Characterization of inhibitory signaling motifs of the natural killer cell receptor Siglec-7: attenuated recruitment of phosphatases by the receptor is attributed to two amino acids in the motifs

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Siglec-7 (p75/AIRM1) is an inhibitory receptor on human natural killer cells (NK cells) and monocytes. The cytoplasmic domain of Siglec-7 contains two signaling motifs: a membrane-proximal immunoreceptor tyrosine-based inhibitory motif (ITIM) (Ile435-Gln-Tyr-Ala-Pro-Leu440) and a membrane-distal motif (Asn458-Glu-Tyr-Ser-Glu-Ile463). We report here that, upon pervanadate (PV) treatment, Siglec-7 recruited the protein tyrosine phosphatases Src homology-2 (SH2) domain-containing protein-tyrosine phosphatase-1 (SHP-1) and SHP-2 less efficiently than did other inhibitory receptors such as Siglec-9 and leukocyte-associated Ig-like receptor (LAIR-1). Alignment of the amino acid sequences of the two Siglecs revealed only three amino acids differences in these motifs. To identify the amino acid(s) critical to recruitment efficiency, we prepared a series of Siglec-7-based mutants in which each of the three amino acids were replaced with the corresponding one of Siglec-9 (I435L, P439S, and N458T mutants). P439S and N458T mutants showed pronounced enhancement of SHP recruitment, but I435L mutant had little effect. A double mutant (P439S, N458T) or triple mutant (I435L, P439S, N458T) recruited SHPs as much as did Siglec-9, indicating that Pro439 in the proximal motif and Asn458 in the distal motif of Siglec-7 attenuate its ability to recruit phosphatases. These amino acids appeared to affect not only phosphatase recruitment but also the subsequent attenuation of Syk phosphorylation.

Key words: inhibitory motif/mutational analysis/Siglec/tyrosine phosphatase

Introduction

Leukocyte activation is controlled by the balance of two groups of receptors that are functionally opposite; that is, one group comprises activating receptors such as the T-cell receptor (TCR), B-cell receptor (BCR), and most Fc receptors (FcRs), and the other comprises inhibitory receptors such as killer cell Ig-like receptors (KIRs), Ig-like transcripts (ILTs), and leukocyte-associated Ig-like receptor (LAIR-1) (Daeron, 1996; Vely and Vivier, 1997). The engagement of activating receptors induces rapid tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tail of the receptor complexes. The phosphorytrosine residue recruits the protein tyrosine kinases Syk and ZAP-70, and their recruitment and phosphorylation initiate several kinase cascades and then the release of intracellular calcium for leukocyte effector responses (Weiss and Littman, 1994). Inhibitory receptors contain at least one immunoreceptor tyrosine-based inhibitory motif (ITIM) within the cytoplasmic tail. The motif is defined by a consensus sequence (I/V/L/SxYxxL/V), which contains three components: (1) a tyrosine residue to be phosphorylated, (2) leucine or valine at position Y+3, and (3) isoleucine, valine, leucine, or serine at position Y−2 (Muta et al., 1994; Burshtyn et al., 1997). When an inhibitory receptor co-engages with an activating receptor, the tyrosine residue in the consensus sequence of the ITIM is phosphorylated, and the resulting phosphorytrosine recruits the Src homology-2 (SH2) domain-containing phosphatases (SHPs) such as the tyrosine phosphatases, SHP-1 and SHP-2, and the inositol phosphatase, SH2 domain-containing inositol 5-phosphatase (SHIP) (Scharenberg and Kinet, 1996). These phosphatases dephosphorylate several signaling molecules, resulting in inhibition of the activating signals induced by ITAM-bearing receptors.

