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Cloning, expression, and genetic mapping of Sema W, a member of the semaphorin family

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ABSTRACT **The semaphorins comprise a large family of membrane-bound and secreted proteins, some of which have been shown to function in axon guidance. We have cloned a transmembrane semaphorin, Sema W, that belongs to the class IV subgroup of the semaphorin family. The mouse and rat forms of Sema W show 97% amino acid sequence identity with each other, and each shows about 91% identity with the human form. The gene for Sema W is divided into 15 exons, up to 4 of which are absent in the human cDNAs that we sequenced. Unlike many other semaphorins, Sema W is expressed at low levels in the developing embryo but was found to be expressed at high levels in the adult central nervous system and lung. Functional studies with purified membrane fractions from COS7 cells transfected with a Sema W expression plasmid showed that Sema W has growth-cone collapse activity against retinal ganglion-cell axons, indicating that vertebrate transmembrane semaphorins, like secreted semaphorins, can collapse growth cones. Genetic mapping of human *SEMAW* with human/hamster radiation hybrids localized the gene to chromosome 2p13. Genetic mapping of mouse *Semaw* with mouse/hamster radiation hybrids localized the gene to chromosome 6, and physical mapping placed the gene on bacteria artificial chromosomes carrying microsatellite markers *D6Mit70* and *D6Mit189*. This localization places *Semaw* within the locus for motor neuron degeneration 2, making it an attractive candidate gene for this disease.**

Development of the nervous system requires that axonal projections reach the correct targets. Within the last few years, a number of molecules have been found that have the ability to guide axonal growth by providing a repulsive signal that inhibits axons from straying outside of their correct paths. Most of these repellant molecules identified to date belong to the semaphorin family. *In vitro*, semaphorin family members collapsin 1 (1) and Sema D (2) can repel axons and cause growth-cone collapse. *In vivo*, grasshopper and *Drosophila* Sema I (3, 4) and *Drosophila* Sema II (5) can influence axon trajectories, and targeted disruption of the gene for Sema D, or its receptor neuropilin 1, leads to severe abnormalities in peripheral nerve projection (6, 7). Recently, new members of the semaphorin family have been described that have effects outside of the nervous system. Human CD100, known as Sema J in the mouse, has been found to function in T cell and B cell aggregation and activation (8, 9). Human Sema E has been found to be involved in the resistance of cancers to radiation and chemotherapeutic drugs (10), and mouse Sema H expression has been found to correlate with the metastatic ability of tumor cell lines (11). In addition, two viral genes bearing significant homology to semaphorin have been described in alcelaphine herpesvirus 1 and vaccinia virus, although the function of these genes is not yet known.

In mammals, 15 members of the semaphorin family of molecules have been described to date and, together with avian and insect semaphorins, can be grouped into seven phylogenetically distinct classes. All semaphorins have a characteristic 500-aa residue sema domain near the N terminus but differ in the composition of their C-terminal portions. Class I and II semaphorins have been found only in insects (3, 12). Class III–VII semaphorins have been found in chickens (where they are known as collapsins) and in mammals. Class I semaphorins have a transmembrane region distal to the sema domain, whereas class II has an Ig-like domain distal to the sema domain and no transmembrane region. Class VI (13–16) and class III semaphorins (1, 2, 10–12, 17–21) resemble class I and class II semaphorins, respectively, in their domain arrangements, but both class VI and class III semaphorins are distinct phylogenetically from class I and class II semaphorins. Class IV semaphorins (8, 17, 21, 22) are composed of a sema domain followed by an Ig-like domain and a transmembrane region. Class V semaphorins (23, 24) are composed of a sema domain followed by a string of type 1 thrombospondin repeats and a transmembrane region. Class VII semaphorins (25, 26) are composed of a sema domain followed by an Ig-like domain and a glycosylphosphatidylinositol linkage signal. Semaphorin homologs have also been described in zebrafish (27), *Caenorhabditis elegans* (28), herpes virus (29), and pox virus (30).

