Immunity, Vol. 8, 473–482, April, 1998, Copyright ©1998 by Cell Press

A Poxvirus-Encoded Semaphorin Induces Cytokine Production from Monocytes and Binds to a Novel Cellular Semaphorin Receptor, VESPR

Michael R. Comeau,[1] Richard Johnson,[2] Robert F. DuBose,[3] Melissa Petersen,[2] Patrick Gearing,[7] Tim VandenBos,[4] Linda Park,[4] Terry Farrah,[3] R. Mark Buller,[5] Jeffrey I. Cohen,[6] Laura D. Strockbine,[1] Charles Rauch,[2] and Melanie K. Spriggs[1,8]

[1]Department of Molecular Biology
[2]Department of Protein Chemistry
[3]Department of Bioinformatics
[4]Department of Biochemistry
Immunex Corporation
51 University Street
Seattle, Washington 98101
[5]Department of Molecular Biology and Immunology
St. Louis University
St. Louis, Missouri 63104
[6]Laboratory of Viral Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland 20892

Summary

The vaccinia virus A39R protein is a member of the semaphorin family. A39R.Fc protein was used to affinity purify an A39R receptor from a human B cell line. Tandem mass spectrometry of receptor peptides yielded partial amino acid sequences that allowed the identification of corresponding cDNA clones. Sequence analysis of this receptor indicated that it is a novel member of the plexin family and identified a semaphorin-like domain within this family, thus suggesting an evolutionary relationship between receptor and ligand. A39R up-regulated ICAM-1 on, and induced cytokine production from, human monocytes. These data, then, describe a receptor for an immunologically active semaphorin and suggest that it may serve as a prototype for other plexin–semaphorin binding pairs.

Introduction

The sophisticated interaction between viruses and their hosts has been the focus of much research for the past several years. Virtually every DNA virus studied encodes viral proteins that interface directly with the host immune system in a manner that facilitates the infectious process or supports viral transmission. Many of these viral immune modulators have significant homology with mammalian genes, suggesting they are of cellular origin (reviewed by Spriggs, 1996). Such viral proteins therefore can be used to initiate studies of host immune regulation, ultimately identifying both phyletic cellular factors and their counterstructures. Examples of this approach include the molecular cloning of the interleukin-17 (IL-17) receptor (Yao et al., 1995) and the leukocyte immunoglobulin-like receptor (LIR-1) (Cosman et al., 1997), which were identified by expression cloning techniques using recombinant versions of virally encoded proteins.

Because virus-specific secreted proteins are not likely to be involved in viral morphogenesis or virus release from infected cells, they are excellent candidates for host immune modulators. To identify secreted proteins encoded by vaccinia virus (VV), a large DNA virus whose genome is fully sequenced, proteins from infected cell supernatants were compared with those of uninfected cells, and the N-terminal sequences of unique proteins were determined. In this fashion, a protein whose VV open reading frame designation is A39R was chosen for further study.

A39R belongs to a family of proteins referred to as semaphorins that contain a large (approximately 500-residue) stretch of loosely conserved amino acids (Kolodkin et al., 1993) called a "Sema" domain. The prototypic members of this family are involved in neuronal development and include the membrane-bound Sema I/fasciclin IV protein of grasshoppers (Kolodkin et al., 1992), whose function includes guidance of sensory growth cones, and the soluble Sema III/collapsin I protein of chickens, also involved in growth cone guidance during development (Luo et al., 1993). Another member of this family is AHVsema (Ensser and Fleckenstein, 1995), which is encoded by alcelaphine herpesvirus type 1 (AHV), the causative agent of malignant catarrhal fever in ruminants. The pathology associated with infection by either VV or AHV (skin lesions at the site of inoculation followed by secondary virus replication in lymphoid organs in the case of VV, and leukopenia and generalized lymphadenopathy in the case of AHV), suggested that the viral semaphorin proteins interact with the host immune system rather than its nervous system.

To determine a potential immune modulating role for A39R, a recombinant chimeric A39R.Fc protein was expressed from mammalian cells and used to affinity purify a human A39R receptor on cells of lymphoid origin. Tandem mass spectrometry of purified receptor peptides was performed, ultimately allowing the identification of overlapping cDNA clones containing the complete coding region of the A39R receptor. This virus-encoded semaphorin protein receptor (VESPR) is a novel, approximately 200 kDa, transmembrane glycoprotein that is a member of the plexin family of molecules, homologues of which have been isolated from *Xenopus*, mouse, and human tissues (Ohta et al., 1995; Satoda et al., 1995; Muller et al., 1996). No ligands have yet been described for the plexins. In this article, data are presented that indicate that VESPR binds to recombinant A39R and AHVsema proteins. Data also are presented that indicate that A39R exhibits biological activity on human monocytes, strongly suggesting that this viral protein can function as an immune modulator during virus infection.

[7]Current address: Coulter Pharmaceuticals, Palo Alto, California 94306.
[8]To whom correspondence should be addressed (e-mail: spriggs@immunex.com).

Results

Identification of Secreted A39R Protein

CV-1 monolayers were infected with VV (Lister strain) and fed with serum-free medium. Approximately 48 hr after infection, cell supernatants were analyzed by two-dimensional gel electrophoresis, and a protein of approximately 55 kDa with a pI of 10 was observed in infected cell medium, but not in uninfected medium (data not shown). The N-terminal sequence of this protein was determined by Edman degradation, and 10 contiguous residues were compared to all predicted open reading frames longer than 100 amino acids encoded by the VV genome (Goebel et al., 1990). A 100% match was found between the sequence derived from the observed 55 kDa protein and residues 15–24 of the VV A39R predicted protein. These amino acids occur just downstream of a putative signal cleavage site, suggesting that they represent the N-terminus of a mature, secreted protein.

Construction of Recombinant Viral Semaphorin Proteins

Oligonucleotide primers were designed to amplify by polymerase chain reaction (PCR) a fragment of the A39R gene containing the mature portion of the protein, and the resulting product was used to construct a chimeric gene containing a portion of the heavy chain of human immunoglobulin G1 (IgG1) upstream of the A39R coding sequence (A39R.Fc). Similarly, a chimeric version of the AHV-derived *sema* gene (*AHVsema.Fc*) was created. Flow cytometric analyses identified an Epstein-Barr virus (EBV)–transformed human cord blood B cell line, CB23 (Benjamin and Dower, 1990), that exhibited significant binding of A39R.Fc and AHVsema.Fc (Figures 1A and 1D). Binding of both could be inhibited by using a 100-fold molar excess of a non-Fc form of recombinant A39R (Figures 1B and 1E), whose C-terminus contains a $[His]_6$ tag, termed A39Rpolyhis (A39R.ph), but not by a heterologous polyhis-containing protein (Figures 1C and 1F). These data indicate that A39R.Fc, A39R.ph, and AHVsema.Fc recognition of CB23 cells occurs through a shared receptor.

