Siglec-5 (CD170) Can Mediate Inhibitory Signaling in the Absence of Immunoreceptor Tyrosine-based Inhibitory Motif Phosphorylation*

Received for publication, February 23, 2005
Published, JBC Papers in Press, March 15, 2005, DOI 10.1074/jbc.M502044200

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Siglec-5 (CD170) is a member of the recently described human CD33-related siglec subgroup of sialic acid binding Ig-like lectins and is expressed on myeloid cells of the hemopoietic system. Similar to other CD33-related siglecs, Siglec-5 contains two tyrosine-based motifs in its cytoplasmic tail implicated in signaling functions. To investigate the role of these motifs in Siglec-5-dependent signaling, we used transfected rat basophil leukemia cells as a model system. Tyrosine phosphorylation of Siglec-5 led to recruitment of the tyrosine phosphatases SHP-1 and SHP-2, as seen in both pull-down assays and microscopy. Siglec-5 could efficiently inhibit FcεRI-mediated calcium fluxing and serotonin release after cross-linking. Surprisingly, a double tyrosine to alanine mutant of Siglec-5 could still mediate strong inhibition of serotonin release in the absence of detectable tyrosine phosphorylation, whereas a double tyrosine to phenylalanine mutant lost all inhibitory activity. In comparison, suppression of Siglec-5-dependent adhesion to red blood cells was reversed by either tyrosine to alanine or tyrosine to phenylalanine mutations of the membrane proximal tyrosine-based motif. Using an in vitro phosphatase assay with synthetic and recombinant forms of the cytosolic tail, it was shown that a double alanine mutant of Siglec-5 had weak, but significant SHP-1 activating properties similar to those of wild type, non-phosphorylated cytosolic tail, whereas a double phenylalanine mutant was inactive. These findings establish that Siglec-5 can be classified as an inhibitory receptor with the potential to mediate SHP-1 and/or SHP-2-dependent signaling in the absence of tyrosine phosphorylation.

Regulation of responses by cells in the hemopoietic and immune systems depends on a balance between activatory signaling and inhibitory signaling. Their relative strengths set an appropriate activation threshold that helps fine-tune the response. Inhibitory signals are typically initiated by receptors containing one or more cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (1). The consensus sequence for ITIMs is (V/I/L)XYXX(L/V), where X is any amino acid. A recent bioinformatics study showed that the human proteome contains ~109 membrane proteins with cytoplasmic ITIMs, and many of these have been shown already to function as inhibitory receptors (2). The established dogma is that Src family tyrosine kinases phosphorylate the tyrosine residue in ITIMs during cellular activation and create high affinity docking sites for SH2 domain-containing phosphatases such as protein-tyrosine phosphatases SHP-1 and SHP-2 and the 5’-inositol phosphatase, SHIP (1). The recruited and activated phosphatases can then dephosphorylate relevant substrates in the vicinity and regulate cellular activation (3).

The human CD33-related siglecs are a recently described subgroup of the siglec family of sialic acid binding immunoglobulin (Ig)-like lectins (4). All eight human CD33-related siglecs are expressed by cells of the hemopoietic and immune systems and contain in their cytoplasmic tails a membrane proximal ITIM and a membrane distal ITIM-like motif. The extracellular region is made up of varying numbers of Ig domains, including an N-terminal V-set Ig domain that mediates binding of sialylated glycans (5). Where studied, CD33-related siglecs can become tyrosine phosphorylated and recruit SHP-1 and SHP-2 after treatment of cells with pervanadate, a protein-tyrosine phosphatase inhibitor (6–11). Some CD33-related siglecs can also inhibit activatory pathways when cross-linked with activating receptors (7, 8, 12, 13). In addition, CD33 and Siglec-7 and -9 have been shown to exhibit enhanced siglec-dependent adhesion after tyrosine mutation of the proximal ITIM (6, 14).

Siglec-5 (CD170) is a typical member of the CD33-related Siglec subfamily, with four extracellular Ig-like domains and two tyrosine-based motifs in the cytosolic region (15). It is expressed on neutrophils, monocytes, dendritic cells, and subsets of tissue macrophages (15–18), but there are currently no reports on the potential inhibitory signaling functions of this receptor. In the present study we have used transfected rat basophil leukemia (RBL) cells as a model system to investigate the inhibitory activity of Siglec-5 and determine the importance of the two tyrosine-based motifs in recruitment of phosphatases, inhibitory signaling, and suppression of Siglec-5-dependent adhesion. We demonstrate that Siglec-5 can recruit SHP-1 and SHP-2 after tyrosine phosphorylation and mediate inhibitory signaling, as measured by calcium flux and serotonin release after co-ligation with the activatory FcεRI. Surprisingly, however, mutagenesis studies showed that inhibition of serotonin release could still occur efficiently after a double tyrosine to alanine substitution, whereas suppression of Siglec-5-dependent adhesion required an intact tyrosine residue at the membrane proximal ITIM. A potential mechanism for tyrosine phosphorylation-independent inhibitory signaling was provided by results of in vitro phosphatase activity.
assays, which demonstrated low level activation of SHP-1 by the Siglec-5 cytoplasmic tail in the absence of tyrosine phosphorylation.

**MATERIALS AND METHODS**

**Reagents—**Unless otherwise specified, all reagents and chemicals were purchased from Sigma-Aldrich. [3H]Serotonin (hydroxytryptamine creatine sulfate, 5-12-3H(Ni)) was from PerkinElmer Life Sciences. [32P]ATP was from Amersham Biosciences. *Vibrio cholerae* sialidase was from Calbiochem.