Here, we report on the molecular cloning, mapping, and functional analysis of a mammalian semaphorin, Sema W. Sequencing of the gene for Sema W indicated that this molecule is a class IV transmembrane semaphorin. Expression studies show that the gene is expressed highly in postnatal brain and lung, and functional studies show that Sema W can collapse retinal ganglion-cell axons. Genetic mapping places the gene for Sema W on chromosome 2p13 in humans and on chromosome 6 in the mouse. Because the gene for Sema W in the mouse resides within the locus for the disease motor neuron degeneration 2 (mnd2), its possible role in this disease will be discussed.

MATERIALS AND METHODS

Sema W cDNA Isolation and Sequence Analysis. The database of expressed sequence tags (EST) at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) was

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Abbreviations: BAC, bacteria artificial chromosome; EST, expressed sequence tag; mnd2, motor neuron degeneration 2.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AB021291 for BALB/c mouse Sema W cDNA, AB021292, and AB022317 for human Sema W cDNA, AB002563 for rat Sema W cDNA, and AB021293 for 129/SvJ mouse genomic Sema W sequence).

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scanned for nucleotide sequences that could encode a 7-aa sequence, Q/R-D-P-Y-C-A/G-W, which is highly conserved among the known semaphorins. This search identified an EST (GenBank accession no. T09073) from human infant brain. A synthesized oligonucleotide based on the 5′ end of the EST then was used to probe a rat adult brain cDNA library following standard procedures (31). Positive clones were cycle-sequenced with an ABI Prism Dye Terminator Cycle Sequencing Reaction Kit (Perkin–Elmer) and analyzed on an ABI Prism 377 sequencing system (Perkin–Elmer). The human *SEMAW* cDNA sequence was determined similarly, by using a rat full-length cDNA as the probe of human adult hippocampus and forebrain cDNA libraries (Stratagene).

The mouse *Semaw* cDNA sequence was determined by direct sequencing of PCR products amplified from adult BALB/c mouse brain cDNA preparations. PCR products for sequencing were made either by using cross-hybridizing rat *Semaw* and human *SEMAW* primers or by amplifying them with by 3′ rapid amplification of cDNA ends (Marathon cDNA Amplification Kit, CLONTECH). To determine the structure of the *Semaw* gene, the rat full-length cDNA and PCR primers based on the rat *Semaw* sequence were used to probe mouse genomic DNA bacteria artificial chromosome (BAC) libraries (Genome Systems, St. Louis). BACs carrying the *Semaw* gene were used as templates for sequencing to identify intron–exon boundaries. Large intron sizes were determined by exon-to-exon PCR with the GeneAmp XL PCR Kit (Perkin–Elmer).

Northern and *in Situ* Hybridization Analysis. Total RNA was prepared from fetal and adult rat tissues with a rapid RNA isolation kit (Nippon Gene, Toyama, Japan) and run on a 1% agarose/formaldehyde gel. The RNA was then blotted onto nylon filters and probed with a ^{32}P-labeled DNA fragment corresponding to amino acids 355–648. For *in situ* hybridization, the same DNA fragment was used to probe sections of brain and spinal cord as described (32, 33).

Genetic Mapping. Mapping of the human *SEMAW* gene was performed with the GeneBridge 4 radiation hybrid panel (Research Genetics, Huntsville, AL); two PCR primer pairs designed from the 3′ end of the human cDNA sequence—hW2 (AGCATCCCTGACTCTCAT) with W4 (CAGAAAG-CAGTTCTGATT) and hW2 with hW10 (TAGGGG-GAAGCCCTGATT)—were used to screen the panel. The results were analyzed by using the radiation hybrid mapping service of the Whitehead Institute for Genomic Research (www.genome.wi.mit.edu). Mapping of the mouse *Semaw* gene was performed with the mouse T31 Radiation Hybrid Panel (Research Genetics). Because no public mapping data for this panel were available, a framework map of the region of mouse chromosome 6 was constructed by screening the panel with microsatellite markers (MapPairs, Research Genetics) and evaluating their order and linkage with the computer program MAP MANAGER QT (34). From genomic sequences spanning intron–exon boundaries, PCR primer sets were designed to amplify mouse *Semaw* differentially. Primers mW1 (CT-GACTGGGTCGTGTGCTAA) and rW4 (ACCGACGTA-AAGTGTGTG) were used to screen the panel, and the results were analyzed with MAP MANAGER QT.

