The myeloid-specific Sialic Acid-binding Receptor, CD33, Associates with the Protein-tyrosine Phosphatases, SHP-1 and SHP-2*

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The myeloid restricted membrane glycoprotein, CD33, is a member of the recently characterized “sialic acid-binding immunoglobulin-related lectin” family. Although CD33 can mediate sialic acid-dependent cell interactions as a recombinant protein, its function in myeloid cells has yet to be determined. Since CD33 contains two potential immunoreceptor tyrosine-based inhibition motifs in its cytoplasmic tail, we investigated whether it might act as a signaling receptor in myeloid cells. Tyrosine phosphorylation of CD33 in myeloid cell lines was stimulated by cell surface cross-linking or by pervanadate, and inhibited by PP2, a specific inhibitor of Src family tyrosine kinases. Phosphorylated CD33 recruited both the protein-tyrosine phosphatases, SHP-1 and SHP-2. CD33 was dephosphorylated in vitro by the co-immunoprecipitated tyrosine phosphatases, suggesting that it might also be an in vivo substrate. The first CD33 phosphotyrosine motif is dominant in CD33-SHP-1/SHP-2 interactions, since mutating tyrosine 340 in a CD33-cytoplasmic tail fusion protein significantly reduced binding to SHP-1 and SHP-2 in THP-1 lysates, while mutation of tyrosine 358 had no effect. Furthermore, the NH2-terminal Src homology 2 domain of SHP-1 and SHP-2, believed to be essential for phosphatase activation, selectively bound a CD33 phosphopeptide containing tyrosine 340 but not one containing tyrosine 358. Finally, mutation of tyrosine 340 increased red blood cell binding by CD33 expressed in COS cells. Hence, CD33 signaling through selective recruitment of SHP-1/SHP-2 may modulate its ligand(s) binding activity.

Over the last few years, a novel family of sialic acid-dependent recognition molecules has emerged. This family, recently designated “siglecs” (sialic acid-binding Ig-related lectin), is a structurally related subgroup of the immunoglobulin superfamily that includes CD22 (siglec-2), sialoadhesin (siglec-1), MAG (siglec-4), CD33 (siglec-3), and the newest member of the family, siglec-5 (1). All siglecs have an NH2-terminal V-set Ig-like domain that contains the sialic acid binding site, followed by varying numbers of C2-set domains. In addition to common structural features that would appear to adapt these molecules for functional protein-carbohydrate cellular interactions, each member exhibits a very specific pattern of tissue distribution. While CD22 is restricted to B cells, sialoadhesin to macrophages, and myelin-associated glycoprotein (MAG) to myelinating oligodendrocytes and Schwann cells, CD33 and siglec 5 are expressed only on cells of the myelomonocytic lineage.

CD22 is perhaps the best characterized member of the siglec family. In addition to being an adhesion receptor for sialic-acid bearing ligands on leukocytes and erythrocytes, CD22 has an important regulatory role as a signal transduction molecule in B cells (2, 3). The cytoplasmic tail of CD22 has six tyrosines, two of which are encompassed within sequences which conform with immunoreceptor tyrosine-based activation motifs (ITAM), while the other four form potential immunoreceptor tyrosine-based inhibition motifs (ITIM). ITAMs and ITIMs are the consensus sequences determined to be necessary for Src homology 2 (SH2) domain binding to phosphotyrosine. Phosphorylation of the ITAM consensus sequence, YXX(L/V)XX(Y/L), when phosphorylated, enables association of SH2 domain-containing phosphatases, SHP-1, SHP-2, or SHIP (5, 6). Phosphorylation of the ITIM sequences of CD22 and subsequent recruitment of SHP-1 results in inhibition of BCR-mediated B cell activation, which then modulates the antigen receptor threshold (2, 3). Hence, CD22 is a member of an expanding superfamily of ITIM-bearing negative co-receptors that also includes the killer-cell inhibitory receptors (KIRs) and FcγRIIB (6–8).