**Plasmids—**Site-directed mutagenesis was performed with sets of mutagenic primers (Table I) using the QuickChange kit (Stratagene, Amsterdam, The Netherlands). The tail deletion form of Siglec-5 (5Δ) was obtained by the PCR using Siglec-5 cDNA (15) as template and the sets of primers described in Table I and cloned into the pcDNA3 vector (Invitrogen). cDNAs encoding native and catalytically inactive forms of human protein-tyrosine phosphatases SHP-1 (C453S) and SHP-2 (C459) were fused at the C terminus with green fluorescent protein (GFP) as described (14). Siglec-5 cytoplasmic tail (CT) constructs (amino acids 468–551) were generated by PCR (Table I) and cloned into glutathione S-transferase (GST) expression vector pGEX-6P-1 (Amersham Biosciences). All PCR reactions were performed using *Pfu* Turbo DNA polymerase (Stratagene), and the presence of introduced mutations was confirmed by DNA sequencing. Human GST-SHP-1 and GST-SHP-2 constructs were kindly provided by B. Neel (Harvard Medical School).

**Antibodies—**The 5A5 (anti-Siglec-5/CD170) monoclonal antibody (mAb) and the affinity-purified sheep polyclonal antibody (pAb) anti-Siglec-5 were described previously (15, 19). The following antibodies were purchased from the suppliers shown: GL183 mAb (anti-CD158b/KIR2DL3) (Immunotech, Marseille, France), OX-18 mAb (anti-rat MHC class I molecules) (Serotec, Oxford, UK), GL183 mAb (anti-MHC class II), anti-Siglec-5 (1A5) (Invitrogen), anti-SHP-1 or anti-SHP-2 (Santa Cruz Biotechnology, Calne, UK). c-Src kinase (Oncogene, Nottingham, UK) in 500 μM X-100. For comparative analysis results were expressed as a mean of specific serotonin release: (cpm test – cpm spont)/cpm max – cpm spont) × 100, where cpm "spont" is the spontaneous release of [3H]serotonin from cells incubated in absence of antibodies, and cpm "max" is the maximum release of [3H]serotonin from cells lysed using 1% Triton X-100. For comparative analysis results were expressed as a mean percentage of inhibition of serotonin release: 100 – (% test × 100%) IgE, where % IgE is the percentage of specific serotonin release: (cpm test – cpm spont)/cpm max – cpm spont) × 100, where cpm "spont" is the spontaneous release of [3H]serotonin from cells incubated in absence of antibodies, and cpm "max" is the maximum release of [3H]serotonin from cells lysed using 1% Triton X-100. For comparative analysis results were expressed as a mean percentage of inhibition of serotonin release: 100 – (% test × 100%) IgE, where % IgE is the percentage of specific serotonin release: (cpm test – cpm spont)/cpm max – cpm spont) × 100, where cpm "spont" is the spontaneous release of [3H]serotonin from cells incubated in absence of antibodies, and cpm "max" is the maximum release of [3H]serotonin from cells lysed using 1% Triton X-100. For comparative analysis results were expressed as a mean percentage of inhibition of serotonin release: 100 – (% test × 100%) IgE, where % IgE is the percentage of specific serotonin release: (cpm test – cpm spont)/cpm max – cpm spont) × 100, where cpm "spont" is the spontaneous release of [3H]serotonin from cells incubated in absence of antibodies, and cpm "max" is the maximum release of [3H]serotonin from cells lysed using 1% Triton X-100.

**Immunoprecipitation with GST-Siglec-5 Cytoplasmic Tail Constructs—**10 μg of GST fusion proteins were incubated with 15 units of c-Src kinase (Oncogene, Nottingham, UK) in 500 μl of tyrosine kinase assay buffer (50 mM HEPES, pH 7.4, 50 mM NaCl, 0.1 mM Na2VO4, 5 mM MgCl2, 5 mM MnCl2, 5 mM ATP) at room temperature for 2 h. 10 μg of GST fusion proteins with or without tyrosine phosphorylation were incubated with 1 ml of U937 cell lysate (1 μg/ml) for 30 min at 37 °C with TRIFC-conjugated rabbit Fab′2 anti-sheep IgG to generate patched Siglec-5. Cells were then treated or not with pervanadate (0.5 mM) for 10 minutes at 37 °C and fixed with 4% formaldehyde. For the FceRI-mediated activation of RBL cells, GFP-transfected cells were stained with mouse IgE (0.1 μg/ml) and 1A5 mAbs (10 μg/ml) for 30 min at 4 °C, incubated with goat Fab′2 anti-mouse Ig (50 μg/ml) for 30 min at 37 °C, and then labeled with sheep anti-Siglec-5 pAb (10 μg/ml) and TRIFC-conjugated rabbit Fab′2 anti-sheep IgG (10 μg/ml) for 15 min at 37 °C to create clusters. Images were acquired using the AxiosVision imaging system (Carl Zeiss, Bieoster, UK) and a Zeiss Axioskope immunofluorescence microscope equipped with a Zeiss X40 objective (Jena, Germany).

