MOLECULAR CLONING OF MIS, A MYELOID INHIBITORY SIGLEC, THAT BINDS PROTEIN
TYROSINE PHOSPHATASES SHP-1 AND SHP-2*

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* This paper is dedicated to the memory of Dr. Matthew L. Thomas.
SUMMARY

We describe the molecular cloning and characterization of a novel myeloid inhibitory siglec, MIS, that belongs to the family of sialic acid-binding immunoglobulin-like lectins. A full length MIS cDNA was obtained from murine bone marrow cells. MIS is predicted to contain an extracellular region comprising three immunoglobulin-like domains (V-set amino-terminal domain followed by two C-set domains), a transmembrane domain and a cytoplasmic tail with two immunoreceptor tyrosine-based inhibitory motif (ITIM) – like sequences. The closest relative of MIS in the siglec family is human siglec8. Extracellular regions of these two siglecs share 47 % identity at the amino acid level. Southern blot analysis suggests the presence of one MIS gene. MIS is expressed in the spleen, liver, heart, kidney, lung and testis tissues. Several isoforms of MIS protein exist due to the alternative splicing. In a human pro-monocyte cell line, MIS was able to bind SH2-containing protein tyrosine phosphatases SHP-1 and SHP-2. This binding was mediated by the membrane proximal ITIM of MIS. Moreover, MIS exerted an inhibitory effect on FcγRI receptor-induced calcium mobilization. These data suggest that MIS can play an inhibitory role through its ITIM sequences.
INTRODUCTION

Leukocyte activation is controlled by a dynamic balance between stimulatory and inhibitory signals through activating and inhibitory receptors, respectively. Engagement of the B and T cell antigen receptors, or Fc receptors on specific cells activate a cascade of biochemical events required for cellular activation. However, the termination of such activation signals also represents a critical component of the natural immune response. Deficiency in inhibitory pathways results in profound immune defects characterized by both decreased activation thresholds and hyperresponsive phenotypes which often leads to autoimmunity and inflammation (1-4). In contrast, prevalence in signaling through an inhibitory receptor can result in abrogation of signal transduction and cell unresponsiveness (5). Inhibitory receptors belong to either of two structural types, the immunoglobulin superfamily with type I membrane orientation or the C-type lectin superfamily with type II membrane orientation (reviewed in (6)). Some inhibitory receptors are ubiquitously expressed (7), while others display a more restricted pattern of expression (8-11). Most inhibitory receptors contain one or more characteristic sequences (V/IxYxxL/V) in the cytoplasmic domain termed an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) (6). Phosphorylation of the tyrosine residue within the ITIM allows the binding of protein tyrosine phosphatases SHP-1 and/or SHP-2, or the inositol phosphatase SHIP through their SH2 domains (12-14). When recruited to the complex, these phosphatases act to block
signal transduction by dephosphorylating key proteins or lipids of a signaling cascade. Thus, regardless of the structural type of an inhibitory receptor, the inhibitory mechanism is similar.

The siglec family is composed of sialic acid-binding immunoglobulin-like type I lectins including sialoadhesin (siglec1), CD22 (siglec2), CD33 (siglec 3), MAG, myelin-associated glycoprotein (siglec-4a), the structurally and functionally-related SMP, Schwann cell myelin protein (siglec 4b), as well as the recently cloned human siglecs 5, 6, 7/p75/AIRM1, and 8 (15-22). Siglec family members have variable numbers of extracellular immunoglobulin-like domains (ranging from two in CD33 to seventeen in sialoadhesin) with the characteristic amino-terminal V-set domain followed by C2-set domains (23, 24). The common feature of these proteins is their recognition of sialic acid residues on cell surface glycoproteins and glycolipids which is mediated by the amino-terminal V-set domain of a siglec (23, 24). Although recognition of sialic acid residues is a hallmark feature of all siglecs, the specificity of binding has been shown for certain family members in terms of their preferences for the position of a sialic acid residue on N-linked oligosaccharides as well as in the binding of ligands expressed on different cell types (23, 25). Each siglec is expressed in a highly restricted fashion (15-22) implying a specific function for each family member. Indeed, it is believed that sialoadhesin is involved in regulation of macrophage function (25); MAG is implicated in myelinogenesis (26); while CD22 serves to inhibit signaling through the B-cell antigen receptor via binding of SHP-1 tyrosine phosphatase to its ITIMs (27, 28) and the lectin function of CD22 is thought to be important in recruiting CD22 to the B-cell antigen receptor (29). Although the function of the
other family members remains unknown, the presence of ITIM sequences in the cytoplasmic tails of some of human siglecs renders them potential inhibitory receptors. Recently, human CD33 has been characterized as an inhibitory receptor by virtue of its ability to bind tyrosine phosphatases SHP-1 and SHP-2 (30, 31).

Although nine human siglec proteins have been discovered, the mouse siglec family has fewer members to date: sialoadhesin, MAG, SMP, CD22 and CD33. Structurally, sialoadhesin, MAG, SMP and CD22 are very similar in mouse and human. In contrast, human and mouse CD33, although somewhat similar in their extracellular domains, are strikingly different in their transmembrane and cytoplasmic regions. Human CD33 contains two ITIM sequences in the cytoplasmic tail whereas mouse CD33 does not. Recent characterization of several ITIM-containing human siglecs implies that there must be murine ITIM-containing siglecs. Here we describe the molecular cloning and characterization of a novel murine ITIM-containing myeloid cell-restricted inhibitory siglec, MIS.

