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Human Semaphorin K1 Is Glycosylphosphatidylinositol-linked and Defines a New Subfamily of Viral-related Semaphorins*

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The semaphorin family contains a large number of secreted and transmembrane proteins, some of which are known to act as repulsive axon guidance cues during development or to be involved in immune function. We report here on the identification of semaphorin K1 (sema K1), the first semaphorin known to be associated with cell surfaces via a glycosylphosphatidylinositol linkage. Sema K1 is highly homologous to a viral semaphorin and can interact with specific immune cells, suggesting that like its viral counterpart, sema K1 could play an important role in regulating immune function. Sema K1 does not bind to neuropilin-1 or neuropilin-2, the two receptors implicated in mediating the repulsive action of several secreted semaphorins, and thus it likely acts through a novel receptor. In contrast to most previously described semaphorins, sema K1 is only weakly expressed during development but is present at high levels in postnatal and adult tissues, particularly brain and spinal cord.

The semaphorins constitute a large family of evolutionally conserved glycoproteins that are defined by a characteristic semaphorin domain of approximately 500 amino acids (1–3). The first vertebrate semaphorin, collapsin-1 in chick, was identified by its ability to induce growth cone collapse (4). Consistent with this function, its mammalian homologue, sema III, has been shown to repel specific subsets of sensory axons (5). As a result of these and other studies, Coll-1/sema III/D has been implicated in the patterning of sensory axon projections into the ventral spinal cord and cranial nerve projections into the periphery (6–11).

Several other semaphorins have also been implicated as repulsive and/or attractive cues in axon guidance, axon fasciculation, and synapse formation (1, 12–17). In addition, members of the semaphorin family have been implicated in functions outside the nervous system, including bone skeleton and heart formation (9), immune function (18, 19), tumor suppression (20–22), and conferring drug resistance to cells (23).

Recent studies have identified the first semaphorin receptor as a member of the neuropilin family. Neuropilin-1 is a high affinity receptor for sema III, E, and IV, whereas neuropilin-2 binds differentially to the subfamily of secreted semaphorins (24–27).

The vertebrate semaphorin family can be classified into several phylogenetically distinct subfamilies (Ref. 15, Fig. 1*B*). Each subfamily has a unique structural arrangement of protein domains. The secreted members of the semaphorin family contain a characteristic semaphorin domain at the NH_2 terminus, followed by an immunoglobulin (Ig) domain and a stretch of basic amino acids in the carboxyl-terminal region. Between the NH_2-terminal semaphorin domain and the transmembrane spanning region, the transmembrane semaphorins contain several alternative structural motifs, including either an Ig domain, a stretch of thrombospondin repeats, or a sequence with no obvious domain homology. Interestingly, semaphorin-like sequences have been identified in the genomes of poxviruses (1) and alcelaphine herpesvirus-1 (28). These virally encoded semaphorins occupy unique branches of the semaphorin phylogenetic tree. Although it has been suggested that these viral semaphorins must have vertebrate homologues, to date none have been reported. Here we report the identification of a GPI[1]-linked human semaphorin, semaphorin K1, which is highly homologous to the semaphorin encoded by alcelaphine herpesvirus-1. The expression and binding properties of sema K1 suggest that it could play an important role in both adult nervous system and in modulating immune function.

EXPERIMENTAL PROCEDURES

Cloning of Sema K1—Four human ESTs, R33537, W47265, R33439, H03806, and one mouse EST, AA260340, were identified that show highest homology with the semaphorin gene in alcelaphine herpesvirus-1 (AHV sema). Oligonucleotides corresponding to the sequences of human ESTs were used to amplify by PCR a cDNA fragment from a human placenta cDNA library (CLONTECH). This PCR fragment corresponds to the central portion of sema K1. The 3′ end was cloned by rapid amplification of cDNA ends using human placenta Marathon-Ready cDNA from CLONTECH (29). The remaining 5′ end was cloned by PCR amplification from a CLONTECH human brain λgt11 cDNA library using an internal primer from sema K1 and an anchor primer corresponding to the λgt11 vector sequence. A specific PCR product corresponding to the 5′ end was identified by Southern blot using sema K1 oligonucleotides as probes. Repeated effort has been made, but failed to yield the remaining 5′ end of cDNA. The full-length cDNA of human sema K1 except the region corresponding to the signal peptide sequence was independently cloned from CLONTECH human placenta λgt10 library by high fidelity PCR amplification and its DNA sequence reconfirmed.

Expression Constructs—Three expression constructs were made that allow the expression of recombinant proteins tagged with either a Myc-His tag at the carboxyl terminus (pEX-mh), an alkaline phosphatase tag at the amino terminus and a Myc-His tag at the carboxyl terminus (pEX-AP), or an Fc domain of human immunoglobulin at the carboxyl terminus (pEX-Fc). Similar expression constructs have been

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF071542.

