, and placenta (Fig. 3B). In lung and placenta, additional Sema6D transcripts were detected, but their natures remain to be elucidated. As Sema6D was expressed predominantly in adult brain, we analyzed the developmental changes in Sema6D expression of the brain (Fig. 3C) and its regional distribution in adult brain (Fig. 3D). Sema6D expression in the brain was detected from E17.5 to 12 months at similarly high levels. Furthermore, Sema6D was expressed ubiquitously in the brain with relatively higher expressions in the thalamus, hypothalamus, midbrain, cerebellum, pons and medulla oblongata, and spinal cord. To further study Sema6D expression patterns, in situ hybridization was performed (Fig. 4). At E13.5 a high Sema6D expression was detected in the neopallial cortex (Fig. 4B, shown in blue). In adult brain

A

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CTCCGCTGACGCTCTGGGTGCGTGATGGGGGTGGGACCCCGAGCCGGGAGCCCGCAGCGC    60
AGCTGTGTGCGGACGCGTGTGTTTGCGTGGGCGTAGCGTCGTAGCTCCGGGAGTTCATT   120
GCTCTCCCAAGCCACACGGTCTCCAGCGGTGCCCGAGGCGCCAGGGGAGGTCTGGCTGAC   180
CAGTCCCAGCTGGCCGGTGGGCGGCCTGGGGGCGGGGCGGGGCAGCTCGGCCCTCCTCCT   240
AGGCGCACCCCTCCTCTGAGGCGCACCCCTAAGGGAAGAAAAGACACCCTGGGCTGCGAG   300
GAAAAGTTTCTTCTTGGCAGGGCCGGCTCGGCGCCGCTCCGCAAGCCGTCAGGGTGAGCC   360
CGCCTTCCTCGCTGCGGGGCTTGGAGCCTCCAGCCCGCCCGCAGCGCAGCCCGGGCCGCC   420
GGGCCAGCTTCGGCCGGGAGAACGCAGGGACAGCCTGGGAGTGCGGAGCCACTGACTGTC   480
CACGCGGGGACCGTGAGCACCCACTCGCGGGTCTCCCAGCCACTCTCCCCCGCAACTTTG   540
CGTGCTTTGTTCTCTGGCTGAAAAAATGCTCCACAGAAGCGCCATAGACCGGGTCTCCTC   600
CAGGCGGCAAGGGCTCGGCCGGGAGACGTGTTACGCTCTCAGCTCCCCATCAATTATGGA   660
TGGAAACAAATAAGGAAAAGTCAATTTTGTATGAGCCGCGTCTTTCGCAGCCAGAGGCGT   720
CTTGTAGCCTGGAGCAGAGGCAGGCAAACCGAGCCCAACAACCAGGTAGCTAAGTGGGAC   780
TTCTGAGGAGGGGGAGCTCAGATTTCTTCTTGGCCAACCATGGGGTTCCTTCTGCTTTGG   840
                                  M  G  F  L  L  L  W              7
TTCTGCGTGCTGTTCCTTCTGGTCTCCAGGTTACGGGCGGTCAGCTTCCCAGAAGACGAT   900
F  C  V  L  F  L  L  V  S  R  L  R  A  V  S  F  P  E  D  D     27
GAGCCCCTCAACACGGTTGACTATCACTATTCAAGGCAATATCCGGTTTTAGAGGACGC   960
E  P  L  N  T  V  D  Y  H  Y  S  R  Q  Y  P  V  F  R  G  R     47
CCTTCAGGCAACGAATCGCAGCACAGGCTGGACTTTCAGCTGATGTTGAAAATTCGAGAC  1020
P  S  G  N  E  S  Q  H  R  L  D  F  Q  L  M  L  K  I  R  D     67
