The Journal of Immunology

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. Crocker

J Immunol 2004; 173:6841-6849; ;
doi: 10.4049/jimmunol.173.11.6841
http://www.jimmunol.org/content/173/11/6841

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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[1]

Tony Avril,* Helen Floyd,* Frederic Lopez,† Eric Vivier,† and Paul R. Crocker[2]*

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 Siglecs-7 and -9 to function as inhibitory receptors and investigate the molecular basis for this. We first demonstrate that Siglecs-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, Siglecs-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 Siglecs-7 and -9, raising the possibility that "inside-out" signaling may occur to regulate ligand binding. ***The Journal of Immunology,*** **2004, 173: 6841–6849.**

Siglecs[3] are sialic acid-binding Ig-like lectins characterized by a homologous N-terminal V-set Ig-like domain and varying numbers of C2-set Ig-like domains (1). In humans, 11 Siglecs have been described: sialoadhesin (Siglec-1, CD169), CD22 (Siglec-2), and the myelin-associated glycoprotein (MAG or Siglec-4) form one group distinct from the other that includes CD33 (Siglec-3) and the more recently described CD33-related Siglecs (Siglecs-5 to -11) (1, 2). The CD33-related Siglecs are expressed differentially in the hemopoietic system (1). Some are broadly expressed as for example Siglec-5 (CD170), which is found in B cells, monocytes, and neutrophils (1, 3). Others have a more restricted distribution, notably Siglec-8, which is present on circulating eosinophils (1, 4). Another feature of this group is the presence of two conserved tyrosine-containing motifs in the cytoplasmic region. The membrane-proximal motif (EI/LXYAXLXF) conforms to the consensus immunoreceptor tyrosine-based inhibition motif (ITIM) I/L/S/VxYxxL/V (5, 6), whereas the membrane-distal motif does not.

ITIMs have been found in the cytoplasmic tail of many inhibitory receptors, such as CD158/killer cell Ig-like receptor (KIR), Ly49, and CD85/Ig-like transport/leukocyte inhibitory receptor molecules expressed on various hemopoietic cells including monocytes and NK cells (reviewed in Refs. 7 and 8). ITIMs were functionally defined as specific amino acid sequences that, once tyrosine-phosphorylated, provide a docking site for the Src homology 2 (SH2)-domain bearing cytoplasmic phosphatases (8). ITIM-related motifs have also been described in many other inhibitory receptors (9). Their amino acid sequences differ from the consensus ITIM sequence by the presence of a threonine in position −2 or an isoleucine in position + 2. For some of the CD33-related Siglecs (CD33, Siglecs-5, -6, and -9), the membrane-distal motif (TEYSEI/VK/R) is similar to the immunoreceptor tyrosine-based switch motif (ITSM) TxYxxI/V (10, 11) found in CD150/signaling lymphocyte activation molecule (SLAM), CD66/carcinoembryonic Ag cell adhesion molecule, leukocyte-associated Ig-like receptor-1 (LAIR-1), and CD31/PECAM-1 molecules (10, 12). The discovery of CD33-related Siglecs and the presence of these two tyrosine motifs raise the possibility that these proteins are involved in regulating cellular activation within the immune system.

Siglecs-7 and -9 share more than 80% sequence identity throughout the extracellular, transmembrane, and intracellular regions (13) but differ in their expression pattern (1). Siglec-7 is highly expressed on NK cells and weakly on monocytes and a minor subpopulation of CD8 T lymphocytes (14, 15). In contrast, Siglec-9 is broadly expressed with high expression on monocytes and low-level expression on neutrophils and subpopulations of NK, B, and T cells (13). They also differ in sialic acid binding specificity: Siglec-7 possesses a unique preference for α(2,8)-linked disialic acids and branched α(2,6)-linked sialic acids,

*Division of Cell Biology and Immunology, The Wellcome Trust Biocentre, University of Dundee, Dundee, United Kingdom; and †Centre d'Immunologie de Marseille-Luminy, Centre National de la Recherche Scientifique-Institut National de la Sante et de la Recherche Médicale-Université de la Méditerranée, Marseille, France

Received for publication July 21, 2004. Accepted for publication September 21, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Research in the PRC laboratory is supported by the Wellcome Trust.

[2] Address correspondence and reprint requests to Dr. Paul R. Crocker, The Wellcome Trust Biocentre, University of Dundee, Dow Street, Dundee DD1 5EH, United Kingdom. E-mail address: p.r.crocker@dundee.ac.uk

[3] Abbreviations used in this paper: Siglec, sialic acid-binding Ig-like lectin; ITIM, immunoreceptor tyrosine-based inhibition motif; ITSM, immunoreceptor tyrosine-based switch motif; KIR, killer cell Ig-like receptor; LAIR-1, leukocyte-associated Ig-like receptor-1; MAG, myelin-associated glycoprotein; PAb, polyclonal antibody; PTP, protein tyrosine phosphatase; RBL, rat basophilic leukemia; SAP, SLAM-associated protein; SH2, Src homology 2; SHP, SH2 domain-containing phosphatase; SLAM, signaling lymphocyte activation molecule; TRITC, tetramethylrhodamine isothiocyanate, WT, wild type.

