Identification of a Novel Transmembrane Semaphorin Expressed on Lymphocytes*

(Received for publication, July 1, 1996, and in revised form, September 18, 1996)

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Semaphorin (also known as collapsin) members are thought to be involved in axon guidance during neural network formation. Here, we report the isolation of a novel member, mouse semaphorin G (M-sema G), which encodes a semaphorin domain followed by a single putative immunoglobulin-like domain, a transmembrane domain, and a cytoplasmic domain. M-sema G is most closely related to M-sema F, which we previously reported, and semB and semC. These four members appear to constitute a transmembrane type subfamily in mouse semaphorins. In contrast to the predominant expression of M-sema F mRNAs in the nervous tissues, M-sema G mRNAs are strongly expressed in lymphoid tissues, especially in the thymus, as well as in the nervous tissues. The mRNAs are also detected in various cell lines from hematopoietic cells. By generating specific antibodies, we confirmed the strong expression of M-sema G proteins on the surface of lymphocytes. These results provide the first evidence that semaphorin is expressed on lymphocytes and suggest that semaphorins may play an important role in the immune system, as well as in the nervous system.

During development, neuronal axons navigate to their appropriate targets. Many factors are involved in the guidance of neural growth cones to their final targets. However, the molecular nature of the mechanism of this navigation remains largely obscure. Adhesion molecules are among the main candidates for signals controlling the direction of growth cone extension. Additional molecules have recently been identified that act as attractive and repellent cues effective in modulating the routes of neurite outgrowth (1–5). Collapasin was identified and purified from adult chick brain using a growth cone-collapsing assay of dorsal root ganglia. A medium of collapasin-transfected COS-7 cells did not induce the collapse of retinal growth cone but did induce the collapse of dorsal root ganglia growth cones (2). A domain of about 500 amino acids of collapasin reveals high similarity to grasshopper Fasciclin IV, later renamed G-Sema I, which is required for the proper guidance and fasciculation of the T1l growth cones in the limb bud of the grasshopper embryo (6). This domain is called the sema domain, and several molecules with this domain constitute the semaphorin family. Kolodkin et al. (7) have identified two members from Drosophila melanogaster, D-sema I and D-sema II. Sema IIIIs identified from human and mouse are most likely to be mammalian homologues of chick collapsin. Puschel et al. (5) have isolated five semaphorin members from the mouse and predicted that all members are secreted proteins. All members share the motifs characteristic of sema domains, including conserved cysteine residues, potential N-glycosylation sites, and immunoglobulin (IgG)-like domains. By the similarity of the sema domains, they divided mouse semaphorins into two groups, group III and group IV. Group III includes sema III (also called semD), semA, and semE, and group IV includes semB and semC (5). We recently isolated an additional mouse semaphorin, M-sema F, which is predicted to encode a transmembrane domain by a hydrophobicity analysis (5, 8).

In the present study, we isolated a novel member that is closely related to M-sema F, semB, and semC named M-sema G, which also encodes a transmembrane domain. Surprisingly, M-sema G mRNAs are very strongly expressed in lymphoid tissues, especially in the thymus, as well as in the brain. Generation of antibodies to M-sema G confirmed strong expression of the protein on lymphocytes. These results suggest that M-sema G may play an important role in the immune system, as well as in the nervous system.

EXPERIMENTAL PROCEDURES

Amplification of cDNA Fragments Encoding the Sema Domain—mRNA was isolated from embryos of ICR mice at embryonic day 14 using Fast Track (Invitrogen, San Diego, CA), and first-strand cDNA was synthesized using Molony leukemia virus reverse transcriptase and random hexamer primers. For polymerase chain reaction (PCR), the following oligonucleotide primers were designed based on the reported alignment of the sema domain: sema5, 5′-TACGACGTC/CT/TN/TT/CT/ATA/CT/GG-3′ (sense); sema3, 5′-TCCCAIGC/CA/CA/AG/AFGG/A/GTC-3′ (antisense) (7). cDNA was amplified with 2 cycles (94°C for 1 min, 37°C for 2 min, 72°C for 3 min) followed by 40 cycles (94°C for 1 min, 42°C for 2 min, 72°C for 3 min) as described previously (8). PCR fragments of expected size (about 300 base pairs) were purified on 1.5% agarose gel and ligated to PCR-Script SK(+) (Stratagene, La Jolla, CA).

