The Membrane-Proximal Immunoreceptor Tyrosine-Based Inhibitory Motif Is Critical for the Inhibitory Signaling Mediated by Siglecs-7 and -9, CD33-Related Siglecs Expressed on Human Monocytes and NK Cells

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The Membrane-Proximal Immunoreceptor Tyrosine-Based Inhibitory Motif Is Critical for the Inhibitory Signaling Mediated by Siglecs-7 and -9, CD33-Related Siglecs Expressed on Human Monocytes and NK Cells

Tony Avril,* Helen Floyd,* Frederic Lopez,† Eric Vivier,† and Paul R. Crocker2*

Siglec-7 and Siglec-9 are two members of the recently characterized CD33-related Siglec family of sialic acid binding proteins and are both expressed on human monocytes and NK cells. In addition to their ability to recognize sialic acid residues, these Siglecs display two conserved tyrosine-based motifs in their cytoplasmic region similar to those found in inhibitory receptors of the immune system. In the present study, we use the rat basophilic leukemia (RBL) model to examine the potential of Siglec-7 and -9 to function as inhibitory receptors and investigate the molecular basis for this. We first demonstrate that Siglec-7 and -9 are able to inhibit the FcεRI-mediated serotonin release from RBL cells following co-crosslinking. In addition, we show that under these conditions or after pervanadate treatment, Siglec-7 and -9 associate with the Src homology region 2 domain-containing phosphatases (SHP), SHP-1 and SHP-2, both in immunoprecipitation and in fluorescence microscopy experiments using GFP fusion proteins. We then show by site-directed mutagenesis that the membrane-proximal tyrosine motif is essential for the inhibitory function of both Siglec-7 and -9, and is also required for tyrosine phosphorylation and recruitment of SHP-1 and SHP-2 phosphatases. Finally, mutation of the membrane-proximal motif increased the sialic acid binding activity of Siglec-7 and -9, raising the possibility that “inside-out” signaling may occur to regulate ligand binding.
whereas Siglec-9 does not bind to α(2,8)-linked diosialic acids but prefers terminal α(2,3)- and α(2,6)-linkages (16, 17). Regardless of the role of sialic acid recognition on their function, Siglec-7 acts as an inhibitory receptor in human NK cells after engagement by Abs (14) or binding with sialic acid-containing ligands (18) and once phosphorylated can recruit the SH2 domain-bearing protein tyrosine phosphatase (PTP) SHP-1 (14). Anti-Siglec-7 Abs also inhibit the proliferation of myeloid cells (19). Concerning the function of Siglec-9, there have been no reports so far, although its high similarity with Siglec-7 strongly suggests Siglec-9 has a similar inhibitory function.

In this study, we demonstrate for the first time that Siglec-9 acts as an inhibitory receptor using the rat basophilic leukemia (RBL) model, which has been widely used to analyze KIR functions in particular. We show that Siglec-9, as well as Siglec-7, can inhibit FcεRI-mediated serotonin release of RBL cells and recruit the tyrosine phosphatases SHP-1 and SHP-2. We then show that the membrane-proximal ITIM is essential for the inhibitory activities of Siglecs-7 and -9, for their tyrosine phosphorylation, and for the recruitment of the PTPs SHP-1 and SHP-2. Furthermore, mutation of the membrane-proximal motif leads to an increase in their RBC binding ability, indicating that signaling involving SHP-1 and SHP-2 may modulate sialic acid recognition mediated by Siglecs-7 and -9.

Materials and Methods
Reagents, plasmids, and Abs
Unless otherwise specified, all reagents and chemicals were purchased from Sigma-Aldrich (Poole, U.K.). [3H]Serotonin ([3H]hydroxytryptamine creatine sulfate, 5-[1,2-[3H][N]])) was obtained from NEN (Hounslow, U.K.). Vibrio cholerae sialidase was obtained from Calbiochem (Nottingham, U.K.).

