mSiglec-E, a novel mouse CD33-related siglec (sialic acid-binding immunoglobulin-like lectin) that recruits Src homology 2 (SH2)-domain-containing protein tyrosine phosphatases SHP-1 and SHP-2

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The sialic acid-binding immunoglobulin-like lectins (siglecs) represent a recently defined distinct subset of the immunoglobulin superfamily. By using the Src homology 2 (SH2)-domain-containing protein tyrosine phosphatase SHP-1 as bait in a yeast two-hybrid screen, we have identified a new member of the mouse siglec family, mSiglec-E. The mSiglec-E cDNA encodes a protein of 467 amino acids that contains three extracellular immunoglobulin-like domains, a transmembrane region and a cytoplasmic tail bearing two immunoreceptor tyrosine-based inhibitory motifs (ITIMs). mSiglec-E is highly expressed in mouse spleen, a tissue rich in leucocytes. The ITIMs of mSiglec-E can recruit SHP-1 and SHP-2, two inhibitory regulators of immunoreceptor signal transduction. This suggests that the function of mSiglec-E is probably an involvement in haematopoietic cells and the immune system as an inhibitory receptor. When expressed in COS-7 cells, mSiglec-E was able to mediate sialic acid-dependent binding to human red blood cells, suggesting that mSiglec-E may function through cell–cell interactions. In comparison with the known members of the siglec family, mSiglec-E exhibits a high degree of sequence similarity to both human siglec-7 and siglec-9. The gene encoding mSiglec-E is localized in the same chromosome as that encoding mouse CD33. Phylogenetic analysis reveals that neither mouse mSiglec-E nor CD33 shows a clear relationship with any human siglecs so far identified.

Key words: glycosylation, inhibitory receptor, sialic acid, tyrosine phosphorylation.

INTRODUCTION

The sialic acid-binding immunoglobulin-like lectins (siglecs) are a recently designated superfamily of cell surface molecules. The siglec family currently comprises nine members, namely sialoadhesin (siglec-1) [1], CD22 (siglec-2) [2,3], CD33 (siglec-3) [4], myelin-associated glycoprotein (siglec-4) [5–7] and the recently identified siglec-5 [8], siglec-6 [9], AIRM1 (adhesion inhibitory receptor molecule 1)/siglec-7 [10,11], siglec-8 [12] and siglec-9 [13,14]. Each member of the siglec family is characterized by an N-terminal V-set immunoglobulin-like domain followed by a variable number of C2-set Ig-like domains, ranging from one in CD33 to 16 in sialoadhesin. All of these siglecs contain an unusual arrangement of conserved cysteine residues in the V-set Ig-like domain that is unique within the Ig superfamily (reviewed in [15]). These give rise to an intrasheet disulphide bond between the B and E β-strands, instead of the intersheet disulphide bridge between the B and F β-strands classically found in immunoglobulins [15].

Site-directed mutagenesis screening [16–19], X-ray crystallography [20] and NMR studies [21] have identified the sialic acid-binding sites in sialoadhesin, CD22 and CD33. The binding sites are located within the V-set Ig-like domain. An arginine residue in the F β-strand forms a salt bridge with the carboxylate of sialic acid, and two aromatic amino acids in the A and G β-strands contribute hydrophobic interactions. The arginine residue and the two aromatic amino acids (Trp, Tyr or Phe) are conserved in all siglec members. The expression of siglec family members is tightly restricted in specific cell types, implying highly specific, non-overlapping functions of these receptor proteins (for reviews, see [22,23]). 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 signalling of the B-cell antigen receptor as an inhibitory co-receptor. CD33 is a marker of early myeloid progenitor cells. AIRM1/siglec-7 is expressed predominantly in natural killer cells, and may negatively regulate natural killer cell functions [10,11,24].

While all members of the siglec family are notable for a high degree of structural similarity in their extracellular domains, some siglecs also share appreciable structural features in their intracellular regions. Most of the siglec members contain one or more immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tails. Studies have shown that, upon tyrosine phosphorylation, ITIM binds to the Src homology 2 (SH2) domains of SHP-1 (SH2-domain-containing protein tyrosine phosphatase-1) and/or SHP-2 (reviewed in [25–31]).

SHPs are key components in the regulation of the signal transduction pathways of multiple receptors, including receptor protein tyrosine kinases, cytokine receptors, T cell receptors, B cell receptors and natural killer cell receptors (reviewed in [25–31]). In particular, SHP-1 negatively regulates the signalling of these receptors by binding to the tyrosine-phosphorylated ITIMs of co-receptors, such as FcγRIIB1 [32], CD22 [33], killer

Abbreviations used: AIRM, adhesion inhibitory receptor molecule; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, haemagglutinin; ITIM, immunoreceptor tyrosine-based inhibitory motif; RACE, rapid amplification of cDNA ends; RBC, red blood cell; SH2, Src homology 2; SHP, SH2-domain-containing protein tyrosine phosphatase; siglec, sialic acid-binding immunoglobulin-like lectin.

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The nucleotide sequence data reported will appear in the GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under accession number AF317298.

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cell inhibitory receptors (reviewed in [28–31]) and paired Ig-like receptor B [34,35], and dephosphorylating the receptors or target proteins recruited by the receptors.

In the present study, we have performed a yeast two-hybrid screen using SHP-1 as bait and identified a novel member of the siglec family, designated mSiglec-E for the fifth mouse siglec. The high level of expression in spleen and the presence of ITIMs in the cytoplasmic tail suggest that mSiglec-E may be involved in the regulation of immunoreceptor signalling. We demonstrate that mSiglec-E is capable of mediating sialic acid-dependent cell–cell interaction and of recruiting both SHP-1 and SHP-2 upon the tyrosine phosphorylation of its ITIM motifs.

MATERIALS AND METHODS

Materials and cell lines

293A, COS-7 and M1 (a transgenic mouse kidney cortical duct cell line) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) fetal bovine serum. Nitrocellulose membrane Hybond-C, horseradish peroxidase-conjugated anti-mouse IgG and an enhanced chemiluminescence (ECL®) kit were from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). Protein A–Sepharose CL-4B and glutathione–Sepharose 4B were from Pharmacia Biotech Inc. (Uppsala, Sweden). Rabbit anti-SHP-1 polyclonal antibody was generated as described previously [36]. Mouse anti-SHP-1 and anti-SHP-2 monoclonal antibodies were obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Anti-phosphotyrosine (4G10), anti-haemagglutinin (HA) (12CA5) and anti-myc (9E10) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). N-glycosidase F and *Vibrio cholera* sialidase were from Boehringer Mannheim (Mannheim, Germany). Tunicamycin and sodium orthovanadate were purchased from Sigma (St. Louis, MO, U.S.A.).