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[1] The abbreviations used are: GPI, glycosylphosphatidylinositol; AHV, alcelaphine herpesvirus; AP, human placenta alkaline phosphatase; PI-PLC; phosphatidylinositol-specific phospholipase C; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; EST, expressed sequence tags.

made for collapsins and semaphorins, and the resulting fusion proteins were shown to be fully functional (7, 10, 23, 24, 30, 31). The multiple cloning site of pSecTagA (Invitrogen) was excised with *Pme*I and *Nhe*I and cloned into pcDNA3.1 (Invitrogen) to make Myc-His tagged vector pEX-mh. This expression vector contains a signal peptide sequence from immunoglobulin κ chain for protein secretion. The cDNA for human placental alkaline phosphatase was PCR-amplified from pSEAP (CLONTECH) and cloned into the *Sfi*I site of pEX-mh maintaining the original reading frame to make the AP-tagged vector pEX-AP. The Fc domain of human IgG1 and an enterokinase cleavage site were PCR-amplified from Signal-pIgplus (Novagen) and cloned into the *Apa*I to *Pme*I sites of pEX-mh maintaining the original reading frame to make the Fc-tagged vector pEX-Fc. Various cDNAs for full-length sema K1, extracellular domain of sema K1 (residues starting from Gly-612 to the carboxyl-terminal end were deleted), sema III, and neuropilin-1 were PCR-amplified from cDNA libraries and subcloned into these expression vectors. The neuropilin-2 expression construct was kindly provided by Dr. Marc Tessier-Lavigne (25).

Cell Surface Staining—COS-7 cells were transiently transfected with the full-length sema K1 in pEX-AP vector using LipofectAMINE (Life Technologies, Inc.). Two days after transfection, cells were washed and treated with or without PI-PLC (Boehringer Mannheim) at 250 milliunits/ml for 1 h at 37 °C. Cells were then fixed in 4% paraformaldehyde for 10 min at room temperature. After phosphate-buffered saline wash, cells were incubated with a rabbit anti-AP antibody (Accurate Antibodies) at a dilution of 1:500 for 1 h followed by a Cy3-anti-rabbit antibody at a dilution of 1:200. The fluorescent images of the transfected cells were photographed in a Zeiss microscope using a 40× lens.

Western Blotting—COS-7 cells were transiently transfected with the full-length sema K1 in pEX-AP vector with LipofectAMINE (Life Technologies, Inc.). Cells transfected with the full-length CD100 in pEX-AP served as a control. Two days after transfection, cells were incubated with or without 250 milliunits/ml of PI-PLC (Boehringer Mannheim) for 1 h at 37 °C. Supernatants and cell lysates were collected and run on a 4–20% SDS-PAGE gel and the AP-tagged sema K1 protein was detected with a horseradish peroxidase-conjugated anti-alkaline phosphatase antibody.

Protein Expression—Stable 293-EBNA cell lines secreting Myc-His-tagged, AP-tagged, or Fc-tagged sema K1 and sema III were derived from transfection of various expression plasmids followed by G418 selection. Conditioned media from stably transfected cell lines were collected and were confirmed for the expression and integrity of recombinant proteins by Western blot using anti-AP, anti-Fc, or anti-Myc antibodies. SDS-PAGE gel demonstrated that sema K1-Fc fusion protein migrates as a dimer linked by the disulfide bonds in the Fc region, whereas the sema K1-mh and AP-sema K1 are monomeric (not shown). Approximately equal amount of AP- or Fc-tagged sema III and sema K1 fusion proteins as judged by Western blot were used in the ligand binding experiments. The amount of active sema III used for the ligand binding experiment was further quantified by a growth cone collapse assay and estimated to be over 80 collapsing units/ml (4, 7).

Ligand Binding Experiments—COS-7 cells were transiently transfected with full-length neuropilin-1 or neuropilin-2 expression constructs with LipofectAMINE (Life Technologies, Inc.). The expression of neuropilin-1 or -2 was confirmed using a monoclonal antibody 9E10 against the Myc tag at the carboxyl-terminal ends of both receptors. After 2 days of transfection, the cells were then incubated with supernatants containing approximately equal amount of sema III-Fc or sema K1-Fc for 1 h. After post-fixing in 1% paraformaldehyde for 10 min, the cells were heat-inactivated at 65 °C for 1 h to destroy the endogenous alkaline phosphatase activity. Cells were then incubated with alkaline phosphatase-conjugated anti-Fc antibody at 1:500 dilution for 1 h and processed for chromogenic AP enzymatic reaction.

For the immune cell staining experiment, P388D1 or RBL-2H3 cells were fixed in 1% paraformaldehyde for 10 min. The suspension cells (A20 and Jurkat) were washed in phosphate-buffered saline once and fixed in 1% paraformaldehyde for 10 min and then cytospun onto glass slides. After blocking for 30 min, AP-sema K1- or AP-sema III-containing supernatants were added to each well and incubated for 1 h. The cells were then post-fixed in 100% methanol for 10 min, and the endogenous AP activity was heat-inactivated at 65 °C for 1 h. Cells were then processed for chromogenic AP enzymatic reactions. AP alone was used as a negative control. For experiments in which sema K1-mh or sema III-mh was used to compete with AP-sema K1 or AP-sema III binding, respectively, sema K1-mh or sema III-mh was incubated with different cell lines for 30 min at room temperature prior to AP-sema K1 or AP-sema III incubation.