The cDNAs encoding human Siglecs-7 and -9 were described in Refs. 13 and 15. Site-directed mutagenesis of Siglecs-7 and -9 cDNAs was performed with sets of mutagenic primers (Table I) using the QuickChange kit (Stratagene, Amsterdam, The Netherlands) according to the manufacturer’s instructions. The tail deletion form of Siglecs-7 (7Δ) and -9 (9Δ) were obtained by PCR using sets of primers described in Table I and cloned into the pcDNA3 vector (Invitrogen, Paisley, U.K.). The cDNA-encoding human tyrosine phosphatases SHP-1 and SHP-2 were a kind gift from Benjamin Neel (Harvard Medical School, Boston, MA) and were cloned into the pEGFP-N1 vector (Clontech, Basingstoke, U.K.). Catalytically inactive forms of SHP-1 (C453S, mutSHP-1) and SHP-2 (C459S, mutSHP-2) were generated as above using sets of mutagenic primers described in Table I. All the PCRs were performed using PFU Turbo DNA polymerase (Stratagene), and the presence of introduced mutations was confirmed by DNA sequencing.

The purified sheep polyclonal Ig and mAbs anti-human Siglec-7 (S7.5a) and anti-human Siglec-9 (K8) were previously described (13, 18). The GL183 (anti-CD158b/KIR2DL3) mAb was obtained from Immunotech (Marseille, France). The IgE-3 mouse IgE was obtained from BD Biosciences (Oxford, U.K.). The goat anti-mouse IgG F(ab′)2 Fragment was obtained from Jackson ImmunoResearch (Luton Beds, U.K.). The FITC-conjugated rabbit anti-mouse Ig F(ab′)2 Fragment was obtained from Dako (Ely, U.K.). The rabbit polyclonal Abs C-19 anti-SHP-1 and C-18 anti-SHP-2 were obtained from Santa Cruz Biotechnology (Calne, U.K.). The 4G10 anti-phosphotyrosine hybridoma was a kind gift from Lars Nitschke (University of Wurzburg, Germany). The HRP-conjugated anti-mouse, rabbit, and sheep IgG Abs were obtained from Vector Laboratories (Peterborough, U.K.).

Cells
The RBL-2H3 cells expressing KIR2DL3 (20) were cultured in DMEM supplemented with 10% FCS, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Stable cell lines expressing wild-type (WT) and mutant forms of Siglecs-7 and -9 were generated by electroporation and selection with geneticin at 1 mg/ml (G418) (Roche, East Sussex, U.K.).

Flow cytometry
All incubations were conducted on ice. Cells were incubated with primary mAbs (10 μg/ml) for 30 min, washed, and incubated with the FITC-conjugated rabbit anti-mouse Ig F(ab′)2 Fragment for 30 min. Cells were analyzed using a FACS Calibur (Becton Dickinson, Oxford, U.K.).

Serotonin release assay
The serotonin release assay was performed as described in Ref. 20. Briefly, [3H]serotonin-labeled RBL cells were incubated with mouse IgE (at 0.1 μg/ml) in the absence or presence of various amount of anti-MHC class I molecules (OX-18), anti-Siglec-7 (S7.5a), anti-Siglec-9 (K8), or anti-KIR2DL3 (GL183) mAbs. After washing, cells were incubated for 30 min at 37°C with goat anti-mouse IgG F(ab′)2 (at 50 μg/ml). Supernatants were collected, and the released radioactivity was analyzed using the WinSpectral liquid scintillation counter (Wallac, Milton Keynes, U.K.). Each assay was set up in triplicate, and the results were expressed as a percentage of specific serotonin release: (cpm test – cpm spont)/(cpm max – cpm spont) × 100, where cpm spont is the spontaneous release of [3H]serotonin obtained with cells incubated in absence of Abs and cpm max is the maximum release of [3H]serotonin obtained with cells lysed using Triton X-100. For comparative analysis, results were expressed as a mean of the percentage of inhibition of serotonin release: 100 – (% test × 100%/% IgE) obtained in experiments performed with different clones, where % IgE is

