Identification and Characterization of S2V, a Novel Putative Siglec That Contains Two V Set Ig-like Domains and Recruits Protein-tyrosine Phosphatases SHPs*

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Zhenbao Yu‡§, Ching-Mei Lai¶, Meryem Maoui‡, Denis Banville‡, and Shi-Hsiang Shen¶¶

From the ¶¶Pharmaceutical Sector, Biotechnology Research Institute, National Research Council of Canada, Montreal, Quebec H4P 2R2, Canada and the ¤Department of Pathology and Microbiology, University of Montreal, Saint-Hyacinthe, Quebec J2S 7C6, Canada

We describe the molecular cloning and characterization of S2V, a novel sialic acid binding immunoglobulin-like lectin. The cDNA of S2V encodes a type 1 transmembrane protein with four extracellular immunoglobulin-like (Ig-like) domains and a cytoplasmic tail bearing a typical immunoreceptor tyrosine-based inhibitory motif (ITIM) and an ITIM-like motif. A unique feature of S2V is the presence of two V-set Ig-like domains responsible for the binding to sialic acid, whereas all other known siglecs possess only one. S2V is predominantly expressed in macrophage. In vivo S2V was tyrosine-phosphorylated when co-expressed with exogenous c-Src kinase. Upon tyrosine phosphorylation, S2V recruits both Src homology 2 (SH2) domain-containing protein-tyrosine phosphatases SHP-1 and SHP-2, two important inhibitory regulators of immunoreceptor signal transduction. These findings suggest that S2V is involved in the negative regulation of the signaling in macrophage by functioning as an inhibitory receptor. When expressed in COS-7 cells, S2V was able to mediate sialic acid-dependent binding to human red blood cells, suggesting that S2V may function through cell-cell interaction.

SHP-1† is a nonreceptor protein-tyrosine phosphatase that contains tandem Src homology 2 (SH2) domains at its N terminus, enabling its association with tyrosine-phosphorylated proteins. It is expressed primarily in hematopoietic cells and epithelial cells and acts predominantly as a negative regulator of a broad spectrum of receptors, including receptor protein-tyrosine kinases such as c-Kit (1), colony-stimulating factor (2), epidermal growth factor (3, 4), and platelet-derived growth factor (5, 6) receptors, cytokine receptors such as erythropoietin (7), interleukin-3 (8) and interferon (9) receptors, and immunoreceptors such as the B cell (10), T cell (11), and natural killer (NK) cell (12, 13) receptors. The importance of the inhibitory signals delivered by SHP-1 is highlighted by the moth-eaten (me) mouse, which has natural defect in SHP-1 expression (14). Mice with this deficiency display widespread autoimmune phenomena caused by an inability to negatively regulate immune responses (14). The inhibitory effect of SHP-1 appears to reflect its capacity to associate with and dephosphorylate the activated receptors and/or the signaling molecules in the receptor complexes such as receptor protein-tyrosine kinase in c-Kit signaling (1), Jak kinase in cytokine receptor signaling (7), and Lck, ZAP70, and Syk in antigen receptor signaling (10, 11).

In addition to direct association with the activated receptor complexes and down-regulation of the signaling of the “growth-promoting” receptors, SHP-1 performs its inhibitory function in many hematopoietic cells by associating with inhibitory receptors that bear the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (reviewed in Refs. 10, 12, and 13). Among the ITIM-bearing proteins are sialic acid binding immunoglobulin-like lectins (siglecs), a recently designated superfamily of cell surface molecules. The human siglec family currently comprises nine members, namely, sialoadhesin/siglec-1 (15), CD22/siglec-2 (16, 17), CD33/siglec-3 (18), myelin-associated glycoprotein/siglec-4 (19–21), and the recently identified siglec-5 (22), siglec-6 (23), AIRM1/siglec-7 (24–26), siglec-8 (27), and siglec-9 (28, 29). Each member of the siglec family is characterized by an N-terminal V-set Ig-like domain followed by a variable number of C2-set Ig-like domains ranging from 1 in CD33 to 16 in sialoadhesin. The sialic acid-binding sites are located within the V-set Ig-like domain (30). An arginine residue in the F-strand forms a salt bridge with the carboxylate of sialic acid, and two aromatic amino acids in the A and G strands contribute hydrophobic interactions (31–34). The expression of siglec family members is tightly restricted to specific cell types, implying highly specific, nonoverlapping functions of these receptor proteins (reviewed in Refs. 35 and 36). For example, sialoadhesin is a macrophage-restricted adhesion molecule. Likewise, CD22 is expressed uniquely in B cells, where it has been implicated in modulating the signaling of the B-cell antigen receptor as an inhibitory co-receptor via binding of SHP-1 to its ITIM (37). CD33 was identified as a marker of early myeloid progenitor cells. Recently, it has been characterized as inhibitory receptor by virtue of its ability to bind SHP-1 and SHP-2 (38–40).