0022-1767/04/$02.00

whereas Siglec-9 does not bind to α(2,8)-linked disialic 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.). [^{3}H]Serotonin (hydroxytryptamine creatine sulfate, 5-[1,2-[^{3}H](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$'$)$_2$ was obtained from Jackson ImmunoResearch (Luton Beds, U.K.). The FITC-conjugated rabbit anti-mouse Ig F(ab$'$)$_2$ 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$'$)$_2$ for 30 min. Cells were analyzed using a FACSCalibur (Becton Dickinson, Oxford, U.K.).

Serotonin release assay

The serotonin release assay was performed as described in Ref. 20. Briefly, [^{3}H]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 [^{3}H]serotonin obtained with cells incubated in absence of Abs and cpm max is the maximum release of [^{3}H]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 *n* different clones, where % IgE is

Table I. *Primers used in this study*[a]

7Y1	Sense	5′-GGAAAGAGAGATCCAGT**T**TGCACCCCTCAGC-3′
	Antisense	5′-GCTGAGGGGTGCA**A**ACTGGATCTCTCTTTCC-3′
7Y2	Sense	5′-GCCACCAACAATGAGT**T**CTCAGAGATCAAGATCC-3′
	Antisense	5′-GGATCTTGATCTCTGAG**A**ACTCATTGTTGGTGGC-3′
7Δ	Sense	5′-TAATACGACTCACTATAGGG*-3′
	Antisense	5′-TCTAGATCATCACCTGCAGGACCTCACTACAATGAAG-3′
9Y1	Sense	5′-GGAGAGCTCCAGT**T**TGCATCCCTCAGCTTCC-3′
	Antisense	5′-GGAAGCTGAGGGATGCA**A**ACTGGAGCTCTCC-3′
9Y2	Sense	5′-GGCCACTGACACCGAGT**T**CTCGGAGATCAAGATCC-3′
	Antisense	5′-GGATCTTGATCTCCGAG**A**ACTCGGTGTCAGTGGCC-3′
9Δ	Sense	5′-TAATACGACTCACTATAGGG*-3′
	Antisense	5′-TCTAGATCATCACCTGCAGGACCTCACTACAACGAAG-3′
SHP-1	Sense	5′-CTCGAGATGGTGAGGTGGTTTCACCG-3′
	Antisense	5′-AAGCTTCTTCCTCT**T**GAGGGAACCC-3′
mutSHP-1	Sense	5′-GGGCCCATCATCGTGCACT**C**CAGCGCCGGCATCGGCCGC-3′
	Antisense	5′-GCGGCCGATGCCGGCGCTG**G**AGTGCACGATGATGGGCCC-3′
SHP-2	Sense	5′-CTCGAGATGACATCGCGGAGATGG-3′
	Antisense	5′-AAGCTTTCTGAAACTTTTCTGCTGTTGC-3′
mutSHP-2	Sense	5′-GGGCCGGTCGTCGTCCACT**C**CAGTGCTGGAATTGGCCGG-3′
	Antisense	5′-CCGGCCAATTCCAGCACTG**G**AGTGCACCACCACCGGCCC-3′

[a] Nucleotide 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 and 9Δ and XhoI/HindIII for SHP-1 and SHP-2) are in italic. Simple underline, initiation codon; double underline, stop codon; asterisk, T7 primer.

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-Siglecs-7 or -9 polyclonal Abs (PAbs), with 4G10 mAb anti-phosphotyrosine or with rabbit PAbs 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, mutSHP-1-GFP, or mutSHP-2-GFP cDNA by electroporation and cultured overnight on coverships (BDH, Lutterworth, U.K.). Cells were then stained with anti-Siglec-7 or anti-Siglec-9 sheep PAbs (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 mIgE (0.1 μg/ml) and S7.5a or K8 mAbs (10 μg/ml) for 30 min at 4°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 PAbs (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 immunofluorescence 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 Siglecs-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 Siglecs-7 and -9 in parallel, we used the well-defined RBL model in which co-engagement of inhibitory receptors and the activatory receptor, FεRI, inhibits serotonin secretion by RBL cells incubated with mIgE. 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. 1*A* 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. 1*A*). 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 mIgE 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. 1*B*). In contrast, cross-linking of Siglecs-7 and -9 substantially reduced the serotonin release of RBL-7WT and -9WT cells