Expression of Sema W in COS7 Cells. To express Sema W protein in mammalian cells, the full-length rat cDNA sequence was introduced into a pUCSRα expression plasmid (35). The plasmid was then transfected into COS7 cells, and the membrane fraction of the cells was isolated according to the method of Luo *et al.* (17). Sema W protein expression was verified by performing a Western blot of the membrane fraction with anti-Sema W polyclonal antibodies prepared in rabbits against an amino acid stretch near the C-terminal domain of the protein (APPSGTTSYSQDPPSPSPEDER).

Growth-Cone Collapse Assay. Growth-cone collapse activity (1) was observed by culturing retinal explants from embryonic-day-6 chicken embryos in a poly-L-lysine and laminin-coated 8-chamber glass slide in F12 growth medium containing 10% fetal calf serum and 20 ng/ml brain-derived neurotrophic factor overnight at 37°C. The next day, membrane fractions from Sema W-expressing or mock-transfected COS7 cells, resuspended in 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate and dialyzed against F12 medium, were added, and the cells were cultured for an additional 60 min. The cells were then fixed in 1% glutaraldehyde, and the proportion of collapsed growth cones was counted under a microscope.

RESULTS

Isolation of the Sema W Transcript. Our search of the database of ESTs identified an EST from a human infant brain library with homology to the semaphorins. Using this EST sequence, we probed an adult rat brain cDNA library and identified several clones. Sequencing the clones gave a full-