Although the biological role of SHP-1 has been much appreciated, the mechanism for the regulation of the cell signaling by SHP-1 remains to be uncovered. This will largely rely on the identification of its authentic targets and the determination of

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† Both authors contributed equally to this work.

‡ To whom correspondence should be addressed: Pharmaceutical Sector, Biotechnology Research Institute, National Research Council of Canada, Montreal, Quebec, Canada H4P 2R2. Tel.: 514-496-6318; Fax: 514-496-6319; E-mail: shi.shen@nrc.ca.

The abbreviations used are: 1 SHP, src homology 2 (SH2) domain-containing protein-tyrosine phosphatase; Ig, immunoglobulin; siglec, sialic acid binding Ig-like lectin; ITIM, immunoreceptor tyrosine-based inhibitory motif; WT, wild type; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RBC, red blood cell; WB, Western blot; PAGE, polyacrylamide gel electrophoresis; NK, natural killer.
the effect of dephosphorylation on the cell signaling. Therefore, we have performed a yeast two-hybrid screen using SHP-1 as bait and identified several SHP-1-binding proteins. One of them is a novel member of the siglec receptor family, designated as S2V. The high level of expression in macrophage and the presence of ITIM in the cytoplasmic tail suggest that S2V may be involved in the negative regulation of the signaling in myeloid cells. S2V is capable of mediating sialic acid-dependent binding to human red blood cells and recruits both SHP-1 and SHP-2 upon the tyrosine phosphorylation of its ITIM motif, and it is an in vivo substrate of SHP-1 but not of SHP-2.

EXPERIMENTAL PROCEDURES

Materials and Cell Lines—The human embryonic kidney epithelial cell line 293A and the African green monkey cell line COS-7 were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Nitrocellulose membrane Hybond-C and anti-mouse IgG-horseradish peroxidase along with enhanced chemiluminescence (ECL) kit were from Amersham Pharmacia Biotech. Protein A-Sepharose CL-4B was from Amersham Pharmacia Biotech. Rabbit anti-SHP-1 polyclonal antibody was generated as described previously (5). Mouse anti-SHP-1 and anti-SHP-2 monoclonal antibodies were obtained from Transduction Laboratories (Lexington, KY). Anti-phosphotyrosine (4G10) and anti-Myc (9E10) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). N-Glycosidase F and Vibrio cholerae sialidase were from Roche Molecular Biochemicals. Tunicamycin and sodium orthovanadate were purchased from Sigma.

Human Red Blood Cell Binding Assay—COS-7 cells growing in 60-mm dishes were transfected with the S2V-Myc expression plasmid using SuperFect™ transfection reagent from Qiagen Inc. (Chatsworth, CA). Twenty-four hours after transfection, the cells in each dish were trypsinized and plated on six 60-mm dishes. Human red blood cells (RBCs) were obtained from healthy donors and washed three times with phosphate-buffered saline containing 0.25% bovine serum albumin. Binding assay was carried out at 48–72 h after transfection. Briefly, the COS-7 cells were washed with phosphate-buffered saline once, and then 1 ml of 0.25% (w/v) suspension of RBCs in Dulbecco's modified Eagle's medium containing 0.2% bovine serum albumin was added to the dish. After 30 min at 37 °C, the nonadherent RBCs were removed by five gentle washes with phosphate-buffered saline containing 0.25% bovine serum albumin. Sialidase pretreatment of COS-7 cells and RBCs was performed by incubating both cells with 0.1 unit/ml V. cholerae sialidase in serum-free Dulbecco's modified Eagle's medium containing 0.2% bovine serum albumin for 2 h at 37 °C followed by three washes with phosphate-buffered saline containing 0.25% bovine serum albumin. The binding was examined under a microscope and quantified by counting the COS-7 cells that bound at least five RBCs. For each dish, 20 fields were counted. Results were expressed as the total number of rosettes from 20 scoring fields.