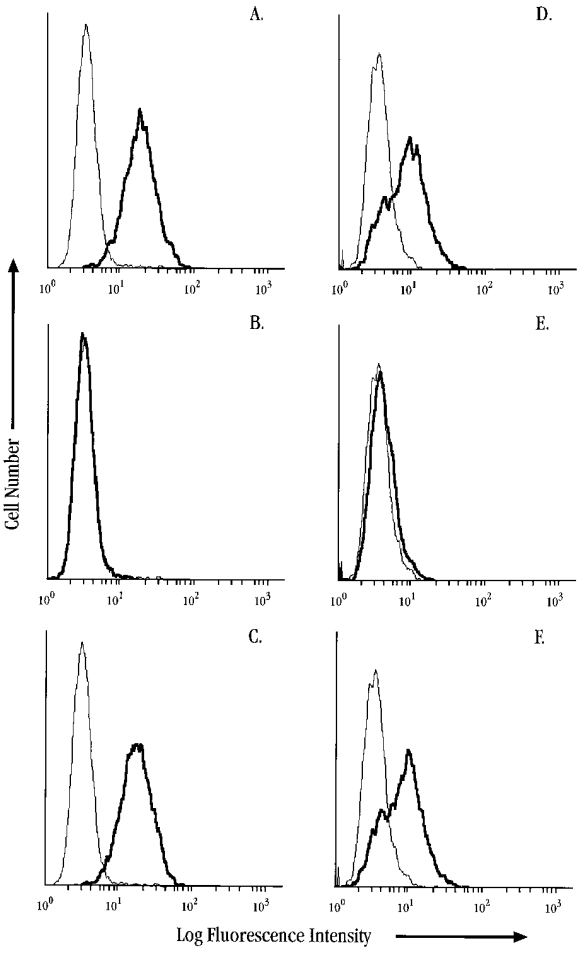

Figure 1. A39R and AHVsema Bind to the Same Receptor on CB23 Cells

Flow cytometric analysis of CB23 cells was performed using 100 ng/ml A39R.Fc (thick lines, A–C) or 5 μg/ml AHVsema.Fc (thick lines, D–F). The binding of the Fc proteins was measured in the presence of a 100-fold molar excess of A39R.ph (B and E) or a 100-fold molar excess of a heterologous polyhis-containing protein (IL-17.ph; C and F). The binding of a heterologous Fc control protein is shown by the thin line in all panels.

Isolation and Molecular Cloning of VESPR from CB23 Cells

The receptor for A39R expressed on CB23 cells was visualized by cell surface iodination (Cosman et al., 1986) and immunoprecipitation using A39R.Fc. A single band that migrated slightly faster than the myosin marker (approximately 220 kDa) was visible in samples that had been precipitated with A39R.Fc (Figure 2A, lane a) but not in samples precipitated with a heterologous Fc-containing protein (lane d). In parallel, precipitates isolated in this manner were digested with N-glycanase, an enzyme that removes N-linked carbohydrate residues. This treatment resulted in a protein that was significantly smaller (Figure 2A, lane c) than the mock-digested control (lane b); however, the deglycosylated protein still migrated well above the 148 kDa standard, indicating that although this receptor contains N-linked sugars, it likely possesses a large polypeptide backbone.

To characterize the A39R receptor at the molecular level, membranes prepared from CB23 cells were subjected to affinity purification, and purified protein was analyzed by polyacrylamide gel electrophoresis (PAGE). This procedure yielded a prominent protein band slightly smaller than the 220 kDa marker (data not shown), similar to that seen when CB23 cells were surface labeled and immunoprecipitated (Figure 2A). This band was excised from the gel, treated with trypsin, and the resulting peptides analyzed by tandem mass spectrometry or N-terminal sequencing or both. Peptide sequences obtained in this fashion that were greater than 10 amino acids in length were used to search public databases. No exact matches were found between the deduced peptide sequences and a nonredundant protein database. However, a 100% match was found between amino acids 420–428 of the sequence shown in Figure 3, and the predicted amino acid sequence of an expressed

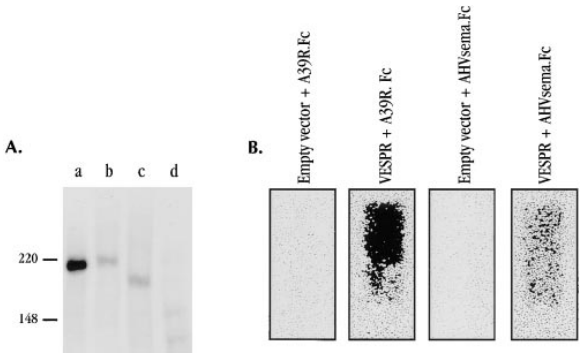

Figure 2. Viral Semaphorins Bind to a 200 kDa Glycoprotein on CB23 Cells, and to Cells Transfected with *vespr* cDNA

(A) CB23 cells were surface radiolabeled and lysates immunoprecipitated using A39R.Fc (lanes a–c) or a control Fc protein (lane d). Precipitates were resolved by SDS-PAGE on 6% gels under reducing conditions and visualized by autoradiography. Parallel samples were digested with N-glycanase to remove N-linked carbohydrates (lane c) or mock digested (lane b).

(B) A cDNA containing amino acids 19–1100 of *vespr*, in which amino acid 19 (leucine) had been mutated to an initiating codon (methionine) and was transfected into CV1/EBNA cells on chambered slides. Two days later, cells were bound with 1 μg/ml A39R.Fc or AHVsema.Fc protein, followed by ^{125}I Fc-specific mouse anti-human IgG. Cells were washed, fixed, and processed for autoradiography as described (Yao et al., 1996).

sequence tag (EST) (GenBank accession number N78220) derived from human placenta. This EST also contained sequences corresponding to amino acids 435–445 and 387–401 of the sequence in Figure 3.

Primers were designed from the EST nucleotide sequence in the regions encoding the deduced amino acids and were used with PCR techniques to amplify specific DNA fragments from cDNA libraries derived from various cell sources. In this way, a human foreskin fibroblast library was identified as containing *vespr* cDNAs. Overlapping *vespr*-containing cDNA clones were isolated from this library by hybridization. The nucleotide sequence for the complete coding region of *vespr* was determined and was verified by sequencing at least two separately isolated cDNAs throughout. That this gene encodes a protein capable of binding to A39R was confirmed by transfection of a single cDNA containing a truncated version of *vespr* that included the extracellular and transmembrane domains and a portion of the intracellular domain into CV1/EBNA cells; subsequent addition of A39R.Fc, AHVsema.Fc, or a heterologous Fc-containing protein; and finally detection of bound Fc proteins with an ^{125}I-labeled anti-Fc monoclonal antibody (mAb) as described (McMahan et al., 1991) (Figure 2B). These experiments confirmed that the *vespr* cDNA encodes a cellular receptor capable of binding to both A39R and AHVsema.