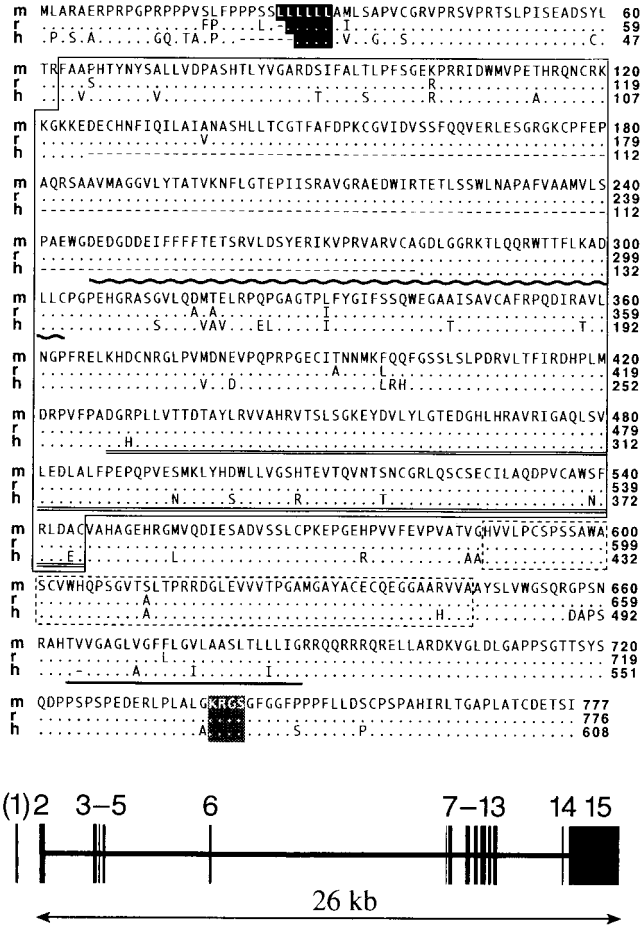

FIG. 1. Amino acid sequence alignment of mouse, rat, and human Sema W and genomic structure of mouse *Semaw*. (*Upper*) Residues in the rat and human sequence that are identical to the mouse sequence are represented by dots; gaps in sequence relative to the mouse sequence are represented by dashes. The sema domain (solid outline), Ig-like domain (dashed outline), and the transmembrane domain (single underline) are shown. A region of homology to the vaccinia virus protein A39R is indicated by a wavy underline. A subdomain of plexin/SEX homology is indicated by a double underline. The string of leucines encoded by the CTG repeat is indicated by black shading, and a cyclic nucleotide-dependent phosphorylation site is indicated by dark-gray shading. (*Lower*) The relative positions and sizes of the *Semaw* exons in the mouse genomic sequence are shown. Exon 1 has been found thus far only in the rat. Intron lengths over 2,000 bp are estimates calculated by comparing intron-spanning PCR products with size markers on an agarose gel.

length cDNA of 4,008 bp encoding a protein of 776 aa, which we designated Sema W. The translated sequence contained a sema domain at approximately amino acid residues 62–567. Within the sema domain, residues 245–302 show 42% identity with the vaccinia virus protein A39R (30), and residues 427–544 show 24% identity with the plexins (36) and the human protein SEX (37). Distal to the sema domain, an Ig-like domain was identified at amino acid residues 586–639, and a transmembrane domain was identified at amino acid residues 663–687. A Prosite pattern search (www.expasy.ch/sprot/prosite.html) also identified a cAMP- and cGMP-dependent protein kinase phosphorylation site at residues 739–742.

To determine the sequence of the human *SEMAW* gene, a full-length rat cDNA probe was used to screen human adult brain cDNA libraries. The first screen gave a clone that, when sequenced, was highly homologous to the rat sequence, although it had a deletion of 193 bases within the region coding for the sema domain. Because this deletion caused a frame shift, we sequenced two additional clones. These clones, surprisingly, had an even larger, albeit in-frame, deletion within the sequence encoding the sema domain, with the deletion totaling 465 bases or approximately one-third of the sema-domain coding region. The locations of these deletions (see below) correspond to the location reported in human *SEMA3F*, where alternative splicing causes a change in the size of the sema domain (18, 20).

To determine the sequence of the mouse *Semaw* gene, we directly sequenced PCR products amplified from adult BALB/c mouse brain cDNA by using primers based on the rat *Semaw* sequence. Rapid amplification of cDNA ends was used to sequence the 3′ untranslated region. An alignment of the mouse, rat, and human amino acid sequences of Sema W is shown in Fig. 1.

Mouse genomic BAC clones were then screened by hybridization with a rat *Semaw* cDNA probe or by PCR with rat *Semaw* primers to determine the genomic structure of the *Semaw* gene. We found four *Semaw* gene-carrying BACs: three from a C57BL/6 BAC genomic library and one from a 129/SvJ BAC genomic library. By sequencing the gene with the BACs as templates, we found that the gene is composed of 15 exons (Fig. 1). Exon 1, which was present in the rat cDNA, has not yet been found in mouse or human. Exon 2 contains the first methionine codon, which is followed by an apparent signal sequence, indicating that it is likely the translation-initiation codon. Exons 3–14 approximately define the sema domain, and exons 5 and 6 or 5–8 are deleted in the human cDNAs. Recently, a search of GenBank for sequence similarity with the human, rat, and mouse sequences turned up a high-throughput genomic sequence of a BAC (BAC b245c12, accession no. AC003061), containing part of the mouse *Semaw* gene. The sequencing of the BAC was not completed at the time of this writing.

Distribution of Sema W mRNA. Northern analysis of RNA from embryonic and adult rat tissue indicated that *Semaw* is expressed abundantly in the adult central nervous system and lung (Fig. 2*a*). In the adult central nervous system, expression was seen in all areas examined thus far (Fig. 2*b*). Little expression was detected in embryonic tissue by Northern analysis (Fig. 2*c*); however, *in situ* hybridization of rat embryonic-day-15 tissue showed expression in the spinal motor neurons, dorsal root ganglia, and sympathetic ganglia (Fig. 2*d*). At the same stage, Sema W mRNA was also detected in retinal ganglion cells and in cells surrounding the optic nerve (Fig. 2*e*).