Yeast two-hybrid screen

The cDNA encoding full-length SHP-1 with a cysteine-455 to serine mutation (SHP-1-C455S) was PCR-amplified from the corresponding plasmid [37,38] and cloned in-frame downstream of the DNA-binding domain of LexA in vector pBTM-116-src [39] to form the bait construct (LexA-SHP-1-C455S) [40]. The human Jurkat cDNA library expressed as fusion proteins with the activation domain of GAL4 in the pACT2 vector was obtained from Clontech Laboratories (Palo Alto, CA, U.S.A.). The bait DNA and library DNA were sequentially transformed into yeast strain L40α, and 1.1×10^7 primary transformants were screened for growth on medium lacking leucine, tryptophan and histidine. The positive colonies were screened further for the expression of β-galactosidase. The plasmid DNA was recovered from $His^+/LacZ^+$ colonies and identified by DNA sequencing.

5′-Rapid amplification of cDNA ends (5′-RACE)

5′-RACE was carried out using the Clontech 5′-RACE system with Marathon-Ready™ cDNA made from mouse kidney (Clontech Laboratories Inc.). The first-round PCR was performed by using the antisense primer 5′-CCAGCCACGGTCATGGCCATGCGGTCC and anchor primer 1 (AP1, provided with the kit). The second-round PCR (nested PCR) was performed by using the antisense primer 5′-CTCCGTGGTAGTGTCCTGTTGCCC and anchor primer 2 (AP2, provided with the kit). The PCR product was cloned into pGEM-T vector (Promega Corp., Madison, WI, U.S.A.) and sequenced.

Northern blot analysis

A mouse multiple-tissue Northern blot containing approx. 2 μg of poly(A)$^+$ RNA per lane was obtained from Ambion Inc. (Austin, TX, U.S.A.). The mSiglec-E cDNA with the full coding region and human β-actin cDNA were labelled with ^{32}P by using the Strip-EZ™ DNA kit from Ambion Inc. according to the manufacturer's instructions. Hybridization was performed in ULTRAhyb™ solution from Ambion Inc. at 42 °C overnight. The blots were washed twice with $2 \times SSC/0.1$ % SDS for 10 min ($1 \times SSC$ is 0.15 M NaCl/0.015 M sodium citrate) and twice with $0.1 \times SSC/0.1$ % SDS for 15 min at 42 °C.

Construction of plasmids for mammalian cell expression

Plasmids expressing SHP-1 and the catalytically inactive mutant with a cysteine-455 to serine mutation (SHP-1-C455S) were constructed as described previously [38]. Full-length mSiglec-E was amplified by reverse transcription–PCR and inserted into the *Hin*dIII site of the pcDNA3.1/myc-His(−) C vector (Invitrogen, Carlsbad, CA, U.S.A.) or the pACTAG-2 vector, which contains three repeats of a HA tag. The constructed plasmids express mSiglec-E with a myc tag or a HA tag respectively at the C-terminus (mSiglec-E–myc and mSiglec-E–HA respectively).

Human red blood cell (RBC) binding assay

COS-7 cells were co-transfected with the mSiglec-E–myc and the green fluorescent protein (GFP) expression plasmids, in the proportions mSiglec-E–myc/GFP = 25:1, by standard calcium phosphate precipitation methods, except that the cells were trypsinized and plated just 2 h before transfection. At 48 h after transfection, the cells were trypsinized and washed three times with serum-free DMEM. Human RBCs were obtained from a healthy donor and washed twice with PBS and once with serum-free DMEM. The cells were mixed in serum-free DMEM (2×10^7 RBCs and 1×10^6 COS-7 cells), and incubated at 4 °C for between 30 and 120 min. The binding of RBCs to COS-7 cells was quantified by counting the green COS-7 cells that bound at least two RBCs. Sialidase treatment was performed by incubating RBCs with 0.1 unit/ml *Vibrio cholera* sialidase in serum-free DMEM containing 1 mM $CaCl_2$ at 37 °C for 2 h. The cells were washed twice with PBS and once with serum-free DMEM after treatment.

Transfection and treatment of 293A cells

293A cells were transfected by standard calcium phosphate precipitation methods. In some experiments, the transfected cells were treated with 0.5 mM sodium pervanadate in regular medium for 30 min. Sodium pervanadate was prepared by mixing 100 mM sodium orthovanadate and 50 mM H_2O_2 and incubating the mixture at room temperature for at least 4 h. Treatment of the cells with tunicamycin was performed by incubating the cells with tunicamycin in regular medium for 24 h. Tunicamycin was dissolved in 95 % (v/v) ethanol at a concentration of 1 mg/ml. For control experiments, ethanol was added to the medium to a final concentration of 1 % (v/v).

N-glycosidase F digestion

MSiglec-E–myc was precipitated with anti-myc antibody from transiently transfected 293A cells. The immunoprecipitates were denatured by boiling in 0.1 M β-mercaptoethanol/0.5 % SDS for 5 min, and then centrifuged (9000 ***g***, 5 min) after the samples had cooled to room temperature. The supernatant was then recovered. Volumes of 25 μl of 0.5 M Tris/HCl buffer (pH 8.0),

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5 μl of 10 % (v/v) Nonidet P40, 2 μl of 10 mg/ml aprotinin and 5 μl of 20 units/ml N-glycosidase F were added to 5 μl of the denatured samples. In a control experiment, the N-glycosidase F was denatured by heating at 100 °C for 15 min before it was added to the samples. The samples were incubated for 24 h at 37 °C. The reaction was stopped by adding an equal volume of 2 × SDS protein loading buffer and then boiling for 5 min.

Immunoprecipitation and immunoblot analysis

293A cells were washed once with cold PBS and lysed in a lysis buffer containing 50 mM Hepes, pH 7.4, 150 mM NaCl, 1 % Triton X-100, 5 mM EDTA, 5 mM β-mercaptoethanol, 0.5 mM sodium vanadate, 1 mM PMSF, 20 μg/ml leupeptin and 1 μg/ml aprotinin (buffer A). The samples were centrifuged at 9000 ***g*** for 10 min at 4 °C. An aliquot of this whole-cell lysate was removed and the remaining lysate was immunoprecipitated and analysed by immunoblotting with appropriate antibodies, as described previously [38,41].

Glutathione S-transferase (GST) fusion proteins and *in vitro* binding assay

The expression and purification of GST and the GST fusion proteins were carried out as described previously [41]. The amount of fusion protein was estimated by SDS/PAGE followed by staining with Coomassie Blue, using BSA as a standard. For the binding assay, 50 μl of 50 % (w/v) suspended glutathione–Sepharose 4B beads with approx. 0.2 μg of bound GST or its fusion proteins was incubated at 4 °C for 16 h with 250 μl of cell lysate. The beads were washed four times with ice-cold lysis buffer (buffer A), and the bound proteins were subjected to Western blot analysis.