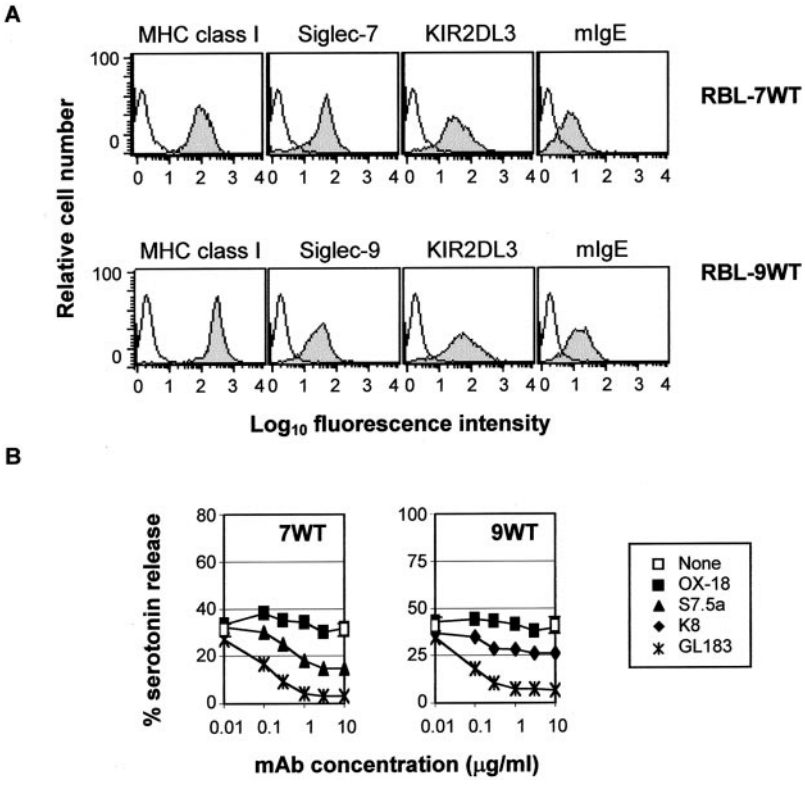

FIGURE 1. Inhibitory function of Siglecs-7 and -9 in RBL cells. *A*, RBL-7WT and -9WT cells were stained or not (open histograms) with OX-18 (anti-MHC class I molecules), S7.5a (anti-Siglec-7), K8 (anti-Siglec-9), GL183 (anti-KIR2DL3) mAbs or mIgE (IgE-3) (shaded histograms), followed by FITC-conjugated rabbit anti-mouse Ig $F(ab')_2$, and analyzed by flow cytometry. *B*, [^{3}H]Serotonin-labeled RBL-7WT and RBL-9WT cells were incubated with mIgE 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.

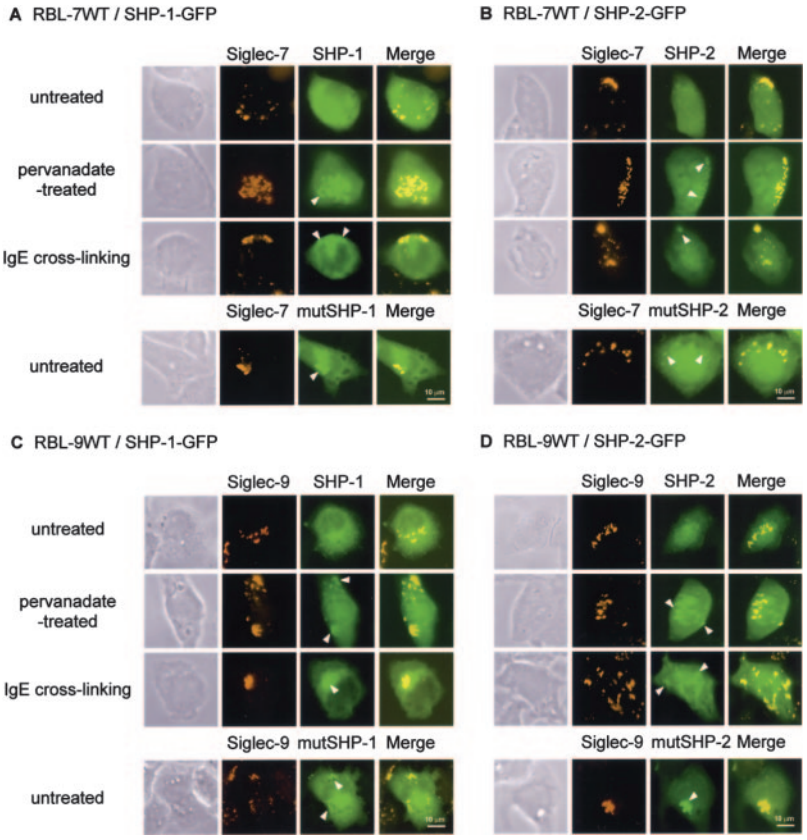

FIGURE 2. Co-localization of WT and catalytically inactive SHP-1-GFP and SHP-2-GFP with Siglecs-7 and -9 in resting and activated RBL cells. RBL-7WT (*A* and *B*) and -9WT (*C* and *D*) were transfected with SHP-1-GFP (*A* and *C*, *lane 1–3*), SHP-2-GFP (*B* and *D*, *lane 1–3*), mutSHP-1-GFP (*A* and *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.

(Fig. 1*B*). These results shows 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