Isolation of cDNA Clones—An adult mouse brain library primed with oligo(dT) (Stratagene) was screened using probes radio labeled by random priming (Pharmacia Biotech, Inc., Uppsala, Sweden). Probes were

* This research was supported by Grant-in-aid 20238702 from the Ministry of Education for Scientific Research, Grant-in-aid 08044282 from the Ministry of Education, the Monbusho International Scientific Research Program, and a grant from the Ichiro Kanehara Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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hybridized to the phage DNA immobilized on nylon filters (Hybond-N, Amersham, Arlington Heights, IL). Cells were washed once with PBS and incubated for 1 h at 37°C with 500 ml of Luria Bertani medium with 500 μg/ml ampicillin, 90 μg/ml XhoI, and 1 μg/ml HindIII restriction enzymes. The sema domain of M-sema G is 51% identical to that of human sema-G (10). The gene fragment was ligated into the pBluescript SK vector. The sequence of the M-sema G-HA fusion protein mouse-semaphorin G (M-sema G) was shown in Fig. 1A. The sequence and estimated amino acid sequence of A18 are shown in Table I. The longest open reading frame starting with an ATG codon matches the consensus sequence of a strong translation initiation site, and ends with a TGA stop codon (Fig. 1A). The amino acid sequence predicted amino acid sequence displayed two stretches of hydrophobic sequence (Fig. 1B). A single putative immunoglobulin-like domain was predicted as described previously (1). Cells were then solubilized with 0.5 ml of lysis buffer containing 5% bovine serum albumin. After preclearing with protein A-Sepharose (Pharmacia), the lysate was immunoprecipitated with antibody to mouse semaphorin G (M-sema G). A hydrophobicity analysis was performed using the ECL system (Amersham Corp.).

RESULTS

Cloning and Structural Features of M-sema G cDNA—Using degenerative primers from conserved motifs of the semaphorin domain (2), cDNAs sharing homology with the collagen and G-sema I genes were amplified by PCR and used as probes to screen an adult mouse brain cDNA library. The cDNA clones isolated, the longest clone, A18, was analyzed. The nucleotide sequence and estimated amino acid sequence of A18 are shown in Fig. 1A. The longest open reading frame starting with the first ATG codon matches the consensus sequence of a strong translation initiation site (14) and ends with a TGA stop codon (Fig. 1A). There are in-frame stop codons preceding the first ATG. The cDNA predicts a protein of 861 amino acids. We named this semaphorin G protein M-sema G. A hydrophobic analysis predicted eight potential sites for N-glycosylation and 13 cysteine residues that are conserved among all vertebrate semaphorins. M-sema G contains a highly acidic region at the C-terminal end that is predicted to be an intracellular domain. The presumed domain of M-sema G is flanked by two repeats of M-sema F, 48% to that of semC, 4% to that of semB, and 40–43% to those of semA, semD, and semE (5). The 295 amino acids within the sema domain are 77% identical to those of plasmid encodes residues 1–860 of M-sema G linked to a tandem repeat of the (KDEL) four-residue buffer (Lys-Asp-Asp-Leu) from the restriction linker and pBlueScript SK vector sequence. The plasmid was transfected into COS-7 cells in a 100-mm tissue culture dish by DEAE-dextran methods (12). After 2 days of culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, cells were harvested, washed twice with PBS, pelleted by centrifugation, and frozen at −20°C before analysis by immunoblotting. After nuclear pellets were removed by centrifugation, lysates were mixed with 2 × sample buffer (2% SDS, 20% glycerol, 100 mM Tris-HCl (pH 6.8), 0.1% bromophenol blue, and 6% 2-mercaptoethanol) and boiled for 5 min. Cell lysates of COS-7 cells were solubilized directly with 1 × sample buffer. The samples (10 μg/lane for murine organs and one-twentieth lysate/lane from 80% confluent culture of transfected COS-7 cells) were electrophoresed in 6% SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp., Bedford, MA). Membranes were blocked in PBS containing 5% nonfat dried milk for 2 h and incubated with affinity-purified anti-M-sema G serum in a 1:1000 dilution. To detect HA-tagged M-sema G fusion proteins by anti-HA Ab, membranes were blocked in Tris-buffered saline (50 mM Tris-HCl, pH 7.6, 150 mM NaCl) containing 5% bovine serum albumin and incubated with anti-HA monoclonal Ab (12CA5; Boehringer Mannheim) at a concentration of 0.5 μg/ml followed by the incubation with affinity-purified rabbit anti-mouse IgG Ab (Cappel, Durham, NC) in a 1:5000 dilution. Membranes were then washed with PBS-0.1% Tween 20 and incubated with horseradish peroxidase-conjugated protein A (Amersham Corp.). After being washed with PBS-0.1% Tween 20, membranes were developed using an ECL system (Amersham Corp.).