The entire nucleotide sequence determined for *vespr* (GenBank accession number AF030339) consists of 5121 base pairs, including 165 base pairs of 3′ noncoding sequence and 249 base pairs of 5′ noncoding sequence. The 5′ noncoding region is GC-rich (>82%, data not shown), a feature that has been noted for several growth factors and oncogenes (Kozak, 1992). A conceptual translation of the coding region of *vespr* is shown in Figure 3. VESPR is predicted to be a 1568 amino acid

```
   1  MEVSRRKAPP RPPRPAAPLP LLAYLLALAA PGRGADEPVW RSEQAIGAIA
  51  ASQEDGVFVA SGSCLDQLDY SLEHSLSRLY RDQAGNCTEP VSLAPPARPR
 101  PGSSFSKLLL PYREGAAGLG GLLLTGWTFD RGACEVRPLG NLSRNSLRNG
 151  TEVVSCHPQG STAGVVYRAG RNNRWYLAVA ATYVLPEPET ASRCNPAASD
 201  HDTAIALKDT EGRSLATQEL GRLKLCEGAG SLHFVDAFLW NGSIYFPYYP
 251  YNYTSGAATG WPSMARIAQS TEVLFQGQAS LDCGHGHPDG RRLLLSSSLV
 301  EALDVWAGVF SAAAGEGQER RSPTTTALCL FRMSEIQARA KRVSWDFKTA
 351  ESHCKEGDQP ERVQPIASST LIHSDLTSVY GTVVMNRFVL FLGTGDGQLL
 401  KVILGENLTS NCPEVIYEIK EETPVFYKLV PDPVKNIYIY LTAGKEVRRI
 451  RVANCNKHKS CSECLTATDP HCGWCHSLQR CTFQGDCVHS ENLENWLDIS
 501  SGAKKCPKIQ IIRSSKEKTT VTMVGSFSPR HSKCMVKNVD SSRELCQNKS
 551  QPNETCTCSI PTRATYKDVS VVNVMFSFGS WNLSDRFNFT NCSSLKECPA
 601  CVETGCAWCK SARRCIHPFT ACDPSDYERN QEQCPVAVEK TSGGGRPKEN
 651  KGNPTNQALQ VFYIKSIEPQ KVSTLGKSNV IVTGANFTRA SNITMILKGT
 701  STCDKDVIQV SHVLNDTHMK FSLPSSRKEM KDVCIQFDGG NCSSVGSLSY
 751  IALPHCSLIF PATTWISGGQ NITMMGRNFD VIDNLIISHE LKGNINVSEY
 801  CVATYCGFLA PSLKSSKVRT NVTVKLRVQD TYLDCGTLQY REDPRFTGYR
 851  VESEVDTELE VKIQKENDNF NISKKDIEIT LFHGENGQLN CSFENITRNQ
 901  DLTTILCKIK GIKTASTIAN SSKKVRVKLG NLELYVEQES VPST***WYFLIV***
 951  ***LPVLLVIVIF AAVGV***TRHKS KELSRKQSQQ LELLESELRK EIRDGFAELQ
1001  MDKLDVVDSF GTVPFLDYKH FALRTFFPES GGFTHIFTED MHNRDANDKN
1051  ESLTALDALI CNKSFLVTVI HTLEKQKNFS VKDRCLFASF LTIALQTKLV
1101  YLTSILEVLT RDLMEQCSNM QPKLMLRRTE SVVEKLLTNW MSVCLSGFLR
1151  ETVGEPFYLL VTTLNQKINK GPVDVITCKA LYTLNEDWLL WQVPEFSTVA
1201  LNVVFEKIPE NESADVCRNI SVNVLDCDTI GQAKEKIFQA FLSKNGSPYG
1251  LQLNEIGLEL QMGTRQKELL DIDSSSVILE DGITKLNTIG HYEISNGSTI
1301  KVFKKIANFT SDVEYSDDHC HLILPDSEAF QDVQGKRHRG KHKFKVKEMY
1351  LTKLLSTKVA IHSVLEKLFR SIWSLPNSRA PFAIKYFFDF LDAQAENKKI
1401  TDPDVVHIWK TNSLPLRFWV NILKNPQFVF DIKKTPHIDG CLSVIAQAFM
1451  DAFSLTEQQL GKEAPTNKLL YAKDIPTYKE EVKSYYKAIR DLPPLSSSEM
1501  EEFLTQESKK HENEFNEEVA LTEIYKYIVK YFDEILNKLE RERGLEEAQK
1551  QLLHVKVLFD EKKKCKWM*
```

Figure 3. Amino Acid Sequence and Structure of VESPR

Computer-assisted sequence analysis predicts a signal peptide cleavage site (arrow), a transmembrane domain (bold italics), and potential N-linked glycosylation sites (shaded). Boxed residues represent tryptic peptides identified by tandem mass spectrometry of purified VESPR.

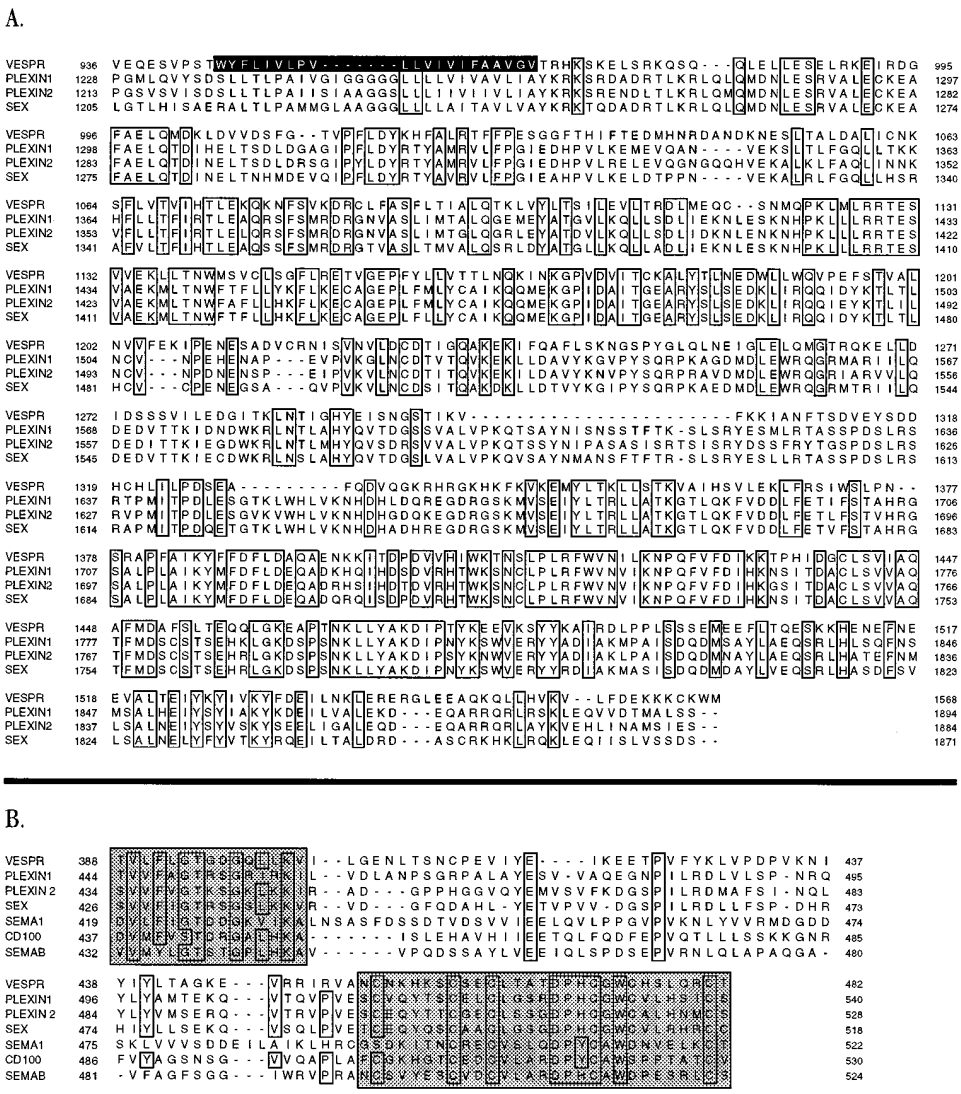

Figure 4. Sequence Alignments of Plexin Family Members

(A) The intracellular domains of VESPR and the plexins are highly conserved. The transmembrane domain of VESPR is shown on a black background.

(B) Shaded residues constitute regions of homology that are shared between the extracellular domain of VESPR, the plexins, and representative semaphorins. Semaphorins included in the alignment are Sema1 (fasciclin IV), CD100, and mouse semaphorin B.

Boxed residues indicate identical amino acids.

type 1 membrane glycoprotein (there are 24 putative N-linked glycosylation sites present in the extracellular domain) with an unmodified molecular mass of approximately 175,740. The cytoplasmic tail contains no obvious motifs that suggest mechanisms by which this receptor may transmit signals.