Table I. Primers used in this studya

| 7Y1 | Sense          | 5′-GGAAAGAAGGATCTGAGTCCTGGACCCCTCAGCC-3′ |
| 7Y2 | Sense          | 5′-GGCCACCAACTAGTCTCAGAGATCAGATCC-3′  |
| 7Δ  | Sense          | 5′-GGATTTGTAGTCTGAGAACTCATATTGTTCTGCG-3′ |
| 9Y1 | Sense          | 5′-GGCAGGCGCGGGGCTCCTGGC-3′          |
| 9Δ  | Sense          | 5′-GGCCACTGACACCGAGTCTCAGGAGATACAGCC-3′ |

| SHP-1 | Sense          | 5′-GGCCAGGGACGAGGCTGTTG-3′          |
| mutSHP-1 | Sense      | 5′-GGGCCCATACATGTCGCGCATCACAGCTACATACAAGG-3′ |
| SHP-2 | Sense          | 5′-GGCCAGGGACGAGGAGTCTGAG-3′          |
| mutSHP-2 | Sense      | 5′-GGGCGGCGCTGCTGCGCATCACAGCTACATACAAGG-3′ |

aNucleotide changes introduced for changing tyrosine to phenylalanine (for 7Y1, 7Y2, 9Y1, and 9Y2 constructs) and cysteine to serine (for mutSHP-1 and mutSHP-2 constructs) are in bold. Nucleotide changes that introduce restriction enzyme sites to facilitate cloning (XbaI for 7Δ/H0004 and mutSHP-1) were described in Table I. All the PCRs were performed using PFU Turbo DNA polymerase (Stratagene), and the presence of introduced mutations was confirmed by DNA sequencing.
the percentage of specific serotonin release obtained with cells incubated with mouse IgE alone.

**Immunoprecipitation and immunoblotting**

Immunoprecipitation and immunoblotting were performed as previously described (21). Briefly, cells were treated or not with pervanadate (0.5 mM) for 10 min at 37°C. Pre-cleared cell lysates were immunoprecipitated with S7.5a (anti-Siglec-7) or K8 (anti-Siglec-9) mAbs and then analyzed by immunoblotting with sheep anti-Siglec-7 or -9 polyclonal Abs (PAsbs), with 4G10 mAbs anti-phosphotyrosine or with rabbit PAsbs C-19 anti-SHP-1 or C-18 anti-SHP-2.

**Fluorescence microscopy**

Siglecs-7 and -9 RBL clones were transiently transfected with SHP-1-GFP, SHP-2-GFP, musSHP-1-GFP, or musSHP-2-GFP cDNA by electroporation and cultured overnight on cover slips (BDH, Lutterworth, U.K.). Cells were then stained with anti-Siglec-7 or anti-Siglec-9 sheep PAsbs (10 μg/ml) for 30 min at 4°C and incubated for 30 min at 37°C with tetramethylrhodamine isothiocyanate (TRITC)-conjugated rabbit anti-sheep Ig F(ab')2, to generate patched Siglecs. Cells were then treated or not with pervanadate (0.5 mM) for 10 min at 37°C, fixed with 4% paraformaldehyde, and analyzed by fluorescence microscopy. For the FcεRI-mediated activation of RBL cells, GFP-transfected cells were stained with mlgE (0.1 μg/ml) and S7.5a or K8 mAbs (10 μg/ml) for 30 min at 37°C, incubated with goat anti-mouse Ig F(ab')2 (50 μg/ml) for 30 min at 37°C and then labeled with anti-Siglec-7 or anti-Siglec-9 sheep PAsbs (10 μg/ml) and TRITC-conjugated rabbit anti-sheep Ig F(ab')2 (10 μg/ml) for 15 min at 37°C to create clusters. Images were acquired using the AxioVision imaging system (Imaging Associates, Bicester, U.K.) and a Zeiss immuno-fluorescence microscope (Jena, Germany).

**RBC binding assays**

RBC binding assays were performed as described in (13). Briefly, RBL 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 RBC were gently washed, and cells were fixed with 0.25% glutaraldehyde and rosetting assessed by microscopy. To quantify binding, the percentage of RBL cells forming rosettes (defined as RBL cells binding >5 RBC) was scored from counting at least 200 RBL cells per field in five different fields per experiment. Results are expressed as a mean percentage, obtained from three independent experiments.

**Statistics**

Values represent the mean ± SD of n different experiments. Student’s t test was applied using a two-tailed distribution of two samples of equal or unequal variances.