Siglec-7 (also termed p75/AIRM1) is a member of the family of sialic acid-binding immunoglobulin-like lectins (Siglecs) and is expressed mainly on human natural killer cells (NK cells) and monocytes (Falco et al., 1999; Nicoll et al., 1999). Siglec-7 binds unique sialoglycans—α2,8 disialyl residue (NeuAcα2,8NeuAcα2,3Gal) and branched α2,6sialyl residue (Galβ1,3[NeuAcα2,6]GlcNAc)—as ligands (Ito et al., 2001; Yamaji et al., 2002; Miyazaki et al., 2004). Antibody cross-linking of Siglec-7 on NK cells attenuated the cytotoxicity of NK cells against FcγRI+ P815 murine mastocyteoma cells, indicating that Siglec-7 functions as an inhibitory receptor (Falco et al., 1999). When human Siglec-3 or mouse Siglec-E, an ortholog of Siglec-7 and Siglec-9, was co-clustered with the activating receptor FcγRI, FcγRI-dependent calcium mobilization was reduced markedly, suggesting that Siglec-7 as well as other Siglecs (especially Siglec-3/CD33-related family members) inhibit cellular activation (Ulyanova et al., 1999; Paul et al., 2000; Ulyanova et al., 2001). These Siglecs contain two conserved
motifs in the cytoplasmic region; the membrane-proximal one is an ITIM and the distal one is an “ITIM-like motif” (Crocker and Varki, 2001; Angata and Brinkman-Van der Linden, 2002). In most cases, the Siglec-dependent inhibitory signal is mediated by the interaction of the motifs with SHP-1 and SHP-2 phosphatases; for example, the proximal ITIMs of CD33, Siglec-7, Siglec-9, Siglec-10, Siglec-11, Siglec-L1, and mouse Siglec-E bound the phosphatases in a tyrosine phosphorylation-dependent manner (Taylor et al., 1999; Ulyanova et al., 1999; Paul et al., 2000; Ulyanova et al., 2001; Whitney et al., 2001; Yu et al., 2001; Angata et al., 2002; Avril et al., 2004).

In this study, we characterized the inhibitory motifs of Siglec-7 in detail and found that two amino acids in the motifs attenuate the recruitment of SHP-1 and SHP-2.

Results

Recruitment of phosphatases by Siglec-7

We prepared an NK cell fraction (defined as CD56-positive cells) from a healthy human donor and subjected it to flow cytometry analysis by using anti-Siglec-7 antibody. The NK cell fraction we used contained 84.2% Siglec-7-positive cells (Figure 1A). The fraction was subjected to an assay for phosphatase recruitment by Siglec-7. We also analyzed another ITIM-containing inhibitory receptor, LAIR-1, as a control. After peripheral NK cells were treated with an inhibitor of tyrosine phosphatase, pervanadate (PV), we immunoprecipitated (IP) Siglec-7 to analyze its phosphorylation. The phosphotyrosine signal of Siglec-7 in the precipitates was more pronounced than that of LAIR-1 (Figure 1B). Siglec-7, however, recruited SHP-1 much less than did LAIR-1, indicating that Siglec-7 recruits SHP-1 at low efficiency in peripheral NK cells. Under the conditions we used, neither SHP-2 nor SHIP was detected in both immunoprecipitates.

To confirm the previous notion that Siglec-7 transduces inhibitory signals in peripheral NK cells, we analyzed the inhibitory activity of Siglec-7 against CD16 (FcγRIIIA)-dependent activation by using the NK cell fraction. CD16 on NK cells is a low-affinity receptor for the Fc portion of IgG, which transduces activating signals to increase the level of intracellular calcium and eventually induces antibody-dependent cellular cytotoxicity. Upon stimulation with anti-CD16 antibody, the intracellular calcium level in NK cells rapidly increased, and co-ligation of Siglec-7 with CD16 partially inhibited the increase (data not shown), showing that Siglec-7 in the NK cell fraction was functional although its poor recruitment efficiency of SHP-1.