RESULTS

Cloning of mSiglec-E cDNA

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 [39]. Full-length SHP-1 with mutation of cysteine-455 to serine (SHP-1-C455S), which lacks protein tyrosine phosphatase catalytic activity but retains the ability to bind to its substrates, was cloned into plasmid pBTM-116-src [40]. Transformation of the plasmid in yeast results in the expression of (LexA DNA-binding domain)–SHP-1-C455S fusion protein and Src kinase [40]. From 1.1×10^7 primary transformants with a human Jurkat cDNA library fused to the GAL4 activation domain in vector pACT2, 124 were positive for both HIS3 and LacZ expression. Sequence analysis revealed that most of these corresponded to human proteins identified previously as interacting with SHP-1, such as LAIR1 (leucocyte-associted Ig-like receptor 1), Grb2 (growth-factor-receptor-bound protein 2), CD31 and PILRα (paired immunoglobulin-like receptor α) [40]. Very surprisingly, seven identical clones showed 100 % identity with a sequence in a mouse lymph node cDNA library found in the EST (expressed sequence tag) database (GenBank accession number AA275188) over 307 bp of overlapping region. To determine whether the sequence was from human or mouse, we carried out PCR experiments using single-strand cDNA and cDNA libraries from several human tissues, human cell lines and one mouse tissue. We failed to amplify the cDNA fragment from any human tissue or cell line tested, including the Jurkat cell line. However, the fragment was amplified from the cDNA of mouse kidney cells (results not shown). These results suggest that the cDNA fragment is most probably from mouse. This was confirmed by the identification of a mouse genomic DNA clone in GenBank (see below). It is not completely understood why the mouse cDNA fragment was identified from a human cDNA library.

The 5′-end of the cDNA was isolated from mouse kidney cDNA using 5′-RACE. Four RACE fragments were sequenced, all of which represented various lengths of the same cDNA. The deduced full-length cDNA is 2011 bp long, with a reading frame encoding a protein of 467 amino acid residues, named mSiglec-E (Figure 1A). The sequence of the coding region was confirmed by sequencing three independent reverse transcription–PCR products. Sequence analysis with the SMART (simple modular architecture research tool) program (http://smart.embl-heidelberg.de) revealed that mSiglec-E is a type 1 transmembrane protein. The N-terminal 18 amino acids constitute a signal peptide. Residues 354–374 are highly hydrophobic, and predict a transmembrane segment. The entire putative extracellular region consists of three homologous Ig-like domains. The N-terminal Ig-like domain is homologous to an Ig V domain, whereas the two C-terminal Ig-like domains resemble an Ig C2 domain. The extracellular domain contains 10 potential N-linked glycosylation sites (Asn-Xaa-Ser or Asn-Xaa-Thr, where Xaa is any amino acid except proline). In the cytoplasmic region, mSiglec-E contains an ITIM consensus sequence [(Ile/Leu/Val)-Xaa-Tyr-Xaa-Xaa-(Leu/Val)] from residues 430 to 435 (Ile-His-Tyr-Ala-Thr-Leu) and a sequence similar to the ITIM consensus sequence from residues 453 to 458 (Thr-Glu-Tyr-Ser-Glu-Ile).

A BLAST search of the non-redundant GenBank database revealed that mSiglec-E is strikingly similar to the members of the recently defined siglec family, and most closely resembles human siglec-9 [13,14] and siglec-7 [10,11], with overall sequence identity of 53 % and 52 % respectively (Figure 1B). The cysteine residues that are conserved among siglec family members are present in mSiglec-E. The three crucial amino acid residues involved in sialic acid binding are also conserved; they are arginine at position 126, phenylalanine at position 25 and tyrosine at position 134.

Chromosomal organization and expression of mSiglec-E

The genomic DNA sequence of mSiglec-E was identified by searching the *htgs* GenBank database. The mSiglec-E gene, consisting of seven exons spanning approx. 9.1 kb, is localized in mouse clone RP23-138K10 (Genbank accession number AC079475) (Figure 2A). The exon–intron boundaries confirm the GT-AG rule. This gene is closely linked to that encoding mouse CD33, which is localized approx. 118 kb downstream of the mSiglec-E gene and consists of six exons spanning approx. 5.1 kb (Figure 2A).

Northern blot analysis revealed the presence of a major mSiglec-E mRNA transcript of 2.0 kb, with the highest level in spleen and moderate levels in lung, heart and liver (Figure 2B). After a longer exposure, the transcript was also detected at very low levels in kidney, thymus, testes and ovary, but not in embryo or brain (results not shown). The cDNA we isolated is almost the same size as that of the major transcript revealed in the blot, and therefore probably represents the full-length species. Additional transcripts of 3–4 kb were seen in the blot. The relative levels of these additional transcripts in different tissues were similar to those of the major transcripts, indicating that the transcripts of 3–4 kb are likely to be alternatively spliced mRNAs of mSiglec-E.

mSiglec-E expressed in 293A cells is a disulphide-linked oligomer

For the expression of mSiglec-E in mammalian cells and the detection of its expression, mSiglec-E was epitope-tagged with