ACACTTTATATTGCTGGCAGGGATCAAGTCTATACAGTGAACTTAAATGAAATCCCCCAA  1080
T  L  Y  I  A  G  R  D  Q  V  Y  T  V  N  L  N  E  I  P  Q     87
ACAGAGGTGATACCAAGCAAGAAGCTGACGTGGAGGTCCAGACAGCAGGATCGAGAAAAT  1140
T  E  V  I  P  S  K  K  L  T  W  R  S  R  Q  Q  D  R  E  N    107
TGTGCTATGAAAGGCAAGCATAAAGATGAATGCCACAACTTCATCAAAGTCTTTGTCCCA  1200
C  A  M  K  G  K  H  K  D  E  C  H  N  F  I  K  V  F  V  P    127
AGAAATGATGAGATGGTTTTTGTCTGTGGTACCAATGCTTTCAACCCGATGTGCAGATAC  1260
R  N  D  E  M  V  F  V  C  G  T  N  A  F  N  P  M  C  R  Y    147
TATAGGTTGAGAACGTTAGAGTATGATGGGGAAGAAATTAGTGGCCTGGCACGATGCCCG  1320
Y  R  L  R  T  L  E  Y  D  G  E  E  I  S  G  L  A  R  C  P    167
TTTGATGCCCGACAAACCAATGTCGCCCTCTTTGCTGATGGAAAACTCTATTCTGCCACA  1380
F  D  A  R  Q  T  N  V  A  L  F  A  D  G  K  L  Y  S  A  T    187
GTGGCTGATTTCCTGGCCAGTGATGCTGTCATTTACAGAAGCATGGGAGATGGATCTGCC  1440
V  A  D  F  L  A  S  D  A  V  I  Y  R  S  M  G  D  G  S  A    207
CTTCGCACAATAAAATACGATTCCAAGTGGATCAAAGAACCACACTTCCTTCATGCCATA  1500
L  R  T  I  K  Y  D  S  K  W  I  K  E  P  H  F  L  H  A  I    227
GAATATGGAAACTATGTCTATTTCTTCTTCAGAGAAATCGCCGTGGAACATAATAACTTA  1560
E  Y  G  N  Y  V  Y  F  F  F  R  E  I  A  V  E  H  N  N  L    247
GGCAAGGCTGTGTATTCCCGCGTGGCTCGCATTTGTAAAAACGACATGGGTGGCTCACAG  1620
G  K  A  V  Y  S  R  V  A  R  I  C  K  N  D  M  G  G  S  Q    267
CGGGTCCTGGAGAAACACTGGACTTCCTTCCTTAAGGCTCGGCTGAACTGCTCCGTTCCT  1680
R  V  L  E  K  H  W  T  S  F  L  K  A  R  L  N  C  S  V  P    287
GGAGATTCCTTTTTCTACTTCGACGTCCTGCAGTCTATAACAGACATAATCCAAATCAAT  1740
G  D  S  F  F  Y  F  D  V  L  Q  S  I  T  D  I  I  Q  I  N    307
GGCATCCCCACTGTGGTTGGGGTCTTCACCACAACGATTCCTGGTTCTGCA           1800
G  I  P  T  V  V  G  V  F  T  T  Q  L  N  S  I  P  G  S  A    327
GTCTGTGCCTTTAGCATGGACGACATTGAGAAAGTGTTCAAAGGGCGGTTCAAAGAGCAG  1860
V  C  A  F  S  M  D  D  I  E  K  V  F  K  G  R  F  K  E  Q    347
AAAACCCCAGACTCTGTTTGGACAGCAGTTCCCGAAGACAAAGTACCAAAACCAAGGCCT  1920
K  T  P  D  S  V  W  T  A  V  P  E  D  K  V  P  K  P  R  P    367
GGCTGTTGTCCAAACACGGCCTCGCAGAAGCTTACAAGACCTCCATCGACTTTCCAGAT  1980
G  C  A  K  H  G  L  A  E  A  Y  K  T  S  I  D  F  P  D      387
GACACCCTGGCTTTCATCAAGTCCCACCCGCTGATGGACTCTGCCGTCCCACCCATTGCC  2040
D  T  L  A  F  I  K  S  H  P  L  M  D  S  A  V  P  P  I  A    407
GATGAGCCCTGGTTCACAAAGACACGGGTCAGGTACAGGTTGACAGCCATCGAAGTGGAC  2100