**Flow Cytometry—**All incubations were carried out on ice. Cells were incubated with primary mAbs (10 μg/ml) for 30 min followed by fluoresein isothiocyanate-conjugated rabbit Fab′2 anti-mouse IgG for 30 min and then analyzed using a FACScalibur (BD Biosciences). Calcium mobilization—Cells were labeled with 4.5 μM Indo-1 (Molecular Probes, Cambridge, UK) in the presence of 0.045% (v/v) pluronic F-127 (Molecular Probes) for 45 min at 37 °C. Cells were washed twice in media, resuspended to a concentration of 1 × 106 cells/ml, and labeled on ice for 30 min with IgE (0.1 μg/ml) alone or in combination with anti-MHC class I, anti-KIR2DL3, or anti-Siglec-5 mAbs (10 μg/ml). After washing cells were warmed to 37 °C for 5 min immediately before analysis, and goat Fab′2 anti-mouse Ig (50 μg/ml) was added to the samples to induce cross-linking of antibodies and cell activation. Calcium mobilization was analyzed using an LSR flow cytometer (BD Biosciences) by measuring the ratio of 400/510 nm fluorescence with FlowJo software (Tree Star Inc., Ashland, OR).
performed as described (19). Briefly, RBL or COS cells were treated or not with sialidase for 1 h at 37 °C, washed, and incubated with human RBC for 1 h at 4 °C. Unbound RBCs were gently washed off, cells were fixed with 0.5% glutaraldehyde, and rosetting was assessed by microscopy. To quantify RBC binding, the percentage of cells forming rosettes (defined as cells binding more than 10 RBC) was scored from counting at least 200 cells per field in 5 different fields per experiment. Results were expressed as the mean percentage obtained in three independent experiments.

**Phosphatase Assay—**Wild type and mutant forms of Siglec-5CT-GST and WT SHP-1-GST were expressed in Escherichia coli BL21pLysS cells (Novagen, Nottingham, UK), and the fusion proteins were purified using glutathione-Sepharose (Amersham Biosciences). Siglec-5CT-GST fusion proteins were cleaved with PreScission protease (Amersham Biosciences), and the Siglec-5CT was purified by gel filtration and either left untreated or phosphorylated with c-Src kinase. Irrelevant peptide from tetanus toxin (amino acids 871–887) was a kind gift from C. Watts (University of Dundee, UK). Non-phosphorylated and double-tyrosyl-phosphorylated synthetic peptides of Siglec-5 (amino acids 514–551) were synthesized by G. Bloomberg (University of Bristol, UK). Peptides and proteins were incubated with purified GST-SHP-1 (0.02 μg) and 32P-labeled Raytid (30,000 cpm/Upstate, Dundee, UK) for 1 h at 37 °C. A constitutively active form of SHP-1 lacking SH2 domains (SHP-1 ΔSH2) was a generous gift from J. Matthews (University of Cardiff, Cardiff, UK) and was used as a control with substrate alone. Phosphatase assays were stopped by the addition of charcoal solution (0.9% HCl, 90 mM sodium pyrophosphate, 2 mM sodium phosphate, 4% (v/v) activated charcoal). After centrifugation, the released radioactivity was analyzed by scintillation counting. Each assay was set up in duplicate, and the results were expressed as the mean of 3 or more different experiments. Student’s t test was applied using a two-tailed distribution of two samples of equal or unequal variances.

**RESULTS**

**Tyrosine Phosphorylation of Siglec-5 and Recruitment of SHP-1 and -2—**In common with other CD33-related Siglecs, the cytoplasmic tail of Siglec-5 contains two conserved tyrosine-based motifs at amino acids 520 and 544, here designated Y1 and Y2. Y1 conforms to the consensus ITIM motif, whereas Y2 is ITIM-like (15). To investigate whether Siglec-5 can become tyrosyl-phosphorylated and recruit SHP-1 and/or SHP-2, Siglec-5-transfected RBL (RBL-5WT) or control non-transfected RBL cells were either untreated or treated with pervanadate, and Siglec-5 immunoprecipitates were analyzed by immunoblotting with antibodies to Siglec-5, phosphotyrosine, SHP-1, or SHP-2 (Fig. 1A). Specific bands of ~100 kDa corresponding to Siglec-5 were observed with antibodies to Siglec-5 (Fig. 1A). Tyrosine phosphorylation of Siglec-5 was undetectable in untreated cells but was readily observed after pervanadate treatment. This was accompanied by recruitment of both SHP-1 and SHP-2 (Fig. 1A).

To visualize the interaction of protein-tyrosine phosphatases with Siglec-5 in living cells, RBL-5WT cells were transfected with SHP-1-GFP or SHP-2-GFP cDNAs and Siglec-5 capped at the cell surface using sheep anti-Siglec-5 pAb followed by TRITC-conjugated rabbit F(ab)2 anti-sheep Ig. As shown in Fig. 1, B and C, upper panels, no co-localization was observed in untreated cells, whereas co-localization was clearly evident when the cells were treated with pervanadate. To investigate if co-localization also occurred after physiological activation signals, Siglec-5 was co-cross-linked with the activatory FcεRI receptor in the absence of pervanadate. Both SHP-1-GFP and SHP-2-GFP co-localized with Siglec-5 under these conditions (Fig. 1, B and C, upper panels).