**EXPERIMENTAL PROCEDURES**

**Cells and antibodies:** EL4 and WEHI231 cells, murine T and B cell lines, respectively, the human monocytic cell line, U937, and human epithelioid carcinoma cell line, HeLa, were obtained from the ATCC (Rockville, MD). The murine B cell line, A20, was a generous gift from Dr. A. C. Chan (Washington University, St. Louis, MO). Murine monocyte/macrophage
cell lines, J774 and WEHI265.1 as well as NK cell lines, KY-1 and KY-2, and the P815 mastocytoma cell line, were a generous gifts from Dr. W. M. Yokoyama (Washington University, St. Louis, MO). The murine T cell hybridoma, 3A9H was a generous gift from Dr. P. M. Allen (Washington University, St. Louis, MO). EL4, A20, WEHI231, J774, and p815 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 0.1 mM non-essential amino acids, and 1 mM sodium pyruvate. WEHI265.1 cells were maintained in RPMI1640 media supplemented with 10% FCS. KY-1 and KY-2 cells were maintained in RPMI1640 media supplemented with 10% FCS, 100 U/ml IL-2. All media were supplemented with 2 mM L-glutamine, 100 µM 2-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin. U937 cells were maintained in RPMI 1640 medium supplemented with 10% FCS and L-glutamine. HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum and L-glutamine.

Mouse bone marrow cells were obtained by flushing the femurs of euthanized mice with phosphate buffered saline (PBS). Murine peritoneal macrophages were aspirated from the peritoneal cavity of the euthanized mice 2 days following intraperitoneal thioglycolate injection.

Antibodies used in this study included: an anti-phosphotyrosine monoclonal antibody (mAb), 4G10 (Upstate Biotechnology, Inc. Lake Placid, NY), the anti-Flag M2 mAb (Sigma, St. Louis, MO), the anti-SHP2 mAb (Santa Cruz Biotechnology, Santa Cruz, CA), the anti-FcγRI mAb (Medarex, Inc.,Annandale, NJ), rabbit anti-vesicular stomatitis virus serum (Access...
Biomedical, San Diego, CA), cross-linking goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), horseradish peroxidase-conjugated secondary antibodies (Cappel Organon Teknika Corp., West Chester, PA). Rabbit anti-SHP-1 antiserum was previously described (32).

RT-PCR: Total RNA was isolated from 1x10^7 mouse bone marrow cells, peritoneal macrophages, or various murine cell lines using RNAzol™ (Tel-Test, Inc, Friendswood, TX) according to manufacturer's instructions. Three micrograms of isolated total RNA were reverse transcribed in 20 μl of buffer comprised of 50mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10mM dithiothreitol, 1 mM dNTP mix, 500 ng of oligo (dT)₁₂₋₁₈ primer (GIBCO BRL, Rockville, MD) or 2 pmol of gene-specific primer and 200 units of SuperScript transcriptase (GIBCO-BRL, Rockville, MD). Three microliters of the resultant cDNA were used in each PCR reaction containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.0 mM MgCl₂, 0.2 μM dNTP mix, 10 pmol of each oligonucleotide primer and 1 unit of Taq polymerase (Promega). PCR amplification was performed for 35 cycles. Each cycle consisted of denaturing at 94°C for 1 minute, annealing at 55°C for 1 minute and elongation at 72°C for 1.5 minutes. After the last cycle, the sample was maintained at 72°C for 10 minutes. Amplification of cDNA ends was performed using 5’ RACE and 3’ RACE (Gibco-BRL) according to the manufacturer instructions. Primers used in each
PCR reaction are listed in the Table 1. PCR-amplified cDNA fragments were cloned into pCR2.1 vector (Invitrogen, Carlsbad, CA) and sequenced.

Southern and Northern blot analysis: Genomic DNA was isolated from C57Bl/6 mouse tail according to standard methods. A 360-bp PCR-amplified fragment of MIS corresponding to the first Ig-like domain of the molecule was labeled by random priming in the presence of $^{32}$P-dCTP and $^{32}$P-dGTP (Amersham Pharmacia Biotech, Piscataway, NJ) and used as a probe. Hybridization was performed in 5X saline sodium citrate (SSC: 150 mM NaCl, 15 mM trisodium citrate), 5X Denhardt’s solution, 1% (wt/vol) sodium dodecyl sulfate (SDS), 50% (vol/vol) formamide, 100 µg/ml denatured herring sperm DNA at 42°C. The blot was washed at 65°C in 0.2X SSC and 0.1% (wt/vol) SDS.