Surface Biotinylation and Immunoprecipitation—Cell surface biotinylation was performed as described previously (13). There are in-frame stop codons preceding the first ATG. The cDNA predicts a protein of 861 amino acids. We named this semaphorin G protein M-sema G. A hydrophobic analysis predicted eight potential sites for N-glycosylation and 13 cysteine residues that are conserved among all vertebrate semaphorins. M-sema G contains a highly acidic region at the C-terminal end that is predicted to be an intracellular domain. The presumed domain of M-sema G is flanked by two repeats of M-sema F, 48% to that of semC, 4% to that of semB, and 40–43% to those of semA, semD, and semE (5). The 295 amino acids within the sema domain are 77% identical to those of...
collapsin-4, which was isolated as a partial cDNA of about 0.9 kilobase from chicken; there is therefore no information on whether coll-4 contains a transmembrane domain (15). A phylogenetic tree was obtained for the complete amino acid sequences of mouse semaphorins (Fig. 1D). Two different groups can be distinguished; one contains semA, semD, and semE, and the other contains M-sema F, M-sema G, semC, and semB. M-sema G is closest to M-sema F and then to semC and semB.

**FIG. 1.** A, cDNA and deduced amino acid sequences of M-sema G clone A18. The coding sequence for M-sema G is 2583 bp, which encodes an 861-amino acid protein. The deduced protein contains a signal sequence (upper dotted letters), a sema domain (double underlined letters), an IgG-like domain (underlined letters), and a transmembrane domain (italic characters). B, hydrophobicity profile of M-Sema G. The algorithm of Kyte and Doolittle was used. The calculations were performed using DNASIS 3.2 software. C, diagram of M-Sema G. ss, signal sequence; TM, transmembrane domain. D, phylogenetic tree for mouse members of the semaphorin family using DNASIS 3.2 program. semA, semD, and semE belong to a subfamily that includes secreted type members, and semB, semC, M-sema F, and M-sema G belong to another subfamily that includes transmembrane type members.
(E14.5) distinctly detected M-sema G mRNA. The hybridization signals were found throughout the nervous tissues, with particularly strong expression in the cortical plate and dorsal root ganglia (Fig. 2). Surprisingly, among tissues other than nervous system, the thymus showed very strong expression for M-sema G mRNAs (Fig. 2). Moderate expression was also detected in the lung and kidney, whereas only background level expression was seen in the liver. The control sections hybridized with sense probes showed no distinct expression. So far there are no other semaphorin family members with strong expression in the immune tissues. For example, the expression of M-sema F mRNAs was very strong throughout the brain and spinal cord but weak in the thymus from E14.5 embryos.

The expression of M-sema G in various tissues from adult animals and cell lines was also studied by RNase protection assay. Radiolabeled antisense RNA, covering the 5' end of M-sema G, was used as a probe. As shown in Fig. 3, M-sema G was detectable in the adult brain, kidney, thymus, spleen, lung, heart, and bone marrow but was undetectable in liver. However, the expression level of M-sema G mRNA was much higher in the immune tissues, especially in the thymus, than in the other tissues. Although a high level of expression was observed by in situ hybridization analysis, the level detected in this assay was much weaker in the brain. This could be due to the maturation-associated decrease in the expression in the brain.

In cell lines, the expression was detectable in T-lineage (EL4 and 2B4), B-lineage (LK, 70Z/3, and NS-1), myeloid (WEHI3 and J774), and mastocytoma (P815) cell lines but was undetectable in thymic and bone marrow-derived stroma (MRL104.8a and BMST) and fibroblast (BALB3T3) cell lines. Thus, M-sema G is a member of the semaphorin family, which is expressed predominantly in hematopoietic cells.

Protein Expression of M-SeMA G—To check the tissue distribution of M-SeMA G at the protein level, we generated a polyclonal Ab against M-SeMA G by immunizing two rabbits with M-SeMA G-GST fusion protein (see “Experimental Procedures”). To confirm whether immunized rabbit serum was directed against M-SeMA G, an epitope-tagged form of M-SeMA G was expressed in transfected cells. COS-7 cells transfected with the M-SeMA G-HA fusion gene were lysed and analyzed by immunoblotting. As shown in Fig. 4A, HA-tagged M-SeMA G immunoblotted with anti-HA monoclonal Ab migrated at about 125 kDa on SDS-PAGE under reducing conditions, although 100 kDa was expected for the core protein of mature M-SeMA G-HA. Because there are eight potential sites for N-linked glycosylation in the deduced protein sequence of M-SeMA G, the difference in the size could be explained by glycosylation at these sites. When HA-tagged M-SeMA G on the same membrane (shown in Fig. 4A) was reprobed with anti-M-SeMA G serum (Fig. 4B), the band migrating at the identical position appeared in the lysate from COS-7 cells transfected with M-SeMA G-HA fusion gene, confirming the specificity of our polyclonal Ab.