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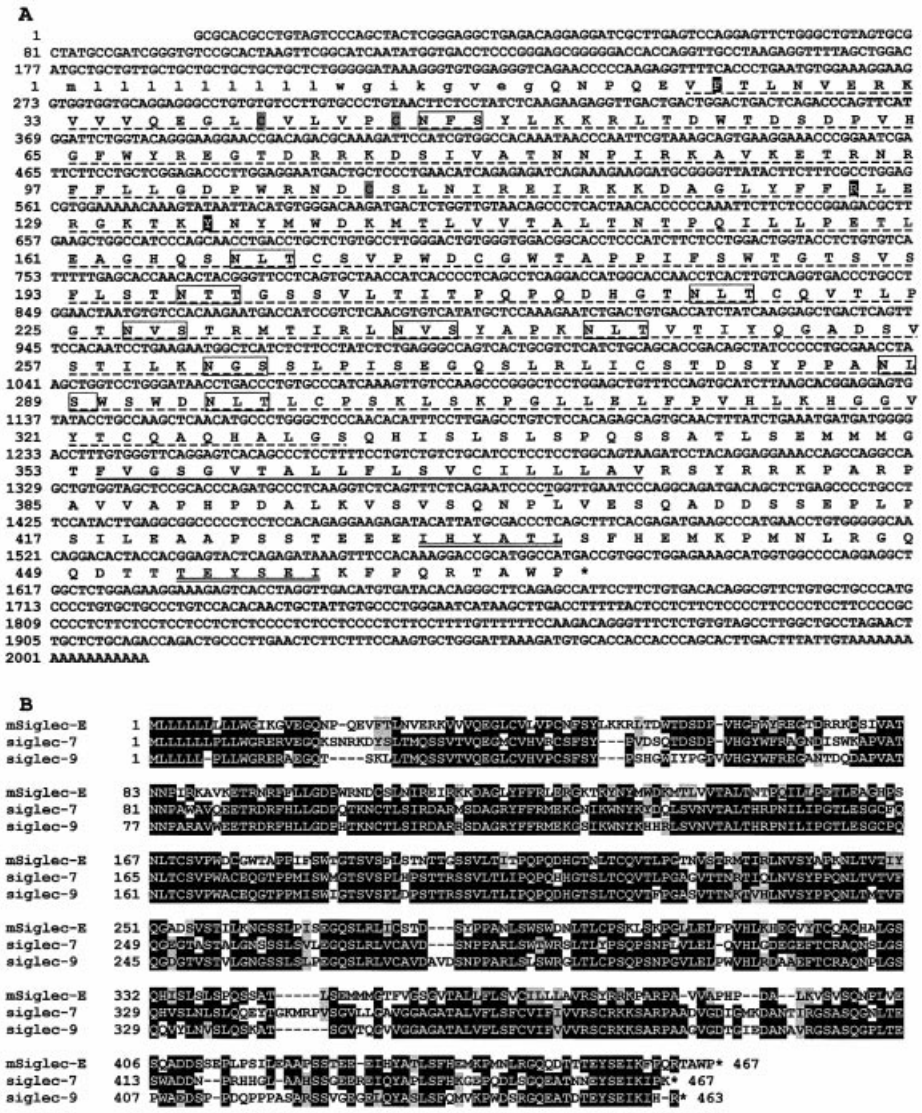

Figure 1 Nucleotide and deduced amino acid sequences of mouse mSiglec-E, and alignment with closely related siglecs

(**A**) The putative signal peptide is indicated in lower-case letters. The transmembrane region is underlined. The three Ig-like domains are underlined with broken lines. Cysteine residues characteristic of siglecs are shown with a grey background. The residues important for the binding of sialic acid are shown with a black background. The potential N-linked glycosylation sites are boxed. The ITIM sequence is double-underlined. (**B**) Residues that are identical in two or three proteins are shown with a black background. Similar residues are shown with a grey background. Dashes indicate gaps inserted to optimize alignments.

myc and HA respectively at the C-terminus (mSiglec-E–myc and mSiglec-E–HA respectively). Western blot analysis revealed two closely spaced bands at an apparent molecular mass of approx. 80–100 kDa under reducing conditions, and two broad bands between 150 and 200 kDa under non-reducing conditions (Figure 3A). This result indicates that mSiglec-E exists in a disulphide-linked complex. To examine whether the 150–200 kDa bands are a homo-oligomer of mSiglec-E or a complex of mSiglec-E with other proteins, we co-expressed mSiglec-E with a myc tag (mSiglec-E–myc) and mSiglec-E with a HA tag (mSiglec-E–HA) in 293A cells, and determined if the two proteins were in the same complex by immunoprecipitating mSiglec-E with antibody against one tag and detecting it by Western blot with antibody against the other tag. As shown in Figure 3(B), mSiglec-E–HA could be detected in the anti-myc immunoprecipitate from cells expressing both mSiglec-E–myc and mSiglec-E–HA, but not from cells expressing only one of them. These results suggest that the mSiglec-E transiently expressed in 293A cells could form a disulphide-linked oligomer containing at least two molecules of mSiglec-E.

mSiglec-E expressed in 293A cells is glycosylated

The apparent molecular mass of mSiglec-E–myc expressed in 293A (Figure 3), COS-7 and M1 (results not shown) cells is 80–100 kDa under reducing conditions, much higher than that predicted from the deduced amino acid sequence (54.8 kDa), suggesting that mSiglec-E–myc is very likely to be modified after its translation. As mSiglec-E contains 10 potential N-linked glycosylation sites 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 molecular mass of

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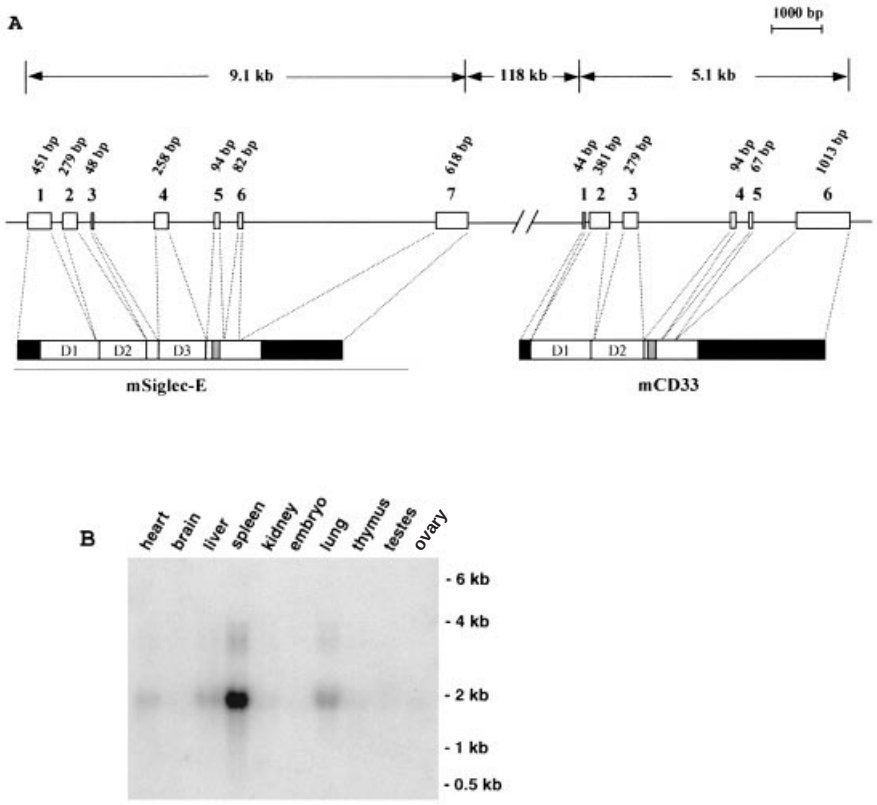

Figure 2 Genomic organization and expression of the mSiglec-E gene

(**A**) Schematic representation of the genes and respective proteins of mSiglec-E and mouse CD33. The number and size of exons are shown above the genes. The Ig-like domains are indicated as D1, D2 and D3. The transmembrane region is shown as a grey box, and the non-coding regions as black boxes. The size of the genes is indicated. The bar represents the scale in bp. (**B**) Northern blot analysis of mSiglec-E in mouse tissues. The full-length coding region of mSiglec-E was used as the probe. Each lane of the blot (Ambion Inc.) contains approx. 2 μg of poly(A)$^+$ RNA from the tissue indicated, and is normalized for the levels of β-actin.