A mouse multiple tissue Northern blot (Clontech, Palo Alto, CA) was hybridized with the full length radiolabeled MIS cDNA, a 0.7 kb SmaI fragment of mCD33A cDNA and a murine β-actin cDNA control probe at 42°C as described above. Following hybridization, the blot was washed at 65°C in 0.2X SSC and 0.1% (wt/vol) SDS.

cDNA reagents and cell transfection: The mouse SHP-1(C453S) cDNA has been previously described (32). MIS constructs were made as fusion proteins composed of the extracellular and transmembrane domains of vesicular stomatitis virus G protein (VSVG) and the cytoplasmic domain of MIS (amino acids 377 - 466). Substitutions of tyrosines by
phenylalanines were introduced by PCR site-directed mutagenesis. All mutations were confirmed by DNA sequencing. SHP-1 and SHP-2 cDNAs were cloned into pBluescript (Stratagene) behind the T7 promoter; CD33 cDNAs were cloned into pGEM-3Z (Promega) behind the T7 promoter. Transfection of HeLa cells was performed using LipofectAce (GIBCO-BRL) as previously described (33). Flag epitope tags were inserted at the amino-terminus of MIS by PCR site overlap extension and into a MscI site at the carboxyl terminus of the MIS.

MIS-Flag cDNA was cloned into the pLZRS, retroviral GFP-containing vector (kindly provided by Dr. D. Link, Washington University, St. Louis, MO). Ecotropic Phoenix cells were transfected with retroviral constructs using the calcium phosphate method, and cell supernatants were used to infect U937 cells. GFP-positive cells were purified by cell sorting (Cytomation).

MIS-Flag cell surface expression was verified by FACS analysis following staining with biotinylated anti-Flag antibody followed by PE-conjugated streptavidin (Jackson Labs).

**Immunoprecipitation and Immunoblot analysis:** Retrovirally-transfected U937 cells overexpressing MIS, and transfected HeLa cells were washed with PBS and, where indicated, treated with 5 mM sodium pervanadate in PBS for 10 min at room temperature. Cells were lysed in 1 ml of lysis buffer (1% NP-40, 50 mM Tris, pH 7.5), 150 mM NaCl, 5 mM NaEDTA, 10 mM sodium fluoride, 10 mM sodium molybdate, 1 mM sodium vanadate, 5 mM iodoacetamide, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM phenylarsine oxide) on ice for 10 min. The lysates were precleared by centrifugation at 12,000
rpm for 10 min at 4°C. An aliquot was removed as the crude lysate sample and the reminder of the lysate was subject to immunoprecipitation with the appropriate antibody.

Immunoprecipitates and crude lysates were resolved by SDS-PAGE under reducing conditions and immunoblot analysis was performed. Proteins were visualized using the enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, USA) according to manufacturer recommendations.

\[\text{[Ca}^{2+}\text{]}_i\text{ measurement:}\] To ensure high expression of FcγRI receptors, parent U937 cells and those overexpressing MIS were treated with 300 U/ml of the recombinant human \(\gamma\)-interferon (a gift from Dr. R. Schreiber, Washington University, MO) for 72 hours prior to the analysis. Cells were loaded with 3 mM Fura-2AM (Molecular Probes, Inc. Eugene, OR) in RPMI 1640/10% FCS for 40 min in the dark at 37°C. Cells (6 X 10^6) were washed once and resuspended in RPMI 1640/10% FCS. Primary antibody was added at a concentration of 8 \(\mu\)g/ml and cells were incubated on ice for 40 min. Cells were washed three times in PBS and resuspended in 2 ml of calcium buffer (25 mM Hepes, pH 7.4, 125 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mg/ml D-glucose, 1 mg/ml BSA, 1 mM CaCl_2, 0.5 mM MgCl_2). Changes in fluorescence, using excitation wavelengths of 340 and 480 nm and the emission wavelength of 510 nm, were measured with a spectrofluorimeter (F-2000, Hitachi Instruments, Danbury, CT) equipped with a thermostatic cuvette holder maintained at 37°C. Cells were warmed to 37°C for 5 minutes prior to analysis. Secondary cross-linking goat anti-mouse IgG antibody (10 \(\mu\)g/ml)
was added to the cuvette at 30 seconds. Intracellular calcium concentrations were calculated as described (34).

RESULTS

Molecular cloning of MIS. Considering the sequence similarity between the extracellular domains of mouse and human CD33 and assuming at least partial similarity between the transmembrane regions of hCD33 and a hypothetical mouse inhibitory receptor, we performed a search within a mouse EST-database using the human CD33 transmembrane amino acid sequence in the query. The search resulted in the identification of one murine cDNA clone with 39% sequence identity to the transmembrane region of hCD33 (Fig. 1 A). Moreover, there were two ITIMs in the sequence of the mouse clone similar to those of hCD33. The cDNA encoded for the transmembrane and cytoplasmic regions of the protein, and also contained a portion of the 3′ untranslated region. RT-PCR was used to obtain a full length cDNA from murine bone marrow cells. The design of the forward primer (mCD33.1, table 1) was based upon highly homologous stretches of cDNA within the first Ig-like domain in mouse and human CD33. The reverse primer (M1, table 1) was based upon the sequence from the EST clone. A 1.4 kb PCR product was obtained and sequenced. To retrieve the 5′ untranslated region and leader peptide, a 5′ RACE strategy was employed using M1 primer for the first strand cDNA synthesis followed by sequential two rounds of PCR amplification with a universal abridged primer and a set of
nested primers, M2 and M3 (Table 1). To obtain the 3′-end, 3′-RACE was performed using oligo-dT primer for the first strand synthesis and M4 (Table 1) and universal primer for the PCR-amplification. In an independent experiment, the full length cDNA was amplified from total RNA isolated from mouse bone marrow cells using M5 and M6 primers deduced from the 5′-RACE and 3′-RACE sequences (Table 1).