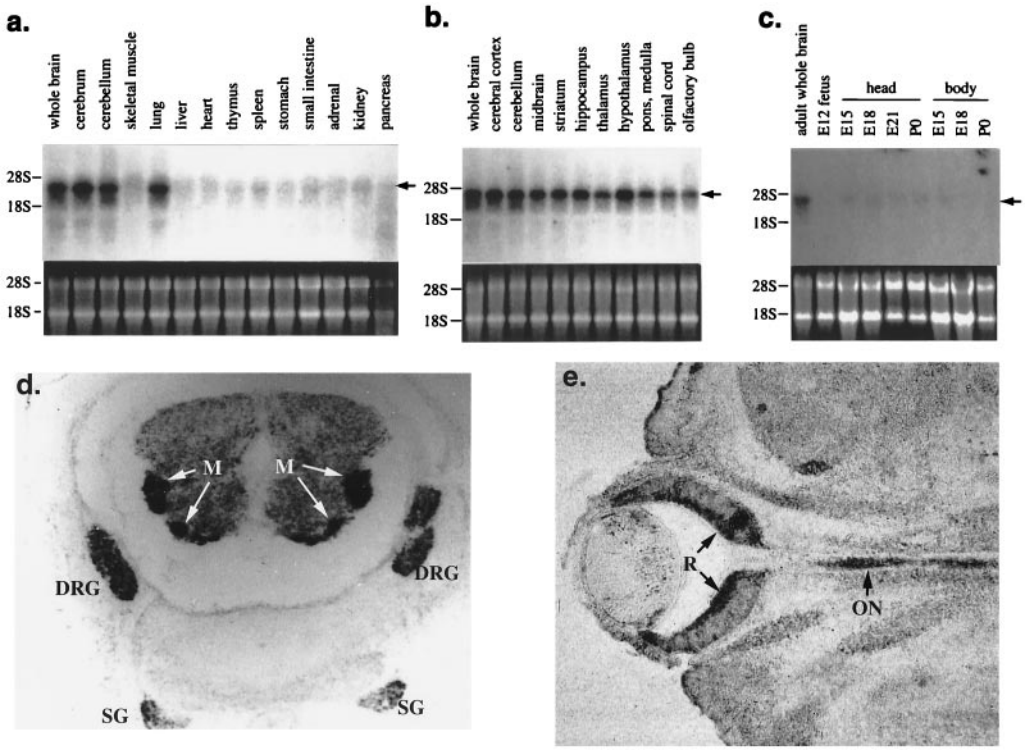

FIG. 2. Northern and *in situ* hybridization analysis of Sema W expression. Northern blots were performed with a rat Sema W probe against a panel of total RNA extracts from various adult rat tissues (*a*), various adult rat central nervous system tissues (*b*), and rat embryonic (E) and postnatal (P) tissues at various stages of development (*c*; given in days post coitus or days after birth, respectively). Below each Northern blot image, confirmation of equivalent RNA amounts by ethidium bromide staining is shown. Locations of 28S and 18S ribosomal RNA are indicated. Sema W bands are indicated with arrows. (*d*) *In situ* hybridization with a rat Sema W probe of a transverse section of an embryonic-day-15 rat spinal column. Darkly staining regions are the motor neurons (M), dorsal root ganglia (DRG), and sympathetic ganglia (SG). (*e*) *In situ* hybridization with a rat Sema W probe of a coronal section of an embryonic-day-15 rat eye. Staining is seen in the retina (R) and along the optic nerve tract (ON).