**Results**

Cross-linking of Siglec-7 and -9 inhibits FcεRI-mediated serotonin release in RBL cells

It has been shown previously that Siglec-7 acts as a NK cell inhibitory receptor in cytotoxicity assays (14, 18). In contrast, there have been no reports on the inhibitory function of Siglec-9. To investigate the potential inhibitory functions of Siglec-7 and -9 in parallel, we used the well-defined RBL model in which co-engagement of inhibitory receptors and the activatory receptor, FcεRI, inhibits serotonin secretion by RBL cells incubated with mlgE. We generated RBL cell lines stably transfected with Siglec-7 or -9 cDNA (designated RBL-7WT and RBL-9WT, respectively) using parental RBL-2H3 cells that had already been transfected with the well-characterized inhibitory receptor KIR2DL3. The expression of KIR2DL3 provided a useful internal reference control in the inhibition assays. As shown in Fig. 1A on a representative clone, expression of Siglec-7 or -9, KIR2DL3, MHC class I (used as a negative control in the inhibition assays), and FcεRI molecules assessed by binding of IgE was demonstrated by flow cytometry analysis (Fig. 1A). RBL-7WT and -9WT clones were then incubated with a range of dilutions of mAbs anti-MHC class I molecules (irrelevant mAb), anti-KIR2DL3 (positive control), anti-Siglec-7 or anti-Siglec-9 in the presence of mlgE and used in a serotonin release assay. As described previously (20), cross-linking of KIR2DL3 with FcεRI dramatically reduced the level of serotonin release of RBL-7WT and -9WT cells in a dose-dependent manner, whereas no significant effect was observed in the presence of an irrelevant mAb that bound MHC class I molecules (Fig. 1B). In contrast, cross-linking of Siglecs-7 and -9 substantially reduced the serotonin release of RBL-7WT and -9WT cells.

**FIGURE 1.** Inhibitory function of Siglecs-7 and -9 in RBL cells. A. RBL-7WT and -9WT cells were stained or not (open histograms) with OX-18 (anti-MHC class I molecules), S7.5a (anti-Siglec-7), K8 (anti-Siglec-9), GL183 (anti-KIR2DL3) mAbs or mlgE (IgE-3) (shaded histograms), followed by FITC-conjugated rabbit anti-mouse Ig F(ab')2, and analyzed by flow cytometry. B. [3H]Serotonin-labeled RBL-7WT and RBL-9WT cells were incubated with mlgE alone (○) or with serial dilutions with an irrelevant mAb OX-18 (■), S7.5a (▲), K8 (●), or GL183 (●) mAbs and then challenged with goat anti-mouse Ig F(ab')2. The serotonin released in supernatants was measured. Results shown are expressed as the mean percentage of specific serotonin release of triplicate wells from one experiment representative of at least three independent experiments.
These results show that Siglec-9 is as potent as Siglec-7 in delivering inhibitory signals to RBL cells. Activation of RBL cells leads to the recruitment of the phosphatases SHP-1 and SHP-2 by Siglecs-7 and -9.

To address the question of whether Siglecs-7 and -9 can recruit the phosphatases SHP-1 and SHP-2 under conditions of activation in RBL cells, we transfected RBL-7WT and -9WT cells with SHP-1-GFP or SHP-2-GFP cDNA and performed co-localization experiments using fluorescence microscopy. Transfected cells were stained with anti-Siglec-7 or -9 sheep PAbs and then incubated with TRITC-conjugated rabbit anti-sheep Ig F(ab')2 for 1 h at 37°C to create patches of Siglec-7 and -9. The cells were then treated or not with pervanadate to inhibit tyrosine phosphatases.

FIGURE 2. Co-localization of WT and catalytically inactive SHP-1-GFP and SHP-2-GFP with Siglec-7 and -9 in resting and activated RBL cells. RBL-7WT (A and B) and -9WT (C and D) were transfected with SHP-1-GFP (A and C, lane 1–3), SHP-2-GFP (B and D, lane 1–3), mutSHP-1-GFP (A and C, lane 4), or mutSHP-2-GFP (B and D, lane 4). After overnight culture, cells were stained with anti-Siglec-7 or -9 sheep PAbs and incubated for 1 h at 37°C with TRITC-conjugated rabbit anti-sheep Ig F(ab')2. Cells were then treated or not with pervanadate, fixed, and analyzed by fluorescence microscopy. For the FcεRI-mediated activation of RBL cells, GFP-transfected cells were stained with mIgE and S7.5a or K8 mAbs followed by goat anti-mouse Ig F(ab')2, then labeled with anti-Siglec-7 (A and C, lane 3) or -9 (B and D, lane 3) sheep PAbs followed by TRITC-conjugated rabbit anti-sheep Ig F(ab')2. SHP1-, mut-SHP-1-, SHP-2-, or mutSHP-2-GFP clusters are indicated by the arrows.