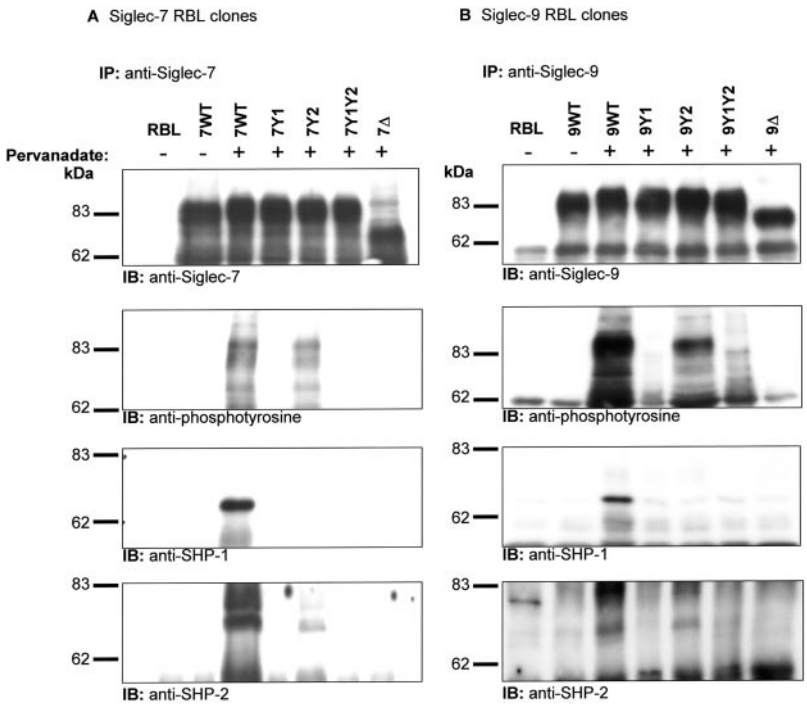

FIGURE 3. Role of the two tyrosine motifs on the tyrosine phosphorylation of Siglecs-7 and -9 and recruitment of the PTPs SHP-1 and SHP-2 in RBL cells. Parental RBL (*A* and *B*, *lane 1*), Siglec-7 (*A*), and Siglec-9 (*B*) RBL clones were untreated or treated with pervanadate, lysed, and immunoprecipitated 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).

Table II. *Constructs used in this study*[a]

		proximal motif / distal motif	
7WT	428	SSGEEREIQYAPLSFHKGEPQDLSGQEATNNEYSEIKIPK	467
7Y1 (Y437F)		---------F----------	
7Y2 (Y460F)		----------F----------	
7Y1Y2 (Y437F/Y460F)		---------F----------F----------	
7WT	363	ALVFLSFCVIFIVVRSCRKKSARPAADVGDIGMKDANTIR	402
7Δ (R380Δ)		ALVFLSFCVIFIVVRSCR*	380
9WT	424	SSVGEGELQYASLSFQMVKPWDSRGQEATDTEYSEIKIHR	463
9Y1 (Y433F)		---------F----------	
9Y2 (Y456F)		----------F----------	
9Y1Y2 (Y433F/Y456F)		---------F----------F----------	
9WT	357	AKVFLSFCVIFVVVRSCRKKSARPAAGVGDTGIEDANAVR	396
9Δ (R374Δ)		ALVFLSFCVIFVVVRSCR*	374

[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.

