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THE EFFECT OF HUMAN SEMAPHORIN SEMA7A ON *IN VITRO* MIGRATION OF T CELLS

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ABSTRACT

THE EFFECT OF HUMAN SEMAPHORIN SEMA7A ON *IN VITRO* MIGRATION OF T CELLS

by Mandana Amiri

Human CDw108/SEMA7A is a glycosylphosphotidylinositol (GPI)-linked membrane glycoprotein in the semaphorin family. SEMA7A and its receptor plexin-C1 are widely expressed on cells of the immune system. It is involved in activation, proliferation, migration, and homing of lymphocytes. The goal of this work was to further characterize the role of human semaphorin SEMA7A in modulating the immune response. We have purified recombinant human SEMA7A and investigated its effect on the migration of T cells in *in vitro* transwell migration assays. We have found that the secreted form of SEMA7A can act as a chemoattractant for migrating MACS-isolated human T cells.

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1. Introduction

1.1. Semaphorin: structure and expression

The semaphorins are a large family of proteins. This family contains secreted, transmembrane, and glycosylphosphotidylinositol (GPI)-linked proteins. Members of the semaphorin family share a Sema domain, which contains 500 amino acids with 15 conserved cysteines. The Sema domain is followed by a class-specific carboxyl terminus. Semaphorins are placed into eight classes or subfamilies. The first seven classes contain invertebrate or vertebrate semaphorins and differ from one another primarily with respect to their C-termini. The eighth class is the viral semaphorins (Kolodkin et al, 1999).

Some of the semaphorins (e.g., Sema 3A and Sema 4A) form homodimers linked by disulfide binds; however, they could possibly make heterodimers where a cell produces more than one class of semaphorins. The dimerization of these semaphorins is required for their function. Functions of secreted and membrane-bound semaphorins can be regulated by furin-dependent proteolytic cleavage. Secreted semaphorins are initially produced in the form of transmembrane proproteins. The proteolytic cleavage of the proproteins at C-termini is necessary for the function of semaphorins because it yields the mature, secreted form of the proteins. However, further cleavage of semaphorins reduces their function by dissociating dimers of the mature proteins. The proteolytic cleavage of membrane-bound semaphorins decreases their *cis*-interactions at the cell surface by removing them from the membrane (Koppel et al., 1998; Adams et al., 1997). Some semaphorins are N-glycosylated and can become phosphorylated at their intercellular

serine/threonine (Ser/Thr) residues. Those semaphorins are favorite substrates for kinases and are likely associated with Ser/Thr kinase pathways (Kolodkin et al., 1993; Elhabazi et al., 1997).

1.2. Semaphorin receptors

To date, three different classes of semaphorin receptors have been reported: neuropilins, plexins, and CD72 proteins.

Neuropilins are transmembrane proteins, which are mostly expressed in CNS (Van Vactor et al., 1999). There are two types of vertebrate neuropilins, including neuropilin-1 (Npn-1) and neuropilin-2 (Npn-2). Npn-1 is a receptor for Sema3A. It was first identified in the nervous system. Later, Npn-1 was found in developing heart and bones as well. Npn-2 is also expressed inside and outside the nervous system (in the limb bud, developing bones, back muscles, and smooth muscles).

The second family of semaphorin receptors is the plexin family. Plexins are transmembrane proteins, which are widely expressed in neurons. The plexins fall into four subfamilies: A, B, C, and D. Plexin-A contains plexin-A1 (Nov), plexin-A2 (Oct), and plexin-A3 (Sex). Plexin-B contains plexin-B1 (Sep), -B2, and -B3. Plexin-C contains plexin-C1 (VESPR). Plexin-D subfamily includes only plexin-D1. Plexin-C1 binds to viral semaphorins and their human orthologue SEMA7A. Plexin-B1 is a receptor for Sema4D, which is expressed in the nervous system and lymphocytes (Tamagnone et al., 1999). Also, plexin-C1 has been identified outside the nervous system on human monocytes, dendritic cells, B cells, T cells, lung, spleen, and placenta

(Comeau et al., 1998). The presence of Sema4D and plexin-C1 on lymphocytes suggests the involvement of semaphorins in the immune system.

Interestingly, complexes of neuropilin and plexin have been detected. In those complexes, plexins act as co-receptors for secreted class 3 semaphorins. Plexins limit the binding ability of neuropilins to their ligands. Consequently, they help neuropilins to distinguish among their different ligands (members of secreted class 3 semaphorins) and to bind to each of them with different affinities. Experiments showed that when the cystoplasmic domain of plexins is deleted, neurons expressing the truncated plexins (with normal Npn-1) do not respond to their ligand Sema3A. This result suggested that plexins are involved in downstream signal transduction cascades and are necessary in the formation of functional receptor complexes (Rohm et al., 2000).

Signaling by semaphorins through plexins has been investigated recently in *Drosophila.* The study showed that members of the flavoprotein monooxygenase family called MICALs regulate Sema-1a axonal repulsion mediated by plexin-A. MICAL is a multidomain protein expressed in axons. It interacts directly with both plexins and cytoskeletal components in axons to regulate the transduction of semaphorin repulsive guidance (Terman et al., 2002).

Recently, the third group of semaphorin receptors CD72 was identified. CD72 belongs to the C-type lectin superfamily. It is a regulatory protein that is primarily expressed on the surface of the B cells during the development (from the pro-B to the mature B cell stage). CD72 is also detected on follicular dendritic reticulum cells, splenic red pulp macrophages, Kupffer cells, a fraction of medullary thymocytes, and

a population of peripheral T cells (in mice). CD72 shows allelic polymorphism. Four allelic forms have been detected in different mouse strains. Among the different forms only $CD72^b$ is expressed on the T cells. The expression of this protein increases on the T cell surface after the cell is activated. CD100, a member of the class 4 semaphorins, can act as a ligand for CD72. Binding of this ligand to CD72 present on the surface of the B cells increases the cells' activities (Parneset et al., 2000).

In summary, semaphorin receptors are present both inside and outside the nervous system. Expression of neuropilins in the lymphoid tissues is not yet known. But plexins (plexin-C1) and CD72 are known to be present in both lymphoid tissues and hematopoetic cells. The presence of the semaphorin receptors in the immune system suggests that semaphorins may have some immunological activities.

1.3. Semaphorins in the nervous system

Semaphorins were initially defined as axon guidance molecules in the developing nervous system. Later, other functions were described for these molecules such as fasciculation, defasciculation, and neuronal path finding. Antibody blocking studies in grasshopper showed that Sema-1a is essential for path finding of limb sensory neurons. Expression of Sema-1a on limb epithelial cells in grasshopper promotes fasciculation of the sensory axons. In *Drosophila*, however, the presence of Sema-1a on motor axons promotes their defasciculation in both CNS and PNS (Van Vactor et al., 1999). Therefore, semaphorins can cause both association and dissociation of neurons depending on their spatial expression.

Semaphorins were first identified as repulsive cues for axon growth in the nervous system. There is now a great deal of evidence available to support that semaphorins are not exclusively repulsive. For example, experiments show that exposure of *Xenopus* spinal growth cones to high levels of cyclic nucleotides (cGMP) can switch their response to Sema3A from repulsion to attraction (Song et al., 1998). In other studies dorsal root ganglion (DRG) neurons exhibit an inhibitory response to SEMA3A alone but their response becomes anti-inhibitory where other semaphorins (such as SEMA3B or SEMA3C) are also present. Finally, *in vitro* experiments suggest that olfactory bulb axons respond in different ways (attraction or repulsion) to different semaphorins (Takahashi et al., 1998).

The above studies indicate that semaphorins are expressed in many different places in the nervous system. They are multifunctional molecules and their functions are cell-specific and state-specific.

1.4. Semaphorins in the immune system

To date, semaphorins have been detected on many different cells and tissues in the immune system. The two most characterized semaphorins in the immune system are SEMA4D (CD100) and SEMA7A (CDw108).

1.4.1. SEMA4D

CD100, also known as a leukocyte semaphorin, is a 150 kDa homodimer linked with two disulfide bonds. The gene belongs to Sema4 subfamily (SEMA4D) that contains an Ig-like domain at the extracellular region. CD100/SEMA4D is expressed on a variety of hematopoietic cells, including T cells, B cells, natural killer cells, monocytes,

and granulocytes. It is also detected in the immune tissues such as spleen, thymus, bone marrow, tonsil, and lymph nodes.

CD100 was originally reported as a T cell activation molecule. A study completed by Herold et al. (1996) showed that CD100 binds to the protein tyrosine phosphatase CD45 after stimulation with an anti-CD100 mAb. This observation suggests that CD100 is involved in T cell activation process through its physical association with CD45, which is a crucial activation molecule on the T lymphocytes. Additionally, CD100 interacts in its cytoplasmic domain with a serine-threonine kinase in the T cells. Enzymatic activities are important factors in stimulating the biochemical cascades during the cell activation process. Therefore, the association of CD100 with the phosphatase and kinase activities in the T cells led scientists to consider this molecule as a regulator of the cell activities in the immune system (Elhabazi et al., 1997). The association of CD100 with the cellular phosphatase activity has also been detected in B lymphocytes. In the secondary lymphoid follicles only the B cells in the germinal center, but not in the mantle zone, express the CD100 molecule. This molecule promotes the adhesion of B cells and increases their viability. Since CD100 is only detected in the germinal center of the follicles and its expression induces cell aggregation, the molecule may also be involved in germinal center formation (Hall et al., 1996).

CD100/SEMA4D is present as either a transmembrane or a secreted protein. The transmembrane form is regulated by a furin-like proteolytic cleavage. The proteolytic cleavage of the transmembrane protein generates the soluble form. This cleavage is metalloprotease-dependent and is regulated by phosphorylation (Elhabazi et al., 2001).

Delaire et al. (1998) examined the effect of the soluble CD100 on the migration of various hematopoietic cells. They used two different expression plasmids for the transfection of Cos-7 cells. One of the plasmids expressed the transmembrane CD100, which could be cleaved by the protease activity. The other plasmid contained only the extracellular domain of the gene so it could only produce the soluble form. The result of the experiment showed that the soluble CD100, obtained from the expression of either of the plasmids, inhibited the migration of human monocytes and B lymphocytes. The inhibitory effect was not observed for the B12 thymic T cell clone. Interestingly, when CD100 was replaced with human SEMA3A, a homologue of the CD100, a similar inhibitory effect on the cell migration was detected. Although SEMA3A had been well studied in the nervous system, nothing was known about its potential function in the immune system. This study provided the first evidence for the possibility of the involvement of SEMA3A in the immune system (Delaire et al., 2001).

In another experiment completed by Bouloc et al. (2000), the effect of soluble CD100 on the migration of blood-derived dendritic cells was studied. The result indicated that soluble CD100 inhibits the spontaneous migration of dendritic cells only at D3-D4 stages of differentiation.

In conclusion, CD100/SEMA4D is involved in the immune system. The effect of this semaphorin on the migration of various immune cells has been studied. The effect is different depending on the type and differentiation stage of the cell.

1.4.2. SEMA7A

SEMA7A, initially called SEMAK1, is the first known GPI-linked semaphorin. The amino acid sequence of this human semaphorin has 45% identity to the semaphorin-like sequence in alcelaphine herpesvirus-1 (AHV). This sequence homology suggests that SEMA7A may have some similar activities to its viral counterpart AHV Sema.

Viral semaphorins are immunomodulators. They interfere with the host immune system to facilitate their own production. AHV Sema is also an immune modulator. Its sema-like gene is not conserved among the herpesviruses. However, the homologue of AHV Sema has been found in its host suggesting that it was originally derived from the host during their evolution (Xu et al., 1998). AHV is not a neurotrophic virus. It can cause lymphoproliferation and transformation of T cells ($CD4^+$ or $CD8^+$) in the host. The DNA sequence information of AHV indicates the presence of a gene that mediates its binding to the host B cells (Ensser et al., 1997). It regulates the expression of ICAM-1 (an adhesion molecule) and induces secretion of cytokines IL-8, TNF-α, and IL-6 from monocytes (Comeau et al., 1998). Since SEMA7A is the human homologue of the AHV Sema, it might also act as an immune modulator. Binding of SEMA7A to the mast cells and macrophages further supports the immune modulating function of this semaphorin (Xu et al., 1998).

SEMA7A/CDw108 is a 75-80 kDa molecule preferentially expressed on the surface of activated lymphocytes and erythrocytes. Angelisova et al. (1999) showed that the surface expression of CDw108 antigen changed during the cell activation process *in vitro*. CDw108 antigen first appears on the surface of all the B cells and most of the T

and NK cells early in activation. The expression of the CDw108 antigen increases rapidly. After 48 hours of activation, CDw108 reaches its maximum expression. Then, the expression decreases gradually, returning to its pre-stimulation level. This finding suggests that CDw108 may be involved in the activation and differentiation of the cells in the immune system (Angelisova et al., 1999). In addition, CDw108, like most of the GPI-linked molecules, interacts with src-family tyrosine kinases. The association of this molecule with kinase activities is further evidence suggesting that CDw108 has a role in the cell activation process (Stefanova et al., 1991).

Finally, Mine et al. (2000) examined the expression pattern of CDw108 on the surface of the thymic T cells during their development. Using flow cytometery, they observed that CDw108 was differentially expressed on the T cells at various stages of development. For example, the CD34+ stem cells that are committed to become T cells and immigrate to the thymus lose the expression of CDw108 protein. But the double positive (CD4+/CD8+) T cells in the thymus express the CDw108 antigen once again. This finding also indicates the contribution of CDw108 in the cell development and activation processes. Additionally, it is possible that this antigen regulates the migration of the lymphocytes and helps them during homing.

Yamada et al. (1999) performed a Northern blot analysis to look at the expression pattern of the CDw108 gene in the normal human tissues. They found that CDw108 mRNA is not only expressed on activated peripheral blood mononuclear cells (PBMCs) but also in the spleen, thymus, testis, placenta, and brain. The mRNA expression is low

in the brain and thymus but high in the other tissues. The expression pattern indicated that CDw108 has a function in a variety of the organs including lymphoid organs.

In summary, SEMA7A is expressed on cells and tissues of the immune system. The expression is different in various cell types depending on their activation state. While the studies discussed above show that SEMA7A mediates the activation and proliferation processes in the immune cell, the effect of this semaphorin on T cell migration has yet to be examined.

Overall, semaphorins are widely expressed in cells and tissues of the immune system and play different roles in immunity. Since SEMA7A is expressed in lymphoid organs (spleen and thymus) and its receptor plexin-C1 is present on the surface of T cells, it is possible that SEMA7A modulates the migration of T cells during their homing and development.

2. Specific aim

The objective of this research project was to investigate the ability of human semaphorin SEMA7A to function as a specific signal molecule (repulsive or attractive) for the migrating T cells. In particular, the chemoattraction effect of soluble human semaphorin SEMA7A on the *in vitro* migration of MACS-isolated human T cells was examined using a fluorescence-based transwell migration system.

3. Experimental overview

The project was completed in four steps (see Appendix A for a flow chart). First, the pEX mammalian expression plasmid containing the SEMA7A extracellular part and a myc-his tag (pEX-SEMA7A-mh, Exelixis Pharmaceuticals, South San Francisco,

California) was amplified by transformation of bacterial cells. After the amplified DNA was isolated, its sequence identity was verified by both restriction mapping and end sequencing. In the second step, the recombinant plasmid was introduced into mammalian Cos-1 cells by transient transfection to produce the SEMA7A-mh tagged protein. Subsequently, the fusion protein produced in the secreted form was purified from cell supernatants using Centricon centrifugal filter devices and nickel-chelating affinity columns. During the third step, the functionality of the purified recombinant protein was examined via monocyte IL-8 release assays and fluorescent-based T cell chemotaxis assays. Finally, results of the migration assays were verified by an immunodepletion experiment.

4. Materials and methods

4.1. pEX-SEMA7A-mh vector

The pEX-SEMA7A-mh plasmid is a mammalian expression vector with a pcDNA3.1 backbone and the multiple cloning site (MCS) from pSECTAG A (Invitrogen, Carlsbad, California). The pSECTAG A component encodes a signal peptide for protein secretion and also incorporates myc-his tags into the product of the expressed gene. These tags allow the detection of SEMA7A proteins on Western blots using anti-myc Ab and the purification of them on nickel columns. The vector was constructed by insertion of the extracellular part of the human SEMA7A gene into the pSECTAG A MCS, so the protein is mostly synthesized in a secreted form in contrast to the endogenous SEMA7A protein (Fig. 1).