Northern Blot Analysis and in Situ Hybridization—A 298-base pair DNA fragment corresponding to the sequence of mouse EST AA260340 was PCR-amplified from a mouse cDNA library. This DNA fragment is predicted to encode a mouse homologue of human sema K1 based on the fact that it shares over 95% amino acid identity with the corresponding region of human sema K1. It was used as a probe in the Northern blot analysis and the *in situ* hybridization experiments. Northern blot was either purchased from CLONTECH (Fig. 4*A*) or prepared as follows (Fig. 4*B*). Adult mouse tissues were homogenized in Ultraspec™ RNA reagent (Biotecx), and total RNA was extracted and precipitated. Poly(A)$^+$ RNA was further selected using FastTrack 2.0 mRNA isolation kit (Invitrogen). Two-microgram poly(A)+ RNA from each tissue was run in formaldehyde gels and blotted onto positively charged nylon membranes (Boehringer Mannheim). DNA probes were labeled with [^{32}P]dCTP (Amersham Pharmacia Biotech) using random prime labeling kit of Life Technologies, Inc. Hybridization was performed in NorthernMax Buffer (Ambion) following the manufacturer's instructions. *In situ* hybridization procedure was performed on cryostat sections of E11, E15 mouse embryos, and on brain and spinal cord sections of P3 and 5-week-old mice (C57BL/6J) as described (32). Tissues were fixed in 4% paraformaldehyde for 4 h at 4 °C and embedded in OCT embedding compound. 20-μm sections were cut and were treated with 1.0 μg/ml proteinase K for 15 min at 37 °C, 0.2 M HCl for 20 min, and then acetylated for 10 min with 0.1 M triethanolamine and 0.25% acetic anhydride. Sections were prehybridized for 1 h at 65 °C, then hybridized with digoxigenin-labeled probes (2 μg/ml) overnight at 55 °C. The hybridization buffer consists of 50% formamide, 5 × SSC, 10% dextran sulfate, 1 × Denhardt's, 0.25 mg/ml tRNA, 0.1 mg/ml single-stranded DNA. After hybridization, slides were washed with 0.2 × SSC for 60 min at 65 °C and detected with an AP-conjugated anti-digoxigenin antibody at a dilution of 1:2000.

RESULTS

Semaphorin K1 Is Highly Homologous to a Viral Semaphorin—In an effort to identify veterbrate homologues of viral semaphorins, we searched existing EST data bases against semaphorin-like sequences found in vaccinia virus and in alcelaphine herpesvirus-1 (AHV sema) using the BLAST algorithm (33). Four human and one mouse ESTs were identified, which encode novel sequences that were most homologous to AHV sema (28). PCR primers were designed based on the EST sequences and were used to obtain a 2.5-kilobase pair cDNA that encodes a candidate semaphorin gene. The cDNA contains all the human EST sequences and predicts a protein of 634 amino acids with a molecular mass of 71.5 kDa (Fig. 1*A*). This protein is named semaphorin K1 (sema K1), since sema A to J has been designated for other known semaphorins in mouse genome data base and sequence analysis indicates that sema K1 is the first member of a new semaphorin subfamily (see below). Hydropathy analysis of the predicted sema K1 sequence (34) indicates that the sema K1 sequence is slightly incomplete, lacking approximately half of the signal peptide sequence required for protein secretion (35). Consistent with this predication, the alignment between AHV sema and sema K1 also showed an eight-amino acid difference at the amino-terminal end of sema K1 (Fig. 1*A*). The hydropathy analysis of sema K1 also identified a long stretch of hydrophobic residues at the carboxyl-terminal end, which resembles a signal peptide sequence required for GPI anchorage (36). Thus, sema K1 is predicted to be the first GPI-linked membrane protein in the semaphorin family.

The sequence of sema K1 is closely related to that of AHV sema. Whereas the sema domain of sema K1 shares 50% amino acid identity with that of AHV sema, it shares less than 30% identity with that of other known semaphorins. In addition, 17 out of 18 cysteine residues and 4 out of 5 potential *N*-linked glycosylation sites are conserved (Fig. 1*A*). The homology extends throughout the entire amino acid sequences of sema K1 and AHV sema except at the carboxyl-terminal end, where only sema K1 contains the signal peptide sequence for GPI anchorage. Thus, sema K1 is predicted to be a GPI-anchored mem-