Transfection and Treatment of 293A Cells—293A cells were transfected with different sets of plasmid DNA by standard calcium phosphate precipitation methods. In some experiments, the transfected cells were treated with 0.5 mM Pefabloc (Calbiochem) 2 h before exposure to tunicamycin. Sodium pervanadate was prepared by mixing 100 mM sodium orthovanadate and 50 mM H2O2 and incubating the mixture at room temperature for at least 30 min. The treatment of the transfected cells with tunicamycin was performed by incubating the cells with tunicamycin in regular medium for 24 h at 37 °C. Tunicamycin was dissolved in 95% ethanol at a concentration of 1 mg/ml. For control experiments, ethanol was added to the medium to a final concentration of 1%.

RESULTS

Cloning of S2V—To identify proteins that interact with SHP-1 in a tyrosine phosphorylation-dependent manner, we carried out a yeast two-hybrid screen with the expression of an exogenous protein-tyrosine kinase in yeast (42). The full-length SHP-1 with mutation of cysteine 455 to serine (SHP-1-C455S), which abolishes the protein-tyrosine phosphate catalytic activity but retains the binding ability to its substrates, was
cloned into plasmid pBTM-116-Src (42). Transformation of the plasmid in yeast results in the expression of LexA DNA binding domain-SHP-1-C455S fusion protein and c-Src kinase (43, 44). From 1.1 x 10^7 primary transformants with a human Jurkat cDNA library fused to the GAL4 activation domain in pACT2 vector, 124 colonies were positive for both HIS3 and LacZ expression. Sequence analysis revealed that most of them correspond to proteins previously identified as interacting with SHP-1, such as LAIR1 (45), Grb2 (46), PECAM-1 (47), and PILRα (48). The other SHP-1-binding proteins identified are SHP-2 and an unknown protein. The unknown protein was identified as a new member of the siglec family (44) showing 83% identity to a sequence with GenBank™ accession number AI132995 in the Expressed Sequence Tag data base over the overlapping region. To clone the full-length cDNA representing the Expressed Sequence Tag fragment, we carried out 5'-RACE experiments. The 5'-end of the cDNA was isolated from human liver cDNA. Four RACE fragments representing various lengths of the same cDNA were sequenced. The deduced full-length cDNA, named S2V (Fig. 1A), is 2145 base pairs in length with a reading frame that encodes a protein of 595 amino acid residues. The sequence of the coding region was confirmed.
through sequencing of three independent reverse transcription-PCR products.

Sequence analysis revealed that S2V is a type 1 transmembrane protein (Fig. 1A). The N-terminal 17 amino acids constitute a signal peptide. The residues 482–502 are highly hydrophobic and predicted a transmembrane segment. The entire putative extracellular region consists of four homologous Ig-like domains. The two N-terminal Ig-like domains are homologous to an Ig V domain, whereas the two C-terminal Ig-like domains resemble an Ig C2 domain. The extracellular domain contains six potential N-linked glycosylation sites (NXST, where X is any amino acid but proline). In the cytoplasmic region, S2V contains an ITIM consensus sequence (I/L/V)YXX(L/V) from residue 563 to 568 (IQYASL) and a sequence similar to the ITIM consensus sequence from residue 586 to 591 (YEYSEI).