VESPR Sequence Comparisons

Searches for proteins sharing sequence homology with VESPR were performed using the BLAST algorithm (Altschul et al., 1990) and the nonredundant public databases. The highest degree of homology was shared with a family of proteins referred to as plexins (Ohta et al., 1995; Maestrini et al., 1996). This family includes a molecule originally isolated from *Xenopus* nervous tissue that was reported to have homotypic adhesion properties (Ohta et al., 1995; Fujisawa et al., 1997); its mouse counterpart, plexin 1 (Kameyama et al., 1996a); two additional related mouse proteins of unknown function (mouse plexins 2 and 3) (Kameyama et al., 1996a); and the human homologues of these mouse plexins, referred to as NOV, OCT, and SEX, respectively (Maestrini et al., 1996). A representative sequence alignment between portions of VESPR, mouse plexins 1 and 2, and the human SEX protein (mouse plexin 3 homologue) is shown in Figure 4A. As noted in previous work (Maestrini et al., 1996),

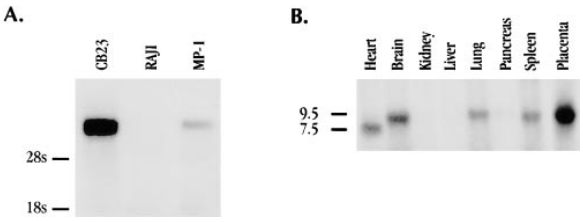

Figure 5. Northern Blot Analysis of *vespr* RNA

(A) Oligo(dT)–selected RNA (5 μg/lane) from the indicated cell lines was electrophoresed on agarose formaldehyde gels, blotted, and hybridized to a ^{32}P-labeled riboprobe that corresponded to amino acids 355–480 of *vespr*.

(B) Northern blot containing total RNA from the indicated tissues was hybridized as in (A). Size markers in kilobases are shown at left.

the intracellular portions of the plexins are strongly conserved. In particular, the regions delineated by amino acids 1065–1201 and 1378–1534 of the VESPR sequence exhibit 44% and 54% identity, respectively, compared to the aligned sequences shown in Figure 4A.

Additional sequence analysis revealed that the extracellular domain of VESPR, and in fact the extracellular domain of all of the plexin family members, contain a region of amino acid homology that is also found within the semaphorin proteins—that is, within the family of proteins that contain the ligand for VESPR. This similarity occurs between residues 380–482 of the VESPR sequence (as numbered in Figure 3) and corresponds to the C-terminal 100 amino acid portion of the canonical semaphorin domain. A partial sequence alignment of representative members of the plexin family and the semaphorin family is shown in Figure 4B. The strongest homology within this stretch of approximately 100 residues occurs in two "subdomains," defined by VESPR residues 388–402 and 454–482, (shaded in Figure 3B). This intriguing observation suggests that this region of the semaphorins and plexins may have arisen from a shared ancestral gene.

Tissue Distribution of VESPR

Flow cytometric analysis using A39R.Fc indicated that this protein bound to a variety of primary cells, including peripheral blood T and B cells, monocytes, and dendritic cells. A panel of predominantly lymphoid or myeloid cell lines also was tested and most bound to A39R.Fc, suggesting a broad distribution of this receptor in these cell types (data not shown). Interestingly, while many EBV-transformed B cell lines bound A39R.Fc, the Burkitt's lymphoma line, Raji, did not. To examine this difference more thoroughly, Northern blot analysis of poly(A)$^+$ RNA obtained from these cell lines was performed using a radiolabeled probe derived from the extracellular portion of VESPR (Figure 5A). A single, strongly hybridizing band that migrated significantly more slowly than the 28S marker was observed in CB23 cells, and a less intense but similarly sized band was seen in RNA derived from the EBV-transformed cell line MP-1. This correlated well with the positive but weaker binding of A39R.Fc to MP-1 cells relative to CB23 cells (data not shown). In contrast, Raji cell RNA showed no hybridization to the VESPR probe, consistent with a lack of A39R.Fc surface binding. Northern analysis using a similar radiolabeled probe on commercially available blots containing total RNA derived from human primary tissues (Figure 5B) indicated two species of VESPR-specific mRNAs, one that migrated just under the 9.5 kb marker (in brain, lung, spleen, and placenta) and one that migrated near the 7.5 kb marker (in heart). Similarly, multiple large mRNA species have been noted for several other members of the plexin family (Kameyama et al., 1996b; Maestrini et al., 1996). The exact relationship of these mRNAs to one another is unclear, but it is possible that the smaller message represents an alternatively spliced form. The results, however, support the idea that the expression of VESPR occurs in multiple human tissues.

Binding Affinity and Inhibition Studies of A39R and AHVsema Proteins

The affinity of A39R.Fc binding to CB23 cells was determined by direct binding assays and Scatchard analysis. A39R.Fc was labeled with ^{125}I as described in Experimental Procedures, and the binding of this protein to CB23 cells was found to be of a biphasic nature. From the average of two experiments, the high-affinity portion showed an association constant (K_a) of $2.9 \pm 0.4 \times 10^9$ M^{-1} with about 2800 ± 100 sites/cell, whereas the measured lower affinity was $1.5 \pm 0.1 \times 10^7$ M^{-1} but with about $18{,}400 \pm 500$ sites/cell. A representative sample of the binding analysis is shown in Figure 6A.

A similar analysis was performed on CV-1/EBNA cells transfected with an expression plasmid containing *vespr* (Figure 6B). In contrast to CB23 cells, the binding to transfected cells did not appear biphasic and showed a single high-affinity K_a of 5.98×10^9 M^{-1} (calculated average of two experiments). The lack of detectable low-affinity sites on these cells compared to CB23 cells may indicate that the CB23 cells express some other plexin molecule capable of binding A39R with lower affinity than VESPR.

To examine the binding affinities of A39R.ph and AHVsema.Fc, binding inhibition assays also were performed using CB23 cells (Figure 6C). In these experiments, the ^{125}I A39R.Fc was used at a concentration that would involve primarily high-affinity binding, and varying amounts of cold protein were titrated into the reactions. A39R.Fc exhibited an inhibition constant (K_i) of $1.5 \pm 0.3 \times 10^{10}$ M^{-1}, whereas the A39R.ph had a lower K_i of $7.1 \pm 2.5 \times 10^8$ M^{-1}. This difference may reflect the lower-order tertiary structure of this recombinant monomeric protein relative to the oligomeric Fc version. The K_i of AHVsema.Fc found on CB23 cells was lower still ($3.2 \pm 1.2 \times 10^7$ M^{-1}), a discrepancy that may be due to species differences, insofar as the AHVsema protein is derived from a virus whose natural host is a ruminant. However, these data clearly indicate that both forms of recombinant A39R protein bind to the receptor expressed on CB23 cells with high affinity and that the AHVsema protein binds to the same receptor but with a lower affinity.

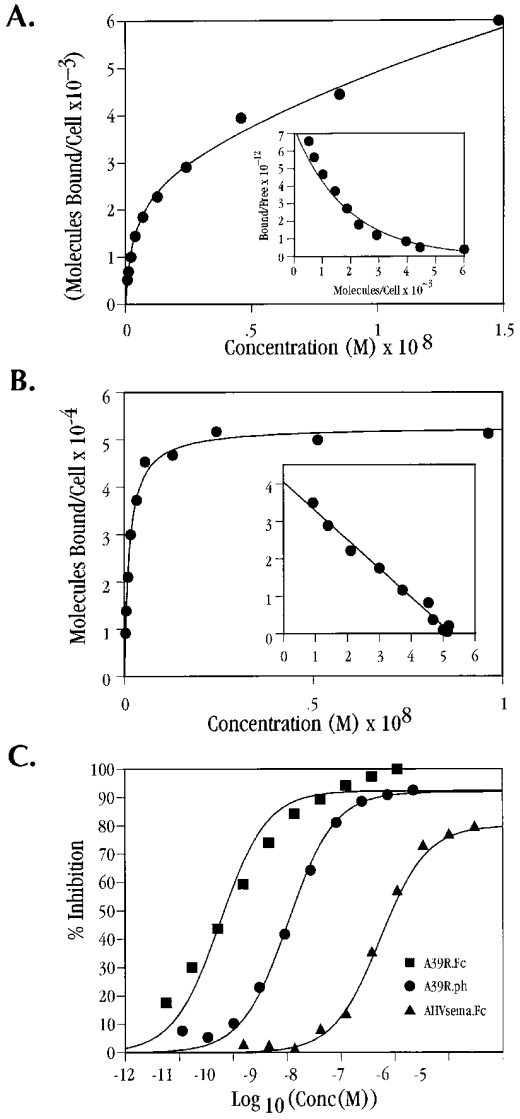

Figure 6. Affinity of Binding of Viral Semaphorin Proteins to VESPR

(A) Various concentrations of ^{125}I-labeled A39R.Fc were incubated with CB23 cells (8.3×10^7 cells/ml) at 37°C for 1 hr, and equilibrium binding determined. Data are corrected for nonspecific binding measured in the presence of a 100-fold molar excess of unlabeled A39R.Fc. Inset, a Scatchard representation of specific binding replotted.