To further characterize the recruitment of phosphatase, we prepared U937 cells that stably expressed Siglec-7. Their expression levels of Siglec-7 were monitored by flow cytometry. Parent U937 cells express endogenous Siglec-7 at low levels (mean fluorescence intensity [MFI] = 20), as shown in the mock transfectant (Figure 2A). Wild-type (WT) Siglec-7 was well overexpressed on U937 cells (MFI = 474). We analyzed the recruitment of phosphatases in the transfectant. When a transfectant that expressed Siglec-7 (U937-WT7) was treated with PV, Siglec-7 was well phosphorylated, similar to endogenous LAIR-1 (U937-WT7/α Siglec-7 versus αLAIR-1, PV[+] in Figure 2B). Recruitment of SHP-1 by Siglec-7, however, hardly occurred, whereas that of LAIR-1 was prominent (lanes U937-WT7/αLAIR-1 and αSig.7, PV[+] of blot for αSHP-1 detection, Figure 2B). Thus the U937-WT7 transfectant also demonstrates poor recruitment of SHP-1 by Siglec-7.

We assessed the inhibitory activities of Siglec-7 against calcium influx by its co-ligating with FcγRI, an activating
Mutational analysis of Siglec-7 inhibitory motifs

receptor on U937 cells. Co-ligation of overexpressed Siglec-7 markedly inhibited calcium influx, whereas that of endogenous Siglec-7 with FcγRI did not have any effect (data not shown), suggesting that overexpressed Siglec-7 is functional but a certain threshold level of Siglec-7 is required for the inhibition.

Among the Siglec family members, Siglec-9 is most homologous to Siglec-7 among all Siglec family proteins (84% similarity) and, like Siglec-7, is expressed on human monocytes. We therefore examined the Siglec-9-dependent recruitment of SHP-1 in an U937 transfectant expressing Siglec-9 (U937-WT9) (Figure 2A). Unlike Siglec-7, Siglec-9 efficiently recruited SHP-1 after PV treatment (lane U937-WT9/αSig.9, PV+[+] in Figure 2B). Siglec-9 recruited also marked amounts of SHP-1 in the absence of PV treatment, suggesting that nonphosphorylated Siglec-9 or a trace amount of phosphorylated Siglec-9 efficiently recruits SHP-1 in U937 cells. In addition, Siglec-9 recruited also a small amount of SHP-2 after PV treatment (see a blot for αSHP-2 detection in Figure 2B).

To characterize the potential for phosphatase recruitment, we transiently expressed either Siglec-7 or Siglec-9 with either SHP-1 or SHP-2 in COS cells. We also overexpressed a catalytically inactive SHP-1 mutant (C455S), a SHP-2 mutant (C459S), or the SH2 domain of SHIP with each of the Siglecs; these mutants were often used for the recruitment assay, because dissociation of the mutants from ITIMs is minimized due to their lack of phosphatase activity. Upon treatment with PV, Siglec-7 recruited trace amounts of WT SHP-1 and its mutant (C455S), whereas Siglec-9 recruited considerable quantities of SHP-1 of both types (see lanes of 7/WT, 9/WT, and C455S [CS] in Figure 3A). Even in the absence of PV, Siglec-9 bound the inactive mutant or a small amount of WT SHP-1. The potential of Siglec-7 for SHP-2 recruitment was better than that for SHP-1, although it was still much less than that of Siglec-9 (see blot for αSHP-2 detection in Figure 3B). Under the conditions we used, neither Siglec recruited the SH2-domain of SHIP (data not shown). Therefore, we conclude that Siglec-7 and Siglec-9 differ greatly from each other in their ability to recruit SHP-1 and SHP-2.