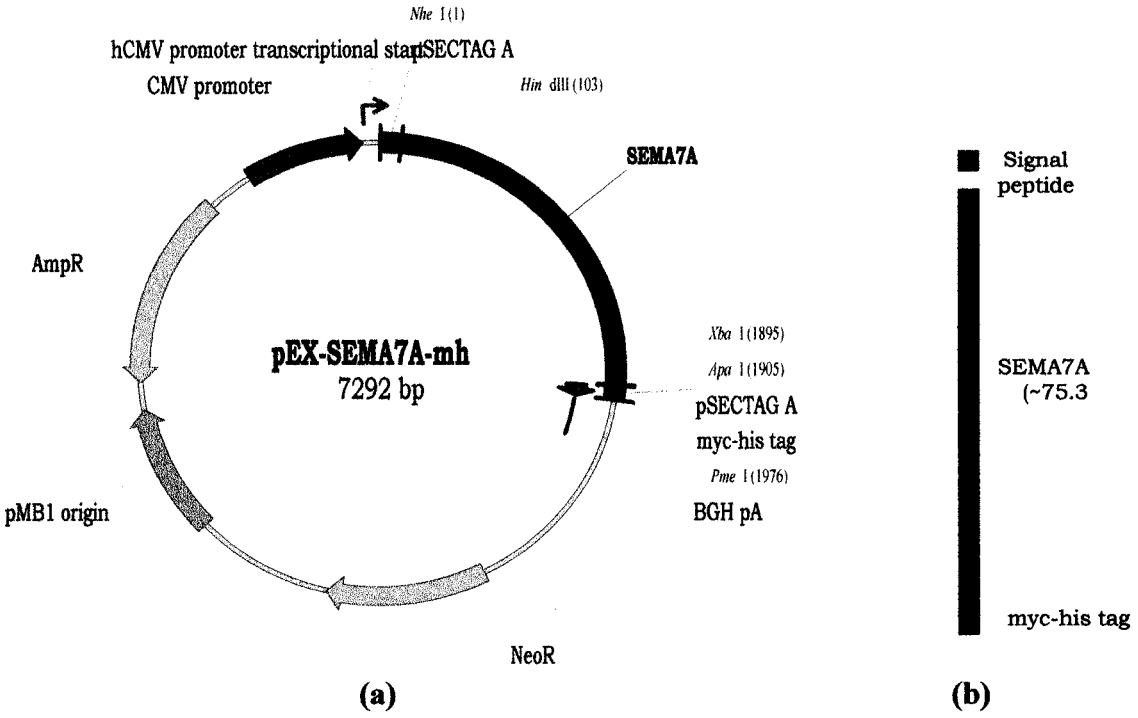


Fig. 1. PEX-SEMA7A-mh construct donated by Exelixis Pharmaceuticals (a) and the protein product (b).

4.2. Amplification of pEX-SEMA7A-mh vector via bacterial transformation

Fifty microliters of DH 5α competent cells (10^7colonies/μg DNA, Invitrogen) were thawed on ice for10 min. Then, 1 μl of SEMA7A containing plasmids (~ 0.1 ng) was added to the cells and the mixture was put back on ice. After 20 min, the cells were heat-shocked for 45 sec in a 42°C water bath and returned to ice for 2 min.

After incubation, 950 μl of room temperature SOC medium were added to the mixture, and the cells were allowed to grow for 1 hr in a 37°C shaker bath. Finally, to select the transformants, 10-25 μl of cell/plasmid mixture were plated on LB/ampicillin (amp) agar plates. The plates were incubated overnight at 37°C.

4.3. Plasmid preparation

Eight colonies were selected from LB/amp plates. Each colony was grown overnight in 3 ml of LB/amp (50 μg/ml) medium in a 37°C shaker. Plasmids were isolated from the cell culture by the alkaline lysis technique. The plasmid preps were digested using endonuclease enzymes presented in Table 1 (p. 15). One of the colonies, which gave the expected restriction pattern, was used to inoculate 100 ml LB/amp medium. The resulting culture was grown overnight in a shaking incubator (255 rpm) at 37°C. After incubation, 500 μl of the culture were added to 500 μl glycerol to prepare glycerol stocks for storage. Plasmids were isolated from the remaining culture using the QIAGEN-tip 500 maxiprep kit (Qiagen, Valencia, California). The isolated plasmids were dissolved in 250 μl of TEN (TE plus 10 mM NaCl, pH 8.0) buffer. The yield and purity of the plasmids were determined by measuring optical density (OD) values at 260 & 280 nm using a Shimadzu UV-visible recording spectrophotometer.

4.4. Plasmid verification by end sequencing

Samples of the plasmid maxiprep were submitted to the PAN Facility at Stanford University for cycle sequencing. Two primers (Operon Technologies, Alameda, California) were used for sequencing the SEMA7A insert from both ends. Each primer was designed in Primer3 program (www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) and examined in Operon (www.operon.com/oligos/toolkit.php) to avoid self-hybridization of the primers or primer-dimerization especially at their 3' ends. Finally, Vector NTI (Informax, North Bethesda, Maryland) was used to ensure that primers would bind to the plasmid only at one place.

The first primer was SEMA7AL1: CTGGCTAACTAGAGAACCCAT (5'-3'). It bound the pSECTAG MCS at the 5' end of the insert. The primer extended from the 5' to 3' end of the insert. The second primer was SEMA7AR1 (BGH): TAGAAGGCAGTCGAGG (5'-3'). This primer bound to the BGH polyadenylation site of the MCS. It extended from the 3'to 5' end of the insert. Each primer revealed the sequence of approximately 100 base pairs from the vector and 300 base pairs from the insert.

The identity of the plasmid was verified by comparing the sequencing data from the PAN facility to the published sequence in the GenBank using BLASTN 2.1.2 program (www.ncbi.nlm.nih.gov/blast).

4.5. Plasmid verification by restriction mapping

The sequence of pEX-SEMA7A-mh was further analyzed via enzymatic digestion. The Webcutter 2.0 program (www.firstmarket.com/cutter/cut2.html) was used to provide a list of the restriction endonucleases that could cut the plasmid up to three times. A few enzymes were chosen from the list based on their cut sites and availability in the laboratory (Table 1). Finally, Vector NTI was used to determine the position of each cleavage in the plasmid.

For each reaction, 3 μg of the plasmid was used. The single digest reaction was prepared by mixing 23 μl plasmid, 5 μl buffer (Roche, Indianapolis, Indiana), 1.5-3 μl enzyme I (30 Units, Bgl II: Stratagene, La Jolla, Californa, Hind III, Xba I, and Apa I: Roche), and 19 μl Millipore water. The double digests contained 23 μl plasmid, 5 μl buffer, 1.5-3 μl enzyme I (30 Units), 1.5-3 μl enzyme II (30 Units, Roche), and 17 μl

water. For a digestion control, enzymes were replaced with water. The reaction mixtures were incubated for 2 hr in a 37°C water bath. At the end of incubation, DNA digestion was stopped by addition of 1 μl EDTA (0.5 M) into each reaction tube, and the tubes were cooled on ice for 30 min. Digestion fragments were analyzed by agarose gel electrophoreses. Samples were prepared by mixing 10 μl reaction digest, 2 μl 10X loading buffer (GibcoBRL, Grand Island, NY), and 8 μl water. The samples were loaded in a 1.2% agarose gel made with Tris-Acetate-EDTA (TAE) buffer containing 3 μg/ml ethidium bromide. After running the gel for 2 hr at 73 V, the image was captured on a Macintosh computer using a Gel Doc Imaging System (BioRad, Hercules, CA).

TABLE 1

RESTRICT DIGESTION OF THE pEX-SEMA7A-mh

Enzyme I (units of activity or U/μl)	Enzyme II (U/μl)	Restriction Buffer	Expected Size of the Restriction Fragment (bp)
----	----	M	7292
Bgl II (10)	----	M	1569, 5723
Hind III (10)	Xba I (20)	M	1792, 5500
Bgl II (10)	Xba I (20)	M	1208, 1569, 4515
Apa I (20)	----	A	7292

4.6. Protein production

4.6.1. Culturing of Cos-1 cells

SEMA7A-mh proteins were synthesized in Cos-1 cells (American Type Culture Collection, Manassas, VA) transfected with pEX-SEMA7A-mh plasmids. The complete medium used for culturing the cells was RPMI 1640 supplemented with 2 mM

L-glutamine, 100 units/ml Penicillin-Streptomycin, and 10% heat-inactivated fetal calf serum (FCS). Twelve milliliters of this medium were added per 1 ml of Cos-1 cells in a Falcon 25-cm^2 tissue culture flask, and the cells were allowed to grow in a 37°C incubator (with 5% CO_2 and humidity of 96%). The cells were examined daily for their health using an inverted microscope. They were fed by renewing the medium whenever the pH of the medium became basic (indicated by the change of the color from pink to yellow).

Incubation was continued for 2 -3 days until the cells became confluent over more than half of the flask. At that time, the cells were subcultured at the ratio of 1:4. The old medium was removed from the flask, and the cells were rinsed with autoclaved-sterilized PBS. Then, the adherent Cos-1 cells were detached from the bottom of the flask with 2 ml of 0.25% trypsin-EDTA (1 mM) solution. The cells were trypsinized for 20 min in the 37°C incubator. After the cells were detached, 6 ml of complete medium were added to inactivate the trypsin. Four new flasks containing 7 ml of the fresh medium were prepared. Two milliliters of the cell suspension were transferred to each flask, and the flasks were returned to the humidified 37°C, 5% CO_2 incubator.

4.6.2. Transfection of Cos-1 cells

Cos-1 cells were transiently transfected at their 3^{rd} or 4^{th} passage using a Lipofectamine Reagent (GibcoBRL). The reagent is composed of the polycationic lipid 2,3-dioleyoxy-N- [2(sperminecarboxamido) ethyl]-N, N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE). The possible mechanism for DNA uptake in this case has been described as

follows. The polycationic bilayer liposomes formed by DOSPA bind the negatively charged phosphate backbone of DNA to produce liposome-DNA complexes. These complexes associate with the anionic cell surfaces and are subsequently taken up by the cells by either phagocytosis or endocytosis (Felgner and Ringold, 1989). The liposome-mediated transfection results in higher efficiency than other methods such as DEAE-dextran and calcium phosphate techniques. In theory with this method 100% of the DNA used for transfection is trapped in the liposome/DNA complexes and delivered effectively into the cells. As a result, the efficiency of the lipofectamine transfection becomes 5 to 100 fold higher than that of DEAE-dextran or the calcium phosphate technique (Felgner et al., 1987). Lipofectamine transfection is routinely performed in our laboratory based on previous optimizations, which also showed that the toxicity of this method was less than that of the calcium phosphate technique.

It is necessary for cells to be in the log phase of growth at the time of transfection. Therefore, Cos-1 cells were plated a day prior to transfection. The cells were passaged as described earlier and counted using Trypan Blue dye and a hemocytometer. Cells (1.5 x 10^6) were seeded in each of 60 x 15 mm tissue culture dishes, and the complete medium was added to the final volume of 3-4 ml. The cells were returned to the 37°C, 5% CO_2 incubator and grown for approximately 18-24 hr until they reached 60-80% confluency.

The medium used for transfection was prepared by adding only L-glutamine (2 mM) to RPMI without any serum or antibiotics. Antibiotics and serum lower the transfection efficiency by either disturbing the cells or interfering with the liposome-mediated mechanism of DNA transfer. Therefore, an hour before transfection the

complete medium was aspirated from the cells. The cells were rinsed with PBS to remove the trace of serum-containing medium. Finally, 2 ml of serum-free medium were added to each plate, and the incubation was continued.

4.6.3. Preparation of the DNA for transfection (DNA optimization)

For each transfection plate, 2 μg of pEX-SEMA7A-mh DNA were diluted into 100 μl of serum-free RPMI medium and vortexed for 1 sec. In another test tube, 7 μl of lipofectAMINE Reagent were mixed with 93 μl of serum-free medium. Finally, the lipofectAMINE and DNA solutions were combined and vortexed again (1 sec). The resulting mixture was incubated for 45 min at room temperature to allow for the formation of liposome-DNA complexes. At the time of transfection, the medium was removed from the cells, and 800 μl of fresh serum-free medium were placed into each plate. Then, 200 μl of liposome-DNA mixture were carefully spread over the medium, and the cells with transfection mixture were incubated at 37°C for 5-6 hr.

Following incubation, 1 ml of medium containing 2X serum was added to each plate to bring the final concentration of serum to that of complete medium. Then, the volume was raised to 4 ml by addition of the complete RPMI medium. The transfected cells were incubated at 37°C for another 12-16 hr until, in theory, they started expressing the transferred gene. At that time, the medium was replaced by 2 ml of VP-SFM (Gibco Invitrogen Corporation, Carlsbad, CA), and the incubation was continued for another 24 hr. VP-SFM is a Viral Production Serum Free Medium with an ultra-low protein content. The medium provides a serum-free environment for the growth of the cell. While the protein concentration in this medium is very low (5 μg/ml), the cell growth is equivalent

to that obtained with serum-supplemented medium. The low protein content of VP-SFM made the downstream SEMA7A-mh protein purification procedures easier. Unfortunately, VP-SFM is known to inhibit the liposome-mediated transfection and therefore it could not be used earlier in the experiment.

At 48 hr posttransfection, VP-SFM containing the recombinant SEMA7A protein was collected and stored in the –80°C freezer. The cells were rinsed with PBS and harvested in 1 ml of PBS using a rubber policeman cell scraper.

4.7. Preparation of extracts

The harvested cells were transferred into a 1.5 ml microfuge tube and centrifuged for 1 min. The supernatant was discarded, and the pellet was resuspended in 5 μl of a protease inhibitor cocktail for mammalian tissues (Cat. # P 8340, Sigma, St. Louis, MO) and 100 μl of the cell lysis buffer. The buffer contained 0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P40, 0.15 M NaCl, 1 mM EDTA, 0.1% gelatin, 0.1% sodium azide, and 0.05 M Tris-HCl (pH 8.0).

Protease inhibitors are added to the crude cell extract to prevent protein degradation by endogenous enzymes. The protease inhibitor cocktail used in this experiment was a mixture of inhibitors specific for proteases that are usually present in mammalian cells. The cell suspension was vortexed for 1 min and incubated on ice for 15 min. Following the incubation, the cell lysate was vortexed for another minute and centrifuged for 15 min. Then, the supernatant containing cell extract was collected and stored at –80°C.

4.8. β-Galactosidase assay

In addition to pEX-SEMA7A-mh transfected cells, two other groups of cells were prepared to serve as controls. 1) Mock-transfected cells (as a negative control): These are cells transfected either with pcDNA3.1 expression plasmids or in the absence of any DNA to provide the protein background in which the semaphorin protein should appear. 2) β-Galactosidase-transfected cells (as a positive control): These are cells transfected with pcDNA3.1 derived β-galactosidase (β-gal) expression plasmids to assess transfection efficiency and protein production.

The reporter plasmids express the *lacZ* gene and produce functional β-gal proteins. The β-gal catalyzes the hydrolysis of ONPG (*ortho*-nitrophenyl-β-D-galactopyranoside) to the ONP anion, which gives a yellow color. The peak absorbance of the product is at 420 nm. The transfection was considered successful if the OD_{420} was greater than 0.2.

For each assayed sample, 3 μl of 100X Mg solution (0.1 M $MgCl_2$ and 4.5M β-mercaptoethanol), 66 μl of ONPG [4 mg/ml ONPG in 0.1 M sodium phosphate (pH 7.5)], 226 μl of 0.1 M sodium phosphate, and 5 μl of cell extract were mixed. The samples were incubated at 37°C. After 30 min of incubation, the mixture turned yellow, and the β-gal activity was stopped by adding 500 μl of 1 M sodium carbonate (Na_2CO_3) to each sample. The samples were added into the wells of a 96-well plate, and OD_{420} values were measured using a BIO-TEK Instruments Microplate Autoreader EC 311.

After the transfection success was verified by the β-gal assay, the extract and supernatant were further examined for the presence of recombinant semaphorins on a Western blot (see section 4.10) using anti-myc mAb (BioRad) and anti-SEMA7A mAb.

4.9. Dot blot analysis

The anti-SEMA7A mAb was generously donated by Dr. Mine from Kurume University (Kurume, Japan). The specificity of the antibody was examined via dot blotting of the pEX-SEMA7A-mh transfected cell extracts. For negative controls, BSA, SEMA3A purified protein, and pEX-mh transfected cell extract (mock extract) were used.

A nitrocellulose membrane was first cut into small pieces. Circles were drawn on the membrane with a pencil. The dry membrane was pre-wetted by sliding it at 45° angle into deionized water. The membrane was then put on a filter paper for 5 min to become dry. Finally, 1 μl of each sample was applied to each circle on the membrane and allowed to dry completely. The membrane was developed as described in the immunoprobing section (4.11).

4.10. Western analysis

SDS-PAGE was used to verify the molecular mass of the recombinant SEMA7A protein product. For the first few experiments large gradient SDS-PA gels (4.5% stacking gels and 7-20% gradient resolving gels) were run overnight in a 4°C cold room. Then, the proteins were transferred from the gel to a nitrocellulose membrane (BioRad) using a Hoefer Trans-Blot apparatus (BioRad). The transfer was performed in the cold room for 1-2 days. The procedure was so long and tedious that minigels and semidry

transfer system were used instead for later experiments. The latter method of Western blotting was fast and easy while it resulted in high resolution and transfer efficiency of the proteins.

The minigels were prepared by adding 4.5% stacking gels (pH 6.8) over 7.5% resolving gels (pH 8.3, 1.5 mm). Ten microliters of extract (or 20 μl of supernatant) were diluted in 10 μl of sample buffer (2% SDS, 7.5% glycerol, 5% 2–mercaptoethanol, 0.1% Bromophenol Blue, 62 mM Tris-HCl, pH 6.8) and heated at 95°C for 8 min. The samples were cooled on ice and loaded into the wells of the gel. The gel was run at 62 V for the first 30 min and then at 125 V for the next hour.