Sequence analysis of the full length cDNA revealed that the protein had an extracellular domain of 331 residues, a hydrophobic transmembrane domain of 27 residues and a cytoplasmic tail of 93 amino acid residues (Fig. 1 B.) The sequence begins with a putative hydrophobic signal peptide. The extracellular portion of the molecule contains three Ig-like domains consisting of an N-terminal V-set domain followed by two C2-set domains. In addition, there are ten potential N-glycosylation sites (Asn-X-Ser/Thr) in the extracellular region of the protein. The cytoplasmic tail contains two potential ITIM sequences. Considering (i) the myeloid cell-restricted pattern of expression of the newly isolated protein (see below), (ii) the similarity of its extracellular domain to the extracellular domains of siglec family members, and (iii) the presence of ITIM-like tyrosine-based signaling motifs, this protein was named MIS, a myeloid inhibitory siglec.

Phylogenetic analysis of the siglec revealed that MIS, siglec8, and siglec7 cluster together, with MIS being mostly closely related to human siglec8. The similarity between MIS protein and other family members ranges from 31% with mCD33 to 47% with human siglec8.
Amino acid sequence alignment of MIS and three other siglecs, human siglecs 3, and 8 and murine CD33 is shown on Figure 2A. Although human siglec 8 is the closest relative of MIS and shares 57% identity with MIS in the extracellular portion of the molecule, their cytoplasmic tails are quite different. There are no ITIM sequences in the siglec8 cytoplasmic region whereas MIS possess two ITIM-like sequences. To this end, the cytoplasmic tail of MIS is more similar to those of the inhibitory human siglecs, siglec3, -5, -6, and –7 (33%, 29%, 29%, and 33% identity, respectively) (Fig. 2B). Thus, these data indicate that MIS belongs to the siglec family and represents a potential inhibitory receptor.

**A single MIS gene.** To determine whether MIS belongs to a family of closely related genes or represents a single MIS gene, genomic DNA from C57BL/6 mouse was digested with a panel of restriction enzymes and subjected to Southern blot analysis. The probe employed represented an exon corresponding to the first V-SET immunoglobulin-like domain of MIS. Only one band was observed for each enzyme digestion (Fig. 3) suggesting the presence of only one MIS gene in the mouse genome.

**Tissue and cell-restricted expression of MIS.** To determine the expression pattern of MIS in adult mouse tissues, Northern blot analysis was performed using the full length MIS cDNA as a probe. As shown on Figure 4A, four mRNA species of approximately 1.6, 1.8, 2.2 and 3.0 kb were detected in different tissues. A transcript of 2.2 kb was expressed (in descending order) in spleen, liver, heart, kidney, and lung (in the latter two tissues this transcript could only be detected after prolonged exposure). In addition to the 2.2 kb transcript, two smaller transcripts...
were detected in these tissues. The 1.8 kb transcript was expressed in liver and kidney, whereas
1.6 kb transcript was expressed in the spleen and heart. When Northern blot analysis was
performed using probes corresponding to the first and third MIS exons that code for the first and
third Ig-like domains, respectively, only a 2.2 kb band was observed. Conversely, analysis with
a probe corresponding to a cytoplasmic exon revealed multiple bands (data not shown). These
data together with results of Southern blot analysis, indicate that there are several MIS variants
due to alternative splicing of a single MIS gene. One transcript of 3.0 kb was observed in testis.
The MIS message could not be detected in murine brain and skeletal muscle.

To determine the pattern of cell expression of MIS, a RT PCR approach was utilized.
cDNA was generated from different mouse cell lines, primary bone marrow cells and peritoneal
macrophages and the expression of MIS, mCD33 and β-actin was analyzed using gene-specific
sets of primers (Fig. 4 B). MIS was detected in bone marrow cells, peritoneal macrophages and
monocyte/macrophage cell lines (upper panel) but was not detected in lymphocytes including T
cells (lanes 3, 4), B cells (lanes 5, 6), or NK cells (lanes 10, 11) or a mastocytoma, P815, cell
line. The overall pattern of MIS expression was similar to that of mCD33 (middle panel). The
efficiency of cDNA synthesis was equivalent in all the samples as judged by the expression of β-
actin (Fig. 4 B, bottom panel). Hence, the expression of MIS is restricted to cells of myeloid
origin.

MIS exists as various isoforms. Alternative mRNA splicing is a common feature among
Ig superfamily genes. Since Northern blot analysis of MIS suggested the existence of multiple
mRNAs, we next examined the possibility that multiple isoforms of MIS did, indeed, exist. Mouse total RNA from bone marrow cells was reverse transcribed using the MIS-specific primer, M1, and then, three different sets of primers corresponding to either the first Ig-like domain (M7 and M8), the first and the second Ig-like domains (M7 and M9), or to all three Ig-like domains of the molecule (M7 and M10) were used to PCR-amplify cDNA fragments (Fig. 5A). Amplification with the former two sets of primers each gave rise to a single products of the expected size. However, the third set of primers (M7+M10) yielded two products, one of the predicted size (the higher band on the gel) and a second which was 0.28 kb smaller (Fig. 5B). Sequencing of the smaller PCR product revealed the absence of the second Ig-like domain in the extracellular region. Combined with the detection of multiple mRNAs by Northern blot analysis, these data suggests that at least two isoforms of MIS exist due to alternative splicing of mRNA within the second Ig-like extracellular domain.