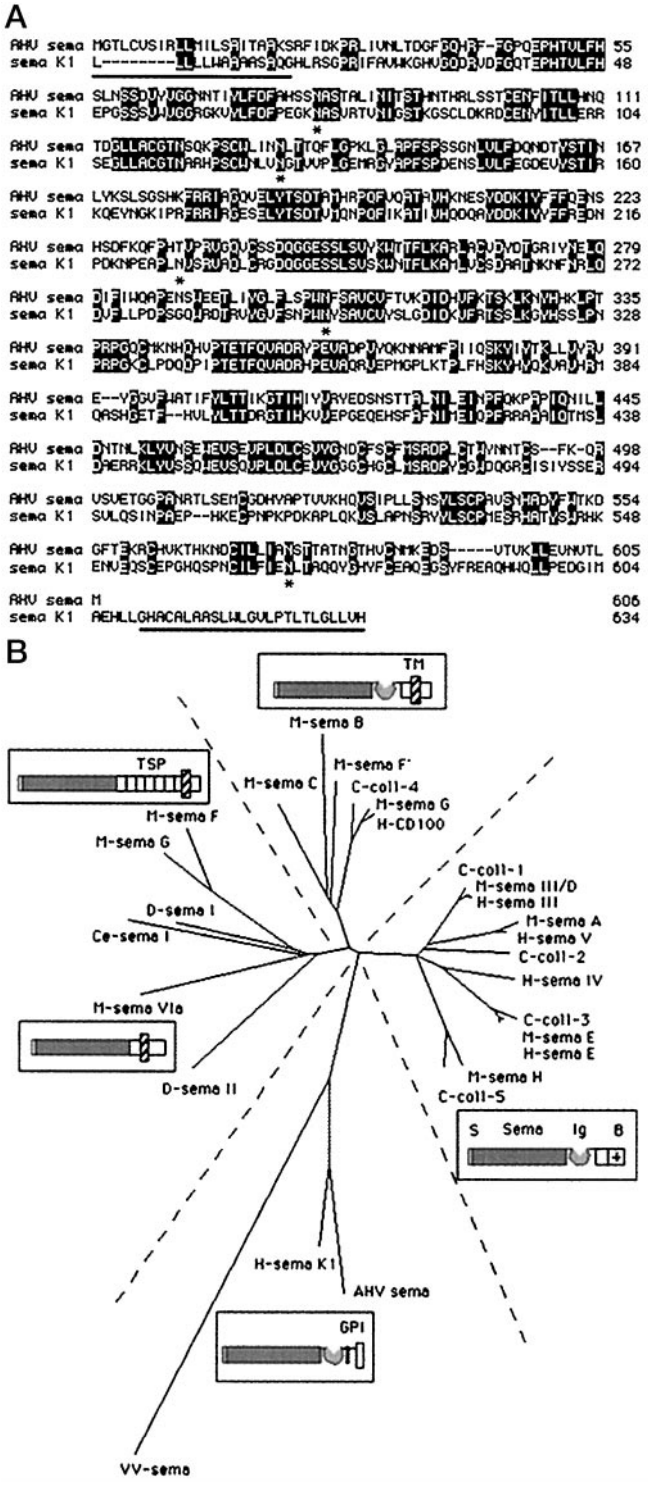

FIG. 1. **Structure of human semaphorin K1.** *A*, alignment of the amino acid sequences of sema K1 and viral AHV sema. The predicted NH_2-terminal and COOH-terminal signal peptide sequences are *underlined*. All asparagine residues likely to be glycosylated are marked by *asterisks*. *B*, dendrogram tree and the predicted structural arrangement of veterbrate semaphorins within each tree branch. A dendrogram tree has been constructed for the indicated semaphorins based on a sequence comparison of sema domains by Clustal W (49). The *dashed lines* separate the four major branches of the tree. Schematic diagrams displaying the domain structures of each subfamily of vertebrate semaphorins is shown within the boxes. *C*, chicken; *H*, human; *M*, mouse; *D*, *Drosophila*; *CE*, *Caenorhabditis elegans*; *AHV*, alcelaphine herpesvirus-1; *VV*, vaccinia virus; *S*, signal peptide; *Sema*, semaphorin domain; *Ig*, immunoglobulin-like domain; *B*, basic region; *TM*, transmembrane domain; *TSP*, thrombospondin repeats; *GPI*, GPI linkage.

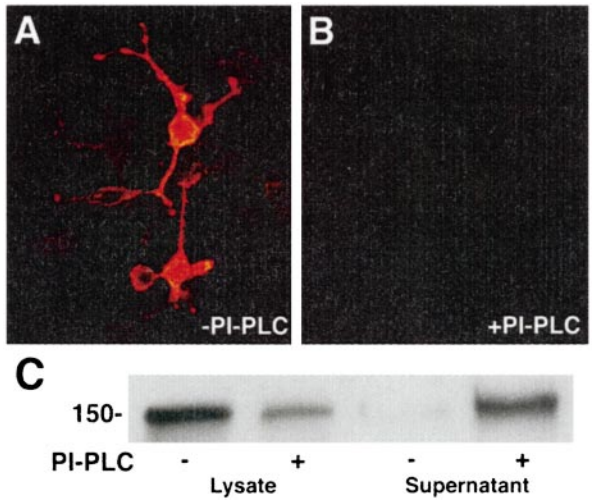

FIG. 2. **Effect of PI-PLC treatment on the cell surface expression of semaphorin K1.** COS-7 cells transfected with the full-length AP-sema K1 fusion construct were treated without (*A*) or with (*B*) PI-PLC. An anti-AP antibody and a Cy3-conjugated secondary antibody was used to detect the AP-sema K1 fusion protein on the cell surface. The cell supernatants and lysates of transfected COS-7 cells treated with or without PI-PLC were run on a SDS-PAGE gel (*C*). The fusion protein was detected with a horseradish peroxidase-conjugated anti-alkaline phosphatase antibody in Western blot. The molecular mass in kilodaltons is marked to the *left*.

brane protein, whereas AHV sema is likely to be a secreted protein. The unique structural arrangement of sema K1 suggests that it may define a new subfamily of vertebrate semaphorins. Consistent with this hypothesis, protein sequence homology analysis showed that sema K1 and AHV sema belong to the same branch of the dendrogram tree and this branch is distinct from that of other semaphorins (Fig. 1*B*). Sequence alignment with other semaphorins also revealed that members of the viral-related semaphorin subfamily lack three tryptophan residues conserved in other semaphorins, suggesting that viral-related semaphorins may contain a structurally distinct sema domain.

Semaphorin K1 Is a GPI-anchored Membrane Protein—To confirm that sema K1 is a GPI-anchored membrane protein, we have transfected COS-7 cells with a sema K1 expression construct and determined the localization of the expressed sema K1 protein. To track sema K1 protein expression, an AP-tagged version of sema K1 was engineered in which the human placenta alkaline phosphatase was fused to the full-length sema K1 at the NH_2 terminus. This fusion protein can be detected with an anti-AP antibody or AP activity. Upon transfection of the expression construct into COS-7 cells, the sema K1 fusion protein was detected on the surface of those transfected cells (Fig. 2*A*). Treatment with PI-PLC resulted in a complete removal of the fusion protein from cell surfaces (Fig. 2*B*). To examine whether the release of sema K1 fusion protein from cell surfaces is a specific action of PI-PLC rather than the result of random proteolysis, we compared the presence of this fusion protein in the supernatant and lysate of transfected COS-7 cells with or without PI-PLC treatment. Supernatants and lysates from PI-PLC treated or untreated cells were subjected to Western blot analysis. A 150-kDa protein corresponding to the predicted size of the fusion protein was detected with the anti-AP antibody. When the transfected COS-7 cells were not treated with PI-PLC, most, if not all, of the fusion protein was found to be associated with the cell lysate (Fig. 2*C*). Treatment of these cells with PI-PLC resulted in significant release of the fusion protein from the cell lysate into the supernatant, without apparent proteolysis (Fig. 2*C*). In a control experiment,