In a previous study (6) it was shown that mAb-induced clustering of CD33 led to transient phosphorylation and recruitment of SHP-1 and -2 followed by dephosphorylation of CD33. To investigate whether the failure to observe co-localization of clustered Siglec-5 with SHP-1 and SHP-2-GFP in untreated cells could be due to SHP-mediated dephosphorylation of Siglec-5, RBL-5WT cells were transfected with catalytically inactive mutants of SHP-1(C453S)-GFP and SHP-2(C459S)-GFP (mutSHP-1-GFP and mutSHP-2-GFP, respectively). These mutants retain the ability to bind substrates via their SH2 domains but are unable to dephosphorylate them (21, 22). Strikingly, clustered Siglec-5 co-localized with mutant SHP-1 and mutSHP-2 in untreated cells (Fig. 1, B and C, lower panels), and this was not significantly enhanced by pervanadate treatment (data not shown). Therefore, in the absence of pervanadate treatment Siglec-5 may become phosphorylated on clustering and recruit SHP-1 and SHP-2, leading to dephosphorylation of Siglec-5 and rapid dissociation.

**Co-cross-linking of Siglec-5 Inhibits FcεRI-mediated Calcium Fluxing and Serotonin Release—**To assess the potential inhibitory function of Siglec-5, we used two assays of cellular activation, namely calcium fluxing and serotonin release. In both systems a putative inhibitory receptor expressed in RBL cells is cross-linked with FcεRI, and the effect on cellular activation measured. The RBL cells used in this study had already been transfected with the well-characterized inhibitor receptor, KIR2DL3, thereby providing a useful internal reference in the inhibition assays (20). As shown in Fig. 2A, similar levels of Siglec-5 and KIR2DL3 were expressed in RBL-SWT. Cross-linking of KIR2DL3 or Siglec-5 with FcεRI reduced the calcium fluxing in RBL-5WT cells in a time-dependent manner when compared with a control antibody that binds MHC class I molecules on RBL cells (Fig. 2, A and B).

The inhibitory activity of Siglec-5 was investigated further using the serotonin release assay (20). Co-cross-linking of Siglec-5 with FcεRI was carried out over a range of anti-Siglec-5 mAb concentrations, revealing a dose-dependent inhibition of serotonin release that was very similar to that produced by co-cross-linking KIR2DL3 (Fig. 2C). No inhibition was seen with the control antibody against MHC class I (Fig. 2C). To define which tyrosine motif(s) on Siglec-5 is required for SHP-1 and SHP-2 association and inhibitory signaling function in RBL cells, we generated tyrosine to alanine mutants at Y1 and Y2 either individually or in combination (Fig. 2A and Table II). With RBL-5Y1A, RBL-5Y2A, and the double tyrosine mutant, RBL-5Y1A2, strong inhibition was still observed after co-cross-linking of Siglec-5 with FcεRI (Fig. 2C). In contrast, truncation of the cytoplasmic tail resulted in a complete loss of inhibition (Fig. 2C). These results show, surprisingly, that the tyrosine residues are not essential for mediating the inhibitory function of Siglec-5.

In immunoprecipitation experiments, mutation of the membrane-proximal tyrosine motif completely abrogated the detectable tyrosine phosphorylation, whereas a significant degree of phosphorylation was retained after mutation of the membrane-distal motif (Fig. 2D). All tyrosine mutants were still able to recruit low levels of SHP-1, similar to untreated RBL-SWT lysates, whereas only the membrane-distal mutant could still recruit significant levels of SHP-2 (Fig. 2D). No SHP-1 or SHP-2 was detectable in blots prepared from RBL-5Δ lysates (Fig. 2D), showing that an intact cytoplasmic tail is required. The interactions of Siglec-5 tyrosine motifs with SHP-1 and SHP-2 were further analyzed using a panel of wild type and tyrosine to alanine mutants of GST-Siglec-5 cytoplasmic tail fusion proteins phosphorylated in vitro with c-Src kinase. SHP-1 and SHP-2 in U937 lysates bound to tyrosyl-phosphorylated Siglec-5, whereas only a very small amount of SHP-2 was observed with unphosphorylated Siglec-5 (Fig. 2E). Interestingly, mutation of the first tyrosine residue (5Y1A) inhibited the binding of SHPs-1 and -2, whereas mutation of the second tyrosine residue (5Y2A) had no effect on SHP-2 binding but significantly reduced SHP-1 binding in a manner similar to
that seen with transfected RBL cells (Fig. 2, D and E).

Tyrosine to Phenylalanine Mutations of Siglec-5 Abrogate the Inhibition of FceRI-mediated Serotonin Release and SHP-1 Recruitment in RBL Cells—The results described above showed, surprisingly, that alanine mutations of both tyrosine residues in the ITIM and ITIM-like motifs of Siglec-5 had no influence on inhibitory signaling. Because most mutagenesis studies of inhibitory receptors employ tyrosine to phenylalanine mutations, we prepared a Siglec-5 mutant, 5Y12F, in which both tyrosines were changed to phenylalanine (Table II). RBL-5Y12F and RBL-5Y12A displayed similar levels of Siglec-5, MHC class I molecules, KIR2DL3, and FcεRI (Figs. 2A and 3A). However, in striking contrast to the double alanine mutant, the double phenylalanine mutant lost all inhibitory activity when tested in the serotonin release assay (Fig. 3, B and C). This correlated with a failure of this mutant to recruit detectable SHP-1 in immunoprecipitation experiments, whereas the double alanine mutant consistently recruited low levels of SHP-1, similar to wild type Siglec-5 not treated with pervanadate (Fig. 3D).