(B) CV-1/EBNA cells were transfected with expression plasmids containing *vespr* and equilibrium binding determined as in (A). Inset, a Scatchard representation of specific binding replotted.

(C) Inhibition of ^{125}I-A39R.Fc binding to CB23 cells by unlabeled A39R.Fc (squares), A39R.ph (circles), or AHVsema.Fc (triangles). CB23 cells (8.3×10^7 cells/ml) were incubated with radiolabeled A39R.Fc (8.18×10^{-10} M) and varying concentrations of the indicated unlabeled proteins. Incubation was for 1 hr at 37°C.

A39R Exhibits Biological Activity on Human Monocytes

Because A39R.Fc protein was shown to bind to primary human monocytes, and because many poxviruses, including those expressing A39R, are believed to spread systemically through infected monocytes in the host, we examined freshly isolated monocytes for their ability to respond to A39R. Monocytes were cultured in the presence of A39R.Fc, A39R.ph, AHVsema.Fc, or heterologous Fc- or polyhis-containing control proteins and monitored for 24 hr. After 6–8 hr, cultures containing these three proteins contained large cellular aggregates, whereas cultures containing control proteins did not (data not shown). These experiments suggested that the viral semaphorins might be acting on these cells to induce cell surface activation antigens.

To address this possibility, flow cytometric analyses using a panel of mAbs were performed on monocytes cultured overnight in the presence of A39R. Recombinant A39R.ph protein was chosen for these analyses to exclude any contribution of monocyte Fc receptor cross-linking that might occur in the presence of Fc-containing fusion proteins. Parallel control cultures included untreated cells, heat-inactivated A39R.ph (a control for the presence of low amounts of endotoxin in protein samples), A39R.ph in the presence of an excess of the A39R blocking mAb m391, or m391 mAb alone. Large aggregates were again noted within 8 hr in cultures containing A39R.ph, but not in control cultures (data not shown). Monocyte cell surface markers examined after overnight culture included major histocompatibility class II, CD86, CD80, CD40, CD49e, CD1a, CD14, and CD54 (intracellular adhesion molecule-1 [ICAM-1]). No consistent change in expression of any marker was seen in response to A39R treatment, with the exception of CD54. In all experiments (six of six donors), CD54 expression was increased on monocytes in cultures containing A39R.ph, compared to that seen on monocytes in cultures containing heat-inactivated A39R.ph (Figure 7A). The average mean fluorescence intensity of CD54 expression on the A39R treated monocytes from these six donors was 137.56 $\pm$ 15.76 compared to 72.01 $\pm$ 9.36 on monocytes treated with heat-inactivated A39R. Monocytes from cultures containing no recombinant protein expressed CD54 at a level indistinguishable from that seen on monocytes treated with heat-inactivated A39R (data not shown).

Because monocytes secrete several proinflammatory cytokines in response to injury or infection, monocyte cultures were tested for the presence of IL-6 and IL-8 after treatment with A39R.ph. IL-6 production was induced in A39R.ph containing cultures in all donors tested (seven of seven), but was not induced in cultures containing cells alone or heat-inactivated A39R.ph (Figure 7B). IL-6 production was significantly reduced, but not completely inhibited, in cultures containing A39R and m391; however, part of the IL-6 production in these cultures appears to have resulted from the addition of m391 alone (Figure 7B). IL-8 also was induced by A39R in all (six of six) donors tested and was significantly inhibited by m391 (Figure 7C).

Discussion

The semaphorin family contains more than 20 members that are classified into six subfamilies based on their predicted structure (reviewed by Keynes and Cook, 1995; Tessier-Lavigne and Goodman, 1996; Zhou et al., 1997). They are best known for their role in neuronal

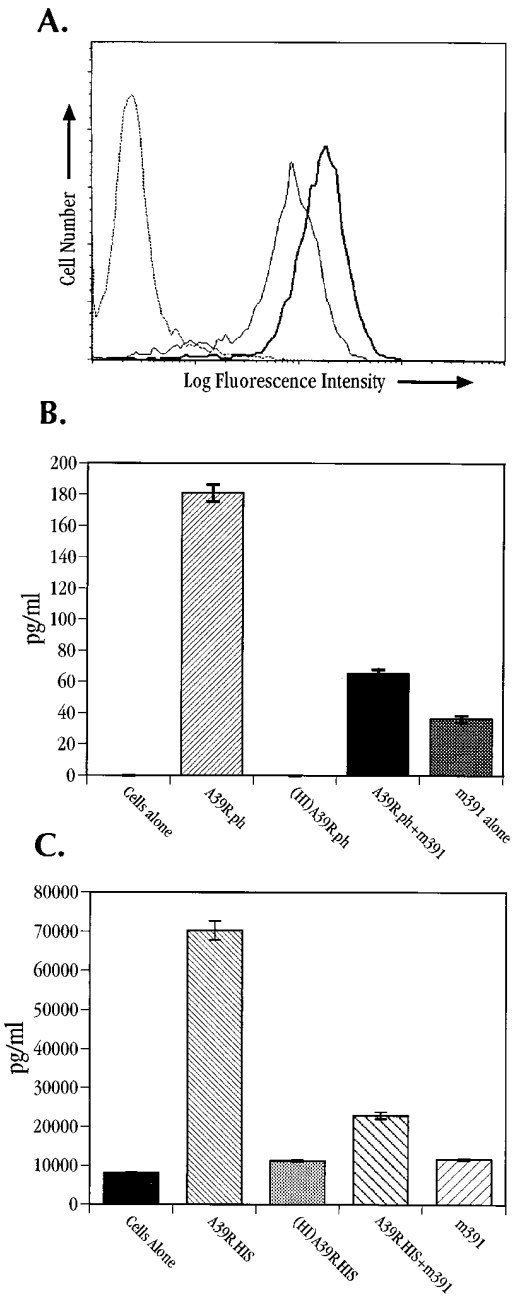

Figure 7. A39R Is Biologically Active on Monocytes

(A) Purified monocytes were cultured for 16 hr with 1 μg/ml A39R.ph (thick line) or 1 μg/ml heat-inactivated A39R.ph (thin line) and stained with PE-conjugated CD54 mAb. The dotted histogram at left indicates the staining pattern seen using a PE-conjugated isotype control mAb.

(B and C) Monocytes were cultured for 16 hr with 1 μg/ml A39R.ph, 1 μg/ml heat-inactivated A39R.ph, 1 μg/ml A39R.ph, and 20 μg/ml mAb391, or 20 μg/ml mAb391 alone, and IL-6 (B) or IL-8 (C) then measured in the supernatants. IL-6 levels were below the level of detectability in supernatants from untreated cells and from cells treated with heat-inactivated A39R.

development; however, one of the semaphorins (CD100) functions immunologically. CD100 and its mouse homologue, Sema G (Furuyama et al., 1996), are expressed on the surface of hematopoietic cells (Bougeret et al., 1992; Hérold et al., 1994, 1996; Hall et al., 1996), and antibodies against CD100 affect T cell activity (Bougeret et al., 1992; Hérold et al., 1994). Interestingly, similar to the activities reported here for A39R, transfected cells expressing CD100 caused the aggregation of human splenic B cells in culture but did not up-regulate CD54 (Hall et al., 1996). In addition to CD100, two other semaphorins have been postulated to function immunologically: they are the virally encoded proteins A39R and AHVsema (Kolodkin et al., 1993; Ensser and Fleckenstein, 1995). In this article, we show that A39R and AHVsema bind to human B cell lines and we describe biological activities for A39R on monocytes. Although A39R and AHVsema are derived from unrelated viruses and share only 29% amino acid identity, they both bind to a novel receptor (VESPR) expressed on a variety of hematopoietic (and, likely, nonhematopoietic) cells.