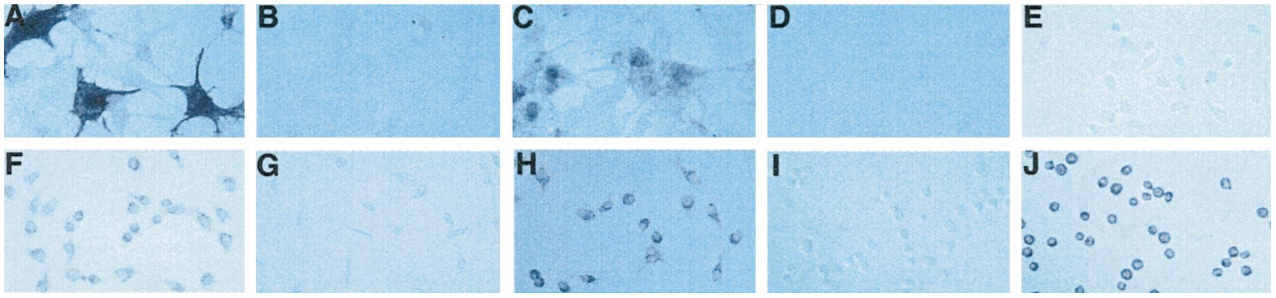

FIG. 3. **Binding of semaphorin K1 to neuropilins and immune cell lines.** COS-7 cells transfected with neuropilin-1 (*A*, *B*) or neuropilin-2 (*C*, *D*) expression constructs were tested for binding with sema III-Fc (*A*, *C*), or sema K1-Fc (*B*, *D*). P388D1 (*E–H*) macrophage and Jurkat T cells (*I*, *J*) were tested for binding with AP control (*E*), AP-sema K1 (*F*, *I*), AP-sema III (*H*, *J*). Binding of AP-sema K1 after preincubation with sema K1-mh is also shown (*G*).

PI-PLC treatment did not release the transmembrane semaphorin CD100 into the cell supernatant (not shown). Furthermore, when a stop codon was introduced immediately NH_2-terminal to the predicted signal peptide sequence for GPI linkage (residues starting from Gly-612 to the carboxyl-terminal end were deleted), the resultant sema K1 protein was released to the cell supernatant in the absence of PI-PLC (not shown). Thus, we conclude that sema K1 is attached to the cell membrane via a GPI linkage.

Semaphorin K1 Binds to Specific Immune Cell Lines—Neuropilin-1 and neuropilin-2 have recently been identified as receptors or components of a receptor complex for sema III and other secreted semaphorins (24–26). To determine whether sema K1 could use neuropilin-1 or -2 as its receptor, we tested the ability of sema K1 to bind COS-7 cells transfected with neuropilin expression constructs. Soluble sema K1 fusion proteins containing either an AP tag at the NH_2 terminus (AP-sema K1), an Fc domain of human IgG1 at the COOH terminus (sema K1-Fc), or a Myc-His tag at the COOH terminus (sema K1-mh) were produced and were used in the ligand binding assay. Similarly arranged AP-sema III, sema III-Fc, and sema III-mh fusion proteins were prepared as controls. To test for interactions with neuropilin-1 or -2, sema K1-Fc, or AP-sema K1 were incubated with neuropilin-expressing COS-7 cells, and ligand binding was detected using an anti-Fc antibody or a chromogenic AP enzymatic reaction. Under conditions where sema III-Fc binds to COS-7 cells expressing neuropilin-1 or -2, the dimerized sema K1-Fc does not bind to either (Fig. 3, *A–D*, note that sema III binds to neuropilin-2 with lower affinity than to neuropilin-1). In this experiment, sema III-Fc does not bind to mock-transfected COS-7 cells and Fc-tagged MUC18, an adhesion molecule served as a control, does not bind to the neuropilin-expressing COS-7 cells (not shown). Both served as negative controls. Similarly, under conditions when AP-sema III can bind to COS-7 cells expressing neuropilin-1 or -2, the monomeric AP-sema K1 does not bind to these cells (data not shown). Thus, sema K1 does not bind neuropilin-1 or -2 with high affinity and may not act through these receptors.

To determine whether or not the soluble sema K1 fusion proteins are competent to bind a cognate receptor and to provide an entry point for investigating the role of sema K1 in modulating immune function, we analyzed several immune cell lines for the presence of sema K1 binding sites. AP-sema K1 or AP-sema III were incubated with Jurkat T cells, A20 B cells, P388D1 macrophages, and RBL-2H3 mast cell lines and the bound ligands were detected with chromogenic AP enzymatic reaction (Fig. 3, *E–J*). AP-sema K1 bound only to the cell surfaces of P388D1 macrophage and RBL-2H3 mast cell lines. This binding is specific, since AP alone does not bind to any of the cell lines, and the binding could be competed by preincubation with sema K1-mh. In comparison, AP-sema III binding was detected on cell surfaces of all four immune cell lines tested. This binding is also specific, since preincubation of these cells with sema III-mh blocks the binding (not shown). The ability of sema III-Fc or sema K1-Fc to bind these four cell lines was also tested and similar results obtained (not shown). We conclude that sema III can bind the four immune cell lines tested, which contrasts with the more selective binding of sema K1 to macrophage and mast cell lines, suggesting the existence of a specific receptor for sema K1 in these cell lines.