Identification of amino acid residues that attenuate the recruitment of SHP-1 and SHP-2

Upon tyrosine phosphorylation, Siglec-9 efficiently recruited SHP-1 and SHP-2, whereas Siglec-7 did so poorly. We speculated that this difference in recruitment ability could be attributed to sequence differences in the proximal and/or distal motifs. Alignment of their amino acid sequences reveals that Siglec-7 and Siglec-9 differ by only three amino acids in these motifs; that is, I435, P439, and N458 in the sequence of Siglec-7 (Figure 4). To identify the amino acid(s) critical to recruitment efficiency, we prepared a series of mutants in which each of the three amino acids in Siglec-7 were replaced with the corresponding one of Siglec-9 (the I435L, P439S, and N458T mutants). We prepared also double or triple mutants that have all the possible combinations of replacements (Figure 4). These Siglec-7-based mutants were transiently expressed in COS cells with inactive mutant SHP-1 (C455S) or SHP-2 (C459S) and their recruitment was assessed.

Among the single-amino-acid mutants, P439S showed the most pronounced enhancement of SHP-1 recruitment
after the treatment with PV (see lane 439, PV[+] in blot for αSHP-1 detection in Figure 5A). The N458T mutant showed moderate enhancement, and little effect was observed with I435L. The results from the double and triple mutants seem to indicate that the effect of the single-amino-acid replacements on SHP-1 recruitment was additive (Figure 5A). Indeed, the recruitment of SHP-1 by one of the double mutants (P439S, N458T) or the triple mutant (I435L, P439S, N458T) was similar to that of WT Siglec-9. The effect of these replacements on SHP-2 recruitment is comparable to that on SHP-1 recruitment (Figure 5B). In light of these combined data, we conclude that Pro439 in the Y+2 position of the proximal motif and Asn458 in the Y−2 position of the distal motif of Siglec-7 attenuate its ability to recruit phosphatases.

Fig. 3. Recruitment of Src homology-2 (SH2) domain-containing protein-tyrosine phosphatase-1 (SHP-1) and SHP-2 by phosphorylated Siglec-7 and Siglec-9 in COS cells. Siglec-7 or Siglec-9 (or neither [—]) was co-transfected with either wild-type (WT) or a C455S (CS) mutant phosphatase (SHP-1 for panel A and SHP-2 for panel B) or neither (—). The cells were incubated in the presence or absence of pervanadate (PV). Siglec-7 (or Siglec-9) in cell lysates was immunoprecipitated (IP) with anti-Siglec-7 polyclonal antibody (Ab) (or anti-Siglec-9 monoclonal antibody [mAb]). The antibody used for detection (i.e., a specific antibody against phosphotyrosine [αP-Tyr], Siglec-7 [αSig.7], SHP-1 [αSHP-1], or SHP-2 [αSHP-2]) is indicated to the left of each panel. The position of immunoglobulin heavy chain is marked with an asterisk.

Fig. 4. Schematic diagram of wild-type (WT) Siglec-7 and Siglec-9 and Siglec-7 mutants. Arrows indicate the positions of residues 435, 439, and 458, where the amino acids in the proximal and distal motifs of Siglec-7 differ from those in Siglec-9. Arrowheads indicate the “Siglec-9-type” amino acids that were substituted for those of Siglec-7. Bold figures indicate amino acid numbers of Siglec-7 and outlined figures indicate those of Siglec-9.

Fig. 5. A: Recruitments of SHP-1 in COS cells. Siglec-7 or Siglec-9 (or neither [—]) was co-transfected with WT or a C455S (CS) mutant phosphatase (SHP-1). The cells were incubated in the presence or absence of pervanadate (PV). Siglec-7 (or Siglec-9) in cell lysates was immunoprecipitated (IP) with anti-Siglec-7 polyclonal antibody (Ab) (or anti-Siglec-9 monoclonal antibody [mAb]). The antibody used for detection (i.e., a specific antibody against phosphotyrosine [αP-Tyr], Siglec-7 [αSig.7], SHP-1 [αSHP-1], or SHP-2 [αSHP-2]) is indicated to the left of each panel. The position of immunoglobulin heavy chain is marked with an asterisk.

Discussion

This work aimed at elucidating the molecular bases that permit Siglec-7 and Siglec-9 to recruit SHP-1 and SHP-2.