The Proximal Tyrosine Motif Modulates Sialic Acid-dependent Adhesion of Siglec-5 in a SHP-2-dependent Manner—It has been shown previously that a mutation of the tyrosine residue in the membrane proximal ITIM of CD33 and Siglecs-7 and -9 can lead to enhanced sialic acid-dependent adhesion to human RBCs (6, 14). To determine whether tyrosine to alanine or tyrosine to phenylalanine mutations of the tyrosine motifs of Siglec-5 similarly affected sialic acid-dependent adhesion, RBL-5WT and mutants were tested in RBC binding assays. As might be expected, no rosettes occurred with untreated RBL cells (Fig. 4A) due to cis interactions between Siglec-5 and sialic acids on RBL cells (15), whereas sialidase-treated RBL-5WT mediated low levels of RBC binding activity. A striking increase in binding was observed with Y1A and Y12A or Y12F mutants, but no effect was observed with the Y2A mutant (Fig. 4A). These results show that the membrane proximal tyrosine motif of Siglec-5 modulates its ligand binding activity similarly to other CD33-related siglecs tested previously.

Because both wild type Siglec-5 and the 5Y2A mutant exhibited low sialic acid binding activity (Fig. 4A) and high levels of SHP-2 recruitment (Fig. 2, D and E), we next investigated whether SHP-2 could negatively regulate the ligand binding activity of Siglec-5. For this purpose we used COS cells that naturally express SHP-2, but not SHP-1, and asked whether the catalytically inactive SHP-2 mutant could reverse the enhanced adhesion mediated by the Y1A and Y12A mutants (Fig. 4B). COS cells showed significant adhesion to RBC in the absence of sialidase treatment that was further increased after pervanadate, lyed, and immunoprecipitated with anti-Siglec-5 mAb. The immunoprecipitates were then analyzed by immunoblotting with a sheep pAb anti-Siglec-5, anti-phosphotyrosine, anti-SHP-1, or anti-SHP-2. B and C, to visualize interactions between Siglec-5 and SHPs in living cells, RBL-5WT cells were transfected with cDNAs encoding SHP-1-GFP (B, upper 12 panels), mutSHP-1-GFP (C453S) (B, lower 4 panels), SHP-2-GFP (C, upper 12 panels), or mutSHP-2-GFP (C459S) (C, lower 4 panels). For untreated and pervanadate-treated cells, Siglec-5 was capped by incubation with sheep anti-Siglec-5 pAb followed by TRITC-conjugated rabbit F(ab’2), anti-sheep Ig and then treated or not with pervanadate before fixation with paraformaldehyde. For IgE cross-linking, and cells were incubated with mouse IgE and anti-Siglec-5 mAb followed by goat anti-mouse Ig F(ab’2) and then labeled with sheep anti-Siglec-5 pAb followed by TRITC-conjugated rabbit anti-sheep Ig F(ab’2). The panels show phase contrast images of selected cells together with TRITC, GFP, and merged fluorescence images. Examples of colocalization of Siglec-5 with SHP-1, mut-SHP-1, SHP-2, or mutSHP-2-GFP are indicated by the arrowheads. Results shown are representative of at least three independent experiments carried out in each case.
sialidase treatment, as reported previously (15). Co-transfection of mutSHP-2-GFP significantly increased the RBC binding activity of Siglec-5 on both sialidase-treated and untreated cells, whereas the wild type SHP-2-GFP had no effect (Fig. 4C). The enhancing effect of mutSHP-2-GFP was specific because it did not affect binding mediated by the 5Y1A mutant (Fig. 4C).

Taken together, these results suggest that SHP-2 recruitment by the membrane proximal ITIM of Siglec-5 is involved in suppression of sialic acid-dependent adhesion.

Non-phosphorylated and Tyrosine-Alanine Mutants of Synthetic and Recombinant Siglec-5 Peptides Activate SHP-1 in Vitro—To explore the molecular basis for the inhibition of serotonin release mediated by the double tyrosine to alanine Siglec-5 mutant, we next asked whether SHP-1 could be directly activated by non-phosphorylated forms of wild type Siglec-5 and the double alanine mutant. This was studied by...
measuring the \textit{in vitro} phosphatase activity of SHP-1 in the presence of polypeptides encompassing wild type or mutant forms of the Siglec-5 cytoplasmic tail (Fig. 5). The addition of a synthetic non-phosphorylated peptide spanning both tyrosine motifs of Siglec-5 (amino acids 514–551) resulted in a dose-dependent activation of SHP-1 (Fig. 5A). This was 100-fold lower than the activation mediated by the corresponding doubly phosphorylated peptide, whereas an irrelevant non-phosphorylated peptide had no activity (Fig. 5A). In a second series of experiments, recombinant full-length Siglec-5 cytoplasmic tail constructs (amino acids 468–551) were tested (Fig. 5B). Weak SHP-1 activation was seen with the non-phosphorylated wild type and double tyrosine to alanine-mutated form of Siglec-5 cytoplasmic tail. In contrast, no significant SHP-1 activation was seen with the tyrosine to phenylalanine-mutated form of Siglec-5 (Fig. 5B). As a control wild type Siglec-5 cytoplasmic tail was phosphorylated \textit{in vitro} by c-Src kinase and tested. This conferred strong SHP-1 activation similar to that seen with the synthetic doubly phosphorylated cytoplasmic tail peptide (Fig. 5, A and B). In conclusion, weak and equivalent activation of SHP-1 was observed with wild type non-phosphorylated and double alanine mutant of Siglec-5 cytoplasmic tail. Furthermore, these results show that the double alanine-substituted form of Siglec-5 cytoplasmic tail is a better non-phosphorylatable mimic of wild type Siglec-5 than the phenylalanine substituted form.