Semaphorin K1 Is Preferentially Expressed in Postnatal and Adult Brain and Spinal Cord—To help define the biological role of sema K1, we examined the expression of sema K1 by Northern blot analysis and *in situ* hybridization. A 298-base pair cDNA corresponding to the mouse homologue of human sema K1 was used as a probe in these studies. This probe does not cross-hybridize with the mRNA of other semaphorins. Northern blot analysis of mRNA isolated from adult mouse tissues revealed a single sema K1 transcript at 4.4 kilobase pairs (Fig. 4). The sema K1 transcript is highly expressed in brain, spinal cord, lung, and testis; moderately expressed in heart, muscle, adrenal gland, lymph nodes, thymus, and intestine; weakly expressed in spleen and kidney; and not detectable in liver, bone marrow, and stomach.

To examine the distribution of sema K1 mRNA in detail, *in situ* hybridization analysis was performed on tissue sections of embryonic day 11 and day 15 embryos, and on the brain and spinal cord sections of postnatal day 3- and 5- week-old mice. A digoxigenin-labeled antisense RNA probe for sema K1 was used in this study. The sema K1 sense probe served as a control, which gave no significant hybridization signal on tissue sections of P3 and adult mice, but gave weak and uniform background signals in E11 and E15 tissue sections (not shown). Sema K1 mRNA does not appear to be expressed significantly in the developing mouse embryo, since no strong hybridization signals were detected in tissue sections generated from entire E11 and E15 embryos except within the spinal cord regions. Above background hybridization signal was detected in the ventral and lateral regions of the spinal cord at E11 (Fig. 5*A*) and E15 (Fig. 5*B*). At P3, the signal became more intense and expanded both dorsally and medially (Fig. 5*C*). By 5 weeks, strong hybridization signals were present in cells scattered throughout the gray matter except in the dorsal region where Rexed lamina layers I and II reside (Fig. 5*D*).

No significant expression of sema K1 mRNA is detected at E11 and E15 in the primodial cerebral cortex and cerebellum (data not shown). At P3, intense expression of sema K1 mRNA becomes evident in the marginal zone of the cerebral neocortex. Moderate levels of expression were detected in the cortical plate and subplate (Fig. 5*E*). In the brain of 5-week-old mice, the expression of sema K1 mRNA becomes widespread throughout the entire cerebral cortex. The level of mRNA ex-

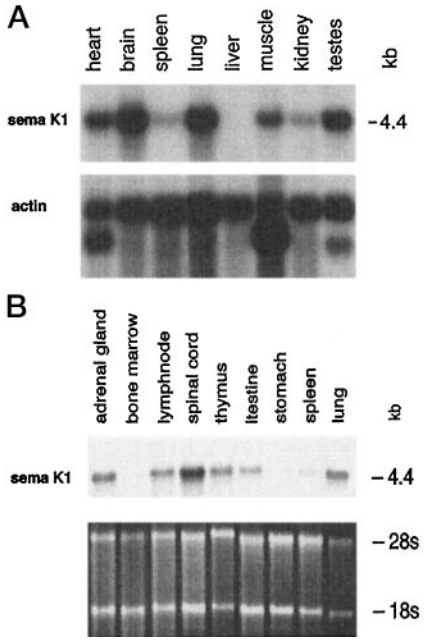

FIG. 4. **Northern analysis of sema K1 expression.** Northern blots made by CLONTECH (*A*) or from various adult mouse tissues (*B*) were probed with the 298-base pair mouse sema K1 probe. Actin or RNA staining serve as controls for the amount of mRNA loaded into each lane.

pression is moderate among all lamina layers except layer I, where no expression is evident (Fig. 5*F*). In the cerebellum at P3, sema K1 message is strongly expressed in the external germinal layer and the primodial Purkinje cell layer (Fig. 5*G*). By 5 weeks, intense expression of sema K1 mRNA is found only in the Purkinje cells (Fig. 5*H*). In addition to the dynamic patterns of expression in spinal cord, cerebellum, and cortex, sema K1 mRNA is found to be present in other structures of adult brain, including the cochlear nucleus, inferior colliculus, hippocampus and dentate gyrus, the olfactory glomerular cell layer and mitral cell layer, and thalamic structures (Table I).

DISCUSSION

GPI-linked Semaphorin K1 Defines a New Subfamily of Viral-related Semaphorins—We have identified a novel human semaphorin, sema K1, that is related to a semaphorin-like sequence in alcelaphine herpesvirus-1 (28). Sema K1 shares 45% amino acid identity with AHV sema but less than 30% with other known semaphorins. Moreover, sema K1 and AHV sema fall into the same branch of a dendrogram tree. Whereas our studies reveal a mammalian counterpart for viral AHV sema, equivalent mammalian homologues for poxvirus semaphorins are still unknown. Nevertheless, since the poxvirus semaphorins occupy a distinct branch of the phylogenetic tree, we suspect that poxvirus-related veterbrate semaphorins might also exist.