Mapping of Sema W in the Human and Mouse. By using the GeneBridge 4 radiation hybrid panel, the human *SEMAW* gene was determined to be located 5.87 cR proximal to the centromere from marker WI-5987 on chromosome 2, placing the *SEMAW* gene within the band 2p13 (Fig. 3). Also residing within this chromosomal band are a recently discovered locus for Parkinson's disease (38) and the locus for the Alström's Syndrome (39).

A region of conserved synteny homologous to human chromosome 2p13 is found on chromosome 6 in mice (40). Because this region contains three disease loci with neurological phenotypes, i.e., truncate (41), *mnd2* (42), and cerebellar deficient folia (43), we mapped the location of mouse *Semaw* by using a mouse/hamster radiation hybrid panel to determine its location within the region accurately. This mapping placed *Semaw* between microsatellite markers *D6Mit71* and *D6Mit9* on chromosome 6, localizing the gene to approximately the same position as the *mnd2* locus (ref. 44; Fig. 3). The four BACs that were found to carry the *Semaw* gene were then screened for the presence of microsatellite markers and ESTs known to map within this region (Mouse Genome Database, www.informatics.jax.org). One marker, *D6Mit189*, was found by PCR analysis to reside on all four of the BACs. A second marker, *D6Mit70*, was found on one of the BACs, and the gene *Dok* was found on two of the BACs. These results confirm and refine the localization of *Semaw* to within the *mnd2* region of chromosome 6 (44).

Functional Studies of Sema W Protein. To study the function of the Sema W protein, the rat *Semaw* gene was expressed transiently in COS7 cells. Expression of the protein was confirmed by Western analysis. This analysis showed a unique band with an apparent molecular mass of 100 kDa (Fig. 4), and, as expected from the predicted domain structure, the band was present only in the membrane fraction. The expressed protein was then used in a growth-cone collapse assay to investigate the effect of Sema W on axon extension. As can be seen in Fig. 5, treatment of retinal ganglion cells with the membrane fraction from cells expressing Sema W caused a significant increase in the percentage of collapsed growth cones when compared with the membrane fraction from control cells. A truncated form of the Sema W protein, produced from a Sema W expression plasmid in which most of the sequence following

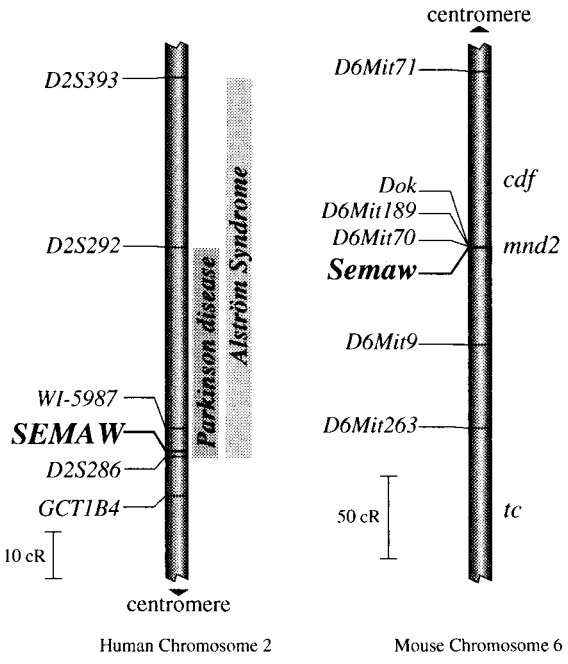

FIG. 3. Location of the gene for Sema W in humans and mice. The human *SEMAW* gene was localized between markers *WI-5987* and *GCT1B4* by radiation hybrid mapping. The locations of disease loci for Parkinson's disease and Alström's syndrome are shown along with the approximate locations of microsatellite markers that define their limits. The relative locations of the markers shown are based on the radiation hybrid map of the human genome mapping project (52). The mouse *Semaw* gene was localized between markers *D6Mit71* and *D6Mit9* by radiation hybrid mapping, and nearby markers *D6Mit189* and *D6Mit70* and the gene *Dok* were localized by physical mapping. The approximate locations of disease loci of cerebellar deficient folia (*cdf*), *mnd2*, and truncate (*tc*) are shown relative to the mapped markers. cR, centirays.