mSiglec-E. Incubation of the cells with tunicamycin resulted in a decrease in the apparent molecular mass of mSiglec-E–myc to approx. 60 kDa, in a dose-dependent manner (Figure 4A). To confirm further the glycosylation of mSiglec-E, we immunoprecipitated mSiglec-E–myc with an anti-myc antibody and treated the precipitated proteins with N-glycosidase F. The apparent molecular mass of the treated mSiglec-E was lowered to 60–70 kDa (Figure 4B).

mSiglec-E mediates sialic acid-dependent binding to human RBCs

To investigate the sialic acid-binding properties of mSiglec-E, we transiently transfected mSiglec-E–myc along with GFP as an expression marker into COS-7 cells. The transfected cells were assayed for binding to human RBCs. The results were expressed as the percentage of green cells that bound at least two RBCs. As shown in Figure 5, 21 % of COS-7 cells transfected with mSiglec-E–myc bound at least two RBCs, while only 5 % of control cells transfected with the empty vector were able to bind at least two RBCs. Pretreatment of RBCs with sialidase resulted in a decrease in the number of RBC-binding COS-7 cells to 3 %, suggesting that the binding is sialic acid-dependent.

mSiglec-E associates with SHP-1 in 293A cells

Having shown that mSiglec-E interacts with SHP-1 in the yeast two-hybrid system in a tyrosine phosphorylation-dependent manner, we further determined whether the two proteins associate in mammalian cells. mSiglec-E–myc was co-transfected with SHP-1 into 293A cells, the transfected cells were treated with pervanadate to prevent the tyrosine-dephosphorylation of cellular proteins, and SHP-1 was immunoprecipitated with an anti-SHP-1 polyclonal antibody. As shown in Figure 6(A), mSiglec-E–myc was detected in the anti-SHP-1 immunoprecipitate of the 293A cells co-transfected with both SHP-1 and mSiglec-E–myc. The immunoprecipitation of mSiglec-E–myc was specifically through its association with SHP-1, and not through non-specific binding to the anti-SHP-1 antibody, since mSiglec-E–myc could not be precipitated by the anti-SHP-1 antibody from cells transfected with mSiglec-E–myc alone (Figure 6A). Reciprocal experiments further confirmed the association of the two proteins (Figure 7A).

The membrane-proximal ITIM of mSiglec-E is sufficient for efficient association with SHP-1

The association of SHP-1 with mSiglec-E in both yeast and mammalian cells is phosphotyrosine-dependent (results not shown). We generated tyrosine-to-phenylalanine mutants of mSiglec-E in order to identify the SHP-1-binding sites. The cytoplasmic tail of mSiglec-E contains three tyrosine residues, Tyr-377, Tyr-432 and Tyr-455. The original clone isolated from the yeast two-hybrid screen expresses the final 45 amino acid residues at the C-terminus (Pro-423 to Pro-467), which contains Tyr-432 and Tyr-455, but not Tyr-377. Both Tyr-432 and Tyr-

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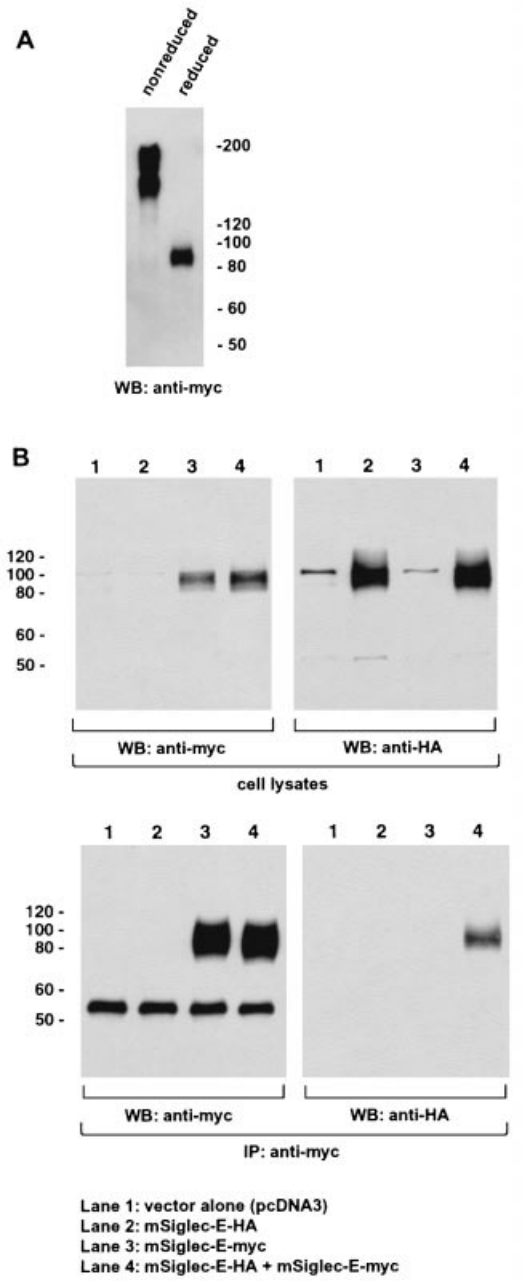

Figure 3 mSiglec-E transiently expressed in 293A cells is a disulphide-linked oligomer

(**A**) 293A cells were transfected with mSiglec-E–myc. At 48 h after transfection, the cells were lysed and subjected to SDS/7.5%-PAGE under either reducing or non-reducing conditions. mSiglec-E–myc protein was detected by Western blot (WB) analysis with an anti-myc antibody. (**B**) 293A cells were transfected with mSiglec-E–myc or mSiglec-E–HA as indicated. At 48 h after transfection, the cells were lysed and subjected to immunoprecipitation (IP) and Western blot analysis. Molecular mass (kDa) is indicated to the right (**A**) or the left (**B**) of the gels.