**DISCUSSION**

In this paper we have shown that Siglec-5 can be tyrosine-phosphorylated, recruit SHP-1 and SHP-2, and inhibit the FceRI-mediated activation of RBL cells. We also show that the membrane proximal ITIM of Siglec-5 is implicated in

**TABLE II**

Constructs used in this study

| Construct | Membrane-proximal motif | Distal-distal motif |
|-----------|-------------------------|------------------|
| 5WT       | 510-PPLEQKPLAYSLFSEM...KREPKDQAPPTTFYSEIKTSK-551 |                  |
| 5Y1A     | (Y520A)                |                  |
| 5Y2A     | (Y544A)                |                  |
| 5Y12A    | (Y520A/Y544A)          |                  |
| 5Y12F    | (Y520F/Y544F)          |                  |
| 5WT      | 448-ALLCICLICLICLIFL...KARRKQAAGRPEKMDDEDPIMGTITSG-488 |                  |
| 5Δ       | (R465Δ)                |                  |

![Fig. 3](link-to-figure)
suppression of its sialic acid dependent binding activity. These results formally demonstrate that Siglec-5 can be classified as an inhibitory receptor (1). Surprisingly, however, our mutagenesis studies clearly showed that inhibition of serotonin release mediated by Siglec-5 may occur in the absence of tyrosine phosphorylation.

The membrane proximal tyrosine-based motif (LHYASL) of Siglec-5 conforms exactly to the ITIM consensus sequence ((V/I/L)XX(L/V), where X is any amino acid) established largely on the basis of mutagenesis studies with other inhibitory receptors (1). By contrast, the membrane distal motif (TEYSEI) is more similar to the immunoreceptor tyrosine-based switching motif (TXXXX(V/I)) described in the CD150/SLAM subfamily (23). Immunoprecipitation experiments with transfected RBL cells together with pull-downs using GST-Siglec-5 fusion proteins showed that phosphorylated WT Siglec-5 and the Y2A mutant were able to recruit both SHP-1 and SHP-2, whereas little or no interaction was seen with the phosphorylated Y1A mutant. These findings show that the membrane proximal motif is dominant over the distal motif for interactions with protein-tyrosine phosphatases and is consistent with previous findings made with other CD33-related siglecs including CD33/Siglec-3, human Siglec-L1, and mouse Siglec-E (6, 7, 9, 12, 13, 24).

Both SHP-1 and SHP-2 contain two SH2 domains that play different roles in phosphopeptide binding and subsequent phosphatase activation (25–27). Interactions of phosphopeptides with the N-terminal SH2 domain lead to its displacement from the catalytic site and trigger phosphatase activation (26). Interactions with the C-terminal domain indirectly affect phosphatase activation by mediating the initial contacts with phosphorylated ligands (27) and by contributing to binding strength (26). Based on a phosphopeptide library screen (28), the membrane proximal motif of Siglec-5 fits the consensus for both the N- and C-terminal SH2 domains (LXpYAXL and (V/I/L)LXpYAXL), respectively, where p indicated phosphorylation), whereas the membrane distal motif fits neither. However, the membrane distal motif is very similar to the equivalent motif in CD33/Siglec-3 (TEpYSEV), which was shown to bind the C-terminal SH2 domain of SHP-1 but not that of SHP-2 (6). This suggests that, similar to CD33, the membrane proximal motif of Siglec-5 is likely to make dominant interactions with both SHP-1 and SHP-2 via the N-terminal domain. In contrast, the membrane distal motif may be important for selectively enhancing interactions with SHP-1.

Despite the clear reductions in SHP-1 and SHP-2 binding after tyrosine to alanine mutations of the tyrosine motifs, these mutations had no effect on inhibitory signaling by Siglec-5. Although it is possible that Siglec-5 inhibitory signaling is mediated via an ITIM- and protein-tyrosine phosphatase-independent pathway, our favored hypothesis is that weak interactions between non-phosphorylated Siglec-5 and SHP-1 and/or SHP-2 are sufficient to mediate inhibitory activity via phos-
phatase activation. In support of this idea, we showed that a non-phosphorylated synthetic peptide or recombinant protein corresponding to the intracellular region of Siglec-5 was able to induce weak SHP-1 phosphatase activation. This was also seen with the double tyrosine to alanine mutant, whereas the double tyrosine to phenylalanine mutant had minimal activity. These results suggest that Siglec-5 is able to recruit and activate SHP-1 in a phosphotyrosine-independent manner in living cells. Similar observations were made recently in study of SHP-2-dependent inhibition of cytotoxicity mediated by KIR2DL4 (29).

Mutation of tyrosine to phenylalanine is widely employed for evaluating the importance of tyrosine phosphorylation in studies of cellular signaling, including many published reports on ITIM-containing inhibitory receptors. This is viewed as the most conservative mutation with just the loss of the hydroxyl and the preservation of the aromatic ring. In our studies it is apparent that the substitution of the tyrosine to alanine rather than phenylalanine is a more functionally conservative mutation. Two explanations are possible. First, the loss of the hydroxyl group could create a hydrophobic patch causing a conformational change or misfolding of the cytoplasmic tail such that the ITIM is not presented correctly for SH2 domain binding. Second, an alanine residue could sit at the edge of the phosphotyrosine binding pocket and not disrupt the network of hydrogen-bonded water molecules that are usually displaced or interacted with by phosphotyrosine. In comparison, the bulky hydrophobic side chain of phenylalanine could be sterically hindered from entering the pocket by the stable network of water molecules. The alanine mutation would, therefore, allow the other ITIM residues to contribute to binding, whereas the phenylalanine mutation would disrupt the docking of the SH2-domain to the entire ITIM.