FIGURE 3. Role of the two tyrosine motifs on the tyrosine phosphorylation of Siglecs-7 and -9 and recruitment of the PTPs SHP-1 and SHP-2 in RBL cells. Parental RBL (A and B, lane 1), Siglec-7 (A), and Siglec-9 (B) RBL clones were untreated or treated with pervanadate, lysed, and immunoprecipitated with S7.5a (anti-Siglec-7, A) or K8 (anti-Siglec-9, B) mAbs. The immunoprecipitates were then analyzed by immunoblotting with anti-Siglec-7 (A, first blot) or -9 (B, first blot) sheep PAbs, 4G10 mAb (anti-phosphotyrosine, A and B, second blot), C-19 rabbit PAb (anti-SHP-1, A and B, third blot), or C-18 rabbit PAb (anti-SHP-2, A and B, fourth blot).
and increase phosphorylation, fixed, and analyzed. As shown in Fig. 2, no co-localization of Siglec-7 or -9 with SHP-1 or SHP-2 was observed with untreated cells, whereas SHP-1 and SHP-2 co-localized with Siglecs-7 and -9 when the cells were treated with pervanadate. To investigate if the co-localization occurs in a more physiological situation, mIgE and anti-Siglec-7 or -9 mAbs were

Table II. Constructs used in this study

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*a* Cytoplasmic tail sequences are shown for Siglecs-7 WT and -9 WT with amino acid substitutions shown for the mutants. Double underlines, putative tyrosine motifs; single underlines, predicted trans-membrane regions; asterisks, stop codon.

FIGURE 4. Role of the two tyrosine motifs on the inhibitory function of Siglecs-7 and -9 in RBL cells. A, Siglecs-7 and -9 RBL clones were analyzed for the expression of MHC class I molecules, Siglec-7, Siglec-9, KIR2DL3, and the binding of mIgE by flow cytometry as described in the legend to Fig. 2. B, Clones expressing similar levels of Siglecs-7 and -9 were used in inhibition assays as described in Fig. 1. C, The bar charts show the mean percentage of inhibition observed with n different clones using mAbs at 10 µg/ml (*, p < 0.05 vs RBL-WT).
Role of the two tyrosine motifs on the RBC binding activity of Siglecs-7 and -9. Siglec-7 (A) and Siglec-9 (B) RBL clones were untreated (gray bars) or sialidase-treated (black bars) and then incubated with human RBC. After washing, cells were fixed and the percentage of RBC cells with rosettes were scored by microscopy as described in Materials and Methods. Results are expressed as the mean percentage obtained in three independent experiments (*, p < 0.05 vs RBL-WT).
and mutSHP-2-GFP and patches of Siglec-7 or -9 were created. Cells were then fixed and analyzed by fluorescence microscopy. No co-localization of Siglec-7 or -9 with mutSHP-1 was observed with any of RBL mutants (data not shown). However, in approximately one-third of RBL-7Y2 or RBL-9Y2 cells, Siglec-7 and -9 clearly co-localized with mutSHP-2 (Fig. 5), whereas no association was observed with the other Siglecs mutants (Fig. 5). These results demonstrate that the membrane-proximal tyrosine motif is essential for the tyrosine phosphorylation of Siglecs-7 and -9 and the recruitment of the PTPs SHP-1 and SHP-2, whereas tyrosine phosphorylation and association with SHP-2 can still occur after mutation of the distal-motif of Siglecs-7 and -9.