Chromosomal Organization of S2V—The genomic DNA sequence of S2V was identified by searching the GenBank database. The S2V gene, consisting of seven exons spanning 8.4–8.5 kilobases (Fig. 2A), is localized in human chromosome 19 clone CTD-3073N11. All exon-intron boundaries strictly conform to the GT-AG rule. The exons ranging from 48 to 447 base pairs are closely related to the protein domains (Fig. 2A). Exon 1 consists of the 5’-terminal non-coding sequence and the sequence encoding the first V-set Ig-like domain. Exon 2, exon 3, and exon 5 encode the second V-set, the first C2-set, and the second C2-set Ig-like domains, respectively. Exon 4, the smallest exon,
encodes a short linker between the first and the second C2-set Ig-like domains. All introns (1, 2, 3, 4, and 5) between the exons that encode the extracellular domains of S2V are shown to be primarily class I, where each exon interrupts the coding sequence between the first and second bases of the codon. This suggests that any alternative splicing between these exons and introns will give a protein that has s distinct number of Ig-like domains but retains the integral transmembrane and intracellular domains. However, we failed to identify any other alternative splicing cDNA fragments by reverse transcription-PCR (data not shown), suggesting that the cDNA we cloned represents the major, if not the only, transcript. Exon 6 encodes the transmembrane domain. Exon 7 encodes the first part of the cytoplasmic domain, and exon 8 encodes the second part of the cytoplasmic domain where the ITIM and ITIM-like motifs are located.

S2V mRNA Is Abundantly Expressed in Macrophage Cells—As a first step in understanding the biological function of S2V, we analyzed the expression pattern of this gene in a variety of tissues. Using Northern blot analysis, we observed a band of $\sim$2.2 kilobases corresponding to the S2V mRNA in spleen (Fig. 2B). We failed to detect any S2V mRNA in any other tissues examined. This suggests that S2V is very likely expressed in hematopoietic cells since spleen is rich in hematopoietic cells. We further analyzed its expression in distinct types of hematopoietic cells. As shown in Fig. 2B, S2V is expressed abundantly in 28SC, a macrophage/megakaryocyte cell line established from human peripheral blood mononuclear cells, and KG-1, a cell line derived from acute myelogenous leukemia and spontaneously differentiated to macrophage and granulocyte-like cells. A very low level of expression was also detected in NK92-Ci, a natural killer cell line, and K-S62, a cell line established from chronic myelogenous leukemia and characterized as highly undifferentiated and of the granulocytic series. However, it was not detected in any of the B cell and T cell lines analyzed. Taken together, S2V is mainly expressed in differentiated myeloid, and it is abundant in macrophage/megakaryocyte. In addition to the main band ($\sim$2.2 kilobases), a band of smaller size (1.8–2.0 kilobases) was observed in NK92-Ci and KG-1 cell lines.

Based on several PCR-based experiments employing several experimental approaches, this smaller transcript appears to be the result of nonspecific binding.

S2V Expressed in 293A Cells Is Glycosylated—For the expression of S2V in mammalian cells and the detection of its expression, S2V was epitope-tagged with Myc at the C terminus (S2V-Myc). Western blot analysis revealed that S2V was expressed in 293A cells with an apparent molecular mass of $\sim$80–100 kDa under reducing conditions. The apparent molecular mass is much higher than that predicted from the deduced amino acid composition ($\sim$70 kDa), suggesting that S2V-Myc is very likely to be modified after its translation. As S2V contains six potential N-linked glycosylation sites (NX(S/T) Where X is any amino acid except proline) in the extracellular region, we first treated the cells with tunicamycin, an inhibitor of N-type glycosylation, and examined the effect of this treatment on the size of S2V. As shown in Fig. 3A, incubation of the cells with tunicamycin resulted in a reduction of the apparent molecular mass of S2V-Myc to $\sim$70 kDa in a dose-dependent manner. To further confirm the glycosylation of S2V, we treated the cell lysate of S2V-Myc-transfected 293A cells with peptide N-glycosidase F. As shown in Fig. 3B, the apparent molecular mass of the peptide N-glycosidase F-treated S2V was reduced to $\sim$70 kDa.