Recently, a receptor (neuropilin-1) was described for the axonal chemorepellent Sema III (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997), and a related receptor, neuropilin-2, was shown to bind to the semaphorins Sema E and Sema IV (Chen et al., 1997). Although the neuropilins are large transmembrane glycoproteins, they share no obvious sequence homology with VESPR. However, VESPR clearly contains many of the structural features of the plexin family of molecules.

The conservation between the cytoplasmic domain of all plexins is pronounced and likely reflects a similarity in the signaling strategies used by these molecules, which are as yet undescribed. With respect to the extracellular domain of VESPR, two striking observations are noted. The first is the identification of a subdomain, highly similar to the C-terminal 100 amino acids of the Sema domain. This subdomain is found in all plexin family members, and although it is not yet known if other plexins bind semaphorins, it strongly suggests that they might. Furthermore, the inclusion of this subdomain in both ligand and receptor is provocative: the structural elements that give rise to the homotypic adhesion properties described for the plexins (Ohta et al., 1995; Satoda et al., 1995; Kameyama et al., 1996b) may be similar to those involved in their receptor-ligand interaction. Second, within the extracellular domain of VESPR is another conserved cysteine-rich motif referred to as a "MET-related sequence" (MRS), so named because the MRS consensus sequence (Cys $X_{(5-6)}$ Cys $X_{(2)}$ Cys $X_{(6-8)}$ Cys $X_{(2)}$ Cys $X_{(5)}$ Cys $X_{(5-6)}$ Cys $X_{(12,15-16)}$ Cys, where X is any amino acid) (Ohta et al., 1995; Maestrini et al., 1996), is also found in the oncoprotein MET (also referred to as hepatocyte growth factor receptor) and the related proteins RON and SEA (Maestrini et al., 1996). MET, RON, and SEA are tyrosine kinase receptors, and their intracellular domains do not share amino acid homology with the plexins. However, the MRS motif appears once in the extracellular domains of MET, RON, and SEA; three times in plexins 1 and 2, and the SEX protein; and twice in VESPR, suggesting some structural commonality between these two families.

The results presented in this article, then, along with those recently described for the interaction between the neuropilins and semaphorins, indicate that although the semaphorins represent a structurally conserved family,

they are diverse in their recognition of cell surface receptors. Another layer of complexity also may exist in that in vitro assays have indicated that neuropilin-1 can bind semaphorins that are related but not identical to Sema III (He and Tessier-Lavigne, 1997), suggesting some promiscuity in receptor–ligand interactions.

The role of A39R and AHVsema in pathogenesis of their respective viral diseases is not clear. Because VESPR is expressed on a variety of cell types, these viral semaphorins may exhibit pleiotropic activities during infection. The ability of A39R to elicit cytokine production from monocytes in vitro suggests that viral semaphorins may contribute to the acute inflammatory response seen in both poxvirus- and AHV-infected hosts.

Many cytokines and cytokine receptors, such as IL-1 and tumor necrosis factor-α, are expressed in both neural and immune cells. The data in this article, along with those published recently for the plexins and neuropilins, suggest that semaphorins and their receptors also may have a dual roles in the development and modulation of both the neural and immunological systems. Further studies will be required to elucidate the complex interactions of semaphorins and their receptors and to understand the role of these interactions both in the naive and in the virus-infected host.

Experimental Procedures

Plasmid Constructs and Protein Production

A cDNA encoding amino acids 15–399 of the mousepox virus Ectromelia (Moscow strain) *A39R* gene was amplified from viral DNA using PCR techniques and oligonucleotide primers whose sequences were based on those published for VV (Goebel et al., 1990). A human Fc region mutated to diminish Fc receptor recognition (Baum et al., 1994) was ligated into a mammalian expression vector pDC304 (Mosley et al., 1989) that contains a signal peptide and a FLAG octapeptide (Hopp et al., 1988). PCR-amplified A39R DNA was then ligated downstream of the Fc moiety. A similar construct was created using a cDNA encoding amino acids 70–653 of the AHV type 1 *sema* gene (strain WCII) (Plowright et al., 1960). The A39R.ph construct DNA contains amino acids 1–399 of A39R and a Gly-Ser-$[His]_6$ termination codon subcloned into the mammalian expression vector pDC409 (Giri et al., 1994). For protein production, plasmids were transfected into CV1/EBNA or COS-1 cells (McMahan et al., 1991) and Fc proteins were purified from supernatants on protein-A Sepharose (Pharmacia, Piscataway, NJ). Polyhis-containing proteins were purified from transfected cell supernatants essentially as described (Hochuli et al., 1987) by binding to nickel columns. Recombinant proteins were N-terminally sequenced to confirm their identity and quantitated by PAGE and comparison to known standards. Endotoxin levels of all proteins were determined by the Limulus assay (Whittaker M. A. Bioproducts, Walkersville, MD).

Immunoprecipitation of Surface-Labeled Protein

CB23 cells (Benjamin and Dower, 1990) were surface labeled using ^{125}I (Amersham, Arlington, IL) as described (Cosman et al., 1986). Radiolabeled lysates were prepared as described (Cosman et al., 1997), and 1–5 μg of Fc fusion protein or antibody was added for 1 hr at 4°C followed by extensive washing with phosphate-buffered saline (PBS) + 1% Triton X-100. Deglycosylation of precipitated VESPR was performed essentially as described (Haziot et al., 1994) and according to the enzyme manufacturer's recommendations (Genzyme, Cambridge, MA).

Affinity Purification of VESPR Protein and Tandem Mass Spectrometry

CB23 cell pellets were suspended in a solution of protease inhibitors that included 1 mM each PMSF, leupeptin, aprotinin, pepstatin A, APMSF, and EDTA in homogenization buffer (10 mM phosphate, 30 mM NaCl [pH 7.4]). Cells were dounced, layered over a solution of 41% sucrose in homogenization buffer, and centrifuged at 25,000 rpm at 4°C for 45 min. Interphases were collected and diluted in cold homogenization buffer, dounced, and centrifuged again.

Membrane pellets were combined into 20 mM Tris, 150 mM NaCl, the protease inhibitors listed above, 1% Triton X-100, and 0.1 mM each of $CaCl_2$, $MgCl_2$, and $MnCl_2$ salts (buffer A); dounced; and centrifuged. The supernatant was placed onto a wheat germ agglutinin column, and proteins specifically bound to the column were then eluted with buffer A containing 0.2 M N-acetyl glucosamine.

Fractions containing protein were pooled and incubated with 100 μg of A39R.Fc for 1 hr at 4°C. The incubated mixture was run through a sepharose column to remove material that bound nonspecifically and then passed through a 0.5 ml column of protein A/Sepharose solid support. After washing, bound protein was eluted in 50 mM citrate (pH 3.0). Fractions containing protein were combined, concentrated, reduced and alkylated using standard dithiothreitol and iodoacetamide procedures. Alkylated proteins were electrophoresed on 8% gels and visualized by Coomassie-G (Sigma, St. Louis, MO), and the approximately 200 kDa band was excised and washed overnight in 100 mM ammonium carbonate. The gel slice was dried, treated with trypsin for 16 hr, and the peptides extracted in 50% acetonitrile, 5% formic acid. The peptides were lyophilized, reconstituted in 50 μl of 0.1% trifluoroacetic acid, and fractionated by reverse-phase high-pressure liquid chromatography (RP-HPLC) on a 500 μm inner diameter $\times$ 25 cm capillary column packed with C-18 reverse-phase packing. The HPLC liquid phase was an acetonitrile/water gradient of 10% after 5 min and 85% after 105 min, and peptides were detected at 215 nm. N-terminal sequence analysis of peptides was performed on a 494 Procise sequencer (ABI, Foster City, CA) according to the manufacturer's instructions.