D  E  P  W  F  T  K  T  R  V  R  Y  R  L  T  A  I  E  V  D    427
CGTTCAGCAGGGCCATACCAAAACTACACAGTCATCTTTGTTGGCTCTGAAGCTGGCGTG  2160
R  S  A  G  P  Y  Q  N  Y  T  V  I  F  V  G  S  E  A  G  V    447
GTACTTAAAGTTTTGGCAAAGACCAGTCCTTTCTCTCTGAATGACAGTGTATTACTCGAA  2220
V  L  K  V  L  A  K  T  S  P  F  S  L  N  D  S  V  L  L  E    467
GAGATTGAAGCTTATAACCCAGCCAAGTGCAGCGCCGAGAGTGAGGAGGACAGAAAGGTG  2280
E  I  E  A  Y  N  P  A  K  C  S  A  E  S  E  E  D  R  K  V    487
GTCTCATTACAGCTGGACAAGGATCACCATGCTTTATACGTGGCCTTCTCTAGCTGCGTG  2340
V  S  L  Q  L  D  K  D  H  H  A  L  Y  V  A  F  S  S  C  V    507
GTCCGCATCCCCCTCAGCCGCTGTGAGCGCTACGGATCGTGTAAAAAGTCTTGCATTGCA  2400
V  R  I  P  L  S  R  C  E  R  Y  G  S  C  K  K  S  C  I  A    527
TCACGTGACCCGTACTGTGGTTGGTTAAGCCAGGGAGTTTGTGAGAGAGTGACCCTAGGG  2460
S  R  D  P  Y  C  G  W  L  S  Q  G  V  C  E  R  V  T  L  G    547
ATGCTGCTGTTAACCGAAGACTTCTTTGCTTTCCATAACCACAGCCCTGGAGGATATGAG  2520
M  L  L  T  E  D  F  F  A  F  H  N  H  S  P  G  G  Y  E      567
CAGGACACGGAGTACGGCAACACAGCCCACCTAGGGGACTGCCACGGTGTACCGTGGGAA  2580
Q  D  T  E  Y  G  N  T  A  H  L  G  D  C  H  G  V  R  W  E    587
GTCCAGTCTGGAGAATCCAATCAGATGGTCCACATGAATGTCCTCATCACCTGCGTGTTT  2640
V  Q  S  G  E  S  N  Q  M  V  H  M  N  V  L  I  T  C  V  F    607
GCCGCTTTGTCTTGGGCGCGTTCATCGCAGGAGTGGCCGTGTACTGCTACCGTGACATG  2700
A  A  F  V  L  G  A  F  I  A  G  V  A  V  Y  C  Y  R  D  M    627
TTCGTTCGGAAGAACAGAAAGATCCATAAAGACGCAGAATCCGCCCAGTCGTGCACAGAC  2760
F  V  R  K  N  R  K  I  H  K  D  A  E  S  A  Q  S  C  T  D    647
TCCAGCGGAAGCTTCGCCAAGCTGAACGGCCTCTTTGACAGCCCCGTCAAGGAATACCAG  2820
S  S  G  S  F  A  K  L  N  G  L  F  D  S  P  V  K  E  Y  Q    667
CAGAACATTGATTCTCCCAAACTCTACAGCAACCTGCTGACCAGTCGGAAGGAACTGCCA  2880
Q  N  I  D  S  P  K  L  Y  S  N  L  L  T  S  R  K  E  L  P    687
CCAAACACGGATACAAAGTCCATGGCCGTGGACCACAGAGGCCAGCCTCCCGAGCTGGCT  2940
P  N  T  D  T  K  S  M  A  V  D  H  R  G  Q  P  P  E  L  A    707
GCTCTCCCCACGCCGGAATCCACACCTGTCCTCCACCAGAAGACCCTGCAGGCCATGAAG  3000
A  L  P  T  P  E  S  T  P  V  L  H  Q  K  T  L  Q  A  M  K    727
AGCCACTCTGAGAAGGCCCACAGCCACGGTGCTTCAAGGAAAGAACACCCCCAGTTTTTT  3060
S  H  S  E  K  A  H  S  H  G  A  S  R  K  E  H  P  Q  F  F    747
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P  S  S  P  P  P  H  S  P  L  S  H  G  H  I  P  S  A  I  V    767