Although phosphotyrosines make key electrostatic interactions with arginine residues within the SH2 domains of SHP-1 (27) and SHP-2 (25, 26), there are a number of additional contacts, particularly hydrophobic interactions involving residues at the Y+3 and Y+5 positions, that are important for high affinity binding (25). In this respect it is noteworthy that the Y+5 residue of the Siglec-5 ITIM is phenylalanine, which would be expected to contribute significantly to phosphotyrosine-independent binding and activation (25, 26).

Besides a role in inhibitory signaling, we also showed that the membrane proximal motif of Siglec-5 is important for suppressing sialic acid-dependent adhesion mediated by Siglec-5. In contrast to the inhibitory assays, however, both YIA and YIF mutations led to a similar increase in sialic acid-dependent binding, suggesting that phosphorylation may play a more important role in this signaling pathway than for inhibition of cellular activation. Similar findings have been made previously for CD33/Siglec-3 (6) and Siglecs-7 and -9 (14), suggesting this is likely to be a general feature of ITIM-containing CD33-related siglec. In the present study SHP-2 was implicated as a negative regulator of Siglec-5 binding since its recruitment correlated with low sialic acid-dependent binding activity, and introduction of a catalytically inactive mutant reversed the adhesion-suppressed phenotype. Although the mechanism by which SHP-2 would mediate this effect is unknown, it is likely to be linked to receptor mobility and clustering since monomeric siglec exhibit low affinity for sialylated ligands and multivalent binding is essential for stable interactions with cellular targets. In this regard SHP-2 has been shown to function as an upstream activator of RhoA and is, therefore, implicated in regulating the actin cytoskeleton organization (30).

The fact that Siglec-5 is expressed on neutrophils, monocytes, macrophages, and dendritic cells raises the question of its potential role as an inhibitory receptor on myeloid cells. Human myeloid cells express several other inhibitory receptors such as CD31/PECAM-1 (31), FeRlb (32), LAIR-1 (33), and several members of immunoglobulin-like transcript receptor family (34). Ligation of these receptors has been shown to modulate a range of leukocyte functions, including migration, proliferation, apoptosis, and cellular activation. Interestingly, ligation of Siglec-5 on neutrophils using intact mAbs was shown to enhance the oxidative burst activity in response to formylmethylleucylphenylalanine (35). Although not examined, it is possible that this effect was due to sequestration of Siglec-5 from “activatory” domains on the neutrophil plasma membrane. Thus, weak constitutive interactions between clustered Siglec-5 and SHP-1 and/or SHP-2 could be important in maintaining leukocytes in the quiescent state until activation is triggered via appropriate receptors. Siglec-5 clustering and inhibitory signaling could be mediated via cis interactions with appropriately sialylated activatory receptors, as shown recently for Siglecs-7 and -9 in transfected human Jurkat T cells (36) and CD33 expressed on human monocytes (37).

By interacting in trans with sialic acids presented on other cells, Siglec-5 could modulate the activation of myeloid cells to prevent damage to “self” tissues in a similar way as the myeloid inhibitory receptors CD200R and SHP-α (38). Siglec-5 has also been shown to bind selectively to sialic acids on the human pathogen Neisseria meningitidis and is, therefore, implicated in host defense functions (39). Further studies are needed to investigate the mechanism and biological significance of the ITIM-dependent adhesion modulation of Siglec-5 demonstrated here, but this is clearly of relevance for the potential of Siglec-5 to mediate both cis and trans interactions with ligands on cells and pathogens. Because there is no obvious orthologue of Siglec-5 in the mouse (40), it will not be possible to use gene disruption experiments to investigate its function in rodent models. However, Siglec-E is the predominant ITIM-bearing siglec expressed on myeloid cells of the mouse (41), so genetic manipulation of this molecule may provide useful insights into the corresponding biological functions of Siglec-5 in humans.

Acknowledgments—We thank Benjamin Neel for the kind gift of SHP-1 and SHP-2 cDNA, Eric Vivier and Frederic Lopez for the KIR2DL3-expressing RBL cells, Mathias Lucas for the mouse IgE mAb, Claire Jones and Kevin Lock for the anti-Siglec-5 antibodies, James Matthews for critical comments on the manuscript.