**MIS associates with protein tyrosine phosphatases SHP-1 and SHP-2.** Since there are two tyrosine-based signaling motifs in the cytoplasmic tail of MIS, the ability of MIS to associate with the SHP-1 and SHP-2 protein tyrosine phosphatases was examined. A Flag epitope-tagged form of MIS was expressed in the U937 human monocytic cell line (Fig. 6). The broad molecular weight range for MIS, as detected by SDS-PAGE, was most likely due to glycosylation in the extracellular region of the protein (there are ten potential N-glycosylation sites in the extracellular region of the molecule). In resting cells MIS was phosphorylated on tyrosine residues, as detected by immunoblotting with an anti-phosphotyrosine mAb (Fig. 6, lane
3). Treatment of the cells with pervanadate, a tyrosine phosphatase inhibitor, resulted in a
dramatic increase in tyrosine phosphorylation of MIS (compare lines 3 and 4, Fig. 6). As MIS
contains two ITIM sequences, it was found to co-immunoprecipitate with both SHP-1 and SHP-
2. While SHP-1 and SHP-2 co-precipitated with MIS in resting cells (Fig. 6 lane 3), the degree
of association correlated with the extent of MIS tyrosine phosphorylation (compare lines 3 and 4
on Fig. 6). Phosphorylated forms of both phosphatases were found to co-precipitate with MIS in
pervanadate-stimulated cells since (i) the lower band on phosphotyrosine immunoblot (line 4)
co-migrates with SHP-1 as was judged by re-probing phosphotyrosine blot with the SHP-1
antibody, and (ii) multiple bands detected by SHP-2 antibody in MIS immunoprecipitate (line 4)
most likely represent differently phosphorylated species of SHP-2 (detecting these species by
phosphotyrosine immunoblot analysis was not possible because of masking effect of tyrosine
phosphorylated MIS band). Thus, the data obtained indicate that MIS shows a basal level of
tyrosine phosphorylation in unstimulated cells and is associated with protein tyrosine
phosphatases SHP-1 and SHP-2. This association increases with an increase in the tyrosine
phosphorylation status of MIS.

The proximal ITIM of MIS is essential for binding to protein tyrosine phosphatases SHP-
1 and SHP-2. To examine which ITIM-like sequence of MIS mediates phosphatase binding,
fusion proteins were made which contained the extracellular and transmembrane regions of
Vesicular Stomatitis Virus protein G (VSVG) and either the wild type cytoplasmic tail of MIS
(YY) or mutants where tyrosyl residues were mutated to phenylalanines either individually (FY: 
first tyrosine mutated; YF: second tyrosine mutated) or in combination (FF). The chimeric proteins were overexpressed in HeLa cells together with either a catalytically inactive SHP-1C453S mutant or wild type SHP-1 or SHP-2. To ensure a high level of tyrosine phosphorylation of cellular proteins, the cells were treated with pervanadate prior to cell lysis. Equivalent levels of the SHP-1 and SHP-2 expression was achieved in each individual cell culture (Fig. 7A, B, C, bottom panels), and a comparable amount of each chimeric protein was immunoprecipitated as judged by an immunoblot analysis with the anti-VSVG antibody. In Hela cells co-transfected with catalytically inactive SHP-1, chimeric proteins bearing the wild type cytoplasmic tail of MIS (YY) and the YF mutant were found to be phosphorylated on tyrosyl residues with the level of phosphorylation of the YF mutant being reduced when compared to that of wild type (Fig. 7A compare lanes 5 and 3). In contrast, both FY and FF mutants were not found to be tyrosine phosphorylated (lanes 4, 6, Fig. 7A). This suggests that (i) there is a sequential order of tyrosine phosphorylation of the ITIMs of MIS where tyrosine 431 needs to be phosphorylated prior to phosphorylation of tyrosine 454, and (ii) both tyrosines are likely to be phosphorylated in wild type MIS. Compatible with the correlation between the tyrosine phosphorylation of MIS and its association with SHP-1, the amount of SHP-1 that co-precipitated with the YF mutant was reduced when compared to that of wild type (compare lines 5 and 3 on SHP-1 immunoblot, Fig. 7A). In addition, no binding of SHP-1 was detected in the FY and FF mutants that are not tyrosine phosphorylated (lanes 4 and 6, Fig. 7A). In cells co-transfected with wild type SHP-1 or SHP-2, the level of tyrosine phosphorylation of wild type
MIS was similar to that in cells co-transfected with SHP-1C453S mutant (compare Fig. 7A, B and C, lanes 1 and 3). In cells co-transfected with wild type SHP-1, tyrosine phosphorylation of the YF mutant was not detectable (Fig.7B, lane 5) whereas in cells co-transfected with wild type SHP-2, the YF mutant was found to be phosphorylated on tyrosine although its tyrosine phosphorylation level was reduced to just a trace amount (Fig. 7C, lane 5). This suggests that the tyrosine of MIS membrane proximal ITIM may serve as substrate for SHP-1 and SHP-2. In agreement with its phosphorylation status, no SHP-1 was associated with YF mutant. In contrast, in cells co-transfected with SHP-2, despite the low level of tyrosine phosphorylation of the YF mutant, the amount of associated SHP-2 was only slightly reduced compared to the wild type (compare lanes 5 and 3, Fig. 7C), suggesting that SHP-2 associates with the MIS membrane proximal ITIM more readily than does SHP-1. No tyrosine phosphorylation nor SHP-1 and SHP-2 binding was detected for both FY and FF mutants. Thus, MIS associates with both SHP-1 and SHP-2 and the proximal ITIM is absolutely required for phosphatase binding.