CTTCCAAACGCCACTCACGACTACAATACATCCTTCTCCAACTCGAATGCCCACAAAGCC  3180
L  P  N  A  T  H  D  Y  N  T  S  F  S  N  S  N  A  H  K  A    787
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E  K  K  L  Q  S  M  D  H  P  L  T  K  S  S  S  K  R  E  H    807
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R  R  S  V  D  S  R  N  T  L  N  D  L  L  K  H  L  N  D  P    827
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N  S  N  P  K  A  I  L  G  E  I  H  M  A  H  Q  T  L  M  L    847
GACCCGGTGGGACCAATGGCTGAGGTCCCACCCAAGGTCCCTAACCGGGAGGCATCTCTA  3420
D  P  V  G  P  M  A  E  V  P  P  K  V  P  N  R  E  A  S  L    867
TACTCCCCTCCCTCCACACTCCCCAGAAATAGTCCAACCAAGAGAGTAGATGTCCCCACC  3480
Y  S  P  P  S  T  L  P  R  N  S  P  T  K  R  V  D  V  P  T    887
ACTCCTGGGGTGCCAATGACTTCTCTGGAAAGACAAAGGGGTTATCACAAAAATTCCTCC  3540
T  P  G  V  P  M  T  S  L  E  R  Q  R  G  Y  H  K  N  S  S    907
CAGAGGCACTCTATATCTGCCGTGCCTAAAAACTTAAACTCACCAAATGGTGTTTTGTTA  3600
Q  R  H  S  I  S  A  V  P  K  N  L  N  S  P  N  G  V  L  L    927
TCTAGACAGCCGAGTATGAACCGTGGAGGCTATATGCCCACCCCAACAGGGGCGAAGGTG  3660
S  R  Q  P  S  M  N  R  G  G  Y  M  P  T  P  T  G  A  K  V    947
GACTATATTCAGGGGACACCGGTGAGTGTTCATCTGCAGCCCTCCCTCTCCAGACAGAGC  3720
D  Y  I  Q  G  T  P  V  S  V  H  L  Q  P  S  L  S  R  Q  S    967
AGCTATACCAGTAATGGCACCCTCCCCAGGACGGGACTAAAGAGGACACCATCCTTAAAA  3780
S  Y  T  S  N  G  T  L  P  R  T  G  L  K  R  T  P  S  L  K    987
CCTGATGTGCCACCAAAGCCTTCCTTTGTTCCGCAAACCACATCTGTCAGACCACTGAAC  3840
P  D  V  P  P  K  P  S  F  V  P  Q  T  T  S  V  R  P  L  N   1007
AAGTACACGTACTAGGCCTCAAGTATGCTATTCCCGTGTGGCTTTATCCTGTCCCTGCTG  3900
K  Y  T  Y  *                                                 1011
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TAAACCAACCAACAATAAGCACTACACATCTGCCACCAACAATGTTGTTTGCATTTACCT  5940
TACCAATATTAATCCCAGCGTGGTAACTCTGTGTGACCCCGATAACATTTTGTAACATTG  6000
TGCTGCCTTAGAGTTTGTACTGTGAGTTCTATCAGTATTTATGTTGAAATTTCTAACATG  6060
GATTCTAGTCTCTATTCTGTTAATTTAATTTTAAATGCTTTATCCATTTGTCAAAGGTA  6120
AACACAGATTGTATCTTTTTTAATGGTACGGCATAAAAAAAATAACCCTAAAGTGAAGTGG  6180
CTCTATACTGTTTTATAGAGTACTTTAACGTGTATAGATATCTTGTAAACTTGTATTGTG  6240
GATGTGTAAATAATATGTACTTTGGGTTTTTAACACCGCATGTAAAGTCAA[AATAAA]ATA  6300
TCCAAGTCATTAAAAAAAAAAAAAAAAAA                                  6330
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B