Following electrophoresis, the proteins separated in the SDS-PA gel were transferred to a piece of nitrocellulose membrane using the semi-dry electroblotting method (or a TransBlot SD Transfer System). Prior to transfer, the membrane was equilibrated in the semi-dry transfer buffer [25 mM Tris base, 150 mM glycine, 10% (v/v) methanol, pH 8.3] for 10 min. Filter papers (8 pieces, Schleicher and Schuell, Inotech, Keene, NH) and the gel were also soaked in the transfer buffer, and then the transfer stack was assembled. Four wet filter papers were placed on the anode (the bottom part of the TransBlot SD Transfer apparatus). The membrane was put on the filters followed by the gel and the other 4 pieces of wet filter papers. Finally, the cathode was placed on the top of the stack and the system was closed. The transfer system was run for 2 hr at 22 V.

After 2 hr of transfer, the gel was exposed to Coomassie Blue stain for 4 hr, and then it was placed in a destaining solution (5% methanol and 7% acetic acid) for 24 hr.

The membrane was also stained after the transfer. The staining was performed in Ponceau stain [0.5% (w/v) Ponceau, 1% (v/v) glacial acid] for 2 min, and then the membrane was rinsed with deionized water. The red protein bands present on the membrane were compared to the bands on the stained gel, and protein transfer was verified.

The SDS-PAGE system was used for Western analysis whenever anti-myc mAb was to be used to detect the fusion protein on the blot. However, when anti-SEMA7A mAb was used as a probe, a native PAGE system was required since the antibody could only bind to the native form of the protein. In the native system all the buffers were prepared as described above without any SDS or mercaptoethanol.

4.11. Immunoprobing

The nitrocellulose membrane was placed in blocking buffer (pH 7.4) for 1 hr at room temperature. The buffer was prepared by adding 2 g of BSA to a liter of PBS containing 0.05% Tween 20. Then, the membrane was incubated with the diluted 1° antibody at 37°C for 2 hr. The 1° antibody was a 1:2000 dilution of anti-myc antibody (monoclonal mouse IgG, BioRad) in blocking buffer. The antibody was removed after 2 hr, and the blot was washed with blocking buffer 5 times over 30 min. After washing, the membrane was incubated with the diluted 2° antibody for 1 hr at room temperature. The 2° antibody was prepared by diluting goat anti-mouse IgG-AP conjugate (BioRad) in blocking buffer (1:2000). After incubation, the membrane was washed 3 times with blocking buffer and twice with PBS/ Tween 20 over 30 min. Finally, the membrane was rinsed with water and developed using the Alkaline Phosphatase Conjugate Substrate Kit

(BioRad) for 30 min. After developing, the membrane was washed in deionized water and then air-dried.

4.12. Protein purification

4.12.1. Centricon centrifugal filtration

After the protein production was verified by both β-gal assays and Western analysis, the cell supernatant was processed for purification of the secreted SEMA7A-mh protein. The fusion protein was first concentrated and partially purified using a Centricon centrifugal filter device (Millipore, Bedford, MA). The Centricon unit had the following parts: a sample reservoir with a YM-50 membrane at the bottom, a filtrate vial, and a retentate vial. Two milliliters of the supernatant were loaded into the sample reservoir of each Centricon unit, and it was covered to prevent evaporation. Then, the filtrate vial was attached to the end of the reservoir, and the setup was placed in a Sorvall SS-34 rotor. The supernate sample was spun at 6500 rpm for 15-20 min. At the end of centrifugation, low molecular weight (MW) proteins had passed through the membrane and collected into the filtrate vial. The YM-50 membrane retained proteins with MW of 50 kDa or higher including the 75 kDa SEMA7A-mh protein. To collect the concentrated supernatant, the retentate vial was put on the top of the sample reservoir, and the setup was inverted and centrifuged again for 3 min at 1500 rpm. Finally, between 80 μl to 100 μl of supernatant containing SEMA7A-mh were recovered from each unit and stored at –80°C. For a control, the supernatant of the mock-transfected cells was also concentrated following the same procedure as described above.

4.12.2. ProBond purification system

The SEMA7A-mh protein was further purified from the Centricon concentrated supernatant using the ProBond purification system (Invitrogen). As mentioned earlier, the fusion protein had six tandem histidine residues on its carboxyl terminus. The polyhistidine-tag has a high affinity for a resin containing nickel ions. Therefore, when the tagged protein was applied to a pre-filled ProBond column, it was captured by the column's nickel-chelating affinity resin. The fusion protein was later eluted with an imidazole gradient under native conditions. Imidazole, with a similar structure to that of histidine, removed the tagged protein from the column by competitive substitution. All the buffers used in the system had a pH of 6 or higher. Therefore, the protein was kept native and functional during the purification scheme.

The ProBond purification of the SEMA7A-mh protein was performed according to the manufacturer's protocol. In brief, the column was pre-equilibrated by washing twice with sterile distilled water and three times with Native Binding Buffer (20 mM sodium phosphate and 500 mM sodium chloride, pH 7.2 to 7.6). Prior to loading, the volume of the concentrated supernatant was raised to 4 ml by adding Native Binding Buffer. Then, the sample supernatant was applied to the column and mixed with the resin for 10 min using a rotating mixer. After the polyhistidine-tagged SEMA7A protein was completely bound to the resin, the column was washed twice with Native Binding Buffer and three times with Native Wash Buffer (20 mM sodium phosphate and 500 mM sodium chloride, pH 6). The washes removed the unbound proteins. Finally, the fusion protein was eluted with an imidazole gradient. The gradient was generated by applying four

Elution Buffers (pH 6) in increasing imidazole concentration (50 mM, 200 mM, 350 mM, 500 mM) into the column. The eluent was collected from the column. The samples were concentrated, and the low MW (68.8 Da) imidazole was removed using Centricon YM-50. For a control, the ProBond purification was also performed on the concentrated supernatant of mock-transfected cells.

After the presence of the fusion protein in the eluent was confirmed by Western blotting, the samples were applied onto fresh ProBond columns a second time to increase the purity of the SEMA7A-mh protein. Prior to loading, the imidazole present in the samples was removed using Centricon units as described earlier. As a result, no interfering factor was present, and the full binding of the tagged proteins to the resin was also achieved in the second run. Finally, the tagged protein was eluted from the column, and 5 ml fractions were collected and concentrated independently. Each fraction was tested for the presence of the purified SEMA7A-mh proteins via SDS-PAGE and Western blotting. The fractions containing purified fusion proteins were pooled together and used for downstream assays.

4.13. Protein quantitation

Protein was quantified by two different methods in this project. The first method was used for measuring the amount of the recombinant protein present in the supernatant of the pEX-SEMA7A-mh transfectants prior to ProBond purification step. The fusion protein was first purified from the Centricon concentrated supernatant using protein G-Sepharose (Pharmacia Biotech, Uppsala, Sweden) and anti-myc mAb.

First protein G-Sepharose was added to the cell supernatant in order to eliminate any antibody already present in the supernatant (100 µl protein G/2 ml supernatant). The mixture was incubated on ice for 15 min and then centrifuged for another 15 min at 4000 rpm. The supernatant was collected and incubated with anti-myc mAb overnight in a rotating shaker at 4°C (15 µl anti-myc mAb/2 ml cell supernatant). At the end of incubation, protein G was added to the mixture and the incubation was continued for 90 min. Then, the pellet was collected by centrifugation for 5 min and washed with cell extraction solution twice. Finally, the pellet was washed with LiCl buffer (0.5 M LiCl, 0.1 M Tris, 0.1% β-mercaptoethanol, pH 8.4) three times. At the end of the washes, 20 µl of loading buffer were added to the pellet and heated for 10 min at 95°C. The sample was then loaded on a SDS-PA minigel.

After the gel was run, it was stained with SYPRO Orange fluorescent dye (BioRad) for 30 min with constant agitation in a dark place. Then, the gel was rinsed with 7.5% acetic acid for 30 sec. Images were captured and analyzed in a Macintosh computer using the Gel Doc 1000 Imaging System and Molecular Analyst image analysis software version 2.1 (BioRad) following the instruction manual. BSA standards (0.125 µg to 8.0 µg) were also analyzed on a SYPRO Orange-stained SDS-PA minigel.

A profile was generated showing both the brightness (counts) and the thickness (mm) of each protein band on the gel. Finally, a BSA standard curve was plotted (counts x mm versus µg of BSA) using the information in the profile. The quantity (µg) of the SEM7A-mh protein was estimated using the BSA standard curve.

The second method for measuring proteins was performed using the NanoOrange Protein Quantitation Kit (Molecular Probes, Eugene, OR). Limited amount of the fusion protein was produced by transiently transfected Cos-1 cells, and a very small quantity was left after the purification procedure. As a result, it was important to use as little protein as possible for quantitation and keep the rest for later assays. The NanoOrange kit containing a reagent with a very high sensitivity, capable of detecting low protein concentrations (100 ng/ml-10 μg/ml), was used. Protein samples were diluted in the 1X NanoOrange protein quantitation reagent using two different dilution factors (100 and 1000). BSA standards were also prepared at different concentrations, ranging from 0 μg/ml to 10 μg/ml, using the NanoOrange reagent. The diluted samples and standards were made in duplicates and heated to 95°C in the dark for 10 min to denature the protein. Then, they were cooled for 35 min at room temperature. When the protein was denatured, the colorless reagent could bind to it and fluoresce.

The fluorescence was measured in a 96-well plate by a PE Biosystems (Foster City, CA) CytoFluor 4000 plate reader using excitation and emission wavelengths of 485 ± 20 nm and 580 ± 50 nm respectively. The gain was set at 30. A BSA standard curve was generated and used for converting the fluorescent readings of the samples to their concentration values. The values read from the standard curve were multiplied by the corresponding dilution factors.

4.14. PBMC isolation

After the SEMA7A-mh protein was purified and quantified, its functionality was studied using both monocyte IL-8 release assay and T cell migration assay. To purify both cell-types (monocytes and T cells), fresh PBMCs were first required.

On the day of each assay, 30 ml of human blood was drawn by venipuncture by a trained, certified phlebotomist from each volunteer donor. Blood was collected into sodium/EDTA Vacutainer Brand tubes to prevent coagulation. Peripheral blood mononuclear cells (PBMC) were isolated from the fresh blood using Ficoll-Paque density gradient centrifugation. First, the blood was diluted with 2 volumes of PBS. Then, 35 ml of the diluted blood were carefully layered over 15 ml of Ficoll-Paque (Pharmacia Biotech) and spun in a clinical centrifuge with a swinging bucket at 1600 rpm (540 g) for 35 min (brake off). The centrifuge had a swinging bucket rotor and its temperature was fixed at 20°C during the whole procedure. Three fractions of blood were obtained at the end of the centrifugation. The upper fraction or plasma layer was aspirated without disturbing the mononuclear cells at the interface. Then, the mononuclear cells (lymphocytes, monocytes, and macrophages) were collected and placed in a new centrifuge tube. The cells were washed with PBS and spun down at 1400 rpm for 10 min (brake on).

Supernatant was removed, and 6 ml of deionized water were added to the pellet. After 20 sec, most of the contaminating erythrocytes were lysed, and PBMCs were returned to isotonicity with 2 ml of 3.4% NaCl. Following erythrocyte lysis, PBMCs were depleted of platelets by centrifugation at low speed. The pellet was resuspended in

PBE buffer (1X PBS, 0.5% BSA, and 2 mM EDTA) and spun at 1100 rpm for 20 min (brake on). The supernatant was drawn off, and the platelet-depletion procedure was repeated. Finally, the PBMCs were resuspended in 5 ml of PBE and counted using 4% Trypan Blue stain (Sigma Chemical Company) and a hemocytometer. The PBE buffer used at the last steps prevented cell aggregation and also nonspecific binding of the cells to the walls of the tubes, resulting in an increased yield.

4.15. Monocyte IL-8 release assay

4.15.1. Monocyte isolation

Monocytes were purified from the fresh PBMCs using the MACS Monocyte Isolation Kit following the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). Briefly, PBMCs were treated with FcR Blocking Reagent (or human Ig) to prevent nonspecific binding of antibodies to monocytes/MQ via Fc receptors. Then, non-monocytic cells were magnetically labeled using a hapten-conjugated antibody cocktail and MACS anti-hapten paramagnetic microbeads. The cocktail contained CD3, CD7, CD19, CD45RA, CD56, and anti-IgE antibodies. After labeling, PBMCs were loaded into the LS^+ column in the magnetic field of the MidiMACS cell separator. Labeled cells including T cells, NK cells, B cells, dendritic cells, and basophils were retained in the column while the unlabeled monocytes were passed through and came out of the column with the effluent. The monocytes were collected and spun down in the clinical centrifuge for 10 min at 1200 rpm (250 g). The PBE buffer was aspirated, and the cell pellet was resuspended in 100 µl of X-VIVO Media15 (BioWhittaker, Walkersville, MD). The cells

were counted in the presence of 4% Trypan Blue. Monocytes (5.6 x 10^6 per 30 ml of blood) were isolated on the LS^+ column and cultured for IL-8 release assay.

Since the IL-8 immunoassay was testing the ability of SEMA7A-mh proteins to induce cytokine secretion from monocytes, it was critical to avoid cell activation and cytokine production by any exogenous factors prior to or during the assay. The MACS isolation with negative selection strategy allowed the separation of untouched monocytes by depleting non-monocytic cells from the PBMC population. As a result, no antibody bound to monocytes, and cell activation was minimized during the isolation procedure. Similar precautions were taken during monocyte cultivation using X-VIVO Media. The medium was designed for growing different cell types, including human monocytes, in a serum-free environment. It was formulated without any exogenous growth factors or artificial stimulators of cellular proliferation, and therefore it was suitable for cell activation studies such as the IL-8 release assay. All together, monocyte isolation by the depletion method and cultivation in the serum-free media eliminated cell activation stimulated by any factor other than the presence of the SEMA7A-mh protein in the monocyte culture.

4.15.2. IL-8 production and quantification

The assay was performed to examine the functionality of the purified fusion protein. AHV Sema, alcelaphine herpesvirus semaphorin, has been shown to induce secretion of the cytokine IL-8 from monocytes (Comeau et al., 1998). Since SEMA7A was the human homologue of the AHV Sema, it was conjectured to have a similar activity to its viral counterpart.

Freshly isolated monocytes (2.24×10^5) were transferred into each well of a 96-well tissue culture plate. The cells were cultured in three different environmental conditions: media alone, media with SEMA7A-mh protein (50 ng/ml), and media with heat- inactivated SEMA7A-mh protein (50 ng/ml). Heat inactivation was achieved by denaturing the protein in a boiling water bath for 10 min. The denatured protein was cooled on ice prior to use. Triplicates of each condition were prepared in the culture plate. The final volume in each well was raised to 200 µl by adding X-VIVO Media containing 1% L-glutamine. The cells were cultivated for 16 hr in a 37°C / 5% CO_2 incubator. Following the incubation, the cell culture supernatant was collected and centrifuged for 1 min so that any floating cell or particulate came down into the pellet. The overlaying supernatant was removed and tested for the presence of IL-8 using an ELISA kit or Quantikine Human IL-8 Immunoassay (R&D Systems, Minneapolis, MN). The assay was performed in triplicate following the protocol provided by the manufacturer.

Prior to the assay, the supernate samples were diluted into 1X Calibrator Diluent (1:1000), which was included in the kit. Duplicates of standards were prepared at seven different concentrations, ranging from 31.2 to 2000 pg/ml, using IL-8 Standard (recombinant human IL-8) also provided in the kit. Following the assay, the OD_{450} values were determined, and the standard curve of OD_{450} versus IL-8 concentration was generated. The concentration of each sample was read from the standard curve and multiplied by the dilution factor (1000).

4.16. T cell migration assay

After the functionality of the purified SEMA7A-mh protein was verified by monocyte IL-8 release assay, the protein was used for T cell migration assays.

4.16.1. T cell isolation

Untouched T cells were purified from fresh PBMCs using a MACS Pan T Cell Isolation Kit (Miltenyi) following the manufacturer's protocol. In general, PBMCs separated by density gradient centrifugation were labeled using a cocktail of hapten-modified CD11B, CD16, CD19, CD36, and CD56 antibodies and MACS anti-hapten paramagnetic microbeads. The labeled cell suspension was applied into a Pre-Separation Filter placed on the top of a LS^+ column to remove the particles and clumps larger than 30 μm. Then, the suspension passed through the LS^+ column in the magnetic field of the MidiMACS cell separator. The labeled non-T cells including monocytes, granulocytes, B cells, platelets, early erythrocyte precursors, and NK cells were retained in the column and depleted. The unlabeled T cells passed through the column and were collected from the effluent by centrifugation.

4.16.2. Fluorescent staining of the isolated T cells

Isolated T cells were labeled using CFSE (carboxyfluorescein diacetate, succinimidyl ester) fluorescent dye (Molecular Probes).

4.16.2a. Fluorescent dye preparation

A stock solution of 1 mM CFSE dye was made by dissolving 50 μg of lyophilized powder into 900 μl of high quality, anhydrous DMSO (Sigma). Small aliquots of the

stock solution were prepared and kept with desiccating beads in a sealed container. The container was stored at –20°C and protected from light exposure.