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

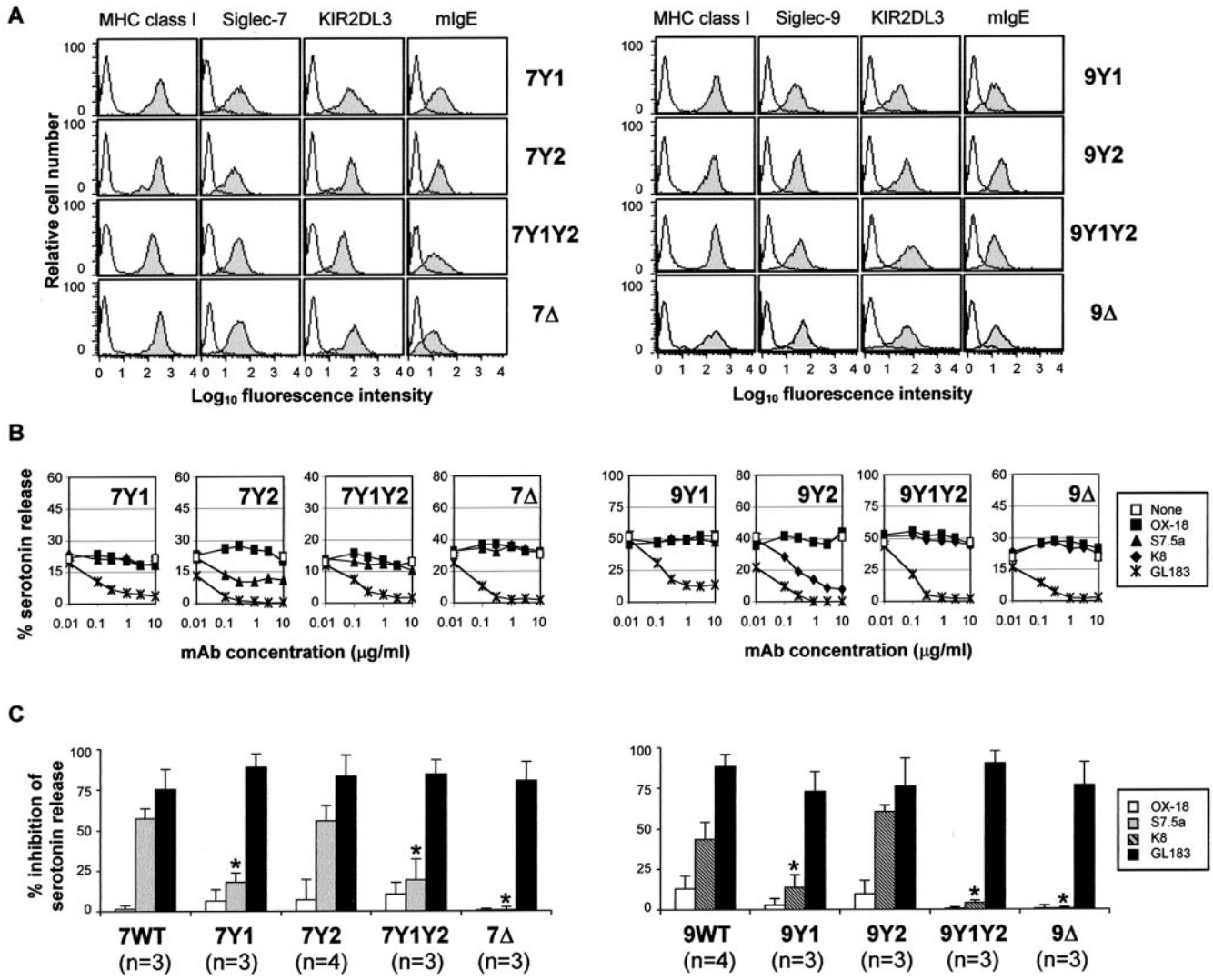

FIGURE 4. Role of the two tyrosine motifs on the inhibitory function of Siglecs-7 and -9 in RBL cells. *A*, Siglecs-7 and -9 RBL clones were analyzed for the expression of MHC class I molecules, Siglec-7, Siglec-9, KIR2DL3, and the binding of mIgE by flow cytometry as described in the legend to Fig. 1. *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).

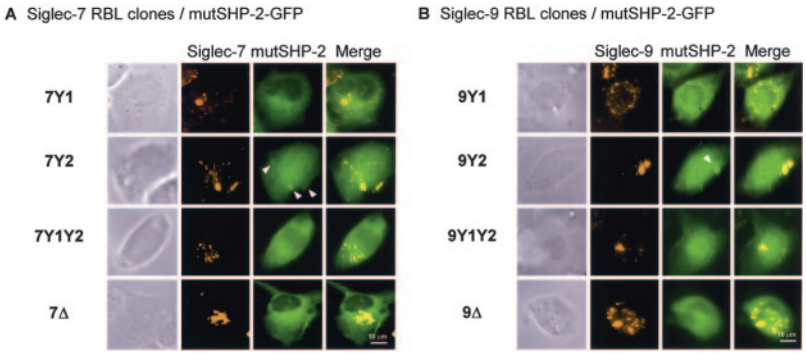

FIGURE 5. Co-localization of catalytically inactive SHP-2-GFP with Siglecs-7 and -9 in RBL clones. Siglec-7 (*A*) and Siglec-9 (*B*) RBL clones were transfected with mutSHP-2-GFP cDNA. After overnight culture, cells were stained with anti-Siglec-7 or -9 sheep PAbs as described in the legend to Fig. 2. Untreated cells were then analyzed by fluorescence microscopy. MutSHP-2-GFP clusters are indicated by the arrows.

cross-linked using GFP-transfected cells in a similar way as in the inhibition assay, and then clusters were created by adding sheep PAbs anti-Siglec-7 or -9 followed by TRITC-conjugated rabbit anti-sheep Ig $F(ab')_2$. As shown in Fig. 2, SHP-1 and SHP-2 co-localized with Siglecs-7 and -9 when the cells were activated via engagement of the FcεRI receptor.

We also used catalytically inactive forms of SHP-1-GFP and SHP-2-GFP (mutSHP-1-GFP and mutSHP-2-GFP), which retain the ability to bind their substrates but are unable to dephosphorylate them. As shown in Fig. 2, Siglecs-7 and -9 co-localized with mutSHP-1 and mutSHP-2 without any activation of RBL cells.

To investigate SHP-1 and SHP-2 association biochemically, parental RBL, RBL-7WT, and -9WT cells were treated or not with pervanadate and Siglec-7 or -9 immunoprecipitates from RBL lysates were then analyzed by immunoblotting with anti-Siglec-7 or -9 PAbs, anti-phosphotyrosine mAb, anti-SHP-1, or anti-SHP-2 PAbs (Fig. 3). As expected, specific bands of ~77 and 78 kDa corresponding to Siglecs-7 and -9, respectively were observed with untreated and pervanadate-treated RBL-7WT and -9WT lysates, whereas no signal was detectable with parental RBL lysates (Fig. 3). Tyrosine-phosphorylated Siglecs-7 and -9 and co-immunoprecipitated SHP-1 and SHP-2 were readily detectable in pervanadate-treated RBL-7WT and -9WT lysates, but no signal was seen with untreated RBL-7WT, -9WT, or the parental RBL lysates (Fig. 3).

Taken together, these results indicate that Siglecs-7 and -9 can be phosphorylated by tyrosine kinases and then recruit the tyrosine phosphatases SHP-1 and SHP-2.

The membrane-proximal tyrosine motif is necessary for the inhibitory function, tyrosine phosphorylation, and recruitment of the PTPs SHP-1 and SHP-2 by Siglecs-7 and -9

To examine the roles of individual tyrosine motifs within the cytoplasmic tail of Siglecs-7 and -9 on the inhibitory function of these molecules, we generated tyrosine to phenylalanine mutants in both the membrane-proximal and membrane-distal tyrosine motifs either individually or in combination (Table II). Truncated forms of Siglecs-7 and -9 in which the cytoplasmic tails were deleted were also used (Table II). RBL clones expressing similar levels of Siglec-7 or -9 were selected for comparative analysis in inhibition assays (Fig. 4*A*). Comparable levels of expression of MHC class I molecules, KIR2DL3, and FcεRI were also confirmed by flow cytometry (Fig. 4*A*). Using RBL-7Y2 and -9-Y2 cells in which the proximal tyrosine motif only was retained, strong inhibition was still observed following co-cross-linking of Siglecs-7 and -9 with FcεRI (Fig. 4, *B* and *C*). In contrast, mutation of the membrane-proximal motif in RBL-7Y1, -7Y1Y2, -9Y1, or -9Y1Y2 or truncation of the cytoplasmic tail resulted in a complete loss of inhibition (Fig. 4, *B* and *C*). These results demonstrate that the membrane-proximal tyrosine motif is both necessary and sufficient to mediate the inhibitory function of Siglecs-7 and -9.