Inefficient recruitment of phosphatases affects inhibition of Syk phosphorylation

Phosphatases recruited by inhibitory receptors dephosphorylate several phosphorylated substrates to block activating signals. We monitored the phosphorylation levels of a substrate protein, Syk, in U937 transfectants; Syk is a kinase crucial for triggering subsequent activation processes. When endogenous FcγRI on U937-WT7 cells was cross-linked with anti-FcγRI antibody, Syk was transiently phosphorylated (Figure 6A). Co-ligation of overexpressed Siglec-7 with FcγRI moderately attenuated the phosphorylation of Syk. The attenuation after co-ligation of WT Siglec-9 with FcγRI was significantly greater than that of Siglec-7 (Figure 6A, B, and E). The difference in the attenuation levels appeared to be attributed to differences in their abilities to recruit SHP-1 and SHP-2. This notion was supported by the results from the triple mutant (U937-I435L, P439S, N458T), in which phosphorylation of Syk was attenuated close to that of WT Siglec-9 (Figure 6D and E). Note that the expression level of the triple mutant (MFI = 378) is lower than WT7 (MFI = 474) (Figure 2A and 6C). These results indicate that these amino acids affect not only the recruitment of phosphatases but also the subsequent attenuation of Syk phosphorylation.
likely, this effect subsequently attenuate the interaction of phosphatases with the signaling motif of Siglec-7. Alternatively, the hydrophobicity of proline can be unfavorable for the interaction. Bruhns et al. clearly demonstrated that the presence of leucine at the Y+2 position enhanced the recruitment of inositol phosphatases by FcγRIIB, which contains a single ITIM (ITYSL/L) (Bruhns et al., 2000). In contrast, recruitment of SHP-1 and SHP-2 by FcγRIIB was inefficient, although the contribution of the Y+2 leucine to the inefficiency was not fully examined in vivo. Whether recruitment of SHP-1 and SHP-2 is attenuated by the presence of a hydrophobic amino acid at the Y+2 position in the ITIMs of Siglec-7 and other inhibitory receptors remains to be investigated.

The involvement of the Y–2 amino acid in inhibitory signaling is consistent with the observation that replacement of valine with alanine at the Y–2 position in the ITIM of KIR abrogated the recruitment of SHP-1 and SHP-2 in vivo (Burshtyn et al., 1999) and in vitro (Burshtyn et al., 1997; Vely et al., 1997). In our substitution experiment, the replacement of asparagine with threonine in the Y–2 position in the distal motif of Siglec-7 enhanced the recruitment of the phosphatases. Several Siglec family proteins other than Siglec-7 contain threonine at the Y–2 position of distal motifs (Figure 7). The presence of a hydroxyl amino acid at the Y–2 position can be favorable for recruitment of phosphatases.

We further demonstrated that the inefficient recruitment of the phosphatases to Siglec-7 also affected the downstream signaling. Cross-linking of FcγRI on U937 cells induced phosphorylation of Syk, which is an upstream signaling molecule for the cellular activation including calcium mobilization. Though co-ligation of Siglec-7 with FcγRII inhibited the phosphorylation of Syk in U937-WT7 cells, the inhibitory activity of Siglec-7 was significantly weaker than that of Siglec-9. The amounts of phosphatase recruited by these Siglecs appeared to correlate with their inhibition potencies. Indeed, introduction of the triple mutation (I435L, P439S, N458T) into the Siglec-7 sequence enhanced phosphatase recruitment and increased the inhibitory activity of the mutant close to the level of Siglec-9. These results suggest that the inhibitory potency of Siglec-7 is weaker than other inhibitory receptors for the SHP recruitment and the following cellular activation. It is hard to say how much the weak recruitment of the phosphatases affect the inhibitory function of Siglec-7 in NK cells and monocytes, but the weak recruitment may partly account for the previous observation by Vitale et al.; that is, Siglec-7 (p75/AIRM1) as well as CD33 had potential to inhibit proliferation of leukemic cells, but, unlike CD33, Siglec-7 did not induce apoptosis (Vitale et al., 2001).