4.16.2b. Fluorescent labeling of the T cells

After T cells were isolated in the MACS column, they were resuspended in cation-free PBS at a concentration of 10^7 cells/ml. The cell suspension was incubated with 3-5 μM CFSE dye for 15 min in a 37°C water bath. After labeling, cells were washed twice with PBE to remove the free dye. The cells were then spun down and the pellet was resuspended in warmed (37°C) RPMI medium without serum. The cell suspension was incubated for another 30 min in the 37°C water bath. The second incubation gave the cells enough time to process the dye completely. Following incubation, the cells were repelleted and the medium was aspirated. The pellet was washed twice with PBE. Finally, the labeled cells were resuspended in pre-warmed RPMI containing 2% FCS. The CFSE-labeled T cells were counted using Trypan Blue and a hemocytometer.

4.17. Transwell system

Cell motility has been typically studied using the Boyden chamber assay developed by Boyden (Boyden, 1962). The chamber consists of top and bottom wells separated by a piece of filter membrane. The cells are normally added to the upper well and then they migrate through the pores of the membrane towards the chemoattractant in the lower well. There are many problems and limitations associated with this system. To evaluate the chemotaxis, the setup must be disassembled. The cells present on the top of the membrane have to be removed, and the migrated cells have to be fixed before staining

and manual counting. The assay is time-consuming, laborious, and insensitive. It also destroys the samples, and therefore does not allow the real-time monitoring of the cell migration.

Using the FluoroBlok transwell system solves these problems. The system is composed of the FALCON HTS FluoroBlok Cell Culture Inserts (BD Labware, Franklin Lakes. NJ) placed in the wells of a 24-well plate. Each insert has a porous membrane (8 μm) impregnated with dye for blocking fluorescence. A fluorescent multiwell plate reader is used which is capable of detecting the fluorescence from underneath the membrane. Therefore, when fluorescent-labeled cells are placed into the insert and allowed to migrate, only those that pass through the pores and go to the other side of the membrane will be detected. The fluorescence of the non-migrating cells will be absorbed or blocked by the dyed membrane. In the transwell system, the plate reader automatically measures the fluorescence of the cells, and the output data can be converted to the cell number using a standard curve. This approach is faster, easier, and more precise than manually counting the cells. Also, there is no need for disassembling the setup or removing the non-migrating cells; therefore, cross-contamination is not a problem in here. Finally, the pre-staining procedure used in the transwell assay is not destructive to the cells. As a result, it allows the real-time analysis of the cell migration (Goldberger et al., 1998).

4.18. Membrane coating with fibronectin

Fibronectin stock solution was prepared by adding 1 ml of sterile distilled water to 1 mg of human fibronectin lyophilized powder (BD Biosciences, Bedford, MA). After

30 min of incubation at the room temperature, the powder was completely dissolved in the water without mixing. Then, aliquots were prepared and stored at –20°C.

Inserts were coated with fibronectin a day prior to each assay. The 1 mg/ml fibronectin stock solution was diluted (1:10) using cation-free PBS (GibcoBRL). The bottom surface or basal side of each insert was incubated with 100 µl of the diluted fibronectin (100 µg/ml). The final coating concentration was 33.3 µg/cm^2 as suggested by the manufacturer. The incubation was continued for 24 hr at 4°C. After coating, the extra fibronectin was aspirated, and the inserts were rinsed with sterile distilled water to remove non-bound material. The inserts were air-dried in a tissue culture hood and stored at 4°C.

4.19. Migration assay

Migration assays were performed in the membrane-coated transwell system. Typically, the membrane used for the T cell migration has a pore size of either 5 µm or 8 µm. Since the 5 µm membrane with fluorescent blocking property was not commercially available, the 8 µm FluoroBlok inserts were used in this project.

On the day of each assay, T cells were isolated from PBMCs and stained with the CFSE dye as described. At the end of the staining, the FluoroBlok insert multiwell plate was loaded as follows. Pre-warmed RPMI containing 2% FCS was first placed in the inserts. Then, the bottom wells were filled with the media (1200 µl/well). Finally, labeled T cell suspension was added to the media in the inserts (2.5 x 10^6 cells/insert) and the final volume of the media in each insert was raised to 400 µl. The cells were added last to avoid migration during the loading of the plate. Immediately after loading, the

plate was placed in a CytoFluor 4000 plate reader (PerSeptive Biosystems) set at 37°C. For 4 hr, readings were taken at 30 min intervals using the bottom read mode, excitation/emission wavelengths of 485/530 nm, and a gain setting of 50.

In each assay, different environmental conditions were considered, and each condition was run in triplicate (Tables 2 and 3). The chemotactic factor was normally added to the wells of the plate unless otherwise stated. For the negative control, the media alone or containing nickel column-purified mock supernatant was used in both inserts and corresponding wells. As a positive control of T cell chemoattraction, RANTES (R & D Systems) was added to the bottom wells at the optimum concentration of 1 ng/ml.

TABLE 2

MEASUREMENT OF THE CHEMOTACTIC EFFECT

Inserts / Wells	*Column purified Mock*	*Column purified SEMA7A-mh*
Column purified Mock	Control of random migration	Testing chemorepulsion
Column purified SEMA7A-mh	Testing chemoattraction	-----

4.20. Immunodepletion experiment

To confirm that any changes in T cell motility observed in migration assays were only due to the presence of the fusion protein in the media, a protein-media mixture that was depleted of SEMA7A-mh was used in the last few assays. Removal of 714 ng SEMA7A-mh proteins from the protein-media mixture was accomplished in four cycles of depletion using anti-myc mAb (1.3-1.6 μg for each cycle) and protein G-Sepharose

(Pharmacia Biotech). In each cycle, anti-myc mAb was added to 100 µl of the media containing nickel column-purified SEMA7A-mh proteins at a ratio of 2.2 µg of Ab per µg of SEMA7A-mh protein. The mixture was incubated, under constant agitation on a horizontal shaker, for 40 min at 4°C. Then, 10 µl of protein G-Sepharose were added to the mixture, and the incubation was continued for another 30 min. Following the incubation, protein G-Sepharose, bound to the Ab and to the fusion proteins, was precipitated by centrifugation (using an Eppendorf 5415C) for 5 min at 4000 rpm in a 4°C cold room. The supernatant was removed and subjected to the next cycle of depletion. Finally, the supernatant obtained at the end of the forth cycle was stored at –80°C until it was tested in a migration assay. Depletion with an unrelated Ab, goat anti-mouse IgG, produced mock-depleted SEMA7A-mh which provided a control for nonspecific binding of the Ab to the recombinant protein (Billard et al., 2000) and to verify that the immunodepletion process does not itself destroy the chemoattraction.

TABLE 3

IMMUNODEPLETION EXPERIMENT

Inserts / Wells	*Media*
Media	Control of random migration
RANTES	Negative control (chemoattraction)
Column purified SEMA7A-mh	Experimental
Depleted SEMA7A-mh	To confirm that chemoattraction is SEMA7A-dependent
Mock depleted SEMA7A-mh	Depletion control

4.21. Data analysis

In the migration assays, each environmental condition was run in triplicate. Two readings were taken and the mean of the readings was obtained. To account for backgrounds, the readings obtained at time zero were subtracted from those taken after four hours of migration. Since the number of measurements for each condition was small, the Q test of Dean and Dixon (Jack, 1995) was used for data evaluation. The Q test rejects any suspect value, which is very different from others in the data set, with 90% confidence. The values retained after the test were used for making the plots.

5. Results

5.1. pEX-SEMA7A-mh vector

The GenBank sequence for SEMA7A (gi: 4504236) is 2661 base pairs (bp) long. Its coding sequence (CDS) has 2001 bp starting from base pair 18 of the mRNA. The first 137 bp of the CDS code for a signal peptide. Those base pairs were deleted from the SEMA7A insert in the Exelixis construct while the sequence encoding the N-terminus of the mature protein was left intact. In the place of the gene's original signal sequence, a 63 bp sequence from the pSECTAG expression vector provides an exogenous secretory signal peptide at the N-terminus of the SEMA7A protein. Seven hundred and twenty one base pairs from the C-terminus of the SEMA7A gene, including the GPI anchor motif, were deleted to provide a 1785 bp long "soluble" SEMA7A insert. The vector also encodes a 66 bp sequence from the pSECTAG so that the secreted protein has the myc-his epitope at its C-terminus. The estimated molecular weight of the unglycosylated mature SEMA7A-mh protein is 75.28 kDa.

5.2. Purity and yield of pEX-SEMA7A-mh plasmid preparations

Two preparations of pEX-SEMA7A-mh plasmid were purified using the QIAGEN maxiprep system. The purity of the plasmid preparations was estimated by measuring the ratio of UV absorbance at 260 & 280 nm (Table 4). The average of the ratios for the two preps was 1.9, which was close to the ideal value of 1.8. Therefore, the preps were considered fairly pure and used for downstream experiments. Since a ratio of 260/280 higher than 1.8 is normally due to the presence of contaminating RNA, an analytical gel was run to evaluate the quality of the plasmid preparations. No RNA impurities were observed in the final product of either preparation.

TABLE 4

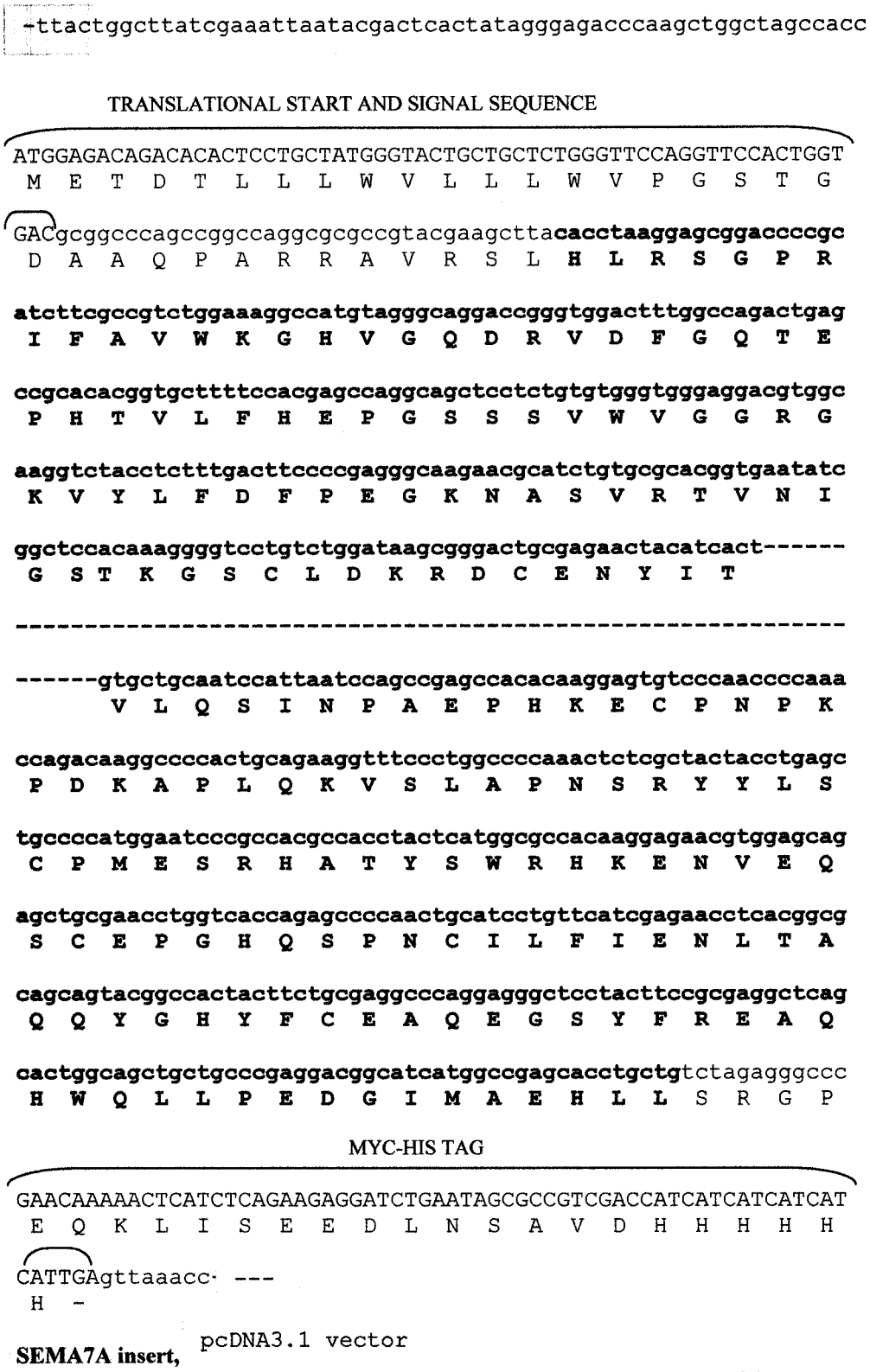
SEMA7A-MH PLASMID PREPARATIONS

pEX-SEMA7A-mh Plasmid	Volume of Culture (ml)	OD_{260}	Concentration (μg/μl)	Purity (260/280)	Total Yield (μg)
Prep # 1 [a]	200	0.638	6.375	1.960	1593.8
Prep # 2	100	0.183	1.825	1.875	456.25

[a] Prep # 1 was used in all experiments reported here.

5.3. Plasmid verification by end sequencing

Partial sequencing and restriction mapping were used to verify the identity of the pEX-SEMA7A-mh construct. Two hundred and fifty five base pairs from the N-terminus and 348 bp from the C-terminus of the insert were sequenced (Fig. 2). The sequence data was compared to the published sequence in the GenBank using the BLASTN 2.1.2 program (www.ncbi.nlm.nih.gov/blast) and revealed a perfect match to the published sequence.



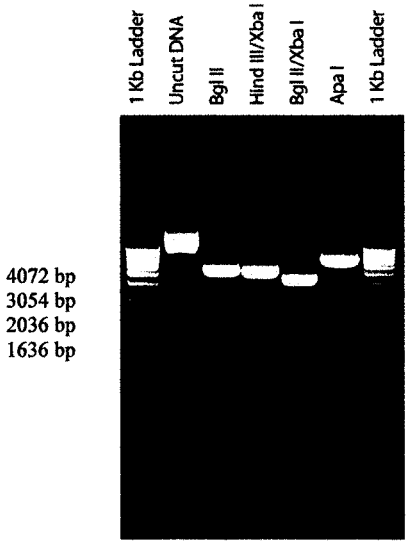
SEMA7A insert,

Fig. 2. The SEMA7A insert in the pEX-mh vector. The sequenced segment of the pEX-SEMA7A-mh is shown above. 1885 bp of the gene encode the ~ 75.28 kDa mature SEMA7A-mh secreted protein.

5.4. Plasmid verification by restriction mapping

The products of the different digestion reactions of pEX-SEMA7A-mh (prep # 1) were analyzed by agarose gel electrophoresis. Restriction fragments appeared on the gel as bands with approximately the predicted sizes (Fig. 3).

The uncut DNA (7292 bp) was run as a negative control for the digestion reaction. Dimers and multimers of the intact plasmid were observed on the gel (Fig. 3, Lane 2). Digestion with Bgl II produced two fragments of 1569 bp and 5723 bp long (Fig. 3, Lane 3). The double digestion with Hind III / Xba I released the complete 1792 bp (Fig. 3, Lane 4). The double digestion with Bgl II / Xba I produced three fragments of 1208 bp, 1569 bp, and 4515 bp long (Fig. 3, Lane 5). Digestion with Apa I linearized the plasmid revealing the total plasmid length of 7292 bp as expected (Fig. 3, Lane 6).



1) 1 kb Ladder
2) Uncut DNA [7292 bp]
3) Bgl II digest [1569 bp and 5723 bp]
4) Hind III / Xba I digest [1792 bp and 5500 bp]
5) Bgl II / Xba I digest [1208 bp, 1569 bp, and 4515 bp]
6) Apa I digest [7292 bp]
7) 1 kb Ladder

Fig. 3. Restriction digests of SEMA7A-mh plasmids run on a 1.2% agarose gel.

After the sequence of the fusion gene in the pEX-SEMA7A-mh construct was verified by both DNA sequencing and restriction mapping, the construct was used for transfection of Cos-1 cells and protein production.

5.5. Transfection of Cos-1 cells

Two hundred and forty eight transfection plates were prepared over the course of this research project. Optimal conditions were determined to increase the efficiency of the Lipofectamine transfection. For example, transfection of Cos-1 cells with β-gal DNA was performed at different passages starting from 2^{nd} to 9^{th}, and the highest efficiency determined by β-gal assay was achieved at the 3^{rd} or 4^{th} passage. Other conditions such as the amount of DNA, the amount of Lipofectamine Reagent, the time of transfection, the type of media, and time of media exchange were also optimized using β-gal assays (see methods section).

5.6. β-Galactosidase assay

Forty-seven β-gal transfection plates were prepared using the plasmid preps indicated in Table 5. Some of these plates were used for transfection optimization purposes. The others were used (as positive controls) to verify the efficiency of transfections prior to protein detection and purification procedures. So, one plate of β-gal transfected cells was prepared for every pEX-SEMA7A-mh transfection experiment, and the cell extract was assayed for β-gal activity.