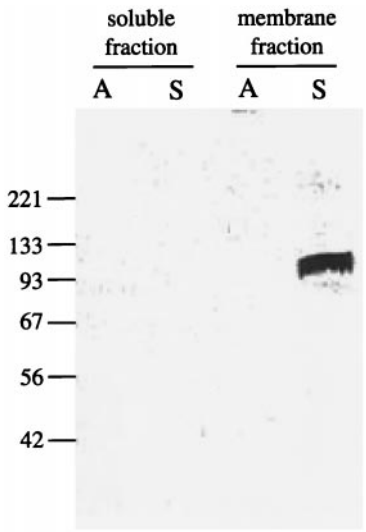

FIG. 4. Expression of Sema W in COS7 cells. Western blotting was performed with an antibody generated against a C-terminal peptide derived from the *Semaw* sequence. Lanes 1 and 2 are blots of soluble fractions from COS7 cells transfected with a *Semaw* antisense or sense plasmid, respectively. Lanes 3 and 4 are blots of membrane fractions from COS7 cells transfected with a *Semaw* antisense or sense plasmid, respectively. A, antisense; S, sense. Molecular mass in kilodaltons is indicated on the left.

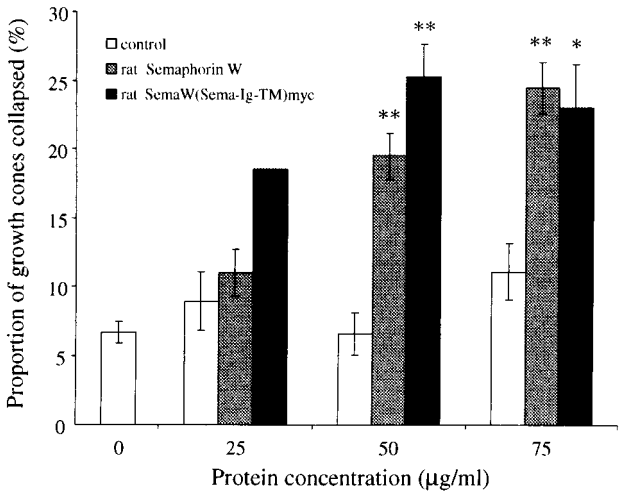

FIG. 5. Growth-cone collapse activity of Sema W. The percentage of collapsed retinal ganglion-cell growth cones in relation to the concentration of total protein added to the growth medium is shown. White bars, membrane fractions from control mock-transfected cells; gray bars, membrane fractions from rat Sema W-expressing cells; black bars, membrane fractions from rat Sema W (Sema-Ig-TM)-myc-expressing cells. The concentrations of the two Sema W forms relative to the total protein concentrations were approximately equal, as determined by Western analysis with an antibody generated against an N-terminal peptide sequence from Sema W (data not shown). *, $P < 0.05$; **, $P < 0.005$, compared with controls.

the transmembrane encoding region was replaced with an myc epitope-encoding sequence and a termination codon [pr-SW(Sema-Ig-TM)myc], also showed similar growth-cone collapse activity (Fig. 5), indicating that the collapse-inducing activity of Sema W resides outside of the C-terminal domain. Sema W also seemed to possess a weak collapsing activity against dorsal root ganglion neurons and spinal motor neurons, but the activity in these cases was not significant compared with controls (data not shown).