Many viruses encode proteins that can function as immune modulators, for example by interfering with antigen presentation, acting as cytokines or cytokine antagonists, inhibiting apoptosis, or interrupting the complement cascade (37). In the case of AHV, the semaphorin-like gene is found within a region of the viral genome that contains genes not conserved among the herpesviruses (38). In many viruses, nonconserved genes often encode homologues of cellular genes that are known to be involved in immunomodulation. For example, poxviruses encode a soluble form of tumor necrosis factor receptor (TNFR), which can bind to TNFα and TNFβ with high affinity (39). Mutation of this viral TNFR results in significantly attenuated virulence, suggesting that the viral TNFR may function to block the antiviral effects of TNF (40).

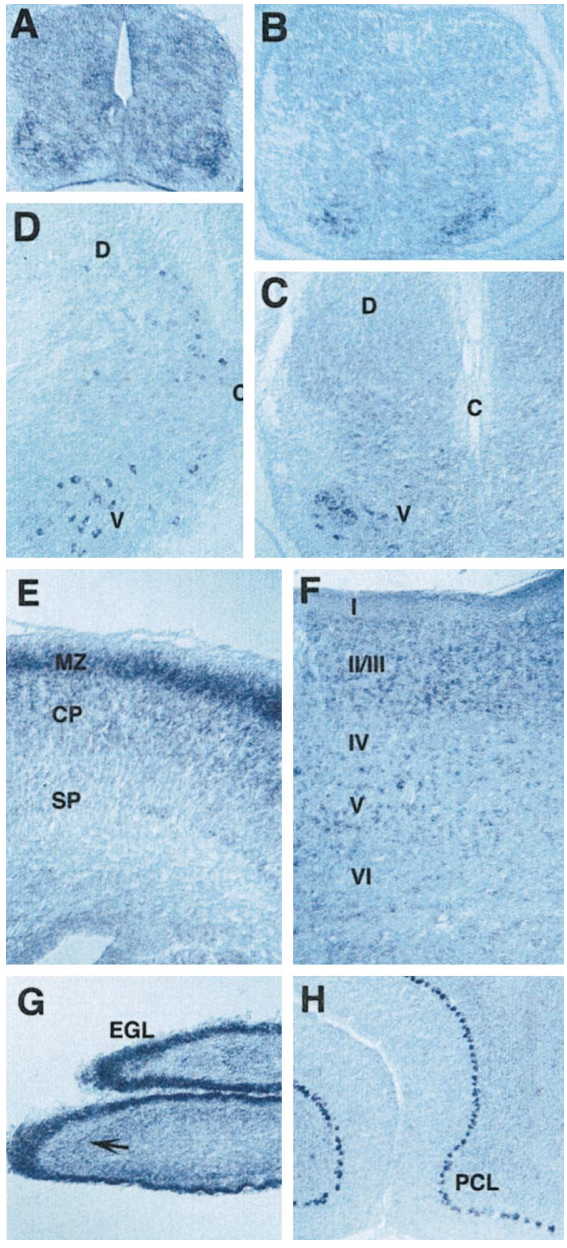

FIG. 5. **Sema K1 mRNA expression in spinal cord, cerebral cortex, and cerebellum.** Coronal sections of mouse spinal cord from E11 (*A*), E15 (*B*), P3 (*C*), and 5-week (*D*)-old mice, horizontal sections of cortex at P3 (*E*) and 5-week (*F*)-old, and cerebellum at P3 (*G*) and 5-week (*H*)-old mice were hybridized with digoxigenin-labeled antisense sema K1 probe. *D*, dorsal spinal cord; *V*, ventral spinal cord; *C*, central canal; *MZ*, marginal zone; *CP*, cortical plate; *SP*, subplate; *EGL*, external germinal layer; *PCL*, Purkinje cell layer. The *arrow* marks the primodial Purkinje cells.

Viral semaphorins have been proposed to be a part of a defense mechanism used by certain viruses to suppress the immune response of the host (1). Since sema K1 is highly homologous to AHV sema, it is tempting to speculate that vertebrate sema K1 could potentially play an important role in modulating immune function. Consistent with this hypothesis, sema K1 can bind to macrophage and mast cell lines. In addition, sema K1 mRNA is detected in several immune tissues, including lymph nodes, thymus, and spleen. A proposed role for semaphorins in immune function is not without precedent. CD100, a transmembrane semaphorin, can act as a T cell co-activation antigen and can promote B cell survival and aggregation (18, 19).

TABLE I
Expression of sema K1 mRNA in adult mouse brain

Moderate to high levels of sema K1 mRNA expression were detected in various regions of adult brain. The areas of sema K1 mRNA expression in the brain are listed.

Medulla	Brain stem (pons and cerebellum)	Midbrain	Diencephalon	Cerebral hemispheres	Olfactory bulb
Cochlear nucleus Gracile nucleus	Lateral lemiscus Spinal trigeminal nucleus Principal sensory trigeminal nucleus Medial lemniscus Purkinje cell layer Deep nuclei of cerebellum Pontine reticular formation	Inferior colliculus	Reticular nucleus Lateral geniculate	Hippocampus Dentate gyrus Cortex layers II-VI	Mitral cells Periglomerular cells

Sema K1 is the first known GPI-linked semaphorin. This conclusion is predicted by the presence of a carboxyl-terminal signal peptide required for GPI-anchorage and confirmed by the ability of PI-PLC to release membrane bound sema K1 into the supernatant. The structural arrangement of sema K1 contrasts with that of viral AHV sema which does not contain a carboxyl-terminal signal peptide sequence and is predicted to be a secreted protein. This structural difference between sema K1 and AHV sema may be of functional significance. It is possible that whereas GPI-linked sema K1 can provide an immune-modulating function in localized regions, the viral AHV sema may act in a diffusible fashion to subvert the host immune response in much broader areas. The identification of sema K1 as a GPI-linked protein demonstrates that members of the semaphorin family can exist in secreted, transmembrane, or GPI-linked forms.