RP-HPLC fractions were dried in a vacuum centrifuge and redissolved in 6 μl of 50% methanol containing 0.5% acetic acid. Two microliters (2 μl) were loaded into nanospray tips (Protein Analysis Company, Odense, Denmark) (Wilm and Mann, 1996). Data were obtained with a Finnigan TSQ700 triple-quadrupole mass spectrometer (San Jose, CA) equipped with a home-built nanospray source. Mass spectra were acquired at unit resolution. For tandem mass spectrometry, the first quadrupole was operated at a resolution sufficient to pass a 3–4 Da wide window, and the third quadrupole was operated at unit resolution. Collision gas was supplied at a pressure of 4×10^{-3} mmHg. Methyl esterification was performed as described (Hunt et al., 1986). Database searches were performed using the Sequest program (Eng et al., 1994).

Tissue Distribution Studies

Northern blot analysis was performed using oligo-dT–purified RNA (5 μg/lane) on 1.2% agarose formaldehyde gels (Sambrook et al., 1989). Multiple-tissue Northern blots containing total RNA were purchased from BioChain Institute (San Leandro, CA). Blots were hybridized and washed under stringent conditions (Sambrook et al., 1989) and probed with a ^{32}P-labeled antisense riboprobe.

Flow cytometry experiments were performed on cells preincubated in fluorescence-activated cell sorting (FACS) buffer (PBS + 3% fetal bovine serum [HyClone Labs, Logan, UT]), 0.1% azide (Sigma), and 3% normal rabbit and goat sera (Gibco–BRL, Grand Island, NY). Fc proteins were added at various concentrations and detected with phycoerythrin (PE)–conjugated Fc-specific anti-human IgG (Rockland, Gilbertsville, PA) in FACS buffer. Cells were washed and analyzed using a FACScan (Becton Dickinson, Bedford, MA). The control Fc-protein used in these studies contained the extracellular portion of the p35 protein of VV.

For binding inhibition studies, A39R.ph or a control ph-containing protein was added to cells at various concentrations prior to the addition of Fc proteins, which were then detected using a PE-conjugated Fc-specific anti-human IgG (Rockland) in FACS buffer.

Receptor Binding Assays

Direct binding assays were performed using a phthalate oil separation method as described (Dower et al., 1984), and ^{125}I-A39R.Fc that had been radiolabled by a modified chloramine-T method (Hunter and Greenwood, 1962; Segal and Hurwitz, 1977). Binding inhibition

studies on CB23 cells were performed essentially as described (Beckmann et al., 1990).

Monocyte Purification and Bioassays

Peripheral blood from healthy donors was diluted 1:1 in low-endotoxin PBS. Diluted blood was layered over Isolymph (Gallard and Schlesinger Industries, Carle Place, NY) and centrifuged. The plasma layer was reserved and the peripheral blood mononuclear cell (PBMC) layer harvested and washed to remove the Isolymph, and cells were resuspended in X-Vivo 15 serum-free media (BioWhittaker, Walkersville, MD). PBMC were seeded in T175 flasks previously coated with 2% gelatin (Sigma) and pretreated for 30 min with reserved plasma, allowed to adhere for 90 min at 37°C, and then rinsed gently. Adhered monocytes were then harvested in Enzyme-Free Dissociation Buffer (Gibco–BRL) and cultured at 5×10^5 cells/ml. Purity of the population was routinely checked by flow cytometry and was greater than 95%. A39R.ph protein was heat inactivated at 100°C for 10 min. The mouse IgG1 A39R-specific mAb, m391, was identified as a blocker by inhibition of binding of A39R.Fc to CB23 cells. IL-6 and IL-8 levels were determined using commercially available ELISA kits (R&D Systems). The minimum levels of detectability were 3.1 and 31 pg/ml, respectively.

Acknowledgments

The authors are indebted to David Cosman and Doug Williams for critical reading of the manuscript, Anne Bannister for expert editorial assistance, Jeanette Bertles, Deborah Silber, Kathy Maggiora and Sonam Dorje for excellent technical assistance, and Robert Klieforth at the Center for Reproduction of Endangered Species (San Diego, CA) for his generous gift of AHV DNA.

Received November 6, 1997; revised March 3, 1998.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403–410.

Baum, P.R., Gayle, R.B. III, Ramsdell, F., Srinivasan, S., Sorensen, R.A., Watson, M.L., Seldin, M.F., Clifford, K.N., Grabstein, K., Alderson, M.R., et al. (1994). Identification of OX40 ligand and preliminary characterization of its activities on OX40 receptor. Circ. Shock *44*, 30–34.

Beckmann, M.P., Schooley, K.A., Gallis, B., VandenBos, T., Friend, D., Alpert, A.R., Raunio, R., Prickett, K.S., Baker, P.E., and Park, L. S. (1990). Monoclonal antibodies block murine IL-4 receptor function. J. Immunol. *144*, 4212–4217.

Benjamin, D., and Dower, S.K. (1990). Human B cells express two types of interleukin-1 receptors. Blood *75*, 2017–2023.

Bougeret, C., Mansur, I.-G., Dastot, H., Schmid, M., Mahouy, G., Bensussan, A., and Boumsell, L. (1992). Increased surface expression of a newly identified 150-kDa dimer early after human T lymphocyte activation. J. Immunol. *148*, 318–323.

Chen, H., Chédotal, A., He, Z., Goodman, C.S., and Tessier-Lavigne, M. (1997). Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. Neuron *19*, 547–559.

Cosman, D., Wignall, J., Lewis, A., Alpert, A., Cerretti, D.P., Park, L., Dower, S.K., Gillis, S., and Urdal, D.L. (1986). High level stable expression of human interleukin-2 receptors in mouse cells generates only low affinity interleukin-2 binding sites. Mol. Immunol. *23*, 935–941.

Cosman, D., Fanger, N., Borges, L., Kubin, M., Chin, W., Peterson, L., and Hsu, M.-L. (1997). A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. Immunity *7*, 273–282.

Dower, S.K., Ozata, K., and Segal, D.M. (1984). The interaction of monoclonal antibodies with MHC class I antigens on mouse spleen cells: I. analysis of the mechanism of binding. J. Immunol. *132*, 751–758.

Eng, J.K., McCormack, A.L., and Yates, J.R. III (1994). An approach to correlate tandem mass spectra data of peptides with amino acid sequences in a protein database. J. Am. Soc. Mass Spectrom. *5*, 976–989.

Ensser, A., and Fleckenstein, B. (1995). Alcelaphine herpesvirus type 1 has a semaphorin-like gene. J. Gen. Virol. *76*, 1063–1067.

Fujisawa, H., Ohta, K., Kameyama, T., and Murakami, Y. (1997). Function of a cell adhesion molecule, plexin, in neuron network formation. Dev. Neurosci. *19*, 101–105.

Furuyama, T., Inagaki, S., Kosugi, A., Noda, S., Saitoh, S., Ogata, M., Iwahashi, Y., Miyazaki, N., Hamaoka, T., and Tohyama, M. (1996). Identification of a novel transmembrane semaphorin expressed on lymphocytes. J. Biol. Chem. *271*, 33376–33381.