**MIS exerts an inhibitory effect on Ca$$^{2+}$$ mobilization following Fc$$\gamma$$RI receptor ligation.**

Upon cross-linking of Fc$$\gamma$$RI, an activating receptor expressed on monocytes, a signaling cascade is initiated that includes tyrosine phosphorylation and rapid Ca$$^{2+}$$ mobilization. To ascertain whether MIS acts as an inhibitory receptor, the effect of its co-ligation with Fc$$\gamma$$RI on Ca$$^{2+}$$ mobilization in monocytes was examined. Flag-MIS was expressed in a human monocytic cell line, U937, by retroviral infection and its expression was detected by cell surface staining (data...
not shown). Cells were pre-incubated with recombinant human γ-interferon for 72 hours to ensure a high level of FcγRI expression. After loading with the fluorescent dye Fura-2AM, cells were incubated with primary anti-FcγRI and biotin-conjugated anti-Flag mAbs either individually or in combination. Ca^{2+} mobilization was measured following receptor cross-linking with secondary anti-mouse IgG antibody. Co-ligation of MIS with FcγRI resulted in a dramatic decrease in Ca^{2+} rise (Fig. 8A), indicating an inhibitory effect of MIS. Similar pattern of Ca^{2+} mobilization was observed when experiments were performed in the presence of 4mM Ca^{2+} chelator, EGTA, to eliminate the impact of extracellular Ca^{2+} (Fig. 8B). Cross-linking of the FcγRI on U937 cells expressing MIS (solid line) or in control cells infected with empty vector (dashed line) resulted in similar increase in Ca^{2+} mobilization (Fig. 8C) indicating that the potential to flux calcium is not affected in MIS-expressing U937 cells. Together, these data suggest that the inhibitory effect of MIS on Ca^{2+} flux is due to blocking Ca^{2+} mobilization from the intracellular stores.

DISCUSSION

Although nine human Siglecs have been characterized to date, there are fewer murine family members (siglecs 1-4). With exception of mCD22, there are no other murine siglecs that possess ITIM sequences. Here we describe a novel murine inhibitory siglec that contains an extracellular region comprised of three Ig-like domains, a transmembrane region and a
cytoplasmic tail with two ITIM-like sequences. Since this newly identified protein showed a pattern of expression restricted to myeloid cells, had ITIM sequences, a characteristic feature of an inhibitory receptor, and had an extracellular region similar to that of some siglecs, we have named this protein MIS, a myeloid inhibitory siglec. MIS associates with protein tyrosine phosphatases SHP-1 and SHP-2 via its tyrosine-based signaling motifs and is able to inhibit signaling initiated through the activating receptor on monocytes.

Although formally MIS has not been shown to bind sialic acid residues, the inclusion of this protein in the siglec family is justified by the structure of its extracellular region (characteristic amino-terminal V-set domain followed by C-set domains) and the fact that the amino acid residues involved in ligand binding in the other siglecs (24) are preserved in the V-set domain of MIS.

The presence of ITIM sequences implies the inhibitory nature of a molecule (35). There are two potential ITIMs in the cytoplasmic tail of MIS protein. The proximal ITIM that is formed around tyrosine 431 (IHY\textsuperscript{431}ATL) contains the SHP-1-binding ITIM consensus sequence (I/V/LxYxxL/V) (35, 36) and is similar to those present in some of human siglecs (17-22), as well as in other inhibitory receptors that associate with SHP-1, including killer cell inhibitory receptor, immunoglobulin-like transcript-3, leukocyte-associated inhibitory receptor-1 (10, 37, 38). The distal tyrosine-based sequence of MIS is formed around tyrosine 454, TEY\textsuperscript{454}SEL. It contains a threonine residue in the –2 position, and is similar to the SAP-docking site (TIYXX(V/I)) found in 2B4 and SLAM, activating receptors, present on NK cells and T/B cells,
respectively (39, 40). SAP was shown to compete with a protein tyrosine phosphatase SHP-2 for binding to SLAM and 2B4 receptors thus regulating their function (41, 42). Similar tyrosine-based signaling motifs are also present in a human inhibitory receptor, siglec 3 (TEY$^{342}$SEV), and in the SHP substrate 1, SIRP/SHPS1, (TEY$^{453}$ASI) that were shown to bind both SHP-1 and SHP-2 phosphatases (31, 43). Our results demonstrate that MIS associates with SHP-1 and SHP-2 protein tyrosine phosphatases. Moreover, this association is sustained in non-activated cells most likely due to a basal level of constitutive phosphorylation of MIS ITIMs. Similar constitutive tyrosine phosphorylation of ITIM sequences and association with a SHP-2-containing tyrosine phosphatase was observed in the PIR-B (44) and in the SHPS-1 inhibitory receptor (43). Constitutive tyrosine phosphorylation of the tyrosine-based signaling motifs suggests an association of MIS with a tyrosine kinase. Indeed, such an association has been shown in case with PIR-B and SHPS-1 with Lyn and PYK2 kinases, respectively (44, 45). The nature of a kinase that may associate with MIS requires further investigation.