Major histocompatibility complex (MHC) class I molecules on cells are recognized by inhibitory receptors like KIRs or CD94/NKG2 (Lanier, 1998). Recognition by these receptors plays a key role in the discrimination between self and nonself. Aberrant cells that fail to express MHC class I molecules are susceptible to NK cell-dependent killing (missing self-recognition) (Ljunggren and Karre, 1990). MHC-recognizing inhibitory receptors likely recruit the phosphatases much more efficiently than does Siglec-7,
to the extent that when MHC inhibitory receptors are functional, Siglec-7 would contribute minimally to inhibitory signaling. Nevertheless, some tumor cells (e.g., melanoma cells) that occasionally express low levels of MHC molecules still survive and form clinically apparent tumors, suggesting that these tumor cells have some mechanism for escaping NK cell-dependent killing. It is tempting to speculate that Siglec-7-dependent inhibition is involved in the MHC-independent mechanism. When GD3 ganglioside, a preferred ligand for Siglec-7, was overexpressed on the target P815 cells, they acquired resistance to cytotoxicity by NK cells in a Siglec-7-dependent manner though sialidase treatment was required to remove sialic acids on NK cells (Nicolle et al., 2003). In addition, melanoma cells often express GD3 ganglioside on the cell surface and secrete the ganglioside (Pukel et al., 1982). The membrane-bound and secreted ligand may transduce a Siglec-7-dependent inhibitory signal to NK cells for tumor survival in vivo. Modulation of
Siglec-7 signaling may lead to a new therapeutic means against tumor cells.

Materials and methods

Antibodies and cytokines

The antibodies used in this study were as follows: mouse anti-CD16 monoclonal antibody (mAb), mouse anti-LAIR-1 mAb, phycoerythrin (PE)-conjugated anti-CD56 mAb, and control rat IgG2a from Becton Dickinson (Mountain View, CA); mouse anti-FcγRI (CD64, clone 10.1) mAb, mouse anti-Syk mAb, and polyclonal rabbit anti-SHP-2 from Santa Cruz Biotechnology (Santa Cruz, CA); polyclonal rabbit anti-human SHP-1 from Upstate Biotechnology (Lake Placid, NY); mouse anti-SHIP mAb and horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine (4G10) mAb from Sigma (St. Louis, MO); control rat F(ab')2 fragment, sheep anti-mouse IgG F(ab')2 fragment, and rabbit anti-rat IgG (Fc specific) from Jackson Immuno Research (West Grove, PA); control mouse IgG1 from Zymed Laboratories (San Francisco, CA); fluorescein isothiocyanate (FITC)-conjugated anti-rat IgG and control rat IgG2b from Southern Biotechnology Associates (Birmingham, AL); FITC-conjugated anti-mouse IgG from Serotec (Kidlington, UK); and HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG from Amersham Pharmacia (Arlington Heights, IL). Polyclonal rabbit and monoclonal rat anti-Siglec-7 antibodies were prepared as described previously (Miyazaki et al., 2004). The polyclonal anti-Siglec-7 antibody weakly cross-reacted with Siglec-9. Monoclonal anti-Siglec-9 was prepared also by the similar method as that for monoclonal anti-Siglec-7. In this study, we used two anti-Siglec-7 antibodies, 2-7-E (rat IgG2a) and 13-3-D (rat IgG2b), and two anti-Siglec-9 antibodies, 5-8-C (rat IgG2a) and 1-3-A (rat IgG2b). Human recombinant interleukin 2 (IL-2) was a generous gift from Dr. Yasunori Kozutsumi (Kyoto University, Kyoto, Japan). Human interferon-γ (IFN-γ) was purchased from Sigma.