All siglecs contain several cysteine residues within their extracellular domain and a single cysteine residue in the transmembrane region, and it has been demonstrated that some siglecs could beimerized through disulfide bond (22, 23, 44).

To determine whether S2V also forms a disulfide-linked dimer, we run a SDS-PAGE gel in nonreduction conditions. As shown in Fig. 3C, the apparent molecular mass of S2V under nonreduction conditions was almost the same as that under reduction conditions. This suggests that S2V does not form a disulfide-linked dimer in 293A cells.

S2V Mediates Sialic Acid-dependent Binding to Human Red Blood Cells—To investigate the sialic acid binding property of S2V, we transiently transfected S2V-Myc or control plasmid (pcDNA3) into COS-7 cells. The transfected cells were assayed for binding to human RBCs. RBCs are rich in sialic acid. The COS-7 cells with bound RBCs were examined under a microscope. The results are expressed as the total number of COS-7 cells that bound at least five RBCs from 20 observed fields. As shown in Fig. 4, the number of RBC binding COS-7 cells in the S2V-transfected cells is 4–5 times more than that in the nontransfected or the empty vector pcDNA3-transfected cells. This result suggests that S2V is able to mediate the interaction of COS-7 cells with RBCs. Pretreatment of RBCs with sialidase resulted in a decrease in the number of RBC binding to COS-7 cells to a level similar to that of the nontransfected cells, suggesting that the binding is sialic acid-dependent. The treatment of COS-7 cells with sialidase did not significantly affect the binding, suggesting that the binding of COS-7 cells with RBCs was not blocked by cis interaction of S2V with the sialic acid existing on the surface of COS-7 cells. The crystal structure of the V-set Ig-like domain of siglec-1 revealed that an
arginine residue in the F β-strand forms a salt bridge with carboxylate group of sialic acid (34). This arginine residue is therefore critical for the binding of siglecs with sialic acid (31–34) and conserved in all siglec members reported. However, in S2V the arginine residue at this position is replaced by cysteine (Cys-122) at the first V-set Ig-like domain and by glutamine (Gln-249) at the second one (Fig. 1B). To determine which V-set Ig-like domain contributes to the interaction, we mutated these two potential critical amino acid residues, Cys-122 and Gln-249 to alanine and examined the binding of the mutants to RBCs. In comparison with the wild type S2V, either the mutation in Cys-122 or the mutation in Gln-249 dramatically reduced but not completely abolished the binding of the transfected COS-7 cells to RBCs (data not shown), suggesting that both of the first and the second Ig-like domains are involved in the interaction of S2V with its ligands.

S2V Associates with SHP-1 in 293A Cells—To determine whether S2V associates with SHP-1 in mammalian cells, we co-transfected S2V-Myc with wild type and catalytically inactive SHP-1 in 293A cells. The transfected cells were treated with pervanadate to prevent the tyrosine dephosphorylation of cellular proteins. SHP-1 was immunoprecipitated with an anti-SHP-1 polyclonal antibody. As shown in Fig. 5B, S2V-Myc was detected by Western blot with anti-Myc antibody in the anti-SHP-1 immunoprecipitant of the 293A cells co-transfected with both SHP-1 catalytically inactive mutant (SHP-1-C455S) and S2V-Myc. The precipitation of S2V was through the specific interaction with SHP-1-C455S because it could not be precipitated from the cells co-transfected with S2V or SHP-1-C455S alone (Fig. 5B). The expression level of both S2V and SHP-1-C455S in each transfected cell is comparable to that shown in Fig. 5A. The interaction is tyrosine phosphorylation-dependent since the two proteins could not be co-precipitated from the cells without treatment with pervanadate (Fig. 5B). Reciprocal experiments further confirmed the association of S2V with the catalytically inactive SHP-1 (Fig. 5C). However, S2V could not be co-precipitated with wild type SHP-1 (SHP-1 WT) by either anti-SHP-1 antibody (Fig. 5B) nor anti-Myc antibody (Fig. 5C). This indicates that S2V is likely to be a substrate of SHP-1, and the rapid dephosphorylation of S2V prevents it from association with SHP-1. Hence we detected the tyrosine phosphorylation level of S2V by Western blot using anti-phosphotyrosine antibody. Fig. 5C shows that the level of the tyrosine phosphorylation of S2V in the SHP-1 WT-transfected cells is indeed much lower than that in the SHP-1-C455S-transfected cells.
by a reduced phosphorylation in S2V-Y565F-Myc as compared with that in S2V-Y588F-Myc. It is due to a lower affinity of SHP-1-C455S for Tyr-588 than that for Tyr-565. Taken together, these results indicate that S2V recruits SHP-1 through the membrane proximal ITIM.