Human CD33 has been characterized as an inhibitory receptor on myeloid cells based upon its ability to bind inhibitory phosphatases, to inhibit signaling initiated from an activating receptor and to repress dendritic cell development (31, 30, 46). The other siglec family member that binds SHP-1, p75/AIRM1, has been shown to inhibit the proliferation of normal or leukemic myeloid cells (47). Similarly, an inhibitory effect of MIS on Ca$^{2+}$ mobilization may be due to the recruitment of SHP-1 and SHP-2 to the activating complex.
The similar structure of ITIM containing cytoplasmic tails of different siglecs dictates the similarity in their inhibitory function. The specificity of siglecs function may be determined either by their unique extracellular regions and/or by their specific expression patterns. Our data indicates that at least two MIS isoforms with either three or two extracellular Ig-like domains exist. In the KIR family, the splicing of one or more Ig-like domains may result in altered ligand recognition (48). In the Siglec family, the amino-terminal V-set domain is responsible for ligand binding (24), therefore the loss of the second Ig-like domain in a smaller MIS isoform most likely will not affect its ligand binding ability. Sialylation of sialoadhesin and CD22 has been found to regulate their ligand binding activity (49, 50). In smaller MIS isoform, the splicing out of the second Ig-like domain which contains the majority of N-glycosylation sites may result in a lower overall oligosaccharide content, and thus, in decreased levels of sialylation of the molecule. Therefore, the the ligand binding of MIS isoforms may be differently regulated. As far as the expression pattern is concerned, the early onset of MIS expression in bone marrow cells suggests the possibility that it may be involved in the regulation of monocyte development.

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Table 1. Oligonucleotide primers used for cloning and RT PCR analysis of MIS

**Murine CD33 primers***
- mCD33.1 (103-132) 5′-AGGAGGGCCTATGTGTCCATGTGCCCTGCA-3′
- mCD33.2 (507-524) 5′-GGGCTTGTGAGCAGGGG-3′
- mCD33.3 (779-761) 5′-CCCAAGAATCAGGAGCTTG-3′

**MIS primers:**
- M1 (1539-1520) 5′-CACAGAAGGAATGGCTCTGA-3′
- M2 (521-506) 5′-GATGGCCAGCTTCAAG-3′
- M3 (370-352) 5′-CTCTCTGATGTTCAAGGGAG-3′
- M4 (1148-1165) 5′-GTAAGATCCTACAGG-3′
- M5 (1-20) 5′-TGAGAGCCTGAAGAGACAG-3′
- M6 (1496-1475) 5′-CTAGGTGACTCTTTTCTTCTCC-3′
- M7 (94-1113) 5′-AGAGTTTTCACCCTGAATG-3′
- M8 (455-436) 5′-GAGTCATCTTTGGCCTTCTCC-3′
- M9 (745-716) 5′-AGACGGATGGTCTCTCTTCTGTTG-3′
- M10 (1065-1046) 5′-AAAGTTGCACTGCTCTGTGG-3′

**Murine β-actin primers:**
- Sense 5′-GCTGTATTCCCCTCCATCGTG-3′
- Antisense 5′-CACGGTTGGCCCTTAGGTTTCAG-3′

* - all mCD33 primers correspond to mCD33A isoform.

**FIGURE LEGENDS**

**Figure 1.** Molecular cloning of MIS. A, Alignment of the amino acid sequence of the transmembrane region of human CD33 and the deduced amino acid sequence of the mouse EST clone (Gene Bank accession number AA275188). Identical amino acids in the transmembrane
regions are shaded. ITIM sequences are underlined. **B**, Deduced amino acid sequence of MIS. The leader peptide is underlined with dashed line. Potential N-glycosylation sites are shaded. The transmembrane region is underlined with a solid line. ITIM-like sequences are boxed. Arrows indicate the start of the Ig-like domains.

**Figure 2.** MIS is a member of the siglec family. **A**, Alignment of the amino acid sequences of MIS (Gene Bank accession number AF329269), human siglec8 (AF195092), human siglec3 (M23197), and murine CD33 A isoform (AAB30843). Amino acids that are identical among all aligned siglecs are shaded in black; in addition, amino acids that are identical in MIS and human siglec8 are shaded in gray. The start of individual Ig-like domain, as well as the transmembrane and cytoplasmic domains are indicated above the sequence. Charged amino acid residues in the transmembrane domain of mCD33 are indicated by asterisks. ITIM-like sequences are underlined. **B**, A schematic representation of members of siglec family excluding siglecs 1-4 that are homologous in mouse and human. Black circles represent ITIMs, rectangles represent transmembrane regions.