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Sema6D  MGFLLLWFCVLFLLVSRLRAVSFPEDDEPLNTVDYHYSRQYPVFRGRPSGNESQHRLDFQ    60
SEMA6D  *RVF**CAYI*L*M**Q*****************************************

Sema6D  LMLKIRDTLYIAGRDQVYTVNLNEIPQTEVIPSKKLTWRSRQQDRENCAMKGKHKDECHN   120
SEMA6D  ************************M*K*****N*************************

Sema6D  FIKVFVPRNDEMVFVCGTNAFNPMCRYYRLRTLEYDGEEISGLARCPFDARQTNVALFAD   180
SEMA6D  ******************************S****************************

Sema6D  GKLYSATVADFLASDAVIYRSMGDGSALRTIKYDSKWIKEPHFLHAIEYGNYVYFFFREI   240
SEMA6D  ************************************************************

Sema6D  AVEHNNLGKAVYSRVARICKNDMGGSQRVLEKHWTSFLKARLNCSVPGDSFFYFDVLQSI   300
SEMA6D  ************************************************************

Sema6D  TDIIQINGIPTVVGVFTTQLNSIPGSAVCAFSMDDIEKVFKGRFKEQKTPDSVWTAVPED   360
SEMA6D  ************************************************************

Sema6D  KVPKPRPGCCAKHGLAEAYKTSIDFPDDTLAFIKSHPLMDSAVPPIADEPWFTKTRVRYR   420
SEMA6D  ************************E**S*****************************

Sema6D  LTAIEVDRSAGPYQNYTVIFVGSEAGVVLKVLAKTSPFSLNDSVLLEEIEAYNPAKCSAE   480
SEMA6D  ****S**H*****************M************************H******

Sema6D  SEEDRKVVSLQLDKDHHALYVAFSSCVVRIPLSRCERYGSCKKSCIASRDPYCGWLSQGV   540
SEMA6D  N***K**I*****************II*************************S

Sema6D  CERVTLGMLLLTEDFFAFHNHSPGGYEQDTEYGNTAHLGDCHGVRWEVQSGESNQMVHMN   600
SEMA6D  *G***P****************AE*******F*************************

Sema6D  VLITCVFAAFVLGAFIAGVAVYCYRDMFVRKNRKIHKDAESAQSCTDSSGSFAKLNGLFD   660
SEMA6D  ************************************************************

Sema6D  SPVKEYQQNIDSPKLYSNLLTSRKELPPNTDTKSMAVDHRGQPPELAALPTPESTPVLHQ   720
SEMA6D  ************************G*****VM*************************

Sema6D  KTLQAMKSHSEKAHSHGASRKEHPQFFPSSPPPHSPLSHGHIPSAIVLPNATHDYNTSFS   780
SEMA6D  *************G*******T************************************

Sema6D  NSNAHKAEKKLQSMDHPLTKSSSKREHRRSVDSRNTLNDLLKHLNDPNSNPKAILGEIHM   840
SEMA6D  ************NI**********D*********************M*D*Q*

Sema6D  AHQTLMLDPVGPMAEVPPKVPNREASLYSPPSTLPRNSPTKRVDVPTTPGVPMTSLERQR   900
SEMA6D  ***N*****M*S*S*************************************************

Sema6D  GYHKNSSQRHSISAVPKNLNSPNGVLLSRQPSMNRGGYMPTPTGAKVDYIQGTPVSVHLQ   960
SEMA6D  ************M*************************************************

Sema6D  PSLSRQSSYTSNGTLPRTGLKRTPSLKPDVPPKPSFVPQTTSVRPLNKYTY          1011
SEMA6D  ****************************************P**********
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Fig. 1. Identification of murine Sema6D gene. (A) The nucleotide and predicted amino acid sequences of murine Sema6D-1. Putative signal sequence and transmembrane domain are underlined. The in-frame stop codon and poly(A) signal sequences are shown in bold letters. (B) Alignment of murine Sema6D-1 (Sema6D) and human SEMA6D proteins.