Giri, J.G., Ahdieh, M., Eisenman, J., Shanebeck, K., Grabstein, K., Kumaki, S., Namen, A., Park, L.S., Cosman, D., and Anderson, D. (1994). Utilization of the β and γ chains of the IL-2 receptor by the novel cytokine IL-15. EMBO J. *13*, 2822–2830.

Goebel, S.J., Johnson, G.P., Perkus, M.E., Davis, S.W., Winslow, J.P., and Paoletti, E. (1990). The complete DNA sequence of vaccinia virus. Virology *179*, 247–266.

Hall, K.T., Boumsell, L., Schultze, J.L., Boussiotis, V.A., Dorfman, D.M., Cardoso, A.A., Bensussan, A., Nadler, L.M., and Freeman, G.J. (1996). Human CD100, a novel leukocyte semaphorin that promotes B-cell aggregation and differentiation. Proc. Natl. Acad. Sci. USA *93*, 11780–11785.

Haziot, A., Rong, G.-W., Bazil, V., Silver, J., and Goyert, S.M. (1994). Recombinant soluble CD14 inhibits LPS-induced tumor necrosis factor-α production by cells in whole blood. J. Immunol. *152*, 5868–5876.

He, Z., and Tessier-Lavigne, M. (1997). Neuropilin is a receptor for the axonal chemorepellent semaphorin III. Cell *90*, 739–751.

Hérold, C., Bismuth, G., Bensussan, A., and Boumsell, L. (1994). Activation signals are delivered through two distinct epitopes of CD100, a unique 150 kDa human lymphocyte surface structure previously defined by BB18 mAb. Int. Immunol. *7*, 1–8.

Hérold, C., Elhabazi, A., Bismuth, G., Bensussan, A., and Boumsell, L. (1996). CD100 is associated with CD45 at the surface of human T lymphocytes: role in T cell homotypic adhesion. J. Immunol. *157*, 5262–5268.

Hochuli, E., Döbeli, H., and Schacher, A. (1987). New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. J. Chromatogr. *411*, 177–184.

Hopp, T.P., Prickett, K.S., Price, V.L., Liggy, R.T., March, C.J., Cerretti, D.P., Urdal, D.L., and Conlon, P.J. (1988). A short polypeptide marker sequence useful for recombinant protein identification and purification. Biotechnology *6*, 1204–1210.

Hunt, D.F., Yates, J.R.I., Shabanowitz, J., Winston, S., and Hauer, C.R. (1986). Protein sequencing by tandem mass spectrometry. Proc. Natl. Acad. Sci. USA *83*, 6233–6237.

Hunter, W.M., and Greenwood, F.C. (1962). Preparation of iodine-131 labeled human growth hormone of high specific activity. Nature *194*, 495–496.

Kameyama, T., Murakami, Y., Suto, F., Kawakami, A., Takagi, S., Hirata, T., and Fujisawa, H. (1996a). Identification of a neuronal cell surface molecule, plexin, in mice. Biochem. Biophys. Res. Commun. *226*, 524–529.

Kameyama, T., Murakami, Y., Suto, F., Kawakami, A., Takagi, S., Hirata, T., and Fujisawa, H. (1996b). Identification of plexin family molecules in mice. Biochem. Biophys. Res. Commun. *226*, 396–402.

Keynes, R., and Cook, G.M.W. (1995). Axon guidance molecules. Cell *83*, 161–169.

Kolodkin, A.L., Matthes, D.J., O'Connor, T.P., Patel, N.H., Admon, A., Bentley, D., and Goodman, C.S. (1992). Fasciclin IV: sequence, expression, and function during growth cone guidance in the grasshopper embryo. Neuron *9*, 831–845.

Kolodkin, A.L., Matthes, D.J., and Goodman, C.S. (1993). The *semaphorin* genes encode a family of transmembrane and secreted growth cone guidance molecules. Cell *75*, 1389–1399.

Kolodkin, A.L., Levengood, D.V., Rowe, E.G., Tai, Y.-T., Giger, R.J., and Ginty, D.D. (1997). Neuropilin is a semaphorin III receptor. Cell *90*, 753–762.

Kozak, M. (1992). Regulation of translation in eukaryotic systems. Annu. Rev. Cell Biol. *8*, 197–225.

Luo, Y., Raible, D., and Raper, J.A. (1993). Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell *75*, 217–227.

Maestrini, E., Tamagnone, L., Longati, P., Cremona, O., Gulisano, M., Bione, S., Tamanini, F., Neel, B.G., Toniolo, D., and Comoglio, P.M. (1996). A family of transmembrane proteins with homology to the MET-hepatocyte growth factor receptor. Proc. Natl. Acad. Sci. USA *93*, 674–678.

McMahan, C.J., Slack, J.L., Mosley, B., Cosman, D., Lupton, S.D., Brunton, L.L., Grubin, C.E., Wignall, J.M., Jenkins, N.A., Brannan, C.I., et al. (1991). A novel IL-1 receptor, cloned from B cells by mammalian expression, is expressed in many cell types. EMBO J. *10*, 2821–2832.

Mosley, B., Beckmann, M.P., March, C.J., Idzerda, R.L., Gimpel, S.D., VandenBos, T., Friend, D., Alpert, A., Anderson, D., Jackson, J., et al. (1989). The murine interleukin-4 receptor: molecular cloning and characterization of secreted and membrane bound forms. Cell *59*, 335–348.

Muller, B.K., Bonhoeffer, F., and Drescher, U. (1996). Novel gene families involved in neural pathfinding. Curr. Opin. Genet. Dev. *6*, 469–474.

Ohta, K., Mizutani, A., Kawakami, A., Murakami, Y., Kasuya, Y., Takagi, S., Tanaka, H., and Fujisawa, H. (1995). Plexin: a novel neuronal cell surface molecule that mediates cell adhesion via a homophilic binding mechanism in the presence of calcium ions. Neuron *14*, 1189–1199.

Plowright, W., Ferris, R.D., and Scott, G.R. (1960). Blue wildebeest and the aetiological agent of bovine malignant catarrhal fever. Nature *188*, 1167–1169.

Sambrook, J., Fritsch, E., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Satoda, M., Takagi, S., Ohta, K., Hirata, T., and Fujisawa, H. (1995). Differential expression of two cell surface proteins, neuropilin and plexin, in *Xenopus* olfactory axon subclasses. J. Neurosci. *15*, 942–955.

Segal, D.M., and Hurwitz, E. (1977). Binding of affinity cross-linked oligomers of IgG to cells bearing Fc receptors. J. Immunol. *118*, 1338–1347.

Spriggs, M.K. (1996). One step ahead of the game: viral immunomodulatory molecules. Annu. Rev. Immunol. *14*, 101–130.

Tessier-Lavigne, M., and Goodman, C.S. (1996). A molecular biology of axon guidance. Science *274*, 1123–1133.

Wilm, M., and Mann, M. (1996). Analytical properties of the nanoelectrospray ion source. Anal. Chem. *68*, 1–8.

Yao, Z., Fanslow, W.C., Seldin, M.F., Rousseau, A.-M., Painter, S.L., Comeau, M.R., Cohen, J.I., and Spriggs, M.K. (1995). Herpesvirus saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. Immunity *3*, 811–821.

Yao, Z., Maraskovsky, E., Spriggs, M.K., Cohen, J.I., Armitage, R.J., and Alderson, M.R. (1996). *Herpesvirus saimiri* open reading frame 14, a protein encoded by a T lymphotropic herpesvirus, binds to MHC class II molecules and stimulates T cell proliferation. J. Immunol. *156*, 3260–3266.

Zhou, L., White, F.A., Lentz, S.I., Wright, D.E., Fisher, D.A., and Snider, W.D. (1997). Cloning and expression of a novel murine semaphorin with structural similarity to insect semaphorin 1. Mol. Cell. Neurosci. *9*, 26–41.