**Figure 3.** Southern blot analysis of MIS. Genomic DNA from C57/BL6 mouse analyzed using the probe corresponding to the first Ig-like domain of MIS.
Figure 4. Tissue and cell expression of MIS. **A**, Northern blot analysis of various mouse tissue mRNA using a full length MIS cDNA probe, or mouse β-actin probe. Arrows indicate various MIS mRNA species. **B**, RT PCR analysis of the murine bone marrow cells (1), peritoneal macrophages (2), T cell lines, EL-4 (3), 3A9H (4), B cell lines, A20 (5), WEHI-231 (6), monocyte/macrophage cell lines J774 (7), WEHI-265.1 (8), NK cells KY-1 (9), KY-2 (10), mastocytoma P815 (11), and a mock reaction (12). cDNA was reverse transcribed using oligo dT primer and amplified using gene-specific sets of primers, mCD33.2 and mCD33.3 for mCD33 and M4 and M6 for MIS.

Figure 5. MIS exists as two alternatively spliced isoforms. **A**, A diagram of MIS protein. Arrow heads indicate the localization of the sequences from which the primers (M7-M10) were designed. TM – transmembrane domain, black rectangles represent ITIMs. **B**, RT PCR analysis of murine bone marrow cells. First strand cDNA was reverse transcribed from bone marrow RNA using MIS-specific primer M1. 1- 100 bp DNA ladder, 2- PCR amplification using M7 and M8 primers, 3- PCR amplification using M7 and M9 primers, 4- PCR amplification using M7 and M10 primers, 5- control without the template cDNA. Arrow indicates the band corresponding to the smaller MIS isoform without the second Ig-like domain.

Figure 6. MIS associates with the SHP-1 and SHP-2 protein tyrosine phosphatases.
U937 cells were retrovirally-infected with either empty vector (vect.) or with Flag-tagged MIS (MIS-flag). Cells were either left untreated or stimulated with sodium pervanadate (PV). Following treatment cells were lysed in 1% NP-40 lysis buffer. Lysates were subjected to immunoprecipitation with anti-Flag antibody. Immunoprecipitates and crude lysates were resolved by 7.5% SDS-PAGE and immunoblotted with the 4G10, anti-Flag, anti-SHP-2 mAbs and an antiserum against SHP-1.

**Figure 7.** The proximal ITIM of MIS is essential for binding to SHP-1 and SHP-2. The wild type cytoplasmic tail of MIS (YY) and the mutants Y431F (FY), Y454F (YF) and YY431,454FF (FF) were expressed as VSVG-fusion proteins (G/MIS) in HeLa cells together with catalytically inactive SHP-1 C453S (A), wild type SHP-1 (B), or wild type SHP-2 (C). Cells were treated with pervanadate for 10 min and then lysed in 1% NP40 lysis buffer; cell lysates were immunoprecipitated with VSVG antibody. Immunoprecipitates and crude lysates were resolved by 7.5% SDS-PAGE and blots were probed with antibodies to VSVG, SHP-1 (in A and B) or SHP-2 (in C), and phosphotyrosine.

**Figure 8.** Calcium mobilization induced by FcγRI receptor cross-linking is inhibited with MIS co-ligation. U937 cells retrovirally-infected with an empty vector (dashed line) or MIS-Flag (black line) were pre-loaded with Fura-2AM and incubated for 30 min either with antibody to FcγRI in combination with anti-Flag antibody (A and B) or with anti- FcγRI antibody alone (C).
\[ \text{[Ca}^{2+}\text{]}, \text{was measured by fluorimetry. Secondary cross-linking anti-mouse antibody was added at the 30 seconds. In some experiments EGTA (4mM) was added at time 0 (B).} \]
Figure 1

A

hS1.1euc3 TH  
EST clone.p  -------------------------------
AGVVHSAGQGVTAIIAALICLRIY  0

hS1.1euc3 TH  
EST clone.p  -------------------------------
ADCSBFLPSILEAAPFSTEEHIHYATLSFHEMKPMNLWGGQQTITTEYSETIFPQRTAWP  0

B

MLLLLLLLLWGIKGVEGQNPOEVFTLNVERKVVPVQEGLCVLPCEFXSYLK  50
KRLTDLTSDLPHGYFWRGRTDDRKSIVATTNNPARKAVKETRNEFFLLG  100
DPWRNDCSLNIREIRKDAHyFFRLERGKTKYMNMDKMTLVVTALTNT  150
PQILLPETLEAGHPSNLTCSVPWDCGWTPAPFIESWGTGVSFLSNTTGS  200
SVLTITPQPOQDHTNLTCQVTLPGTNTVRMTIPLNYTAYPKNLTVITIQ  250
GADSVSTILNGSLPISEGQLSLRCLSTCSYPNALSWSWDNLITCPSK  300
LSKPGGLLELFVPVHLKHSVGVYTCQAIHGLG3QHLISLSLSPQSSATLSMMN  350
GFTYUAGVTLFLSVCILLAVRSYRKPARPAVFAPVPHDLAKVSQON  400
PLVESQADDSEPLPSILEAAPFSTEEH4YAT1SFHEMKPMNLWGGQDT  450
TEYSETIFPQRTAWP  466
Figure 2
Figure 3
Figure 4
Figure 6
Figure 7
Molecular cloning of MIS, a myeloid inhibitory siglec, that binds protein tyrosine phosphatases SHP-1 and SHP-2

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