REFERENCES

1. Ravetch, J. V., and Lanier, L. L. (2000) Science 290, 84–89
2. Staub, E., Rosenthal, A., and Hinzmann, B. (2004) Cell Sigil 16, 435–456
3. Stebbins, C. C., Watzl, C., Billadeau, D. D., Leibson, P. J., Burstyn, D. N., and Long, E. O. (2003) Mol. Cell. Biol. 23, 6291–6299
4. Crocker, P. R., and Varki, A. (2001) Trends Immunol. 22, 337–342
5. Crocker, P. R. (2002) Curr. Opin. Struct. Biol. 12, 609–615
6. Taylor, V. C., Buckley, C. D., Douglas, M., Cody, A. J., Simmons, D. L., and Freeman, S. D. (1999) J. Biol. Chem. 274, 15150–15152
7. Ulyanova, T., Shah, D. D., and Thomas, M. L. (2001) J. Biol. Chem. 276, 14451–14458
8. Paeo, M., Biasetti, R., Bottino, C., Vitale, M., Sivori, S., Augugliaro, R., Moretta, L., and Moretta, A. (1999) J. Exp. Med. 190, 783–802
9. Yu, Z., Maoui, M., Wu, L., Banville, D., and Shen, S. (2001) Biochem. J. 353, 483–492
10. Angata, T., Kerr, S. C., Greaves, D. B., Varki, N. M., Crocker, P. R., and Varki, A. (2002) J. Biol. Chem. 277, 24466–24474
11. Kitzig, F., Martinez-Barriocanal, A., Lopez-Botet, M., and Sayos, J. (2002) Biochem. Biophys. Res. Commun. 296, 355–362
12. Ulyanova, T., Biasioli, J., Woodford-Thomas, T. A., and Thomas, M. L. (1999) Eur. J. Immunol. 29, 3440–3449
13. Paul, S. P., Taylor, L. S., Stansbury, E. K., and McVicar, D. W. (2000) J. Immunol. 164, 6841–6849
14. Avrit, T., Floyd, H., Lopez, F., Vivier, E., and Crocker, P. R. (2004) J. Immunol. 173, 6841–6849
15. Cornish, A. L., Freeman, S., Forbes, G., Ni, J., Zhang, M., Cepeda, M., Gentsch, R., Augustus, M., Carter, K. C., and Crocker, P. R. (1996) Blood 92, 2123–2132
16. Connolly, N. P., Jones, M., and Watt, S. M. (2002) Br. J. Haematol. 116, 221–238
17. Virgo, P., Denning-Kendall, P. A., Erickson-Miller, C. L., Singha, S., Evley, R., Howes, J. M., and Freeman, S. D. (2003) Br. J. Haematol. 123, 420–424
18. Lock, K., Zhang, J., Lu, J., Lee, S. H., and Crocker, P. R. (2004) Immunology 209, 199–207
19. Zhang, J. Q., Nicoll, G., Jones, C., and Crocker, P. R. (2004) Immunobiology 209, 199–207
20. Blery, M., Delon, J., Trautmann, A., Cambiaggi, A., Olcese, L., Biassoni, R., Moretta, L., Chavrier, P., Moretta, A., Daeron, M., and Vivier, E. (1997) J. Biol. Chem. 272, 8989–8996
21. Fischer, E. H., Charbonneau, H., and Tonks, N. K. (1991) Science 253, 401–406
22. Milarski, K. L., and Saltiel, A. R. (1994) J. Biol. Chem. 269, 21239–21243
23. Shlapatska, L. M., Mikhala, S. V., Berdeva, A. G., Zelensky, O. M., Yun, T. J., Nichols, K. E., Clark, E. A., and Siderenko, S. P. (2001) J. Immunol. 166, 5480–5487
24. Yu, Z., Lai, C. M., Masui, M., Banville, D., and Shen, S. H. (2001) J. Biol. Chem. 276, 23816–23824
25. Lee, C. H., Kominos, D., Jacques, S., Margolis, B., Schlessinger, J., Shoelson, S. E., and Kurzyn, J. (1994) Structure 2, 423–438
26. Hof, P., Pluskey, S., Bhe-Paganon, S., Eck, M. J., and Shoelson, S. E. (1998) Cell 92, 441–450
27. Yang, J., Liu, L., He, D., Song, X., Liang, X., Zhao, L. J., and Zhou, G. W. (2005) J. Biol. Chem. 278, 43117–43125
28. Beebe, K. D., Wang, P., Arabaci, G., and Pei, D. (2000) Biochemistry 39, 13251–13260
29. Yusa, S., Catina, T. L., and Campbell, K. S. (2002) J. Immunol. 168, 5047–5057
30. Schoenwaelder, S. M., Petch, L. A., Williamson, D., Shen, R., Feng, G. S., and Burridge, K. (2000) Curr. Biol. 10, 1523–1526
31. Jackson, D. E. (2003) FEBS Lett. 540, 7–14
32. Tridandapani, S., Steffek, K., Teilza, J. L., Carter, J. E., Wewers, M. D., and Anderson, C. L. (2002) J. Biol. Chem. 277, 5082–5089
33. Meyard, L., Adema, G. J., Chang, C., Woollatt, E., Sutherland, G. R., Lanier, L. L., and Phillips, J. H. (1997) Immunity 7, 283–290
34. Dietrich, J., Nakajima, H., and Colonna, M. (2000) Microbes Infect. 2, 323–329
35. Erickson-Miller, C. L., Freeman, S. D., Hopson, C. B., D’Alessio, K. J., Fischer, E. I., Kikly, K. K., Abrahamson, J. A., Holmes, S. D., and King, A. G. (2003) Exp. Hematol. 31, 382–388
36. Ikehara, Y., Ikehara, S. K., and Paulson, J. C. (2004) J. Biol. Chem. 279, 43117-43125
37. Lajaunias, F., Dayer, J. M., and Chizzolini, C. (2005) Eur. J. Immunol. 35, 243–251
38. Nathan, C., and Muller, W. A. (2001) Nat. Immunol. 2, 17–19
39. Jones, C., Virji, M., and Crocker, P. R. (2003) Mol. Microbiol. 49, 1213–1225
40. Angata, T., Margulies, E. H., Green, E. D., and Varki, A. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 13251–13256
41. Zhang, J. Q., Biedermann, B., Nitschke, L., and Crocker, P. R. (2004) Eur. J. Immunol. 34, 1175–1184
Siglec-5 (CD170) Can Mediate Inhibitory Signaling in the Absence of Immunoreceptor Tyrosine-based Inhibitory Motif Phosphorylation

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*J. Biol. Chem.* 2005, 280:19843-19851.
doi: 10.1074/jbc.M502041200 originally published online March 15, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M502041200

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