Preparation of F(ab')2 fragments

Anti-Siglec-7 (13-3-D) or anti-Siglec-9 (1-3-D) was incubated with pepsin beads (Pierce, Rockford, IL) in 20 mM sodium acetate (pH 4.5) for 4–6 h. F(ab')2 fragment in the supernatant was separated from undigested antibody and Fc fragment by using anti-rat IgG (Fc specific) antibody-immobilized agarose beads. The beads were prepared by using the Seize Protein A Immunoprecipitation Kit (Pierce) according to the manufacturer’s protocol.

Construction of plasmids

Primers we used are shown in Table I. cDNA fragments of human Siglec-7 and Siglec-9 were amplified by polymerase chain reaction (PCR) using pcDNA3.1-Siglec-7-myc and pcDNA3.1-Siglec-9-myc (Yamaji et al., 2002), respectively, as templates and s.XhoI-ATG and 9 a.s.stop-NheI (Siglec-7) and s.XhoI-ATG and 7 a.s.stop-NheI (Siglec-9) as primers. Each PCR product was digested with XhoI and NheI and then ligated into the XhoI–NheI site of a modified pCXN2 (Niwa et al., 1991), of which a multcloning site was inserted into the EcoRI site. Several Siglec-7 mutants were prepared by using the primers shown in Table I through the same method as that described previously (Yamaji et al., 2002).

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<td>s.XhoI-ATG</td>
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<td>7 a.s.stop-NheI</td>
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<td>SHP-1 a.s.STOP-168L</td>
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The initiation and stop codons are underlined, the restriction enzyme sites are italicized, and the mutation sites are bold.
cDNA fragments encoding various phosphatases and their mutants were prepared as follows. Fragments of human SHP-1 and SHP-2 were PCR amplified from human spleen cDNA (Human Immune System cDNA, Clontech, Palo Alto, CA) by using the primers SHP-1 s.ATG and SHP-1 a.s.STOP (SHP-1) and SHP-2 s.ATG and SHP-2 a.s.STOP (SHP-2), respectively. Each PCR product was ligated into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) by thymine adenine (TA) cloning. Each construct was digested with XhoI and SacI, and the resulting fragment was ligated into the XhoI-SacI site of the modified pCXN2. SHP-1 C455S and SHP-2 C459S were prepared by using the primers shown in Table I through the same method as above. A cDNA fragment encoding the SH2 domain (amino acids 1 through 168) of human SHIP was PCR amplified from human spleen cDNA by using the primers SHIP-1 s.ATG and SHIP-1 a.s.STOP-168L. Each PCR product was ligated into pGEM-T-Easy (Promega, Madison, WI) by TA cloning. Each construct was digested with SpeI, and the resulting overhangs were blunted using Pyrobest polymerase (Takara, Ohtsu, Japan). The product was further digested with NotI, and the generated fragment was ligated into the EcoRV–NotI site of the modified pCXN2.

Purification of NK cells
Human peripheral blood mononuclear cells (PBMC) were prepared from a healthy volunteer by using Ficoll-Paque Plus (Amersham Pharmacia). The PBMC were cultured in RPMI1640 containing 10% heat-inactivated fetal calf serum (FCS) and 1000 U/mL human IL-2. NK cells were prepared from the PBMC by using the NK Cell Isolation Kit for an magnetic activated cell sorting (MACS) system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instruction.

Flow cytometric analysis
Cells were incubated with the appropriate primary antibody or an isotype-matched control Ab; then they were incubated with FITC-conjugated secondary antibody or F(ab')2 fragment. PE-conjugated anti-CD56 mAb was used for NK cell staining. The stained cells were analyzed with an EPICS XL cytometer by using Expo32 software (Beckman Coulter, Hialeah, FL).

Transfectants
COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated FCS. The construct carrying the cDNA of Siglec-7 or Siglec-9 was co-transfected with that carrying phosphatase cDNA into COS-1 cells by using Fugene 6 (Roche, Mannheim, Germany) according to manufacturer’s instruction.