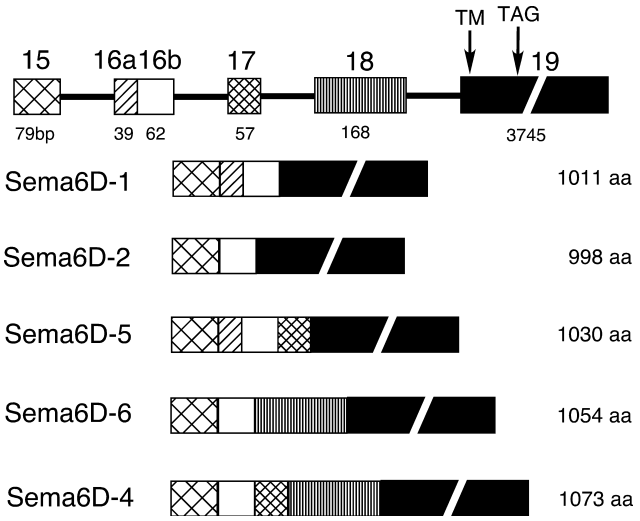

Fig. 2. Schematic diagram of generation of Sema6D isoforms. The boxes and lines represent exons and introns, respectively. The length of introns is arbitrary. The exons 15–19 and the length (bp) of their exons are shown, and the coding exons 2–14 are identical in all isoforms and omitted from the figure. The nomenclature of isoforms was adjusted with human Sema6D [23]. TM represents a putative transmembrane region. The stop codon TAG is also indicated.

Sema6D transcripts were detected in the olfactory bulb, dentate gyrus, and other regions (Figs. 4D and F, shown in blue), suggesting important roles in these regions.

Expression pattern of Sema6D isoforms

To clarify expression profile of Sema6D isoforms in various tissues, RT-PCR was performed by a common primer set designed on exons 15 and 19 (see Materials and methods) (Fig. 5). Most of the Sema6D isoforms were detected in all tissues, but their expression level differed depending on tissues. It is noteworthy that all isoforms were expressed in the brain, while Sema6D-2 and Sema6D-6 were the major transcripts in other tissues. Thus, it is possible that individual isoforms play both redundant and distinct biological functions.

Discussion

In the present study, we identified and cloned murine Sema6D cDNAs. Predictable protein of Sema6D-1 is 1011 aa and showed a 95.3% identity with that of human SEMA6D (KIAA1479). We also identified four Sema6D isoforms. These isoforms were generated by alternative splicing (Fig. 2). In all other class 6 semaphorins, isoforms were reported [19,22,24,25]. Sema6C isoforms are generated by alternative splicing and the isoform expression is controlled in a tissue- and stage-dependent manner, though functional differences between the isoforms have not been reported [22]. We found that all