The cell extract was obtained either by a freeze and thaw method or by using a cell extraction solution. The former method resulted in higher β-gal activity. However, since the SEMA7A-mh protein is not as robust as the bacterial β-gal protein and could

get degraded during the freeze and thaw procedure, the cell extraction solution was used for obtaining the fusion protein. For consistency between the control and the experimental, the same solution was used for preparing β-gal extractions.

The transfection was considered successful if the OD_{415} of the β-gal extract was greater than 0.2. This value was high for most of the experiments ranging from 1.5 to 2.7. The major transfection experiment, which produced the fusion protein used in downstream assays, had an OD_{415} of 2.361 for the extract of its β-gal control. This result suggested that the recombinant protein was produced with high efficiency in pEX-SEMA7A-mh transfected cells.

TABLE 5

PLASMID PREPS USED FOR β-GAL TRANSFECTIONS.

β-gal Plasmid	OD_{260}	Concentration (μg/μl)	Purity (260/280)	Yield (μg)
Prep # 1	0.642	6.42	2.0	1605
Prep # 2	0.497	2.5	1.85	625

5.7. Western analysis of SEMA7A-mh protein using anti-myc mAb

After verification of transfection success by β-gal assays, the extract and concentrated supernatant of pEX-SEMA7A-mh transfectants were analyzed by Western blotting. The samples were run on a 7.5% SDS-polyacrylamide minigel and blotted to a nitrocellulose membrane. After the blot was developed (Fig. 4) a band of approximately 75.36 kDa (I) was detected in both samples using anti-myc mAb. The two other bands (II, III) observed in the supernatant sample were fragments of anti-myc mAb used during the concentrating procedure by protein G-Sepharose.

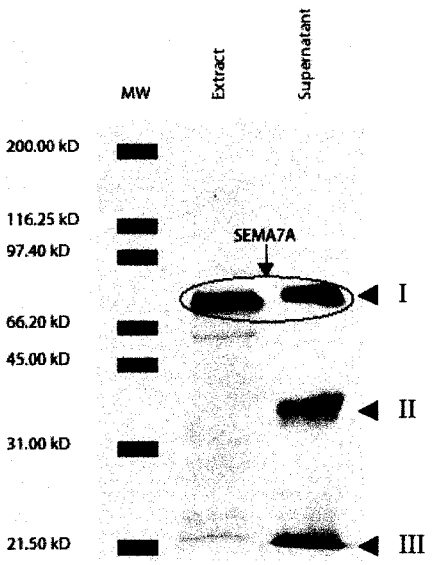


Fig. 4. SEMA7A-mh stained by anti-myc mAb on the Western blot of a reduced SDS-PA gel.

5.8. Verification of SEMA7A-mh protein production using anti-SEMA7A mAb

5.8.1. Testing the specificity of anti-SEMA7A mAb by dot analysis

To confirm that the protein detected by anti-myc mAb on the Western blot was in fact the SEMA7A protein fused to the mh tag, anti-SEMA7A mAb was used. The antibody was first tested in dot blotting since the method was faster, simpler, and more sensitive than Western blotting.

Nitrocellulose membranes spotted with the extract of pEX-SEMA7A-mh transfected cells showed darkly stained dots upon exposure to AP color development reagent. The dark color dots did not appear on negative control blots that were spotted with BSA, pEX-mh-transfected cell extract (mock extract), or SEMA3A purified proteins. Thus, the antibody bound the SEMA7A-mh protein specifically.

After seeing positive results on the dot blots, the anti-SEMA7A mAb was used in Western blotting to verify its binding to the ~75 kDa myc- tagged protein.

5.8.2. Western analysis of SEMA7A-mh protein production using anti-SEMA7A mAb

The anti-SEMA7A mAb is a conformational Ab that only binds to the native form of the fusion protein. Therefore, a native PAGE system was used for the Western analysis. The blot was cut in half and probed with the two different antibodies: anti-myc mAb and anti-SEMA7A mAb. Both antibodies recognized proteins of the same molecular weight (Fig. 5).

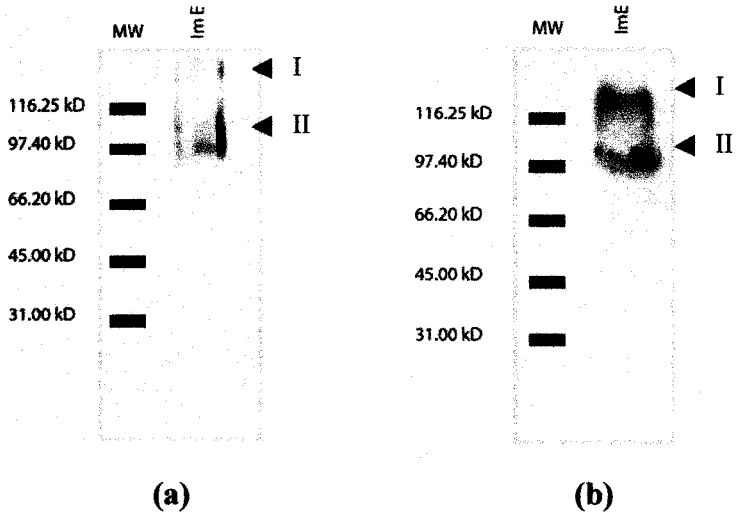


Fig. 5. Western blotting of the nickel column-purified SEMA7A-mh protein (ImE - Imidazole Elutent). A native PAGE system was used for the above blots. The non-reduced samples were probed with anti-myc mAb (a) or anti-SEMA7A mAb (b).

Two protein bands (I: 140.22 kDa, II: 98.78 kDa) appeared on Western blots of the non-reduced SDS gels. The bands did not correspond to the estimated size of the SEMA7A-mh proteins obtained with the reducing system. The apparent molecular mass of the fusion protein on the blots may have been increased using the native PAGE system for a variety of reasons. For example, the bands could represent the dimeric SEMA7A-mh protein formed by intermolecular disulfide bond(s) between the two monomers. The bonds would also be formed between the SEMA7A-mh protein(s) and

other cysteine-containing proteins. The ORF of SEMA7A has 16 cysteine residues that can form intra-peptide disulfide bonds under native conditions that produces a monomer (or dimer), which is smaller than the less compact, partially folded protein. That could explain the appearance of the lower band on the blots. Finally, aggregates of SEMA7A-mh protein and some contaminating proteins might have been formed under the conditions of the discontinuous system used in this experiment since the stacking gel of the system induces protein aggregations. Using a continuous system might solve the problem. The above data demonstrate that the pEX-SEMA7A-mh transfectants produced the fusion protein and that the protein could be detected using the anti-myc Ab.

It was desirable to use anti-SEMA7A mAb (KS.2) for all Western analysis but the antibody was not commercially available and only a limited amount was donated by Dr. T. Mine from Kurume University Research Center in Japan. Therefore, after it became clear that the protein recognized by the anti-myc mAb was the same as that recognized by the anti-SEMA7A antibody, the anti-myc antibody was used in subsequent Western blot experiments.

5.9. Protein quantitation on a SYPRO Orange-stained SDS-PA minigel

The amount of the fusion protein present in the supernatant of pEX-SEMA7A-mh transfected cells was measured prior to the ProBond nickel column purification step. For this purpose, the protein was first isolated from Centricon concentrated supernatant using protein G-Sepharose and anti-myc mAb. Then, the quantity of the purified protein was estimated on a SYPRO Orange-stained SDS-PA minigel using a BSA standard curve as

described in the methods section (Fig. 6). The results showed that the supernatant contained ~780 ng/ml of soluble SEMA7A-mh protein, less than 1% of the total proteins.

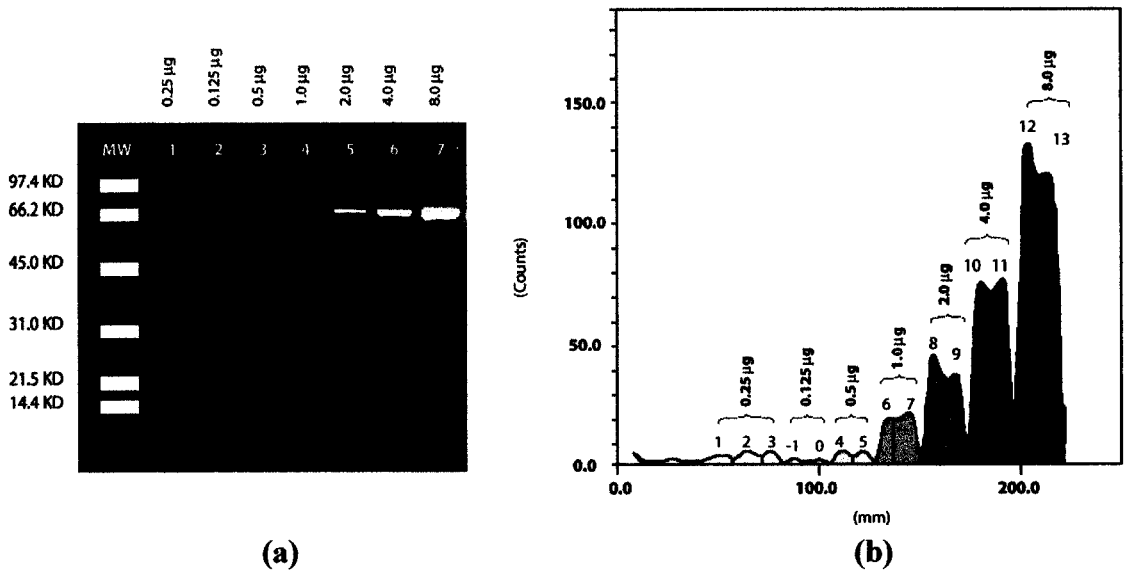


Fig. 6. Quantification of the SEMA7A recombinant protein in the supernatant of pEX-SEMA7A-mh transfectants using the SDS-PAGE and Molecular Analyst software. a) BSA standards run on a reducing SDS-PA minigel stained with SYPRO Orange dye. b) BSA standard profile generated by Molecular Analyst software as described in the methods section. The BSA standard curve (counts x mm over µg of protein) was generated using the information obtained from the above figures.

5.10. Protein purification

The purification scheme of the SEMA7A-mh protein is shown in Figure 7. In brief, supernatant of the cells transfected in the absence of any DNA (mock-transfectants) was used for the control. Values related to the control are indicated in parentheses. Eighty milliliters (23 ml) of the cell supernatant containing SEMA7A-mh protein were collected from 38 (14) transfection plates. The fusion protein in the supernatant was concentrated and partially purified using a Centricon YM-50 centrifugal filter device

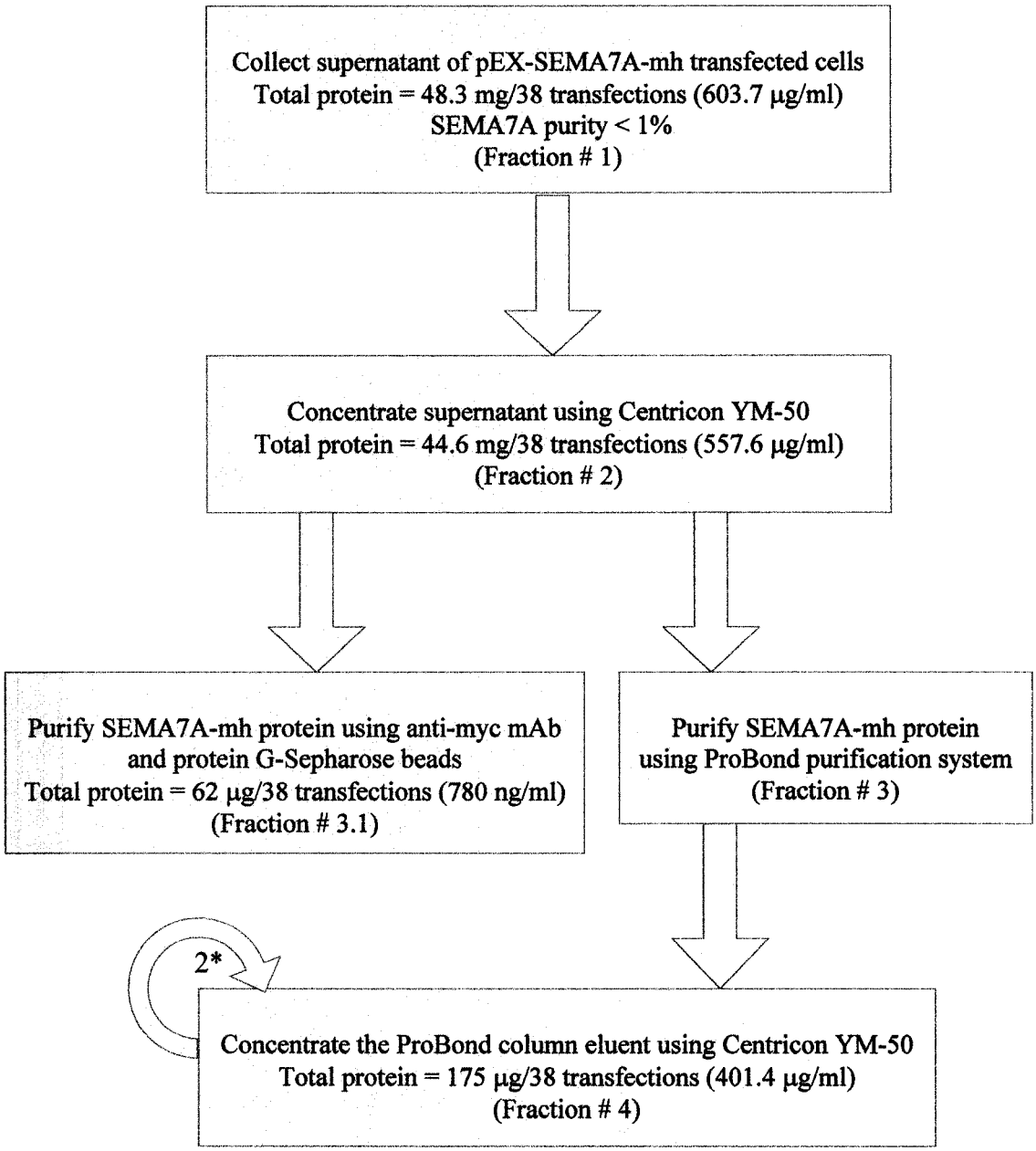


Fig. 7. Purification Scheme. Quantification of the total protein present in fractions # 1, 2, 4 using NanoOrange Protein Quantitaion Kit, and fraction # 3.1 by SDS-PAGE and Molecular Analyst software.

(Millipore). The total protein content of the supernatant decreased from 48.3 mg to 44.6 mg after the Centricon partial purification step. The SEMA7A-mh protein comprised less than 1% of the total proteins present in the 1.346 ml (817 μl) of the concentrated supernatant. As a result, after the concentrated sample was applied to the ProBond nickel column with binding capacity of 5 mg fusion proteins, all the SEMA7A-mh protein was bound. The protein was eluted and Centricon concentrated to the final volume of 830 μl (70 μl). The fusion protein was once again passed through the ProBond column and eluted with different concentrations of imidazole (Im). Five-milliliter fractions were collected and Centricon concentrated (Table 6). Fractions 2 and 3, containing approximately 175 μg of purified SEMA7A-mh protein, were pooled together and used for all subsequent experiments.

TABLE 6

PROBOND PURIFICATION OF THE HIS-TAGGED PROTEIN FROM THE CONCENTRATED SUPERNATANT OF SEMA7A-MH AND MOCK TRANSFECTED CELLS.

ProBond & Centricon Purified Proteins	Fraction 1 Eluted by 50 mM Im.	Fraction 2 Eluted by 200 mM Im.	Fraction 3 Eluted by 350 mM Im.	Fraction 4 Eluted by 500 mM Im.	Final Volumes
mock	88 μl	60 μl	75 μl	75 μl	135 μl
SEMA7A-mh	715 μl	395 μl	70 μl	70 μl	465 μl

5.11. PAGE and Western analysis of the SEMA7A-mh protein in the purification scheme

Samples collected at each step of the purification procedure were analyzed using both reducing and non-reducing PAGE systems followed by Western blotting. The unpurified supernatant of transfectants contained proteins produced by the cells and

proteins provided by the medium (VP-SFM). When transfected-cell supernatants were run on the gel, most of the proteins including SEMA7A-mh were in low concentrations and thus could not be observed clearly (Fig. 8a, Lane 1). Only one major band was detected that corresponded to a dominant contaminating media protein.

The first step in the purification process was performed using Centricon columns. The membrane used in these columns eliminated any protein smaller than 50 kDa while leaving others including SEMA7A-mh protein untouched. When the Centricon concentrated / partially purified proteins were separated on a native gel, the protein with the lowest molecular weight was ~ 65 kDa (dominant contaminating protein), and no bands at the range of 50 kDa or smaller were observed on the gel. However, proteins in the same sample displayed a very different pattern on the denaturing gel (Fig. 8a). Under reducing conditions of the SDS-PAGE system, protein complexes dissociated liberating proteins of 50 kDa or smaller on the gel (Fig. 8a, Lane 5).