To investigate how the mutations of the tyrosine motifs affect phosphorylation of Siglecs-7 and -9 and their capacity to recruit the PTPs SHP-1 and SHP-2, RBL mutants were treated with pervanadate and Siglec-7 or -9 immunoprecipitates from RBL lysates were analyzed by immunoblotting with anti-Siglec-7 or -9 PAbs, anti-phosphotyrosine mAb, anti-SHP-1, or anti-SHP-2 PAbs (Fig. 3). As expected, no tyrosine phosphorylation was detected with RBL-7Y1Y2, RBL-7Δ, RBL-9Y1Y2, and RBL-9Δ lysates (Fig. 3). In addition, mutation of the membrane-proximal tyrosine motif completely abrogated the tyrosine phosphorylation, whereas substantial phosphorylation was retained with the mutation of the membrane-distal motif (Fig. 3). Concerning the recruitment of the PTPs, all mutations completely abrogated the recruitment of SHP-1, whereas only the membrane-distal mutation could still recruit significant levels of SHP-2 (Fig. 3).

To confirm our findings in co-localization experiments, Siglecs-7 and -9 RBL mutants were transfected with mutSHP-1-GFP

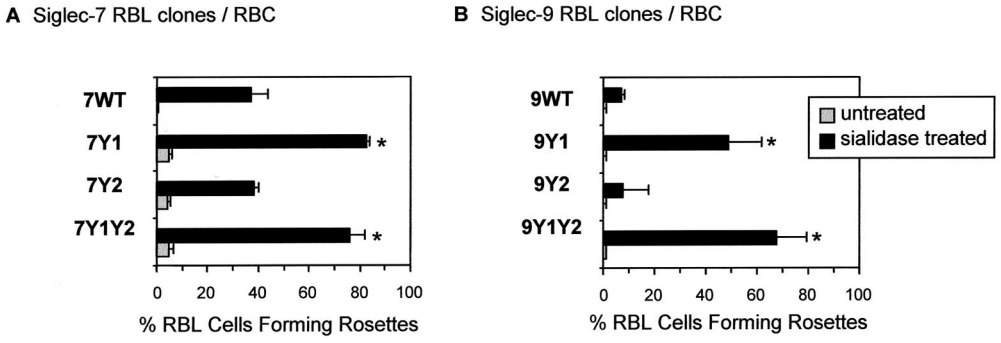

FIGURE 6. Role of the two tyrosine motifs on the RBC binding activity of Siglecs-7 and -9. Siglec-7 (*A*) and Siglec-9 (*B*) RBL clones were untreated (gray bars) or sialidase-treated (black bars) and then incubated with human RBC. After washing, cells were fixed and the percentage of RBC cells with 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, Siglecs-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-phoshorylation 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 CD158/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, VTYAQL, 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 phospho-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,

39–41). 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 Siglecs-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 Siglecs-7 and -9, raised the possibility that interactions of this phosphatase could negatively regulate the ligand binding activity of Siglecs-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 Siglecs-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 the inhibitory receptor superfamily and that the membrane-proximal ITIM is essential for the inhibitory function of both Siglecs-7 and -9 molecules.

Note added in proof. A recent publication demonstrated the inhibitory activity of Siglec-7 and Siglec-9 in transfected Jurkat cells (Ikehara, Y., S. K. Ikehara, and J. C. Paulson. 2004. Negative regulation of T cell receptor signaling by Siglec-7 (p70/AIRM) and Siglec-9. *J. Biol. Chem. 279:43117.*).

Acknowledgments

We thank Benjamin Neel for the kind gift of SHP-1 and SHP-2 cDNA, Lars Nitshke for providing the anti-phosphotyrosine mAb, Mathias Lucas for the mouse IgE Abs, Claire Jones for the sheep anti-Siglec-9 Abs, Gavin Nicoll and Kevin Lock for the anti-Siglec-7 Abs, Jiquan Zhang for the anti-Siglec-9 mAb, and Andrew Ferenbach and Faye Wesley for the SHP-1 and SHP-2-GFP constructs.