Tyrosine Phosphorylation of S2V Can Be Mediated by c-Src—The phosphorylation of S2V by the inhibition of the activity of cellular protein-tyrosine phosphatases could be mediated by any protein-tyrosine kinases expressed in the cells. We then determined which protein tyrosine kinases are involved in the phosphorylation of S2V in mammalian cells. Because several ITIM-containing proteins, such as LAIR-1, PECAM-1, PILRa (43), and mSiglec-E (44) were identified as SHP-1-binding proteins in our modified yeast two-hybrid screen with the expression of c-Src in yeast, we first tested if c-Src is also involved in the in vivo phosphorylation of S2V in 293A cells. To do so, we co-transfected S2V-Myc and SHP-1-C455S with two ubiquitously expressed Src family kinases, c-Src and Fyn, respectively, in 293A cells and detected the phosphorylation level of S2V-Myc and other cellular proteins by Western blot with anti-phosphotyrosine antibody. As shown in Fig. 7A, S2V-Myc was tyrosine-phosphorylated by co-expressing c-Src in 293A cells (lane 8). However, S2V-Myc could not be phosphorylated by Fyn (Fig. 7A, lane 9), although several other proteins (Fig. 7A, lane 5) including SHP-1-C455S (Fig. 7A, lane 6) were phosphorylated by Fyn, as shown in the Western blot of whole cell lysates (WCL) and anti-SHP-1 precipitant with anti-phosphotyrosine antibody. Meanwhile, we assessed the ability of the c-Src-phosphorylated S2V to bind to SHP-1. As shown in Fig. 7B, S2V-Myc was detected by Western blot with anti-Myc antibody in the anti-SHP-1 immunoprecipitant from the 293A cells that was co-transfected with S2V-Myc, SHP-1 C455S, and c-Src (lane 5) but not from the cells without exogenous expression of c-Src (lane 4 and 6). This interaction was further confirmed by reciprocal experiments, although the level of SHP-1-C455S co-precipitated with S2V-Myc by anti-Myc antibody is very low (Fig. 7C, lane 8). The expression levels of both siglec-Myc and SHP-1-C455S in each of the transfected cells were comparable, as shown in Fig. 7, B and C (lanes 1, 2, and 3). S2V-Myc and SHP-1-C455S were more easily co-precipitated by anti-SHP-1 antibody than by anti-Myc antibody, probably due to the fact that the Myc tag is very close to the ITIM (SHP-1-binding site) in S2V-Myc, and hence, the binding of SHP-1 to this ITIM may affect the antibody to access the Myc tag. Taken together, these data suggest that c-Src is involved in the phosphorylation of S2V, which leads to the interaction of S2V with SHP-1.

S2V Associates with SHP-2—SHP-1 and SHP-2 are the only two SH2 domain-containing protein-tyrosine phosphatases identified in mammalian cells. In many cases, they bind to tyrosine-phosphorylated proteins with similar consensus sequences. We determined whether S2V also associates with SHP-2. As shown in Fig. 8A, SHP-2 was co-precipitated with

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**Fig. 6.** S2V binds to SHP-1 through the membrane proximal ITIM. 293A cells were transfected with S2V-Myc or its mutants and SHP-1 C455S as indicated. At 48 h after transfection, the cells were treated with 0.5 mM pervanadate for 30 min. Whole cell lysates were subjected to immunoprecipitation (IP) and Western blot (WB) analysis as described under "Experimental Procedures." Molecular mass (kDa) was indicated to the left of the gel. pTyr, Tyr(P).