The Centricon concentrated supernatant was then applied to the ProBond column. The majority of the protein impurities did not bind to the ProBond resin. Three polypeptides, however, remained bound after all the washes: 75.36 kDa, 65.23 kDa, and 50.93 kDa (Fig. 8a, Lane 4). Most of the 65.23 kDa dominant contaminating protein came down in the pass-through fraction during the washing step and appeared as a single band on the gel (Fig. 8a, Lane 3). The wash sample did not contain SEMA7A-mh protein detectable on the subsequent gels or Western blots, indicating that no fusion protein was released from the column in the washing step (Fig. 8a, Lane 3; Fig. 8b, Lane 2). The desired protein was eluted from the column using a series of imidazole elution buffers.

Three protein bands in the pooled eluent were detected on the denaturing gel. Among the three only the upper band contained SEMA7A-mh protein, which was detected on the Western blot (Fig. 8a, Lane 4; Fig. 8b, Lane 3).

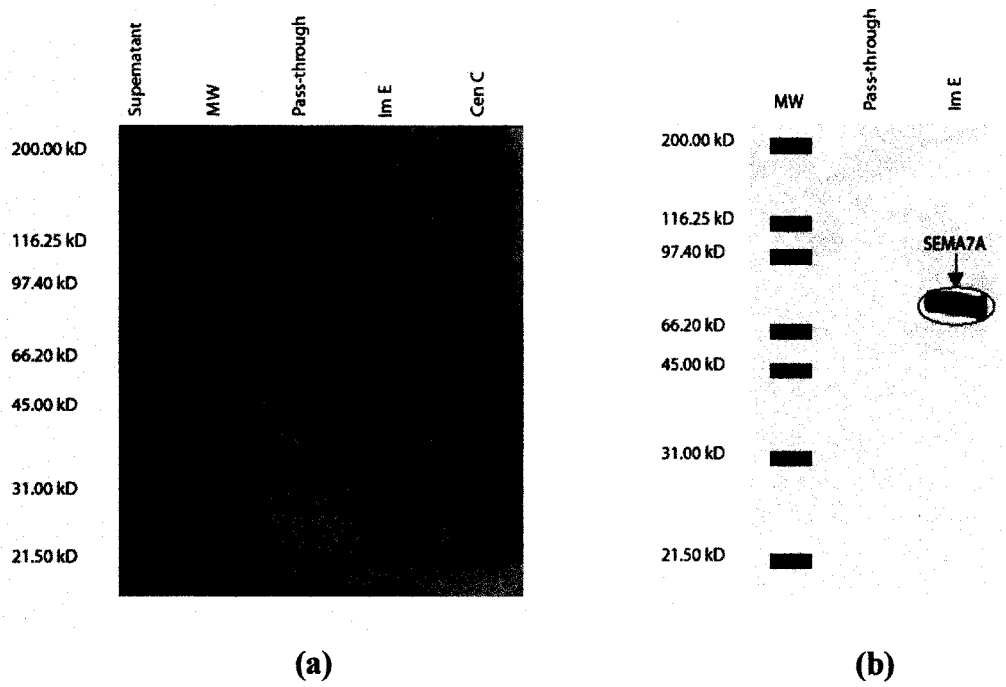


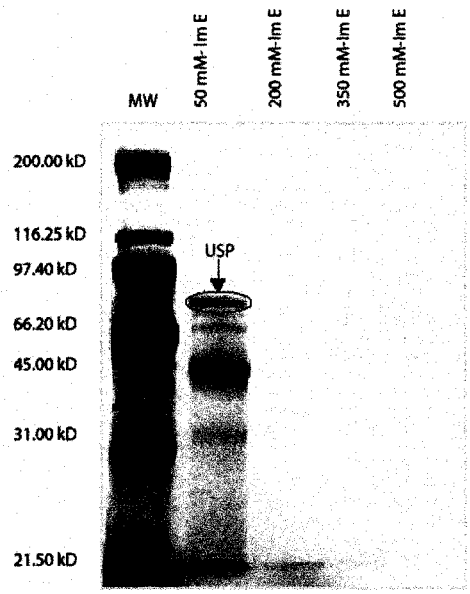
Fig. 8. Protein purification scheme.
a) Samples collected at each step of the purification procedure run on a reduced SDS-PA gel (Im E- Imidazole eluent, Cen C- Centricon concentrated supernatant). b) SEMA7A fusion protein detected in the imidazole eluent on a nitrocellulose membrane blotted from a reduced SDS-PA gel.

In an effort to isolate the SEMA7A-mh from the three contaminating proteins, each imidazole eluent was collected separately in another round of purification. The 20 ml of eluent from the previous purification was concentrated in the Centricon columns to a volume of 650 µl and loaded to the nickel column. The proteins were released using a series of 5 ml imidazole elution buffers (50 mM, 200 mM, 350 mM, 500 mM). The 5 ml

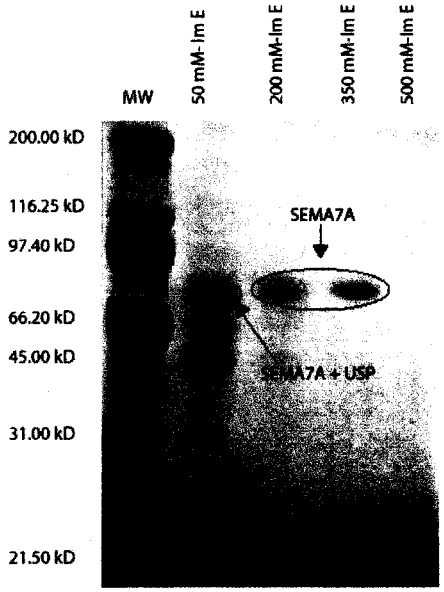
eluents were collected separately, and a sample of each eluent was analyzed using gel electrophoresis and Western blotting. For a negative control, supernatant of the mock-transfected cells was also prepared in the same procedures as described above.

The first eluted fractions (50 mM imidazole eluent) from both experimental and control ProBond columns resulted in three bands at the range of 75.36 kDa, 65.23 kDa, and 50.93 kDa on the reducing gel. But only the eluents from the SEMA7A-mh column contained proteins in the second (200 mM imidazole eluent) and third fractions (350 mM imidazole eluent). Neither experimental nor control samples showed any band in the last fraction on the gel (Fig. 9a, Fig. 9b). When the gels were blotted and probed with anti-myc Ab, SEMA7A-mh protein was detected in the first three fractions of the tested samples and none of the controls as expected (Fig. 9c).

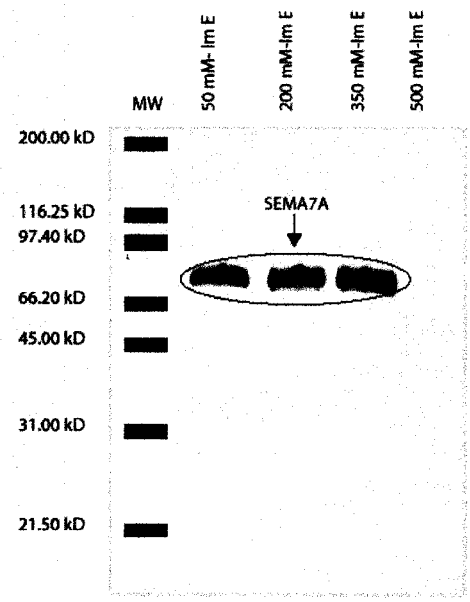
Therefore, almost all the impurities plus some of the SEMA7A-mh protein (present in the upper band) came down in the first fraction. One reason for seeing the fusion protein along with contaminating proteins in the first fraction could be the formulation of the VP-SFM. After the experiments were completed, the producer (Gibco) informed us that the media contained approximately 50 times the normal amount of ionic chelators, which could have inactivated some of the nickel ions in the ProBond resin. As a result, some of the fusion proteins were weakly bound to the resin and eluted with the low concentration (50 mM) of imidazole. The second and third fractions had the most purified fusion protein since they gave a single band at ~ 75 kDa in the tested sample and no bands in the control. These two fractions were pooled together and used as a source of the purified protein for downstream experiments.



(a)



(b)



(c)

Fig. 9. Purification of SEMA7A-mh protein by ProBond columns. a) Imidazole eluents (Im Es) from the control column in a reduced SDS-PA gel. b) Imidazole Eluents containing SEMA7A-mh from the experimental column in a reduced SDS-PA gel. c) Nitrocellulose membrane blotted from the gel in Figure 9b and probed with anti-myc mAb.

5.12. Protein quantitation using the NanoOrange Protein Quantitation Kit

After the second and third fractions eluted from the ProBond column were pooled together, their protein content was determined using the NanoOrange Protein Quantitation Kit. To have a consistent system for protein quantitation the same kit was used for measuring the total amount of protein present in the unprocessed supernatant and Centricon concentrated supernatant.

As shown in Figure 7 (p. 49), the total protein in the supernatant, collected from 38 pEX-SEMA7A-mh transfection plates, decreased from 48.3 mg to 44.6 mg after semi-purification of the fusion protein using the Centricon filter device. This amount was then decreased to175 μg in the pooled fraction (second and third eluents) obtained after a second round of ProBond purification process. The total protein content in the supernatant of mock-transfected cells was also measured as above to control for background proteins. The result showed that the purity of SEMA7A-mh protein increased from < 1% in the supernatant to > 93% in the pooled final fraction.

5.13. Monocyte IL-8 release assay

The monocyte IL-8 release assay was used to test the functionality of the SEMA7A-mh protein after the purification procedure.

5.13.1. Monocyte isolation

PBMCs (3.7×10^7) were obtained from 28 ml of freshly drawn whole blood by density gradient centrifugation followed by red blood cell lysis and platelet depletion by differential sedimentation. Untouched monocytes (5.6×10^6) were isolated from the fresh

PBMCs on the MACS LS^+ column using a cocktail of CD3, CD7, CD19, CD45RA, CD56, and anti-IgE antbodies.

5.13.2. IL-8 production and quantitation

Because the SEMA7A protein has been shown to bind to monocytes, and its viral homologue is capable of inducing IL-8 secretion from these cells, monocyte cultures were examined for the presence of IL-8 after incubation with the SEMA7A-mh fusion protein.

The cultures of the monocytes incubated with the unprocessed supernatant of mock or pEX-SEMA7A-mh transfected cells were also tested in this assay. The assay was repeated five times using different samples, and there was no discernable effect (data not shown). The unprocessed supernatant contained a large amount of protein impurities (Fig. 8a, lane 1) that could have interfered with either the SEMA7A activity or the IL-8 detection system used in the ELISA kit.

As described in the methods section, 2.24×10^5 freshly isolated monocytes were incubated with 50 ng/ml of the purified SEMA7A-mh protein or heat-inactivated purified SEMA7A-mh protein. For a negative control, the monocytic cells were cultured alone. After 16 hr of incubation, IL-8 concentration in the cell supernatant was determined using an IL-8 ELISA kit.

Even though precautions were taken to prevent cell activation prior to the assay (see methods section), the background IL-8 production was high in cultures of both the control cells and cells treated with the inactivated fusion protein (~443064 pg/ml). The

activation probably happened during the isolation of PBMCs since the hypotonic lysis of the erythrocytes used in this process is believed to induce cell activation.

The concentration of IL-8 released was increased by 60%, however, when cells were incubated with the purified SEMA7A-mh protein (Fig. 10). This result was very close to the 64.3% value reported by Comeau et al. (1998) for the monocytes treated with a recombinant *viral* semaphorin.

The data demonstrated that the SEMA7A-mh protein, similar to its viral counterpart, is able to upregulate IL-8 production by monocytes. The result was obtained using the nickel column-purified protein indicating that the fusion protein remained functional even after the purification procedure and was suitable for functional assays such as T cell migration assays.

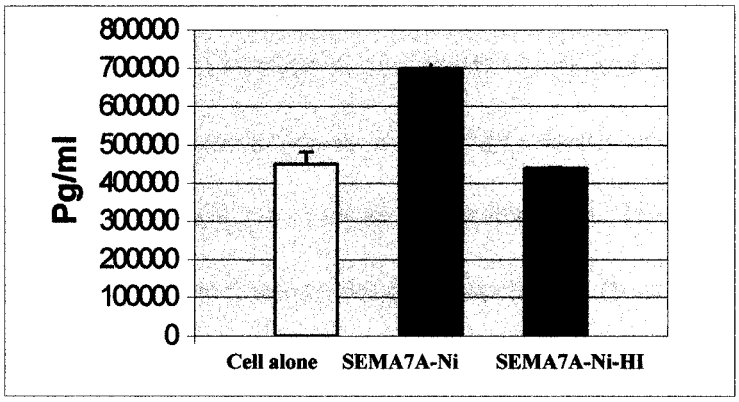


Fig. 10. IL-8 upregulation in SEMA7A-mh treated monocytes determined using ELISA kits (SEMA7A-Ni - nickel column purified SEMA7A, SEMA7A-Ni-HI - heat inactivated nickel column purified SEMA7A). Data represents the mean of triplicates + SE.

5.14. T cell migration assay

5.14.1. Fluorescent staining of T cells

Many different criteria had to be considered before choosing the fluorescent dye for staining the cells. T cell migration assays are usually lengthy, often requiring 4 to 16 hr for the cells to complete their *in vitro* migration. Also, the percentage of the cells migrating in response to the chemoattractant RANTES has been reported to be low. Therefore, only a dye that stays in the cells for at least 16 hr and has a high sensitivity or high minimum detection limit is suitable for these long assays. To select that dye, three different fluorochromes typically used for live lymphocyte staining were examined. Calcein-AM is the most popular fluorochrome used in chemotactic assays since it does not have any adverse effect on the migration of the cell. However, this dye has short cellular retention time and starts leaking out of the cells within 90 min of staining. CMFDA (5-chloromethylfluorescein diacetate) is another fluorescein-based dye that remains in the cells for at least 24 hr but its sensitivity is reported to be low. Finally, CFSE (carboxyfluorescein diacetate, succinimidyl ester) mostly used for *in vivo* T cell migration assays stays in cells (*in vitro*) for at least a few days. However, the dye can decrease the motility of the cells if used in high concentration (9 μM). Taken together, selecting the suitable dye for the T cell migration assay presented a difficulty since each dye resulted in a different problem. An experiment was performed to test the sensitivity of a low concentration of CFSE dye compared to the two other dyes. The data showed that the CFSE dye at a concentration of 5 μM had the highest sensitivity of all three.

Subsequently, the flourochrome was used in the migration assay and no problems were encountered.

5.14.2. Analysis of T cell migration in a FluoroBlok transwell system

After the labeled T cells were loaded in the fibronectin-coated inserts, they were allowed to migrate for 16 hr at 37°C. The degree of migration was measured in real time using a CytoFluor plate reader that detected the fluorescence from below the membrane every half-hour.

The result of this real-time analysis showed that the rate of chemotaxis was time-dependent and reached its highest level after 4 hr of incubation. Any fluorescence observed at the beginning of the assay was considered to be background. To account for this background, the mean of readings taken at time zero and 30 min was calculated and subsequently subtracted from the reading measured after 4 hr of migration. The average of the fluorescence values from the two time points was used since the data collected for the first 30 min were not consistent from assay to assay.

The migration assay results showed that in the negative control, where both the insert and the well contained only media (or column-purified mock supernatant), some cells were passed through the membrane randomly. However, when the T cell chemoattractant RANTES was added to the well (as a positive control), the rate of migration increased significantly as the cells moved toward the chemotactic factor in a directed motion.

Eleven migration assays were performed. The two data sets shown are representative of the eleven assays. Consistent results were obtained whenever the assay

controls worked. The results were rejected when either the positive control failed, or the fluorescence values for different environmental conditions were all similar to that of the negative control.

5.14.3. Measurement of the chemotactic effect

A chemoattraction effect of the soluble SEMA7A on the T cells isolated from human peripheral blood was investigated in this experiment. The effect was measured when the cells in column-purified mock supernatant were loaded into the insert, and purified SEMA7A was placed in the bottom well. For a negative control, the purified mock supernatant was used in the place of SEMA7A. The fluorescence intensity of the cells that migrated through the filter into wells was analyzed statistically as described earlier.

The result of the analysis (Fig. 11) showed that the fluorescence intensity and, therefore, cell migration increased by 38.5% in the presence of SEMA7A when compared to the negative control. However, when the SEMA7A was added into the upper insert

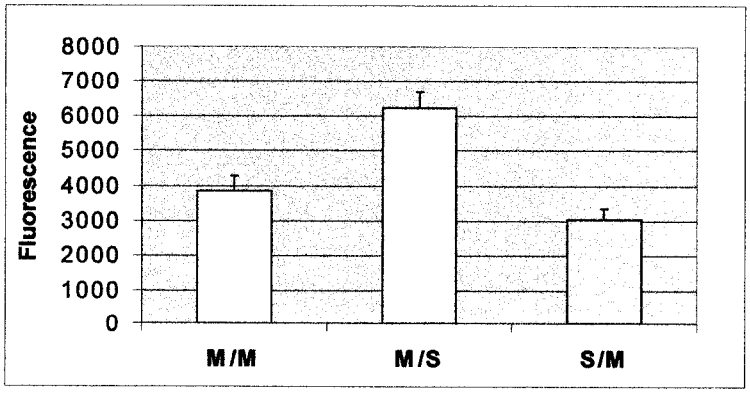


Fig. 11. Chemotactic effect of SEMA7A (S) on the MACS purified human T cells (M - mock). Data represents the mean of triplicates + SE.

instead of the bottom well, fewer cells migrated through the membrane in comparison to that of negative control. Therefore, soluble SEMA7A was able to act as a chemoattractant for MACS purified human T cells in the FluoroBlok migration system.