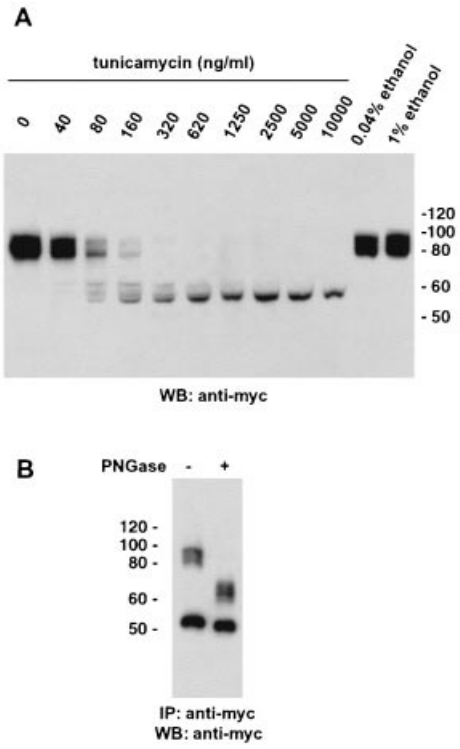

Figure 4 mSiglec-E transiently expressed in 293A cells is an N-linked glycoprotein

(**A**) 293A cells, growing in a 100 mm dish, were transfected with mSiglec-E–myc. At 18 h after transfection the cells were collected and plated in 6-well plates. Following a further 6 h, the cells were incubated with regular medium in the absence or presence of tunicamycin for 24 h. Total-cell lysates were prepared and subjected to SDS/PAGE and Western blot (WB) analysis. (**B**) 293A cells were transfected with mSiglec-E–myc. At 48 h after transfection, the cells were lysed and proteins were immunoprecipitated (IP) with an anti-myc antibody. The immunoprecipitated proteins were treated with N-glycosidase F (PNGase) as described in the Materials and methods section, and examined by Western blotting. Molecular mass (kDa) is indicated to the right (**A**) or to the left (**B**) of the gel.

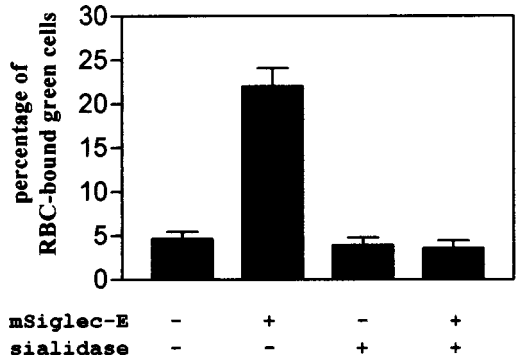

Figure 5 COS-7 cells transfected with mSiglec-E bind human RBCs

COS-7 cells were transfected either with mSiglec-E–myc and GFP expression plasmids or with the GFP plasmid alone. At 48 h after transfection, the RBC-binding assay was carried out as described in the Materials and methods section. The data are presented as the percentage of the green cells that bound at least two RBCs relative to the total number of green cells scored. The results shown are means ± S.E.M. from four independent binding assays performed in two separate transfection experiments.

455 reside in potential ITIM sequences. Therefore we mutated each of them, or both, to phenylalanine (mSiglec-E-Y432F–myc, mSiglec-E-Y455F–myc and mSiglec-E-Y432F/Y455F–myc). The mSiglec-E mutants were co-transfected with SHP-1-C455S into 293A cells, and immunoprecipitation and immunoblotting experiments were performed as described above. In the immunoprecipitates of anti-SHP-1, mSiglec-E-Y455F–myc was detected at the same level as wild-type mSiglec-E–myc, whereas mSiglec-E-Y432F–myc was detected at a much lower level than the wild type (Figure 7A). The double mutation (Y432F/Y455F) completely abolished the association. Similar results were observed in

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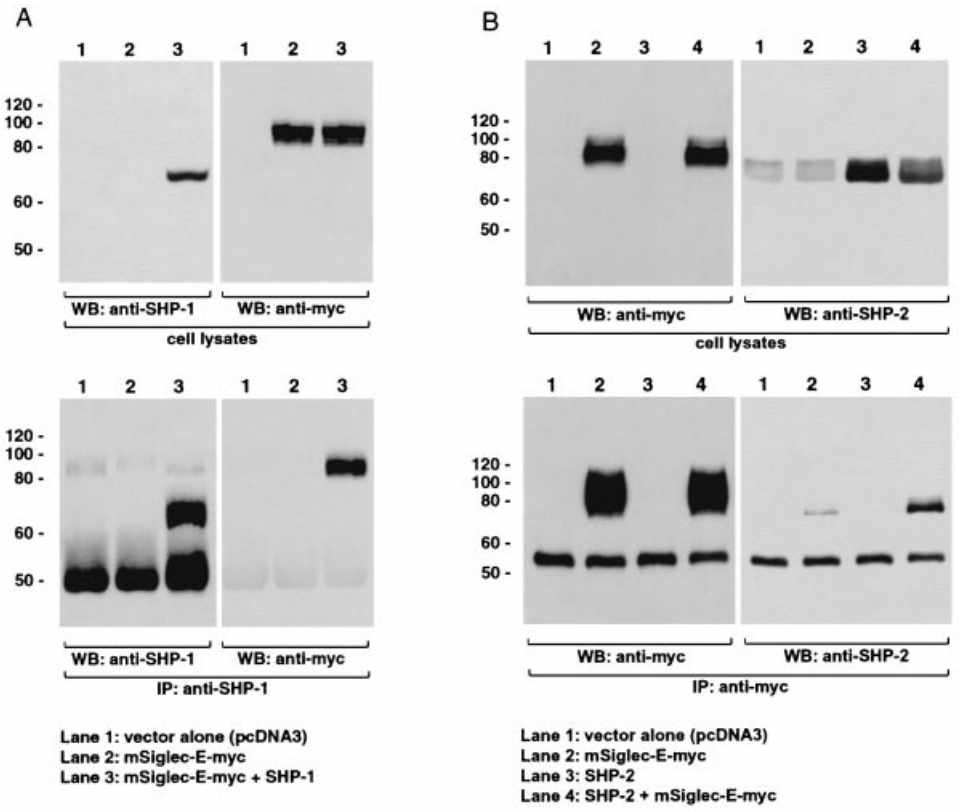

Figure 6 mSiglec-E associates with SHP-1 and SHP-2 when co-expressed in 293A cells

293A cells were transfected with mSiglec-E–myc and SHP-1 (**A**) or SHP-2 (**B**) 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 in the Materials and methods section. Molecular mass (kDa) is indicated to the left of the gels.

the reciprocal experiments (Figure 7A). The levels of tyrosine phosphorylation of mSiglec-E-Y432F–myc and mSiglec-E-Y455F–myc were similar, as shown in the anti-phosphotyrosine blot of anti-myc precipitants. This suggests that the decreased association of mSiglec-E-Y432F–myc with SHP-1-C455S was not caused by decreased phosphorylation of Tyr-455 in mSiglec-E-Y432F–myc in comparison with that of Tyr-432 in mSiglec-E-Y455F–myc. A lower affinity of SHP-1-C455S for Tyr-455 than for Tyr-432 is the likely explanation for these observations. Taken together, these results indicate that mSiglec-E recruits SHP-1 through either the membrane-proximal ITIM (centred at Tyr-432) or the membrane-distal ITIM (centred at Tyr-455), and that the membrane-proximal ITIM alone is sufficient for binding, since mutation of Tyr-455 exerts no detectable effect on the association.