Immunoblotting analysis using lysates from various organs revealed that M-SeMA G was highly expressed in the thymus, in contrast to no expression in liver (Fig. 4C), which is consistent with the expression level of mRNA. Although low and faint
expressions of M-Sema G were also observed in the brain and kidney, respectively, the size detected in the brain seemed to be smaller than that detected in the thymus. The reason for the different sizes of M-Sema G between thymus and brain is thus far unknown, but posttranslational modification of M-Sema G could vary with tissues.

Cell Surface Expression of M-Sema G—Because the sequence of M-Sema G encoded a transmembrane domain, it could be expressed on the cell surface. To verify this possibility, thymocytes were surface biotinylated and cell surface proteins were immunoprecipitated with anti-M-Sema G Ab. As shown in Fig. 5, the protein that migrated at the same position as detected by immunoblotting analysis was immunoprecipitated with anti-M-Sema G Ab, demonstrating that M-Sema G is expressed on the surface of thymocytes.

DISCUSSION

The first member of the semaphorin family was identified by different methods in vertebrates and invertebrates. Sema I (known previously as FasIV) was cloned by using a monoclonal antibody recognizing special axonal tracts of the grasshopper embryos. Then several genes were cloned using a PCR method with degenerative primers for conservative amino acids, and a domain with about 500 amino acids was shown to be very conserved among invertebrates and vertebrates; it was named the sema domain (7). A member of the sema family was shown to be a human homologue of chick collapsin, which was independently identified as a protein collapsing a growth cone of dorsal root ganglion neurons (2). More members of the semaphorin family were cloned; semA, B, C, D, and E and M-sema F from mouse and coll-1, 2, 3, 4, and 5 from chick (5, 15, 8). Most of these members have been predicted to be secreted (5, 15), and our hydrophobicity analysis suggests that M-sema F and SemB and SemC of group IV are transmembrane proteins (8).

In the present study, we identified a novel member of the semaphorin family from mouse brain using a PCR method with degenerative primers; we named this semaphorin M-sema G. This cDNA deduces a protein that has a sema domain, an IgG-like domain, and a transmembrane domain. We also obtained evidence that this protein is a transmembrane type by a surface labeling analysis. This is the first evidence that transmembrane-type members of the semaphorin family exist in vertebrates and are different from invertebrate transmembrane-type members in that the latter have no IgG domain.

A BLAST search of a data base revealed that a fragment of chick coll-4 showed the highest homology, has 77% identical amino acids in the sema domain of M-sema G, and is thought to be a chick homologue of M-sema G. In addition, M-sema F, semC, and semB also showed high similarity to M-sema G, in that order, and are predicted to have a transmembrane domain from a hydrophobicity analysis. These results suggest that M-sema G and these members constitute a transmembrane-type subfamily of semaphorins. However, the expression of M-sema G is unique among this subfamily, because the immune tissues, especially the thymus, displayed very strong expression for this member. Our results provide the first evidence that semaphorin is expressed on lymphocytes.

It will be intriguing to elucidate the functional role of M-sema G in lymphocytes. Our preliminary results demonstrated that the protein expression of M-sema G is much higher in thymocytes than in peripheral T and B cells. Moreover, in situ hybridization analysis using adult thymus revealed that M-sema G is expressed predominantly in the thymic cortex, espe-
cially in the outer cortex. The outer cortex contains thymic lymphoblasts, which give rise to more mature thymocyte populations, i.e. the small cortical thymocytes and the medullary thymocytes (16). Along with the progression of maturation, thymocytes move from the outer cortex to the medulla through the deep cortex. The mechanism responsible for the transport of thymocytes from the cortex toward the medulla is thus far unknown. Because semaphorins contribute to the guidance of axons on growth cones (17), the function of M-Sema G on thymocytes could be related to a maturation-dependent movement of immature thymocytes. Alternatively, because semaphorins have been shown to have repellant activity in axonal guidance of the nervous system, thymocytes may repel each other by expressing M-Sema G on the cell surface to interact efficiently with the thymic stromal cells that did not express M-Sema G (Fig. 3). The precise distribution of M-Sema G on lymphocytes of various developmental stages must be determined to address these possibilities. To this end, we are currently generating a monoclonal Ab that can react with the extracellular portion of M-Sema G using M-Sema G-human IgG fusion proteins. Although the functions of M-Sema G in lymphocytes are currently unknown, our present results suggest that semaphorins are not a family of proteins whose functions are limited to the guidance of growth cones.

Acknowledgments—We thank Drs. T. Suda and S. Nagata for kindly providing us with pEF-Fc and Dr. T. Takagi for advising for us concerning the experiments.

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