5.15. Immunodepletion experiment

To confirm that the chemoattraction effect observed in the T cell migration assay was SEMA7A-dependent, immunodepletion of the recombinant protein from the SEMA7A-media mixture was performed using anti-myc mAb and protein G-Sepharose. For the depletion control, goat anti-mouse IgG was used in the place of anti-myc mAb.

The immunodepleted mixtures were subsequently tested in the migration assay. The fluorescence intensity of the labeled cells that migrated in response to SEMA7A-depleted mixture was compared with that of depletion control or undepleted protein-media mixture. As before, chemoattraction of T cells was obtained with the undepleted SEMA7A-containing media. Similar chemotactic effect was detected in the depletion control; however, this effect was no longer observed after SEMA7A was removed from the protein-media mixture (Fig. 12).

To show the significance of SEMA7A-induced response, the chemotaxis of the T cells in the above experiment was expressed as a chemotactic index (stimulated migration / random migration) in Figure 13. Chemotactic Index of > 2 is considered significant according to the literature. This value for SEMA7A is 4.02 and therefore highly significant.

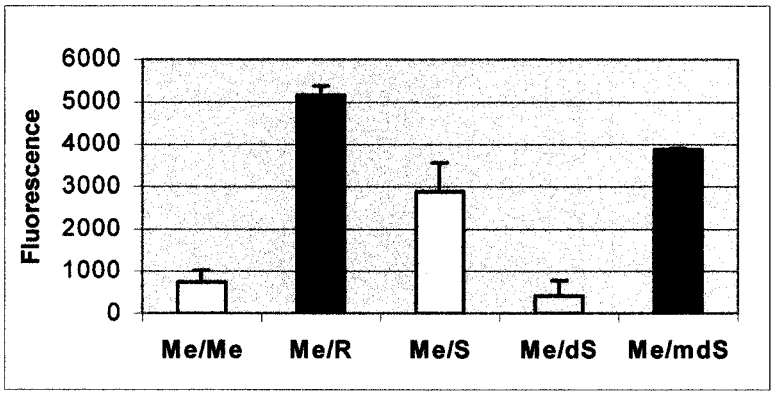


Fig. 12. Chemotactic effect of SEMA7A confirmed by immunodepletion experiment (Me - media, dS - protein-media mixture depleted of SEMA7A, mdS - control or mock depleted SEMA7A, S - undepleted media-protein mixture). Data represents the mean of triplicates + SE. Suspect values were rejected with 90% confidence using Q test of Dean and Dixon.

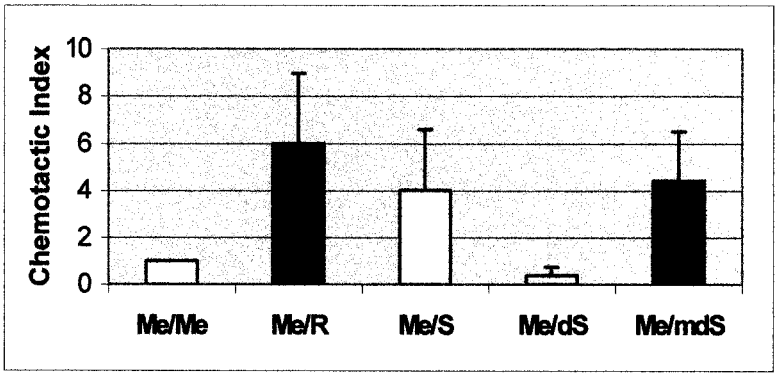


Fig. 13. Index of chemoattraction (Me - media, dS -protein-media mixture depleted of SEMA7A, mdS - control or mock depleted SEMA7A, S - undepleted media-protein mixture). Data represents the mean of triplicates + SE. Suspect values were rejected with 90% confidence using Q test of Dean and Dixon.

6. Discussion

The goal of this research was to identify the effect of human semaphorin SEMA7A on the *in vitro* migration of MACS isolated human T cells. In the first part of the project, the pEX-SEMA7A-mh vector was verified by end sequencing and restriction

mapping. In the second part, the SEMA7A recombinant protein was produced by transient transfection of Cos-1 cells with the vector, and the secreted protein was subsequently purified from the supernatant of the cells using Centricon centrifugal filter devices and nickel columns. In the third part of the project, the functionality of the purified protein was tested using both monocyte IL-8 release assay and T cell migration assay. Finally, the T cell chemotactic effect of the purified SEMA7A-mh protein observed in FluoroBlok transwell migration assays was verified by the immunodepletion experiment.

6.1. Immunological properties of SEMA7A

In the present study, the immunological functions of soluble SEMA7A were investigated. The reported data demonstrated that soluble SEMA7A is a potent stimulator of both human monocytes and T cells. In the monocyte IL-8 release assay, SEMA7A was able to upregulate IL-8 production from monocytes by 60% when compared to the control. The observed role of SEMA7A on monocyte activation has been recently confirmed by the work of Holmes et al. (2002). Their study showed that SEMA7A could also stimulate monocyte and neutrophil chemotaxis. This thesis research, however, examined the chemotactic effect of the protein on human T cells. The data obtained from transwell migration assays demonstrated that SEMA7A has a significant chemotactic effect on the migration of T cells *in vitro*. This protein acts as a chemoattractant to MACS-purified (untouched) human T cells. This result suggested that SEMA7A has a similar chemotactic effect on both myeloid and lymphoid cells.

The T cells used in the migration assays were purified from fresh PBMCs by depletion of non-T cells, monocytes included, using the MACS system. The isolated T cells were relatively pure (> 98%), as determined by flow cytometry and microscopic analysis, suggesting that the significant migration observed in the transwell assays was mainly related to the T cells rather than any contaminating cells such as monocytes.

6.2. The effect of SEMA7A on T cell migration

The expression of Sema7A mRNA in murine lymphoid tissues has been recently studied in our laboratory using *in situ* hybridization. The results showed that Sema7A is expressed in the lymphoid follicles in lymph node, inner cortex of thymus, and germinal centers of the white pulp in spleen where lymphocytes are concentrated. The regional pattern of Sema7A expression in these tissues suggested that this semaphorin might have a role in patterning lymphoid organs and regulating lymphocyte migration. The SEMA7A induced T cell chemotaxis, observed in this project, further supported the above hypothesis.

The recent findings suggested that SEMA7A is possibly acting as a T lymphocyte trafficking molecule *in vivo*. This protein has a T cell chemoattraction effect, which can be modulated in the presence of other chemotactic and adhesion molecules such as IL-8 and collagen, respectively.

The T cell chemotactic effect of SEMA7A was opposite that of viral semaphorins previously reported in the laboratory. The viral semaphorins have been shown to inhibit the migration of T cells in a microchemotaxis chamber by 70% when compared to the control. This observation suggested that the viral semaphorins are capable of blunting the

immune response possibly by either interfering with the action of host semaphorins or directly sending inhibitory signals of leukocyte migration. Following viral infections, the cells probably release viral semaphorins, which compete with endogenous SEMA7A and bind to their common receptor plexin-C1 on the surface of the T cells. After binding, they send signals for inhibition of T cell migration rather than SEMA7A-induced chemoattraction. By this mechanism, the virus will survive the host immune system and continue its proliferation in the host.

In sum, this study demonstrated that soluble SEMA7A is a potent chemoattractant for freshly isolated human T cells *in vitro*. Thus, this semaphorin might act as a trafficking molecule involved in the regulation of T cell migration both developmentally and physiologically during infection and inflammation. Further experiments are needed to study the exact biological role of SEMA7A with respect to T cell migration *in vivo*.

6.3. Problems that can occur with FlouroBlok migration assays

In the first few migration assays, the fluorescent values for all the different environmental conditions were similar to that of the negative control. This was most likely due to the fact that during the T cell isolation procedure, most of the cells formed clumps that were very hard to break apart and, therefore, could not probably pass through the pores of the insert's membrane. All the fluorescent values measured for these assays were very low, indicating that no detectable T cell migration had occurred. This problem was later solved by addition of 2 mM EDTA to PBS during the cell washing steps that allowed the preparation of a single cell suspension.

In some later assays, all the different conditions resulted in very high fluorescent values and, therefore, it was hard to detect any cell migration against such high backgrounds. One reason for the above difficulty might have to do with a high concentration of CFSE dye (5 μM) initially used for staining the human T cells. After processing the dye, labeled cells released the extra dye into their media, generating a high fluorescence background. This problem was eliminated by both decreasing the dye concentration to 3 μM and increasing the number of washes at the end of the labeling. The high background was also detected when significant cell migration occurred prior to the beginning of the assay. To prevent this issue, T cell suspension was added last into the inserts when loading the transwell system. This precaution was taken for the last few assays, the results of which are reported here.

Occasionally positive controls failed to show any chemotactic response probably due to the clumping issue, already mentioned, or improper fibronectin coating. Another possibility for the failure of the positive control is described as follows. T cells might become activated during the cell isolation process and secrete different chemokines such as RANTES. The chemokine could modulate or desensitize its receptor on the T cell surface and, therefore, reduced the cell's responsiveness to this chemokine (Taub and Oppenheim, 1994).

In some assays, one data point was very different from the rest (by > 3-fold) and, thus, rejected using the Dean and Dixon's Q test which is designed for as few data points as three. Since a damaged insert was occasionally found, in which the membrane

contained large visible pores (> 8 μm), the suspected fluorescence values might have been obtained from the damaged inserts.

It was desirable to have more than three data points per condition to have a better evaluation of data reliability and reproducibility. However, the assay was lengthy and tedious, required a large number of freshly isolated T cells due to a large surface area of the membrane, and needed highly purified SEMA7A protein which was prepared only in a limited quantity. Thus, performing the assay in more replicates than three or repeating the experiment more times than mentioned presented difficulties.

Though the number of collected data points could have been higher, the immunodepletion experiment verified and reinforced the earlier chemotaxis assay findings. When the protein-media mixture depleted of SEMA7A fusion protein was used for migration assays, cells fail to migrate which was indicated by a significant decrease in the fluorescence intensity compared to that of SEMA7A containing media. Since the T cell migration was also obtained with the depletion control (media mixture depleted with an unrelated antibody), the depletion experiment did not destroy the chemoattraction property of SEMA7A. Therefore, the high fluorescence values detected in the presence of SEMA7A were largely due to the directed movement of the T cells in response to this protein.

In summary, the FlouroBlok transwell system is an effective technique for studying *in vitro* cell migration assays. The result of this analysis method was reproducible as shown in the depletion experiment. However, the assay had failed many times during this project because of a variety of reasons. Some problems and limitations

associated with this method were recognized and addressed in this section. Further optimizations of the system are necessary to provide a more precise and robust analysis of the cell motility.

6.4. Future directions for basic research

6.4.1. T cell migration in vivo

A soluble SEMA7A protein was used in this project as opposed to a GPI-linked protein, which is the main form present in the human body. Xu et al. (1998) have suggested that a GPI-linked carboxyl-terminal signal peptide, which is missing in the secreted form, might have some functional significance. Therefore, it is an interesting issue for future studies to investigate the role of the GPI-linked SEMA7A in the T cell migration assay.

Although SEMA7A is widely expressed as a GPI-linked protein, it can be also released from the cell surface by proteolysis (Angelisova et al., 1999) to produce a soluble form of the protein. This soluble semaphorin, present in physiological fluids in the human body, might act as a chemoattractant factor for guiding T cells during their development. Furthermore, if the GPI-anchored SEMA7A protein does not exhibit a chemoattraction activity, it can be concluded that the cells release the soluble form only during the course of an immune response in order to attract T cells to the site of inflammation or infection.

Migration of T cells in the body may be mediated by a combination of factors. Here, the *in vitro* effect of SEMA7A protein on spontaneous T cell migration was studied in the presence of the cell adhesion molecule fibronectin. *In vivo,* however, other

extracellular matrix proteins, for example collagen and laminin, are also present which promote the chemotactic activity. Moreover, cytokines such as IL-8, RANTES, macrophage inflammatory protein-1 α (MIP-1 α), and MIP-1 β are produced by different cell types which induce T cell migration by both directed chemoattraction activity and increase of cell adhesion to the matrix (Taub and Oppenheim, 1994). These environmental factors have to be considered when studying the role of SEMA7A in immune cell migration in the body. It is predicted that the observed chemoattraction activity of SEMA7A will be enhanced on the chemokine-induced T cells in the presence of different adhesion molecules *in vivo*.

Furthermore, it is important to look at the T cell chemotactic effect of different semaphorins expressed in the immune system individually or in a group. Previous reports have shown that monocyte migration is stimulated by SEMA7A while inhibited by SEMA4D (Holmes et al., 2002; Delaire et al., 2001). This observation suggested that various semaphorins exert different effects on the immune cell migration. Based on recent studies in the laboratory, other semaphorins than SEMA7A including SEMA4D and SEMA4F present regional expression patterns in the lymphoid organs known to be important in T cell maturation. Thus, finding a correlation between the expression pattern of these semaphorins in lymphoid tissues and their T cell chemotactic activity is necessary for further understanding of the cell movement during development in the human body.

6.4.2. The SEMA7A receptor

In the present work soluble SEMA7A acted as a chemoattractant agent for freshly isolated human T cells. However, it remains unknown which T cell subtypes were responsible for this observation. Plexin-C1, which is the receptor or a component of the receptor for SEMA7A protein, is present on the surface of T cells (Comeau et al., 1998). Yet the expression pattern of this receptor on different subsets of T cells has not been reported. It is predicted that, as SEMA3A in the nervous system acts as a chemorepellent for DRG cell axons stimulated by neuron growth factor but not by neutrophin-3 (Messersmith et al., 1995), SEMA7A also induces a diverse chemotactic response depending on the subtype of T cells. The cell-specific response to SEMA7A signal is likely regulated by the differential expression of the SEMA7A receptor plexin -C1 on various cell types. Therefore, future experiments are necessary to study the expression profile of the receptor plexin-C1 in various T cell subtypes including $CD4^+$ or T helper cells, $CD8^+$ or T cytotoxic cells, $CD45RO^+$ or memory T cells, $CD45RA^+$ or naïve T cells, and CD2/CD2R-stimulated or activated T cells. Alternatively, the effect of SEMA7A protein on the migration of each T cell subtype can be investigated in transwell migration assays.

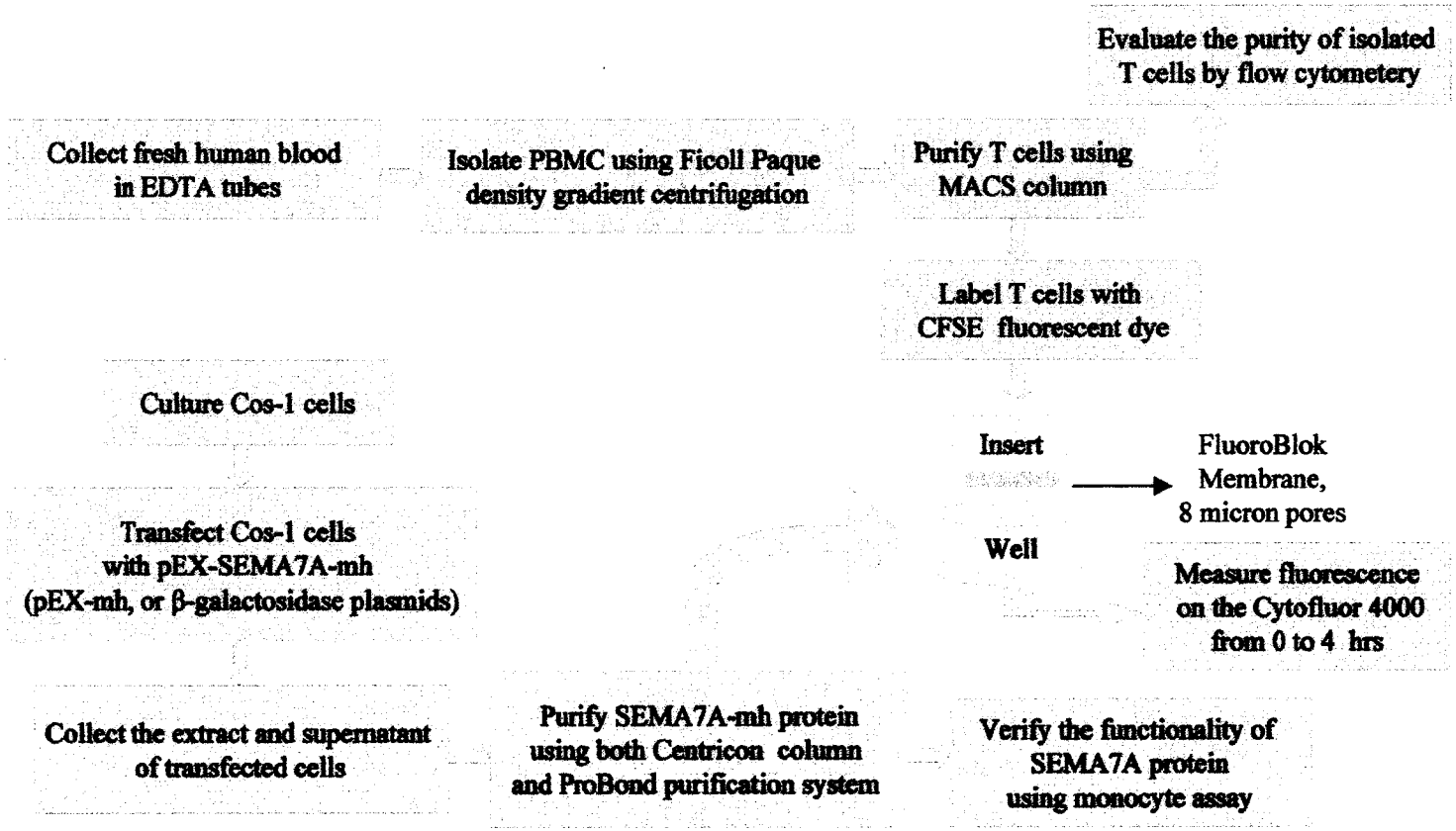
This thesis project provided the first evidence for the role of soluble SEMA7A in stimulating T cell chemotaxis. This semaphorin-induced chemoattraction was highly significant with Chemotactic Index of > 2. While interaction of SEMA7A with its receptor on the surface of the T cells seems necessary for sending the chemotactic signals, there is no evidence supporting this binding. Although SEMA7A receptor

plexin-C1 is present on most of the T cells, ligand binding studies have failed to demonstrate the binding of soluble SEMA7A protein to Jurkat T cells (Xu et al., 1998). One explanation for the above discrepancy is that the binding of the plexin-C1 to its ligand might be regulated by a cofactor or a co-receptor whose expression is altered in the Jurkat T cell line as opposed to the freshly isolated human T cells. Obviously, untouched T cells, isolated from fresh blood using MACS system, more closely resemble the T cells *in vivo* than established cell lines do. However, further investigations are required to elucidate the ligand-receptor interaction, factors that regulate this binding, and the downstream signal transduction events.