Both SH2 domains of SHP-1 interact with mSiglec-E

In order to identify which SH2 domain of SHP-1 interacts with mSiglec-E, we performed an *in vitro* binding assay using GST fusion proteins containing the first SH2 domain (GST–SHP-1-SH2N) or the second SH2 domain (GST–SHP-1-SH2C) of SHP-1. As shown in Figure 8, both GST–SHP-1-SH2N and GST-SHP-1-SH2C bound wild-type mSiglec-E–myc and mSiglec-E-Y455F–myc. GST–SHP-1-SH2N, but not GST–SHP-1-SH2C, also bound mSiglec-E-Y432F–myc. Neither GST–SHP-1-SH2N nor GST–SHP-1-SH2C bound mSiglec-E-Y432F/Y455F–myc. These results suggest that each SH2 domain of SHP-1 is able to bind to the membrane-proximal ITIM, whereas only the first (N-terminal) SH2 domain is able to bind to the membrane-distal ITIM.

mSiglec-E associates with SHP-2

SHP-1 and SHP-2 are the only two SH2-domain-containing protein tyrosine phosphatases that have been identified in mammalian cells. In many cases, they bind to tyrosine-phosphorylated proteins with similar consensus sequences. We determined whether mSiglec-E also associated with SHP-2. As shown in Figure 6(B), SHP-2 was co-precipitated with an anti-myc antibody from 293A cells transfected with mSiglec-E–myc, but not from untransfected cells. Unlike SHP-1, SHP-2 is expressed endogenously at high level in 293A cells. The association of mSiglec-E with endogenous SHP-2 was also detected. Transient overexpression of SHP-2 increased the amount of SHP-2 co-precipitated with mSiglec-E–myc. Experiments with mutated mSiglec-E–myc revealed that the manner of the association of SHP-2 with mSiglec-E is similar to that of SHP-1 (Figure 7B).

DISCUSSION

The consensus (Leu/Ile/Val)-Xaa-Tyr-Xaa-Xaa-(Leu/Val) sequence defined as an ITIM mediates the association of receptors with SHP-1 and SHP-2. A growing number of ITIM-bearing receptors have been identified, although the ligands and/or functions of many of them are unknown. In the present study we have identified a mouse ITIM-bearing receptor which we have

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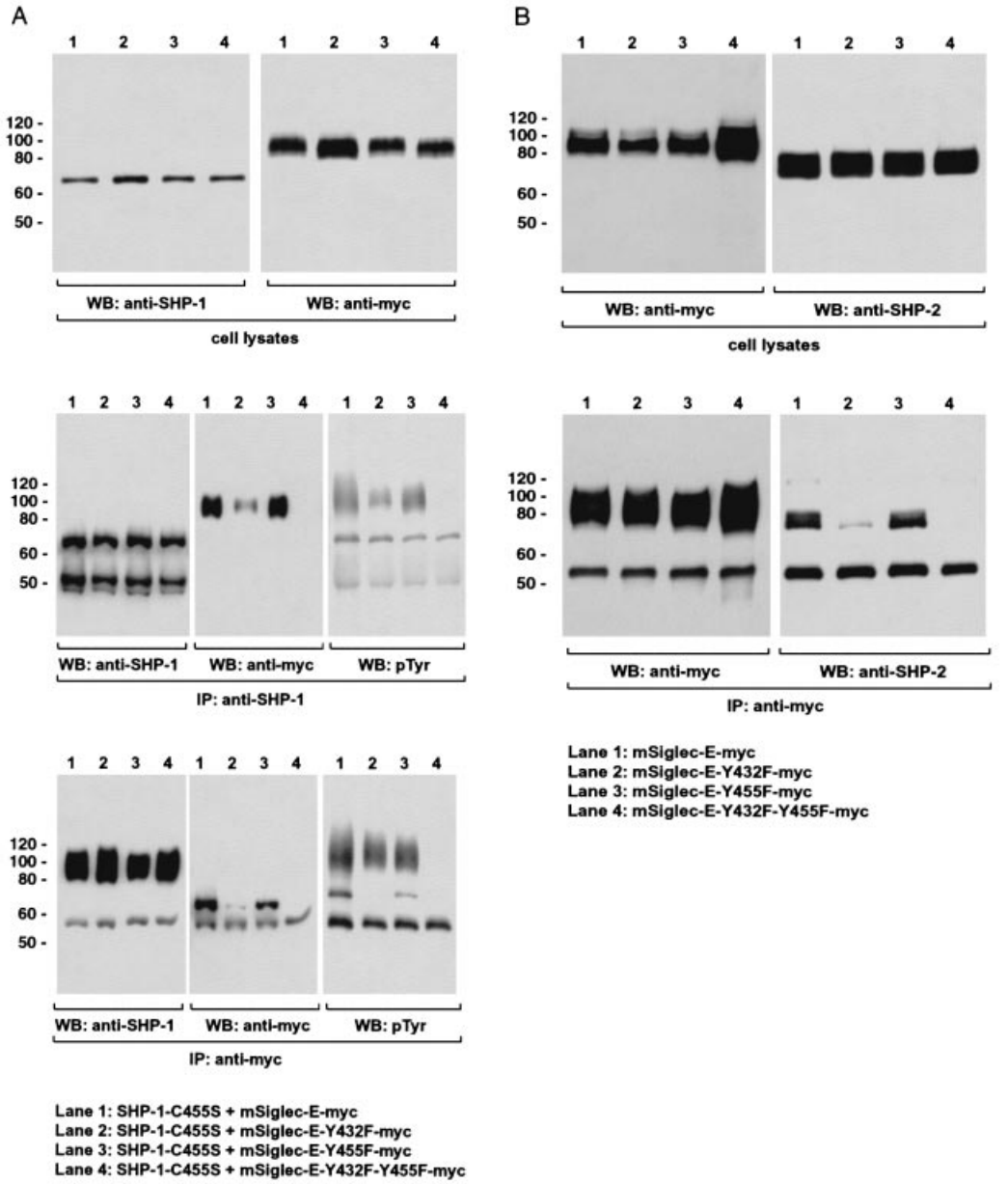

Figure 7 mSiglec-E binds to SHP-1 and SHP-2 through both membrane-proximal and membrane-distal ITIMs

293A cells were transfected with mSiglec-E–myc or its mutants (**A** and **B**) and SHP-1-C455S (**A**) 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 in the Materials and methods section. pTyr, phosphotyrosine. Molecular mass (kDa) is indicated to the left of the gels.