**Fig. 7.** Tyrosine phosphorylation of S2V by c-Src in 293A cells. 293 cells were transfected with S2V-Myc, SHP-1 C455S, and c-Src or Fyn as indicated. The cells were grown in regular Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum for 48 h after transfection and then lysed without any treatment. SHP-1-C455S and S2V-Myc were immunoprecipitated (IP). Whole cell lysates (WCL, lanes 1, 2, and 3), anti-SHP-1 precipitands (lanes 4, 5, and 6) and anti-Myc precipitants (lanes 7, 8, and 9) were subjected to Western blot (WB) analysis with anti-phosphotyrosine antibody (A), anti-Myc antibody (B), and anti-SHP-1 antibody (C) as described under "Experimental Procedures." Molecular mass (kDa) was indicated to the left of the gel.
S2V, a Myeloid Siglec

Fig. 8. S2V associates with SHP-2. 293A cells were transfected with S2V-Myc and wild type SHP-2 (SHP-2 WT) or catalytically inactive SHP-2 (SHP-2 C459S) as indicated. At 48 h after transfection, the cells were optionally treated with 0.5 mM pervanadate for 30 min. Whole cell lysates were subjected to immunoprecipitation (IP) and Western blot (WB) analysis as described under “Experimental Procedures.” Molecular mass (kDa) is indicated to the left of the gel.

Discussion

In this study, we identified S2V, the first siglec that contains two V-set Ig-like domains. Because siglecs bind their ligands sialic acids through the V-set Ig-like domain and all siglecs identified previously have only one V-set Ig-like domain, the presence of dual V-set Ig-like domains in S2V indicates that S2V has a unique feature in the ligand binding specificity. Accumulating data revealed that an arginine residue in the Fβ-strand of V-set Ig-like domain of siglecs forms a salt bridge with the carboxylate group of sialic acid, and two aromatic amino acids in A and G β-strands contribute hydrophobic interactions (31–34). The arginine is conserved in all siglec members identified before. However, the arginine residues are not conserved in either the first or the second V-set Ig-like domains of S2V. They are substituted by cysteine (Cys-122) in the first V-set Ig-like domain and glutamine (Gln-249) in the second one. The guanidino group of arginine 97 in sialoadhesin/siglec-1 forms a salt bridge with the carboxylate of sialic acid (34). At present, the interaction mechanism of S2V with sialic acid is not clear. Interestingly, a similar salt bridge can be formed through the glutamine and the carboxylate of sialic acid (Scheme 1). However, if Cys-122 also contributes to the interaction, the mechanism must be different since cysteine is not able to form such a salt bridge with carboxylate.

The biological significance of the existence of dual V-set Ig-like domains in S2V remains to be investigated. Each member of the siglec family shows a distinct specificity for the type of sialic acid with which it interacts. Sialic acids occur naturally in ~30–40 different forms. It has been shown that siglecs exhibit a preference for the glycosidic linkage of sialic acid to adjacent sugars (reviewed in Refs. 35 and 36). Sialoadhesin/siglec-1, CD33/siglec-3, and myelin-associated glycoprotein/siglec-4 bind preferentially to oligosaccharides terminating with α2,3-linked sialic acid, whereas CD22/siglec-2 binds only to the α2,6-linkage (35, 36). Determining which V-set Ig-like domain in S2V binds sialic acid and its preferential type of oligosaccharide linkage is currently under investigation.