Appendix A

Experimental Overview

EXPERIMENTAL OVERVIEW



Appendix B

Addendum to Human Subjects Protocol

David Matthes, Ph.D.
Department of Biological Sciences
San Jose State University
One Washington Square
San Jose, CA 95192-010

Addendum to Human Subjects Protocol
May 23, 2002

Title of Research: Investigation of Semaphorins as Immunomodulators.

[Note for the IRB Reviewer:
all items marked "on file" are unchanged from
the protocol approved and on file with SJSU-IRB.
(from) E.Fortin, IRB Coordinator]

1. Completed HS-IRB cover sheet
(on file)

2. Succinct statement of purpose and justification
(on file)

3. Hypotheses or question(s) to be addressed.
(on file)

4. Methods section including the following:

Subjects
a) Number, age and type of subjects.

The migration assays require the isolation of low-abundance subtypes of peripheral blood T cells, B cells, natural killer (NK) cells and other leukocytes. A typical experiment requires up to 150 mls of blood which is usually obtained by drawing 30 mls of blood from each of up to five volunteers. Donors can be anywhere from 18 to 65, any ethnic background and either gender. They must, however, be healthy, non-pregnant, and weigh at least 50 kg (110 lbs).

b) Procedure for selecting subjects. Include, in layman's language, how subjects will be recruited. Stipulate who and where the potential subjects are, how they were identified as potential subjects, how they will be contacted, and what will be said to recruit them and by whom. If a subject pool is to be used, include a sample of sign-up posting. If media advertisement is to be used, include a copy of text.

Potential blood donors are selected from among Biology department students, staff and faculty. They must be healthy and willing to contribute blood. Many have donated blood for research or for the American Red Cross in the past and so are familiar with the process. Candidates are asked simply "would you consider donating blood for a NIH-sponsored research project?" If they would, they are then given more information on the kinds of experiments that will be done with the blood gathered. No flier or advertisement is used as the total amount of blood needed is small. Repeat donors are permitted if they have given less than 120 mls in the preceding 30 days; most donors given no more than three times in that period. Volumes of previous donations are able to be determined from notations on Informed Consent forms. This maximum amount is far below the Federally recommended donation limit of 550 mls of blood per 8 weeks, also with a 50 kg minimum weight, twice per week maximum donation frequency. (Department of Health and Human Services publication, "Categories of research that may be reviewed by the IRB through an expedited review process" which can be found at the following site: http://ohrp.osophs.dhhs.gov/humansubjects/guidance/expedited98.htm).

c) Rationale for employing the type of subjects selected for the study.
(on file)

d) Are subjects expected to benefit from participation in the study? If so, how?

Subjects are told that they will receive no benefit from participation other than the satisfaction of having contributed to a research project of significant scientific interest.

e) Are any risks to the subjects anticipated from participation in the study? If so, what are they?

Subjects are told of the minimal risks they might anticipate from participation in the study on the "informed consent" form that each is given to sign (with a second copy given for them to keep).

Regarding safety of blood work-up after donation, we also take steps to ensure safety for the investigator and public. We typically carry blood from the health center to our laboratory in Duncan Hall in a styrofoam test-tube rack within a sealed styrofoam cooler. The Vacuutainer tubes are unopened until the investigator has returned to the lab. The investigator who processes the blood has been trained in the safe handling of human blood and is aware of the hazards inherent therein. Our lab is frequently inspected by Department and College staff in charge of lab safety and by county health inspectors and has always received favorable reports. Biohazard waste containers are used and disposed according to Department guidelines.

f) Will any compensation be awarded to subjects for participation in the study? If so, what and why?

No.

g) Will subjects be identified with the data? If subjects are to be identified with the data, the consent form must say so, and indicate the extent to which the subject's name or other identifiers will be used.

No. It is not important from whom the blood is gathered. Blood is pooled and no record is kept of the donor other than their signed informed consent form.

Materials and Devices
a) Describe any test materials to be used, such as psychological, educational, or evaluative tests. Please include copies of the above materials with the protocol, and/or provide representative examples of computer stimulus materials.

None.

b) Include a full description of any devices to be employed.

A 21-23 gauge needle, butterfly, and EDTA-containing vacuutainers, each used only by a licensed phlebotomist and once only (i.e. the whole set-up is changed after each donor).

Procedures
a) Describe what the subjects will do, step by step.

Subjects review the informed consent form, are given an opportunity to ask any questions they might have about the process or project, and then are brought to the donation site (typically the Student Health Center, otherwise a properly prepared blood donation station in the Department of Biological Sciences) where their blood is drawn following standard phlebotomy protocol.

b) State where, when, and by whom the research will be conducted. If treatment is to be done, state qualifications of the practitioner.

Only state-licensed phlebotomists draw blood. Most blood-draws are done by phlebotomists at the Student Health Center on SJSU's campus. Others are done by phlebotomists in the Biology Department.

Confidentiality
Describe mechanisms for maintaining confidentiality. Specify how materials collected will be kept safe and who will have access to materials. If subjects' identities are to be connected with the data during the collection period (e.g., to keep track of several interviews with the same person) the key must be maintained in a secure location and be destroyed following the collection period.

Beyond the signed informed consent form, no record of blood donors is kept or attached to any experimental data.

5. Consent form
(on file)

6. Copies of all data collection instruments

None.

Appendix C

NIH-MBRS Human Subjects Protocol

NIH-MBRS Human Subjects Protocol
Funding period: 9/1/00-8/30/04

Title of Study: Semaphorins as Modulators of Lymphocyte Migration

Purpose: To determine the functional response of human leukocytes to recombinant viral and human semaphorins. Initial work suggests that some semaphorins are produced by cells in the lymph node, spleen, and thymus, as well as by cells infected by variola pox virus or alcelaphine herpes virus. An understanding of the role of semaphorins as guidance cues for migrating leukocytes during development and the immune response requires an investigation of semaphorin response in *in vitro* migration assays using freshly prepared human leukocytes. It is expected that if leukocytes are repelled by semaphorins *in vitro*, it may be suggestive of a similar function exisiting *in vivo*.

The data from this study will guide future work investigating the function of semaphorins in human tissue and will lay the foundation for future *in situ* functional studies in the mouse (not proposed here).

Rationale for Not Using Alternatives to Human Subjects: Functional migration studies require cells that can respond to the molecules presented to them. Human cell lines behave abnormally in many ways and don't serve as a precise model of untransformed human cells. Mouse leukocytes, though as interesting as human cells, are available in much smaller quantities, insufficient for the assays we perform that require 50,000 purified leukocytes per experimental condition. Hence, human blood donors provide the most appropriate cells to study, and the cells in sufficient number that can be donated without harming the subject.

Hypothesis: (1) Semaphorins are essential mediators of cell migration in the immune system both developmentally, during lymphocyte maturation, and physiologically, during the response to infection. And (2), semaphorins work combinatorially with other chemotactic and adhesion molecules to provide leukocytes with complex but unambiguous navigational instructions during extravasation or chemotaxis. We expect that different classes of leukocytes will respond to different semaphorins, some being repelled or inhibited in their migration, some not being affected at all, and some, perhaps, that will be attracted by the semaphorins.

Methods and Procedures:

Isolation of lymphocytes

The T cell subtypes required by this study can all be isolated readily from whole blood by standard blood separation methods. Thirty milliliters of blood will be drawn from volunteers into Vacutainers (Becton-Dickinson) by licensed phlebotomists in the biology department or at the student health

center at San José State University (SJSU). Volunteers will each be provided with an "Agreement to Participate in Research" form to read, sign, and keep a copy of for their records (see attached). Protocols for handling of human blood samples are established in the biology department and meets NIH and OSHA guidelines for handling potential blood-borne pathogens. All laboratory workers handling blood are required to attend a seminar outlining the risks posed by blood-borne pathogens and the procedures for working safely with blood.

Cells isolated from fresh blood have advantages over established cell lines since they are obtainable in large numbers, are not transformed, and most closely resemble the differentiated state found *in vivo*. Each cell type can be obtained at >90% purity in a procedure that takes less than four hours.

PBMCs from a 46% Percoll-gradient pellet can be further separated into different lymphocyte subtypes by mixing them with magnetic beads conjugated to T cell subtype-specific antibodies (MACS systems; Miltenyi Biotech, Inc.). This method is preferable to the use of nylon wool since the lymphocytes are generally not activated during the isolation. For some experiments, T cells will be incubated with anti-CD3 antibodies to cross-link the T cell receptor to examine the effect of activation on semaphorin responsiveness. For others "untouched" T cells will be isolated by selective depletion of all non-T cells.

The yield and purity of viable lymphocytes will be determined by staining cells with cell-specific antibodies (Accurate Antibodies). Acridine orange and ethidium bromide staining provide an accurate estimate of viable cells. Flow cytometric will be done to obtain precise cell counts and enrichment analysis as necessary. The expected yield of lymphocytes per 30 ml of blood is approximately 8×10^6 cells.

Anticipated Results: In the nervous system where semaphorins have been examined by *in situ* hybridization, regional expression is the norm, and is highly correlated with axon guidance functions in the nervous system. It is expected that *in vitro* functional studies of the effect of semaphorins on leukocyte migration will correlate well with the *in situ* expression studies that we propose to perform.

If semaphorins can be shown to be chemorepellent or inhibitory guidance cues for cells in the immune system, it would be a first in the field of lymphocyte guidance, and an important milestone on the road to fully characterizing the set of cues that work in combination to guide developing and activated lymphocytes through the body.

Appendix D

The Human Subject Institutional Review Board

San José State
UNIVERSITY

Office of the Academic Vice President
Associate Vice President Graduate Studies and Research
One Washington Square
San José, CA 95192-0025
Voice: 408-924-2480
Fax: 408-924-2477
E-mail: gstudies@wahoo.sjsu.edu
http://www.sjsu.edu

TO: David Matthes
Biological Sciences
San Jose State University
San Jose, CA 95192-0100

FROM: Nabil Ibrahim, N. Ibrahim
AVP, Graduate Studies & Research

DATE: September 25, 2000

The Human Subjects-Institutional Review Board has approved your request to use human subjects in the study entitled:

"Semaphorins as Modulators of Lymphocyte Migration"

This approval is contingent upon the subjects participating in your research project being appropriately protected from risk. This includes the protection of the anonymity of the subjects' identity when they participate in your research project, and with regard to any and all data that may be collected from the subjects. The approval includes continued monitoring of your research by the Board to assure that the subjects are being adequately and properly protected from such risks. If at any time a subject becomes injured or complains of injury, you must notify Nabil Ibrahim, Ph.D., immediately. Injury includes but is not limited to bodily harm, psychological trauma and release of potentially damaging personal information. This approval is in effect for one-year and data collection beyond September 25, 2001 requires an extension request.

Please also be advised that all subjects need to be fully informed and aware that their participation in your research project is voluntary, and that he or she may withdraw from the project at any time. Further, a subject's participation, refusal to participate, or withdrawal will not affect any services the subject is receiving or will receive at the institution in which the research is being conducted.

If you have any questions, please contact me at (408) 924-2480.

Appendix E

Agreement to Participate in Research

Mandana Amiri

Dr. David J. Matthes

Shahin Aslam

Taji Zarinnal

Leila Amiri

Kourosh Amiri

Dr. Harris Goonewarden

San José State
UNIVERSITY

College of Science
Department of Biological Sciences
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Fax: 408-924-4840
http://www.sjsu.edu

Agreement to Participate in Research

Responsible Investigator: Dr. David J. Matthes
Protocol: Blood draw for cell fractionation and testing of blood cell behavior in response to treatment with test compounds.

1. I have been asked to participate in a research study investigating the effect of recombinant Semaphorins on white blood cells.

2. I will provide 30 milliliters (or amount I specify) of blood to be drawn by a licensed phlebotomist using a syringe needle and Vacuutainer. Standard blood drawing and handling procedures will be followed.

3. There may be slight discomfort upon sticking with the needle. There is a possibility of hematoma formation (bleeding under skin) resulting from the blood draw.

4. There are no benefits to me consequent to participation in this study aside from the feeling of having contributed to a research program.

5. No alternative procedures will be done.

6. The results of this study may be published but no information that could identify the subject will be included.

7. There will be no compensation for participation in this study.

8. Questions about the research may be addressed to the principal investigator at San Jose State University, David Matthes (408) 924-4872. Complaints about the research may be presented to the Biology Department Chair, Sally Veregee (408) 924-4880. Questions or complaints about the research, subjects' rights, or research-related injury may be presented to Nabil Ibrahim, Ph.D, Associate Academic Vice President for Graduate Studies and Research(408) 924-3968.

9. No service of any kind, to which one is otherwise entitled, will be lost or jeopardized if I choose not to participate in the study.

10. Consent is given voluntarily. I may refuse to participate in the study or in any part of the study. If I decide to participate in the study, I am still free to withdraw at any time without prejudice to my relation to San Jose University or any other participating institution.

11. I have received a signed and dated copy of the consent form.

- A signature of a subject on this document indicates agreement to participate in the study.
- The signature of a researcher on this document indicates agreements to include the above named subject in the research and attestation that the subject has been fully informed of his or her rights.

Subject's Signature — Date: 08/06/01

Investigator's Signature — Date: 08/06/01

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UNIVERSITY

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Department of Biological Sciences
One Washington Square
San José, CA 95192-0100
Voice: 408-924-4900
Fax: 408-924-4840
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- The signature of a researcher on this document indicates agreements to include the above named subject in the research and attestation that the subject has been fully informed of his or her rights.

D. J. Matthes — 8/6/01
Subject's Signature — Date

D. J. Matthes — 8/6/01
Investigator's Signature — Date

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Agreement to Participate in Research

Responsible Investigator: Dr. David J. Matthes
Protocol: Blood draw for cell fractionation and testing of blood cell behavior in response to treatment with test compounds.

1. I have been asked to participate in a research study investigating the effect of recombinant Semaphorins on white blood cells.

2. I will provide 30 milliliters (or amount I specify) of blood to be drawn by a licensed phlebotomist using a syringe needle and Vacuutainer. Standard blood drawing and handling procedures will be followed.

3. There may be slight discomfort upon sticking with the needle. There is a possibility of hematoma formation (bleeding under skin) resulting from the blood draw.

4. There are no benefits to me consequent to participation in this study aside from the feeling of having contributed to a research program.

5. No alternative procedures will be done.

6. The results of this study may be published but no information that could identify the subject will be included.

7. There will be no compensation for participation in this study.

8. Questions about the research may be addressed to the principal investigator at San Jose State University, David Matthes (408) 924-4872. Complaints about the research may be presented to the Biology Department Chair, Sally Veregee (408) 924-4880. Questions or complaints about the research, subjects' rights, or research-related injury may be presented to Nabil Ibrahim, Ph.D, Associate Academic Vice President for Graduate Studies and Research(408) 924-3968.

9. No service of any kind, to which one is otherwise entitled, will be lost or jeopardized if I choose not to participate in the study.

10. Consent is given voluntarily. I may refuse to participate in the study or in any part of the study. If I decide to participate in the study, I am still free to withdraw at any time without prejudice to my relation to San Jose University or any other participating institution.

11. I have received a signed and dated copy of the consent form.

- A signature of a subject on this document indicates agreement to participate in the study.
- The signature of a researcher on this document indicates agreements to include the above named subject in the research and attestation that the subject has been fully informed of his or her rights.

T. Mal — 8/6/1
Subject's Signature — Date

M. Matthes — 8/6/01
Investigator's Signature — Date

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