**The proximal tyrosine motif modulates the sialic acid recognition of Siglecs-7 and -9.**

As members of the Siglec family, Siglecs-7 and -9 have the ability to recognize sialic acid residues, either in cis on the Siglec-expressing cell, or in trans on other cells (reviewed in Ref. 1). In a recent study, we have shown that the Siglec-7 binding site is masked on NK cells, but can be unmasked following sialidase treatment to destroy the cis-interacting sialic acids (18). Human RBC are a very convenient indicator of sialic acid-dependent binding of most Siglecs, including Siglecs-7 and -9 (13, 15). To investigate if mutations in the tyrosine motifs affect recognition of sialylated glycans by Siglecs-7 and -9, RBL-7WT, -9WT, and respective mutants were treated or not with sialidase and tested in RBC binding assays. As expected, no rosettes occurred with untreated RBL cells (Fig. 6), suggesting that cis-interactions between Siglec-7 or -9 and sialic acids occurs on the membrane of RBL cells and abrogate any trans-interaction. In contrast, sialidase-treated RBL-7WT and -9WT mediated robust RBC binding activity. A striking increase in binding was observed with Y1 and Y1Y2 mutants, but no effect was observed with Y2 mutants (Fig. 6). These results indicate that the membrane-proximal tyrosine motif modulates the ligand binding activity of Siglecs-7 and -9. One explanation could be that the Y1 and Y1Y2 mutations directly affect the distribution of Siglecs-7 and -9 on the membrane of the cells. To investigate this possibility, RBL-7WT, -7Y1Y2, -9WT, and -9Y1Y2 cells were treated or not with sialidase, fixed, stained with anti-Siglec-7 or -9 mAbs, and analyzed by confocal microscopy. No obvious difference was observed between RBL-7WT or -9WT cells and RBL-7Y1Y2 or -9Y1Y2 cells, respectively (data not shown).

**Discussion**

In this study, we show for the first time that Siglec-9 can function as an inhibitory receptor. Taken altogether with the fact that Siglec-9 contains an ITIM within its cytoplasmic domain, inhibits the FcεRI-mediated activation of RBL cells, is tyrosine-phosphorylated after pervanadate-treatment, and recruits the PTPs SHP-1 and SHP-2 after pervanadate-treatment and FcεRI-mediated activation, it fulfills the criteria for inclusion in the inhibitory receptor superfamily (5, 7, 8).

Previous reports on CD33 (22–24) and mouse Siglec-E (25, 26), demonstrated the importance of the membrane-proximal tyrosine motif in recruitment of SHP-1 and SHP-2 by co-immunoprecipitation, but this study on Siglecs-7 and -9 provides the first analysis of the role of tyrosine-based motifs in the inhibitory function for CD33-related Siglecs. Mutation of the membrane-proximal tyrosine motif that conforms to the consensus ITIM led to a complete abrogation of tyrosine-phosphorylation of the receptors, recruitment of the PTPs SHP-1 and SHP-2, and the inhibition of FcεRI-mediated activation of RBL cells. Similar results have been obtained with CD158/KIR molecules that contain two ITIMs, demonstrating that the proximal ITIM is critical for the inhibition of T cell activation by CD158a/KIR2DL1 (27), the FcεRI-mediated activation of RBL cells by CD158b/KIR2DL3 (28) or NK cytotoxicity function by CD158b/KIR2DL3 (29), CD158e1/KIR3DL1 (30), and CD158b/KIR2DL5 (31). Some of these studies also showed that tyrosine phosphorylation of these receptors and recruitment of the PTPs were dependent on the membrane-proximal motif (28–30). In addition, mutation of the membrane-proximal ITIM of LAIR-1 molecule, which also has two tyrosine-based motifs, resulted in abrogation of its inhibitory function in NK cytotoxicity using a NK cell line (32), whereas both motifs contributed equally to the inhibition function using RBL cells. Both motifs contributed to the inhibition mediated by NKG2A, which has also two ITIMs, using the RBL model (33), with a dominant role of the membrane-distal motif probably due to the opposite orientation of the ITIMs in type II vs type I transmembrane proteins (33). Interestingly, the fact that tyrosine phosphorylation of Siglecs-7 and -9 did not occur after mutation of the membrane-proximal motif strongly suggests that this motif is the main binding site of tyrosine kinases. However, we cannot exclude the possibility that the anti-phosphotyrosine mAb used is unable to detect the tyrosyl-phosphorylated membrane-distal motif present in the Y1 mutants. Although the identity of the kinase(s) involved remains unknown, it is worth noting that Siglec-3/CD33 can be phosphorylated in vitro and in vivo by Src family kinase c-Src (22) and Lck (24), the membrane-proximal motif playing a key role (24)