designated mSiglec-E, as it shares a significant sequence identity with the siglec family of receptors and is the fifth mouse siglec to be identified. Several human siglecs have been identified either by searching homologous sequences in DNA databases, such as for siglec-5 [8], siglec-7 [11], siglec-8 [12] and siglec-9 [13,14], or by expression cloning, such as for siglec-6 [9] and AIRM1/siglec-7 [10]. In comparisons of primary structures with those of previously reported members, all of these newly identified members are more closely related to CD33 (siglec-3) than to sialoadhesin (siglec-1), CD22 (siglec-2) or myelin-associated glycoprotein (siglec-4). For instance, CD33 and all of the new members except siglec-8 contain a cytoplasmic tail with 83–93 amino acid residues, which bears a membrane-proximal ITIM (Glu-Leu/Val-His/Gln-Tyr-Ala-Xaa-Leu) and a membrane-distal ITIM (Thr/Asn-Glu-Tyr-Ser-Glu-Ile/Val). The membrane-distal ITIM, with Thr/Asn instead of Leu/Ile/Val at position -2, is different from, but similar to, the typical ITIM consensus sequence (Ser/Leu/Ile/Val)-Xaa-Tyr-Xaa-Xaa-(Leu/Val). Although each of the two ITIMs in mSiglec-E was able to bind the SH2 domains of SHP-1 and SHP-2, the membrane-proximal ITIM (centred at Tyr-432) alone was sufficient for the interaction. Thus the manner of the interaction of mSiglec-E with SHP-1 and SHP-2 is similar to that of human CD33/siglec-3 with SHP-1 [42], but different from that of human CD22/siglec-2 with SHP-1. For CD22, at least two ITIMs are required for optimal binding to SHP-1 [43].

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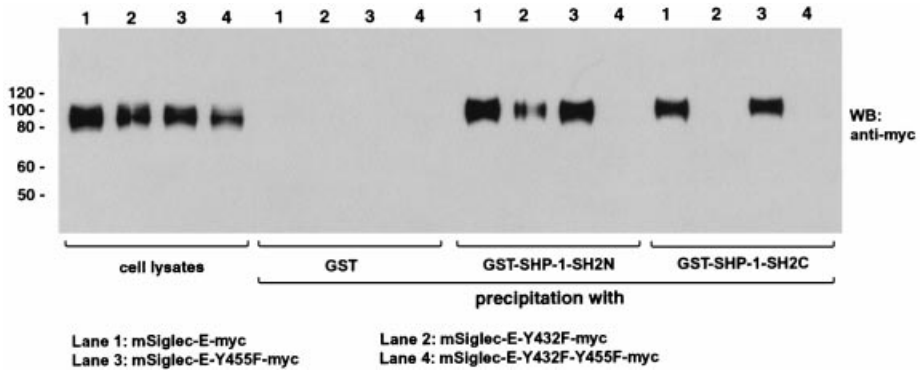

Figure 8 Both the N-terminal and the C-terminal SH2 domains of SHP-1 interact with mSiglec-E

293A cells were transfected with mSiglec-E–myc and its mutants 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 GST fusion protein binding and Western blot (WB) analysis as described in the Materials and methods section. Molecular mass (kDa) is indicated to the left of the gels.

The two SH2 domains of SHP-1 display different binding specificities for tyrosine-phosphorylated proteins. For example, the N-terminal SH2 domain binds activated c-Kit [44] and interleukin-3 receptor [45], whereas the C-terminal SH2 domain binds tyrosine-phosphorylated ITIMs of FcγRIIB [32] and the killer cell inhibitory receptor [46]. With more and more ITIM-bearing receptors being identified, it has been established that some ITIM-bearing receptors interact with SHP-1 through its N-terminal SH2 domain instead of the C-terminal SH2 domain [47], while others can bind to both the N-terminal and the C-terminal SH2 domains [48,49]. In the present study, we found that both SH2 domains of SHP-1 could bind to the membrane-proximal ITIM of mSiglec-E, but only the N-terminal SH2 domain could bind to the membrane-distal ITIM. In contrast, it was reported that only the C-terminal SH2 domain of SHP-1 was able to bind to the membrane-distal ITIM of human CD33 [42].

It has been reported that some ITIM-bearing proteins associate with both SHP-1 and SHP-2 [42,50], whereas others associate with only one of them [10,51,52]. Two members of the CD33-like siglecs have so far been reported to recruit the SHPs. Human CD33 associates with both SHP-1 and SHP-2 [42], whereas AIRM1 associates only with SHP-1, and not with SHP-2 [10]. In the present study we have shown that mSiglec-E associates strongly with both SHP-1 and SHP-2. Since SHP-1 and SHP-2 have distinct functions in the regulation of signal transduction, further analysis of the specificity of the association of each siglec with SHP-1 and SHP-2 will help us to understand the mechanisms of signal transduction of individual siglec members.

In accordance with its structural similarity with human siglecs, mSiglec-E was also found to bind RBCs in a sialic acid-dependent manner, suggesting that the function of mSiglec-E is involved in cell–cell interaction. Except for siglec-4, which is expressed in the nervous system, human siglecs are generally expressed in haematopoietic cells and the immune system. The high transcript levels in spleen indicate that mSiglec-E is probably expressed in haematopoietic cells, since this organ is rich in leucocytes. The lack of mSiglec-E transcripts in embryos further indicates that it is likely to be present in lymphocytes. Many ITIM-bearing transmembrane proteins have been identified as inhibitory receptors in the immune system. Since mSiglec-E is able to recruit SHP-1, a major negative regulator of immune signalling, it would be interesting to determine whether mSiglec-E is indeed an inhibitory receptor.

All of the recently identified siglecs (siglec-5, -6, -7, -8 and -9) are from human. They are closely related to CD33/siglec-3, and are therefore grouped as 'CD33-related'. The genes for CD33-related siglecs are all localized on human chromosome 19q13.3–4, suggesting that they arose by repeated gene duplication during evolution. Apart from mSiglec-E, only one mouse CD33-related siglec (mouse CD33) has been found. Phylogenetic analysis revealed that mouse CD33 does not show a clear relationship with human CD33/siglec-3 [14]. One explanation may be that the true mouse orthologue of human CD33 and the human orthologue of mouse CD33 have not yet been identified. Another possibility is that some of the genes in this cluster duplicated at the early stage of evolution and then the human and mouse genes further duplicated independently [14]. Like mouse CD33, the second mouse CD33-related siglec, mSiglec-E, does not show high sequence similarity with any particular human siglecs. The highest similarity is with siglec-7 and siglec-9, with which it shows approx. 50 % identity. This suggests that mouse mSiglec-E is not an orthologue of any human siglecs yet identified. It will be very useful to isolate more mouse siglecs for the study of the evolution of this family of proteins and the characterization of their specific functions.

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