Recently, several members of the human siglec family were identified either by searching homologous sequences in DNA data bases such as siglec-5 (22), siglec-7 (25, 26), siglec-8 (27), and siglec-9 (28, 29) or by expression cloning such as siglec-6 (23) and AIRM1/siglec-7 (24). In comparisons of primary structures with those of the previously reported members, all the newly identified members more closely related to CD33/siglec-3 than to sialoadhesin/siglec-1, CD22/siglec-2, or myelin-associated glycoprotein/siglec-4. For instance, CD33/siglec-3 and all of the new members, with the exception of siglec-8, contain a cytoplasmic tail with 83–93 amino acid residues, which bears a membrane proximal ITIM (L/V)(H/Q)YA(N/T/P/Y)AXYXX(L/V). The membrane distal ITIM-like domain, with T/N/Y instead of L/I/V/S at position −2, is different from but similar to the typical ITIM consensus sequence (S/L/V/Y)EYXX(I/V). It has been proved that both the membrane proximal ITIM and the membrane distal ITIM-like motifs in CD33/siglec-3 and mouse mSiglec-E are able to bind to the SH2 domains of SHP-1 and SHP-2 (39, 44). However, in this study, we could not detect the binding of SHP-1 and SHP-2 to the membrane distal ITIM-like motif of S2V. A probable explanation is that the amino acid residue at position −2 is tyrosine in S2V but threonine in CD33 and mSiglec-E. Compared with tyrosine, threonine is relatively close in structure to leucine, isoleucine, valine, and serine. In most members (siglec-3, -5, -6, -9) of the CD33-related siglec subfamily except for siglec-7 and S2V, the amino acid residue at the −2 position of the membrane distal ITIM-like motif is threonine. In siglec-7, it is asparagine (24–26). Although it has been reported that siglec-7 is able to associate with SHP-1 (24), it is unknown whether the membrane distal ITIM can also mediate this interaction. If the CD33-related siglecs share the same mechanism in the intracellular signal transduction, the major function of the membrane distal ITIM-like motif is un-
likely to recruit SHP-1 and SHP-2, and it may mediate the interaction of siglecs with other proteins.

It has been reported that some ITIM-bearing proteins associate with both SHP-1 and SHP-2 (39, 48), whereas others associate only with one of them (24, 49, 50). Three members of the CD33-like siglecs have so far been reported to recruit the SH2 domain-containing protein-tyrosine phosphatases. Human CD33/siglec-3 and mouse siglec-E associate with both SHP-1 and SHP-2 (39, 44), whereas AIRM1/siglec-7 only associates with SHP-1 (24). In this study, we have shown that S2V strongly associates with both SHP-1 and SHP-2. However, an obvious difference is that S2V can be rapidly dephosphorylated by SHP-1 but not by SHP-2. This suggests that SHP-1 and SHP-2 have different effects on the S2V-mediated signaling. Different from S2V, both CD33 and mSiglec-E could be precipitated with wild type SHP-1, suggesting that S2V is more easily dephosphorylated by SHP-1 than are CD33 and mSiglec-E. Since SHP-1 and SHP-2 have distinct functions in the regulation of signal transduction in conjunction with their substrate specificity, further analysis of the association of each siglec with SHP-1 and SHP-2 will help us to understand the function of individual siglec members in the signal transduction.

The expression of siglec family members is tightly restricted to specific cell types, implying highly specific, nonoverlapping functions of these receptor proteins (reviewed in Refs. 30 and 31). For example, sialoadhesin is a macrophage-restricted adhesion molecule. Likewise, CD22 is expressed uniquely in B cells where it has been implicated in cell adhesion and in modulating the signaling of the B-cell antigen receptor as an inhibitory co-receptor (37). CD33 is a marker of early myeloid progenitor cells and probably an inhibitory receptor (38–40).

AIRM1/siglec-7 is expressed predominantly in NK cells and may negatively regulate NK cell functions (24, 25). In this study, we found that S2V is highly expressed in macrophage. Since S2V contains an ITIM in the cytoplasmic domain and is able to recruit SHP-1, a major negative regulator of immune signaling, it is very possible that S2V is an inhibitory receptor and negatively regulates macrophage functions. So far, several ITIM-containing inhibitory receptors such as PIR-B, SHPS-1, LIR-1, and LIR-2 have been identified in macrophage. By recruiting SHP-1 to the membrane, these ITIM-containing receptors in macrophage may share similar intracellular signal transduction processes, but the diversity of the structure in the extracellular regions can be attributed to the protection of undesirable self-reactivity of many different cell types with a broad spectrum of molecules on their surface.

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