References

1. Crocker, P. R., and A. Varki. 2001. Siglecs, sialic acids and innate immunity. *Trends Immunol. 22:337*.
2. Crocker, P. R. 2002. Siglecs: Sialic-acid-binding immunoglobulin-like lectins in cell-cell interactions and signalling. *Curr. Opin. Struct. Biol. 12:609*.
3. Cornish, A. L., S. Freeman, G. Forbes, J. Ni, M. Zhang, M. Cepeda, R. Gentz, M. Augustus, K. C. Carter, and P. R. Crocker. 1998. Characterization of siglec-5, a novel glycoprotein expressed on myeloid cells related to CD33. *Blood 92:2123*.
4. Floyd, H., J. Ni, A. L. Cornish, Z. Zeng, D. Liu, K. D. Carter, J. Steel, and P. R. Crocker. 2000. Siglec-8. A novel eosinophil-specific member of the immunoglobulin superfamily. *J. Biol. Chem. 275:861*.
5. Vivier, E., and M. Daeron. 1997. Immunoreceptor tyrosine-based inhibition motifs. *Immunol. Today 18:286*.
6. Ravetch, J. V., and L. L. Lanier. 2000. Immune inhibitory receptors. *Science 290:84*.
7. Lanier, L. L. 1998. NK cell receptors. *Annu. Rev. Immunol. 16:359*.
8. Long, E. O. 1999. Regulation of immune responses through inhibitory receptors. *Annu. Rev. Immunol. 17:875*.
9. Staub, E., A. Rosenthal, and B. Hinzmann. 2004. Systematic identification of immunoreceptor tyrosine-based inhibitory motifs in the human proteome. *Cell Signal. 16:435*.
10. Shlapatska, L. M., S. V. Mikhalap, A. G. Berdova, O. M. Zelensky, T. J. Yun, K. E. Nichols, E. A. Clark, and S. P. Sidorenko. 2001. CD150 association with either the SH2-containing inositol phosphatase or the SH2-containing protein tyrosine phosphatase is regulated by the adaptor protein SH2D1A. *J. Immunol. 166:5480*.
11. Sidorenko, S. P., and E. A. Clark. 2003. The dual-function CD150 receptor subfamily: The viral attraction. *Nat. Immunol. 4:19*.
12. Meyaard, L., G. L. Adema, C. Chang, E. Woollatt, G. R. Sutherland, L. L. Lanier, and J. H. Phillips. 1997. LAIR-1, a novel inhibitory receptor expressed on human mononuclear leukocytes. *Immunity 7:283*.
13. Zhang, J. Q., G. Nicoll, C. Jones, and P. R. Crocker. 2000. Siglec-9, a novel sialic acid binding member of the immunoglobulin superfamily expressed broadly on human blood leukocytes. *J. Biol. Chem. 275:22121*.
14. Falco, M., R. Biassoni, C. Bottino, M. Vitale, S. Sivori, R. Augugliaro, L. Moretta, and A. Moretta. 1999. Identification and molecular cloning of p75/AIRM1, a novel member of the sialoadhesin family that functions as an inhibitory receptor in human natural killer cells. *J. Exp. Med. 190:793*.
15. Nicoll, G., J. Ni, D. Liu, P. Klenerman, J. Munday, S. Dubock, M. G. Mattei, and P. R. Crocker. 1999. Identification and characterization of a novel siglec, siglec-7, expressed by human natural killer cells and monocytes. *J. Biol. Chem. 274:34089*.
16. Blixt, O., B. E. Collins, I. M. van den Nieuwenhof, P. R. Crocker, and J. C. Paulson. 2003. Sialoside specificity of the siglec family assessed using novel multivalent probes: Identification of potent inhibitors of myelin-associated glycoprotein. *J. Biol. Chem. 278:31007*.
17. Yamaji, T., T. Teranishi, M. S. Alphey, P. R. Crocker, and Y. Hashimoto. 2002. A small region of the natural killer cell receptor, Siglec-7, is responsible for its preferred binding to α2,8-disialyl and branched α2,6-sialyl residues. A comparison with Siglec-9. *J. Biol. Chem. 277:6324*.
18. Nicoll, G., T. Avril, K. Lock, K. Furukawa, N. Bovin, and P. R. Crocker. 2003. Ganglioside GD3 expression on target cells can modulate NK cell cytotoxicity via siglec-7-dependent and -independent mechanisms. *Eur. J. Immunol. 33:1642*.
19. Vitale, C., C. Romagnani, M. Falco, M. Ponte, M. Vitale, A. Moretta, A. Bacigalupo, L. Moretta, and M. C. Mingari. 1999. Engagement of p75/AIRM1 or CD33 inhibits the proliferation of normal or leukemic myeloid cells. *Proc. Natl. Acad. Sci. USA 96:15091*.
20. Blery, M., J. Delon, A. Trautmann, A. Cambiaggi, L. Olcese, R. Biassoni, L. Moretta, P. Chavrier, A. Moretta, M. Daeron, and E. Vivier. 1997. Reconstituted killer cell inhibitory receptors for major histocompatibility complex class I molecules control mast cell activation induced via immunoreceptor tyrosine-based activation motifs. *J. Biol. Chem. 272:8989*.
21. Angata, T., S. C. Kerr, D. R. Greaves, N. M. Varki, P. R. Crocker, and A. Varki. 2002. Cloning and characterization of human Siglec-11. A recently evolved signaling that can interact with SHP-1 and SHP-2 and is expressed by tissue macrophages, including brain microglia. J. *Biol. Chem. 277:24466*.
22. Taylor, V. C., C. D. Buckley, M. Douglas, A. J. Cody, D. L. Simmons, and S. D. Freeman. 1999. The myeloid-specific sialic acid-binding receptor, CD33, associates with the protein-tyrosine phosphatases, SHP-1 and SHP-2. J. *Biol. Chem. 274:11505*.
23. Ulyanova, T., J. Blasioli, T. A. Woodford-Thomas, and M. L. Thomas. 1999. The sialoadhesin CD33 is a myeloid-specific inhibitory receptor. *Eur. J. Immunol. 29:3440*.
24. Paul, S. P., L. S. Taylor, E. K. Stansbury, and D. W. McVicar. 2000. Myeloid specific human CD33 is an inhibitory receptor with differential ITIM function in recruiting the phosphatases SHP-1 and SHP-2. *Blood 96:483*.
25. Ulyanova, T., D. D. Shah, and M. L. Thomas. 2001. Molecular cloning of MIS, a myeloid inhibitory siglec, that binds protein-tyrosine phosphatases SHP-1 and SHP-2. *J. Biol. Chem. 276:14451*.
26. Yu, Z., M. Maoui, L. Wu, D. Banville, and S. Shen. 2001. 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. *Biochem. J. 353:483*.
27. Fry, A. M., L. L. Lanier, and A. Weiss. 1996. Phosphotyrosines in the killer cell inhibitory receptor motif of NKB1 are required for negative signaling and for association with protein tyrosine phosphatase 1C. *J. Exp. Med. 184:295*.
28. Bruhns, P., P. Marchetti, W. H. Fridman, E. Vivier, and M. Daeron. 1999. Differential roles of N- and C-terminal immunoreceptor tyrosine-based inhibition motifs during inhibition of cell activation by killer cell inhibitory receptors. *J. Immunol. 162:3168*.
29. Burshtyn, D. N., A. S. Lam, M. Weston, N. Gupta, P. A. Warmerdam, and E. O. Long. 1999. Conserved residues amino-terminal of cytoplasmic tyrosines contribute to the SHP-1-mediated inhibitory function of killer cell Ig-like receptors. *J. Immunol. 162:897*.
30. Yusa, S., and K. S. Campbell. 2003. Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2) can play a direct role in the inhibitory function of killer cell Ig-like receptors in human NK cells. *J. Immunol. 170:4539*.
31. Yusa, S., T. L. Catina, and K. S. Campbell. 2004. KIR2DL5 can inhibit human NK cell activation via recruitment of Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2). *J. Immunol. 172:7385*.
32. Verbrugge, A., T. T. Ruiter, H. Clevers, and L. Meyaard. 2003. Differential contribution of the immunoreceptor tyrosine-based inhibitory motifs of human leukocyte-associated Ig-like receptor-1 to inhibitory function and phosphatase recruitment. *Int. Immunol. 15:1349*.
33. Kabat, J., F. Borrego, A. Brooks, and J. E. Coligan. 2002. Role that each NKG2A immunoreceptor tyrosine-based inhibitory motif plays in mediating the human CD94/NKG2A inhibitory signal. *J. Immunol. 169:1948*.
34. Yusa, S., T. L. Catina, and K. S. Campbell. 2002. SHP-1- and phosphotyrosine-independent inhibitory signaling by a killer cell Ig-like receptor cytoplasmic domain in human NK cells. *J. Immunol. 168:5047*.
35. Burshtyn, D. N., A. M. Scharenberg, N. Wagtmann, S. Rajagopalan, K. Berrada, T. Yi, J. P. Kinet, and E. O. Long. 1996. Recruitment of tyrosine phosphatase HCP by the killer cell inhibitor receptor. *Immunity 4:77*.