DISCUSSION

In this study, we report the identification of a member of the semaphorin family, Sema W. Sema W can be classified into the class IV group of semaphorins based on its phylogenetic and structural similarity to other class IV semaphorins. Functionally, Sema W is able to induce the collapse of growth cones of retinal ganglion neurons. This ability, combined with its expression in the embryo around the optic nerve and in the retina, indicate that Sema W may play a role in the development of the visual system. In the development of optic nerves, Eph receptors and their ligands, the ephrins, which are expressed in the developing nervous system, have been shown to play an important role in achieving topographical projection of retinal axons to the target area of the brain, tectum, and superior colliculus (45). However, the guidance mechanism that brings the retinal axons to the target area where synapse formation occurs is still unclear. Sema W may play an important role in preventing growing retinal axons from deviating from their proper paths during development. Temporal-expression analysis shows that Sema W is expressed at the highest levels postnatally, and therefore, in contrast to other semaphorins that may be involved in the development of the nervous system, Sema W might also play a postdevelopmental role in remodeling during learning, regrowth after injury, or in the maintenance and growth inhibition of established neural pathways.

Thus far, our studies on the biological activity of the Sema W protein, which is membrane bound, show that it resembles soluble semaphorins in its ability to induce growth-cone collapse. There are two invertebrate transmembrane semaphorins, grasshopper and *Drosophila* Sema I, that are also thought to be able to provide repulsive signals to growing axons (3, 4), although evidence for an attractive or permissive function for axonal growth has also been found (46, 47). In addition, *Drosophila* Sema I has been shown to induce the defasciculation of axons (4). Sema W may function similarly as a cell-surface ligand to influence the direction of axonal growth.

Neural guidance by semaphorins has been described primarily as a repulsive unidirectional signal toward an axon. A human transmembrane semaphorin, CD100, which, like Sema W, is also a class IV semaphorin (although CD100 has no known neural guidance function), associates with a serine kinase activity, indicating that it can transduce signals back into the cell in which it is expressed (9). This receptor-like role of CD100 is supported further by antibody-binding studies that show that CD100 can provide comitogenic signals to T cells (48). The presumed cytoplasmic tail of Sema W is 89 aa in length, which is sufficient to have signaling function. It is also rich in proline residues, similar to other transmembrane semaphorins, suggesting that it may interact with intracellular signaling or cytoskeletal proteins (22, 49). Sema W contains a cyclic nucleotide-dependent protein kinase phosphorylation site, which is of interest, because intracellular cyclic nucleotides recently have been shown to have effects on the response of neurons to Sema III (50). Sema W may therefore, like CD100, possess the ability to transduce signals back into the cell on which it is expressed.

The localization of the *Semaw* gene in the mouse places it on chromosome 6, within a small region known to harbor the gene linked to the murine disease mnd2. mnd2 is a disease characterized by rapidly progressive paralysis, muscle wasting, thymus and spleen regression, and early death (42). The motor neurons of affected mice appear swollen and stain weakly. The localization of the *Semaw* gene within the *mnd2* locus and its expression in spinal motor neurons and dorsal root ganglia (which also contain neurons that innervate muscle) make this gene an especially attractive candidate gene for the disease. It is feasible that a mutation in the *Semaw* gene could affect the survival of motor neurons, for example, through inappropriate signaling that could cause the regression of established neuron-to-muscle connections, through direct toxicity to motor neurons or to muscles, or through defective axon targeting that results in insufficient trophic support for the neurons. It is particularly interesting that the gene for Sema W contains a CTG-CAG trinucleotide repeat near its 5′ end that shows variation in length between mice, rats, and humans (see Fig. 2). CTG-CAG trinucleotide-repeat expansions have been implicated in a number of neurodegenerative diseases, such as Huntington's disease, and in the muscular disease myotonic dystrophy (51). Sequencing of the *Semaw* gene in mnd2 mice will be necessary to determine whether *Semaw* carries a defect that could implicate it in the mnd2 phenotype.

Sema W may play a role in axon guidance during development. However, the expression of Sema W primarily in the adult makes it a potentially important molecule in nervous system maintenance and repair. Therefore, it will be of interest to explore whether this molecule plays a role in the response to traumatic or ischemic nervous system injury. Its potential role in the murine disease mnd2 also merits further study. It is hoped that the elucidation of the functions of Sema W will provide us with possible treatment strategies for nervous system injury and for neurodegenerative diseases.

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