U937 transfectants, on which Siglec-7, Siglec-9, and Siglec-7 mutant were stably overexpressed, were prepared through the same method as described previously (Miyazaki et al., 2004).

Calcium mobilization
The IFN-γ-treated U937 transfectant cells (1 × 10^7) or human NK cells (3.5 × 10^7) were incubated at 35°C for 30 min with 3 μM Fura-2 AM (Dojindo, Kumamoto, Japan) in RPMI1640 containing 10% FCS. The cells were washed three times with RPMI1640 containing 10 mM Hepes (pH 7.4) and 0.1% bovine serum albumin (BSA). The cells were then incubated on ice for 30 min with 10 μg/mL antibody against an activating receptor together with 10 μg/mL anti-Siglec-7 or control F(ab')2 fragment. After being washed twice in a buffer comprising 25 mM Hepes (pH 7.4), 140 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl_2, 0.49 mM MgCl_2, 12 mM NaHCO_3, 0.37 mM Na_2HPO_4, 5.6 mM glucose, and 0.1% BSA, the cells were resuspended in 2 mL of the same buffer. The cells were kept at 35°C for 5 min and then added to a cuvette of a spectrofluorometer (FP-6500, Jasco Corporation, Hachioji, Japan) that was equipped with a thermostatic holder for keeping a cuvette at 35°C. We monitored the signal ratio of emission at 500 nm upon excitation at 340 nm versus that at 380 nm, every 2 s. The activation signal was induced by cross-linking receptors with 10 μg/mL sheep anti-mouse IgG F(ab')2 fragment, which also cross-reacted with rat IgG.

Stimulation with sodium PV
NK cells and U937 transfectants were washed twice with RPMI1640 containing 20 mM Hepes (pH 7.4). COS cells were washed twice with Dulbecco’s modified Eagle’s medium (DMEM) containing 20 mM Hepes (pH 7.4). The cells (1 × 10^7 cells/mL) were incubated at 37°C for 15 min in the same buffer containing 1 mM sodium PV. After washing with cold phosphate-buffered saline (PBS) containing 1 mM Na_3VO_4, the cells (1 × 10^7 cells/mL) were lysed on ice in a lysis buffer comprising 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, and 5 mM ethylenediaminetetraacetic acid (EDTA) with protease inhibitor cocktail (Roche) and phosphatase inhibitor II (Sigma).

Cross-linking of receptors on cell surface
IFN-γ-treated U937 transfectants (2 × 10^7 cells) were resuspended in RPMI1640 containing 20 mM Hepes (pH 7.4) and 0.1% BSA. The cells were incubated on ice for 30 min with 10 μg/mL anti-FcγRI mAb together with 10 μg/mL anti-Siglec-7, anti-Siglec-9, or control F(ab')2 fragment. The cells were washed twice and then resuspended in the medium. An aliquot of the cell suspension (2 × 10^6 cells/125 μL) was kept at 37°C for 5 min and then incubated with 10 μg/mL of sheep anti-mouse IgG F(ab')2 fragment that cross-reacted with rat IgG. After washing with cold PBS containing 1 mM Na_3VO_4, the cells were treated with the lysis buffer.

Immunoprecipitation and western blotting
The cell lysates prepared as described in the above paragraphs were centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant was incubated with an appropriate antibody followed by the incubation with protein G Sepharose. The beads were resuspended in sodium dodecyl sulfate (SDS) sample buffer containing 5% 2-mercaptoethanol. The resulting immunoprecipitates or whole-cell lysates underwent SDSPolyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to a nitrocellulose membrane. The membrane was incubated with HRP-conjugated anti-phosphotyrosine mAb or an appropriate primary antibody followed by HRP-conjugated anti-rabbit IgG. Antigen signals were detected by the use of Supersignal West Dura (Pierce) and a LAS-1000 luminescent image analyzer (Fujifilm,


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