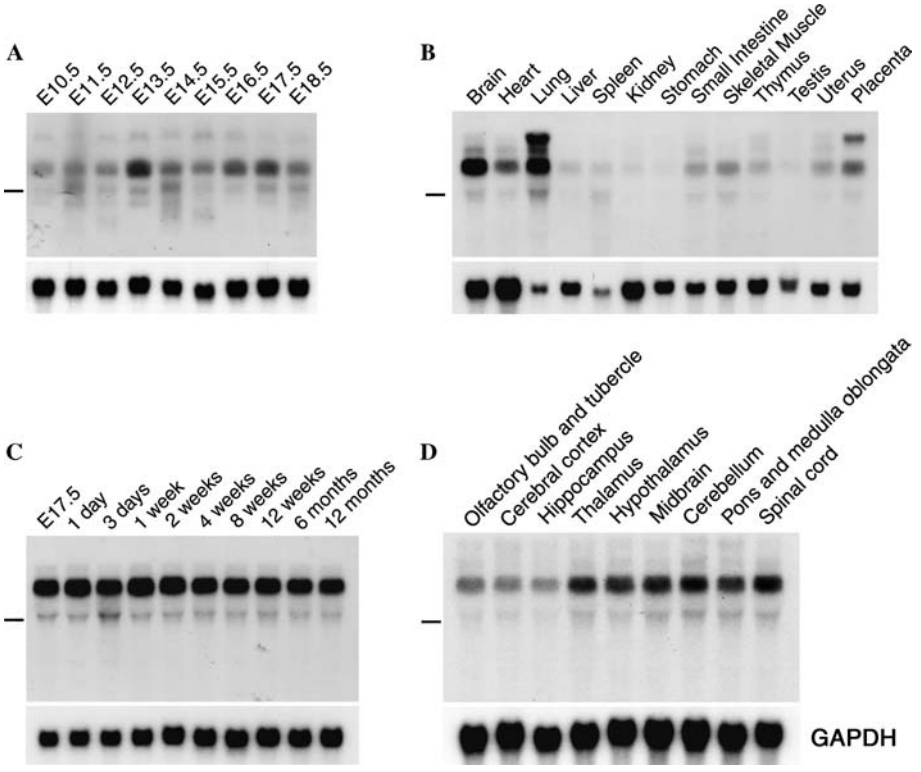

Fig. 3. Analysis of Sema6D expression by Northern blotting. Each lane contained 20 μg of total RNA. (A) Sema6D expression of the whole embryo during murine embryogenesis. (B) The distribution of Sema6D in adult murine tissues. (C) Sema6D expression in murine brain at various developmental stages. (D) Regional distribution of Sema6D in adult rat brain. The lines show the location of murine 28S ribosomal RNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression patterns are also shown as references.