36. Burshtyn, D. N., W. Yang, T. Yi, and E. O. Long. 1997. A novel phosphotyrosine motif with a critical amino acid at position -2 for the SH2 domain-mediated activation of the tyrosine phosphatase SHP-1. *J. Biol. Chem. 272:13066*.
37. Philosof-Oppenheimer, R., C. S. Hampe, K. Schlessinger, M. Fridkin, and I. Pecht. 2000. An immunoreceptor tyrosine-based inhibitory motif, with serine at site Y-2, binds SH2-domain-containing phosphatases. *Eur. J. Biochem. 267:703*.
38. Veillette, A. 2002. The SAP family: A new class of adaptor-like molecules that regulates immune cell functions. *Sci. STKE (120):PE8*.
39. Sayos, J., C. Wu, M. Morra, N. Wang, X. Zhang, D. Allen, S. van Schaik, L. Notarangelo, R. Geha, M. G. Roncarolo, H. Oettgen, J. E. De Vries, G. Aversa, and C. Terhorst. 1998. The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. *Nature 395:462*.
40. Poy, F., M. B. Yaffe, J. Sayos, K. Saxena, M. Morra, J. Sumegi, L. C. Cantley, C. Terhorst, and M. J. Eck. 1999. Crystal structures of the XLP protein SAP reveal a class of SH2 domains with extended, phosphotyrosine-independent sequence recognition. *Mol. Cell 4:555*.
41. Morra, M., J. Lu, F. Poy, M. Martin, J. Sayos, S. Calpe, C. Gullo, D. Howie, S. Rietdijk, A. Thompson, A. J. Coyle, C. Denny, M. B. Yaffe, P. Engel, M. J. Eck, and C. Terhorst. 2001. Structural basis for the interaction of the free SH2 domain EAT-2 with SLAM receptors in hematopoietic cells. *EMBO J. 20:5840*.
42. Howie, D., M. Simarro, J. Sayos, M. Guirado, J. Sancho, and C. Terhorst. 2002. Molecular dissection of the signaling and costimulatory functions of CD150 (SLAM): CD150/SAP binding and CD150-mediated costimulation. *Blood 99:957*.
43. Latour, S., G. Gish, C. D. Helgason, R. K. Humphries, T. Pawson, and A. Veillette. 2001. Regulation of SLAM-mediated signal transduction by SAP, the X-linked lymphoproliferative gene product. *Nat. Immunol. 2:681*.
44. Kitzig, F., A. Martinez-Barriocanal, M. Lopez-Botet, and J. Sayos. 2002. Cloning of two new splice variants of Siglec-10 and mapping of the interaction between Siglec-10 and SHP-1. *Biochem. Biophys. Res. Commun. 296:355*.