Semaphorin K1 Binds to a Receptor Distinct from Neuropilin-1 or -2—The mechanism of action of sema K1 remains unknown. Neuropilin-1 is a receptor mediating the repulsive action of sema III (24, 25). Neuropilin-1 and -2 bind to members of secreted semaphorins (16, 25, 26). It was reported that sema III binds with high affinity to neuropilin-1 but not neuropilin-2, although it was pointed out that the possibility of sema III binding to neuropilin-2 with low affinity could not be excluded (26). Our study clearly demonstrates that sema III can indeed bind to neuropilin-2, albeit with apparent low affinity. Whether neuropilin-1 or -2 can bind to members of other subfamilies of semaphorins remains unknown. It has been reported that in initial experiments, sema VIa and *Drosophila* sema II do not interact with either of the neuropilins (26). We showed here that sema K1 does not interact with these neuropilins either. One explanation is that our recombinant sema K1 is not functionally active, but this appears unlikely since sema K1 can bind to macrophage and mast cell lines (but not T or B cell lines). This binding profile is different from that of sema III, which interacts with all the cell lines tested. These results support the notion that sema K1 possesses binding sites in macrophage and mast cell lines that are distinct from those of sema III, suggesting that sema K1 acts through an as yet unidentified receptor.

In this study, we showed that sema III possesses binding sites on T cell, B cell, macrophage, and mast cell lines. These results raise the possibility that sema III might have a role in immune modulation. One possibility is that sema III might function in the immune system in a manner similar to that in the nervous system, acting as a guidance cue to regulate lymphocyte migration and homing.

Functional Implications of Semaphorin K1 Expression—The abundant expression of sema K1 in brain and spinal cord suggests that sema K1 is likely to play an important role in the nervous system. Sema K1 is weakly expressed in developing embryos but abundantly expressed in postnatal and adult brain and spinal cord. The expression pattern of sema K1 stands in contrast with that of previously known semaphorins and neuropilins, which are strongly expressed in the developing nervous system (2, 8, 10, 15, 26, 41–46). Sema K1 expression is particularly striking in spinal cord, cerebellum, and cortex, where the expression pattern is dynamically regulated. In spinal cord, sema K1 expression contrasts with the expression pattern of sema III and sema VIa, which are expressed in similar regions of the ventral spinal cord embryonically, with expression significantly reduced by birth (3, 43, 45). It is possible that sema K1 might be active at later developmental stages when final innervation patterns are being formed. The expression pattern of sema K1 mRNA also contrasts with that of sema VIa in the cerebellum and cortex, where sema VIa is highly expressed in early cortical and cerebellar development but is greatly reduced by birth (45). The high level of sema K1 expression in the cerebellar external germinal layer and primordial Purkinje cells at P3, the stage of active granule neuronal migration, raises the possibility that sema K1 could regulate migration processes in the primordial cerebellum. Similarly, the presence of sema K1 mRNA in regions of marginal zone, cortical plate, and subplate at a time of active neuronal migration in the cortex is also potentially consistent with this proposed role.

The widespread and abundant expression of sema K1 mRNA in adult brain suggests that it could play a role in the maintenance and plasticity of connections in the adult nervous system. Many members of the semaphorin family are implicated as repulsive guidance cues that repel growing axons during development. If sema K1 is functionally analogous to those semaphorins, it could help maintain established neuronal circuits in the adult nervous system by inhibiting unwanted neurite sprouting. Regulated expression of sema K1 and its receptor in regions of plasticity could contribute to the remodeling of neuronal circuits. The presence of such maintenance factors in the adult brain may also contribute to the failure of central nervous system axons to regenerate upon injury. At present we do not know whether sema K1 can inhibit the extension of axons of adult central nervous system neurons.

The evidence that sema K1 binds to macrophage and mast cell lines and that high level of sema K1 mRNA expression is found in the adult brain also raises the possibility that sema K1 might be involved in neural-immune interaction. Of particular relevance is the possibility that sema K1 affects microglial cells, the resident macrophages in the brain. Microglial cells are thought to arise from the blood monocytes of the monocyte-macrophage series, which invade the brain during embryonic and postnatal life. Between the second and third postnatal week they transform into ramified resting microglia, which are marked by their diminished phagocytic activity and mitotic capability (47). The fact that sema K1 shows strong homology to a possible viral immune suppresser (AHV sema), is highly expressed in postnatal and adult brain when microglia become ramified, and contains binding sites in macrophage cell lines are all consistent with the hypothesis that sema K1 might contribute to the ramification of microglia and maintenance of

their resting state. Last, it has been known that nerve injury in the peripheral nervous system is always followed by rapid and massive invasion of macrophages, whereas macrophage infiltration to the injured areas of the central nervous system is very limited. The lack of macrophage infiltration may in part explain the failure of axon regeneration in the central nervous system (48). It is possible that members of the semaphorin family, including sema K1, could contribute to the limited migration and infiltration of macrophages into immune privileged sites such as brain. Thus, the possible regulation of lymphocyte migration and response both within and outside of the nervous system appear to represent reasonable biological targets for future investigation of sema K1 and sema III activity.

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Human Semaphorin K1 Is Glycosylphosphatidylinositol-linked and Defines a New Subfamily of Viral-related Semaphorins

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