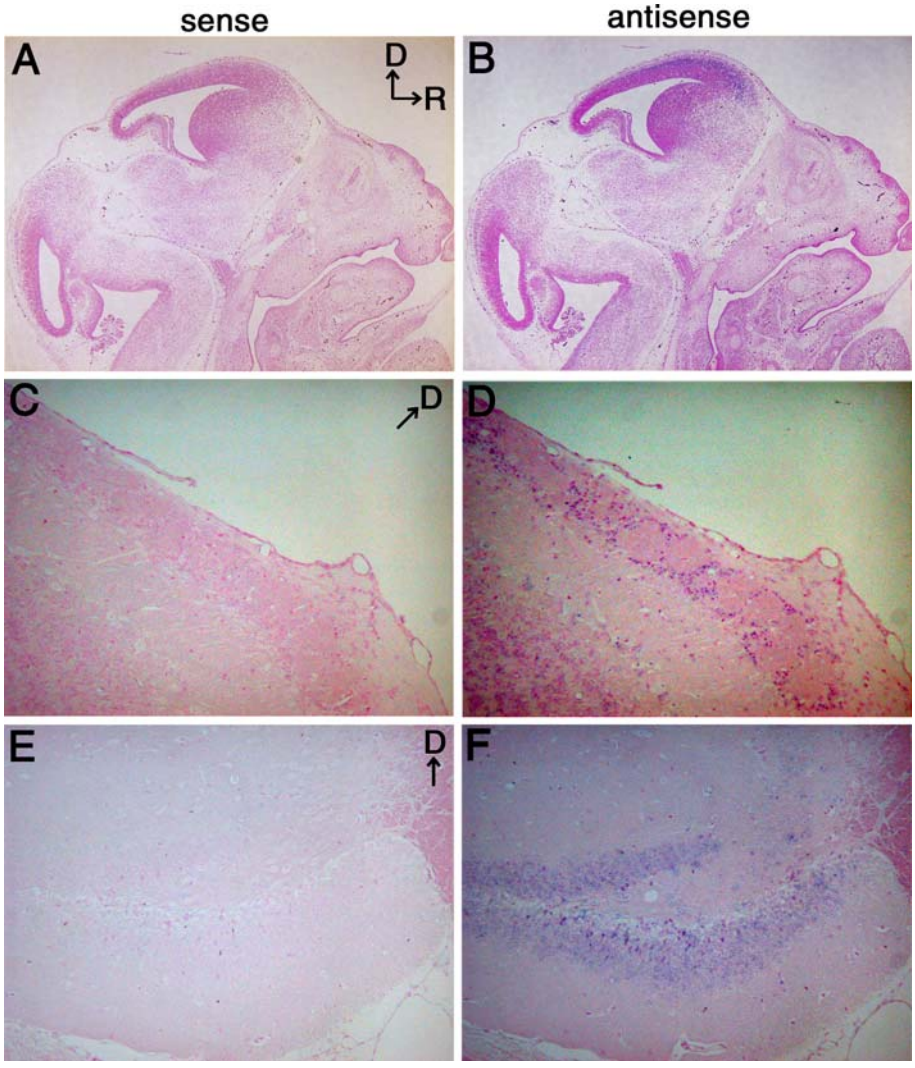

Fig. 4. Sema6D expression pattern of sagittal sections by in situ hybridization with sense riboprobe (A,C,E) and antisense riboprobe (B,D,F). (A,B) are from murine E13.5 and (C–F) are from adult murine brain. (B) At E13.5 Sema6D was expressed highly in the neopallial cortex (shown in blue). (D,F) In adult brain Sema6D transcripts were detected in the glomerular layer of the olfactory bulb and dentate gyrus (shown in blue). D and R represent dorsal and rostral, respectively.

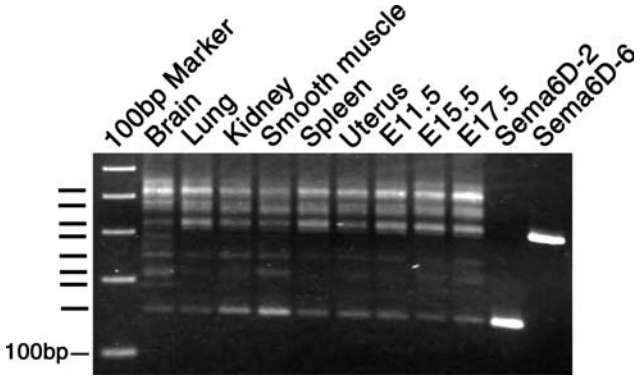

Fig. 5. Expression pattern of Sema6D isoforms. RT-PCR was performed with PCR primers designed on exons 15 and 19. Putative isoform bands are shown by thick bars at the left. The sizes of Sema6D isoforms are as follows: Sema6D-2 (155 bp), Sema6D-1 (194 bp), Sema6D-5 (251 bp), Sema6D-6 (323 bp), and Sema6D-4 (380 bp). Several unidentified bands are also seen.

Sema6D isoforms were identified in adult brain (Fig. 5). Northern blot analysis showed that in brain Sema6D transcript exhibits a single band (Fig. 3), suggesting that either the lengths of Sema6D isoform transcripts are similar to that of Sema6D-1 transcript, or the content of other variants is minute. To clarify the expression profile of individual transcripts, RT-PCR was performed with various tissues (Fig. 5). In the brain all isoforms are expressed, while Sema6D-2 and Sema6D-6 are predominant transcripts in other tissues. These results suggest that the alternative splicing is regulated in a tissue-specific manner, although the biological function of each isoform remains to be revealed.

Sema6D was expressed during embryogenesis (Fig. 3A), suggesting that Sema6D may function in neurogenesis and morphogenesis like Sema3A [6,26,27]. It was expressed predominantly in the brain and its

ubiquitous expression in the brain continued from embryo to adulthood (Figs. 3B, C, and D). Thus, Sema6D may function in neurogenesis and plasticity in the brain. It was also expressed in the lung (Fig. 3B) like other semaphorins, but the function of semaphorins in the lung has not been analyzed except for Sema3A [28]. Sema6D was expressed in the heart and placenta. Sema3A and Sema3C function during cardiac development [17,26]. Class 3 semaphorins control vascular morphogenesis [29]. In fact, neuropilin-1 is a class 3 semaphorin receptor and also a vascular endothelial growth factor (VEGF) co-receptor [30], functioning in vasculogenesis [31]. Plexin-A2 is a component of a Sema3A receptor complex with Neuropilin-1 [14,32] and may function during cardiac development [33]. Thus, semaphorins and their respective receptors play roles in vasculogenesis and angiogenesis besides the axon guidance.

During the preparation of this manuscript, human SEMA6D was reported independently [23]. They showed that human SEMA6D induced the growth cone collapse of DRG and hippocampal neurons in culture. The information of murine gene would be useful for further functional analysis of this molecule in vivo.

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