We have also shown that mutation of the membrane-distal motif did not affect the inhibitory function of Siglecs-7 and -9. Interestingly, with this mutation, we were still able to observe tyrosine phosphorylation and SHP-2 recruitment using both immunoprecipitation- and fluorescence microscopy-based approaches, whereas SHP-1 was not recruited. Although an involvement of other signaling partners cannot be excluded, it is tempting to speculate that the inhibition observed after mutation of the membrane-distal motif is mediated by SHP-2. Such a mechanism involving SHP-2 rather than SHP-1 in inhibitory signaling has been suggested in a previous study with KIR2DL3 (28) and has been demonstrated recently with KIR2DL4 in RBL cells (34) and KIR3DL1 and KIR2DL5 in NK cells (30, 31). Based on these studies and the work on LAIR-1 (32), it has been proposed that the conserved sequence, VTVQQL, is sufficient for the recruitment of SHP-2 and the inhibitory capacity of the receptor (32). The involvement of SHP-2 rather than SHP-1 in the inhibitory function could also apply to Siglecs-7 and -9, although they have different ITIM sequences (IQYAPL and LQYASL, respectively) compared with the proposed consensus. Studies using peptides from KIR molecules indicated that the position –2 from the tyrosine was critical for SHP-1 and SHP-2 binding (5, 35, 36). Furthermore, a study using peptides from mast cell function-associated Ag, which has a single ITIM, clearly demonstrates that the nature of the amino acid residue present at position –2 is important for differential binding of SHP-1 or SHP-2 (37). Indeed binding of SHP-1 was only observed with the native phosphate peptide SIpYSTL and the mutation serine to valine, whereas binding of SHP-2 was maintained after mutation of the serine to several amino acid residues and in particular to isoleucine and leucine (37), residues present in Siglecs-7 and -9 ITIMs. Taken together, our results support the concept that SHP-1 requires two functional tyrosine-based motifs, whereas SHP-2 tolerates only one ITIM (28, 29, 32).

Another feature of some CD33-related Siglecs is that the membrane-distal motif is similar to the ITSM TxYxxI/V found in CD150/SLAM and SLAM-related molecules (10, 11, 38). It has been shown that the ITSM of CD150 is involved in recruitment of SLAM-associated protein (SAP) or EAT-2 in T and B cells (10, 39).
39–41). This prevented an interaction with the phosphatase SHP-2 (10, 39, 41, 42) and facilitated the recruitment of the phosphatase SHIP (10, 43) or the Src family kinases FynT (43). Although this issue has not been addressed directly in this study, it is unlikely that this ITSM serves as a docking site for SAP-family adaptor molecules. Indeed Siglec-10, which contains two membrane-proximal ITIMs and one membrane-distal ITSM-like motif, does not bind to the SAP molecule in a three-hybrid system (44).

Finally, we have shown here that mutation of the membrane-proximal ITIM of Siglec-7 and -9 leads to an increase in the sialic acid-dependent binding of RBC, whereas mutation of the membrane-distal motif has no effect. Similar results have been reported previously with Siglec-3/CD33 using transiently transfected COS cells (22) and may be a general feature of human CD33-related Siglecs. The fact that SHP-2 was only recruited with Y2 mutants, which displayed low levels of RBC binding similar to the WT forms of Siglec-7 and -9, raised the possibility that interactions of this phosphatase could negatively regulate the ligand binding activity of Siglec-7 and -9. Binding of sialic acid residues by monomeric Siglecs is generally of very low affinity and therefore clustering of receptors within the plasma membrane is essential for high-avidity interactions with ligands on other cells. Although we failed to see gross changes in distribution of Siglec-7 or -9 comparing WT and mutant forms of the proteins expressed on transfected RBL cells by confocal light microscopy, there could be subtle changes that would require higher resolution imaging techniques for their visualization.

In conclusion, we demonstrate that Siglec-9 is a new member of the inhibitory receptor superfamily and that the membrane-proximal ITIM is essential for the inhibitory function of both Siglec-7 and -9 molecules.


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References