CD33, the smallest member of the siglec family, is a 67-kDa transmembrane glycoprotein with only one V-set and one C2-set Ig-like domains (9). CD33 is expressed by the earliest myeloid progenitors and continues to be present during myelomonocytic differentiation until it is down-regulated on granulocytes, although retained on monocytes. This specific expression pattern has meant that monoclonal antibodies (mAbs) directed against CD33 are extremely useful clinical reagents. These mAbs are extensively used both in the immunodiagnosis of leukemias and for therapeutic targeting and purging in acute myeloid leukemia (10–14). However, despite the clinical importance of CD33, little is known of its role in myeloid cells, except that it may be involved in sialic acid-dependent cell interactions. Determining the function of CD33

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¶ The abbreviations used are: ITIM, immunotyrosine-based inhibition motif; ITAM, immunotyrosine-based activation motif; Ig, immunoglobulin; SH2, Src homology 2; N-SH2, NH2-terminal SH2 domain; C-SH2, COOH-terminal SH2 domain; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; BCR, B cell receptor; NK, natural killer, KIR, killer inhibitory receptor; MOPS, 4-morpholinepropanesulfonic acid.
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has become of even greater relevance, since the recent identification of novel molecules sharing high sequence homology to CD33 indicates the existence of a distinct subfamily of CD33-like molecules (15, 16).

Interestingly, the cytoplasmic tail of CD33 contains two tyrosine-based motifs (LXY\textsuperscript{340}XXL and TXY\textsuperscript{358}XXV), both potential ITIMs, although only the first matches the consensus sequence exactly. This raised the possibility that, similar to CD22 in B cells, CD33 might act as an inhibitory receptor in myeloid cell signaling.

We report here that CD33 becomes tyrosine-phosphorylated in myeloid cells after both pervanadate treatment and CD33 receptor cross-linking, and these stimuli result in recruitment of the tyrosine phosphatases SHP-1 and SHP-2. Furthermore, we show that the first cytoplasmic tyrosine residue of CD33 is dominant in SHP-1/SHP-2 binding and that mutation of this same tyrosine enhances CD33-mediated adhesion.

EXPERIMENTAL PROCEDURES

Materials—SeeBlue-pretained SDS-PAGE markers were purchased from Novex. The Hybond-P membrane, enhanced chemiluminescence Western blot detection kit, and Biotek GST Purification Module were obtained from Amersham Pharmacia Biotech. Purified human recombinant c-Src kinase came from Upstate Biotechnology, Inc. Tyrosine kinase inhibitors specific to the Src and Syk family kinases, PP2 and PP4, were obtained from Calbiochem. All other chemicals were obtained from Sigma.

Antibodies—The murine anti-CD33 monoclonal antibodies 1C7/1 and 3D6/1 were produced by the Imperial Cancer Research Fund (London, United Kingdom) using CD33-Fc recombinant protein as the immunogen. The mouse IgG1 control was purchased from Becton Dickinson. Goat anti-mouse IgG came from Sigma. Monoclonal anti-phosphotyrosine antibody, PY20, and monoclonal anti-SHP-1 were obtained from Transduction Laboratories. A polyclonal rabbit anti-SHP-2 (C-18) antibody was from Sigma. Rabbit anti-goat horseradish peroxidase-conjugated secondary antibodies were obtained from Dako. Rabbit anti-goat horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. Polyclonal anti-SHP-1 and SHP-2 (N-16) were obtained from Upstate Biotechnology, and GST-Y340/358A, and GST-N-SH2-SHP1, GST-C-SH2-SHP1, and GST-N-SH2-C-SH2-SHP1, were used to transform Escherichia coli BL21 cells. CD33 fusion proteins were produced by inducing log-phase 500-ml cultures with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside, and purified using the Btik GST Purification Module following the manufacturer’s instructions.

Tyrosine Phosphorylation of CD33-Fusion Construct—100 µg of GST or GST-CD33 fusion protein were incubated with 15 units of c-Src kinase in 500 µl of tyrosine kinase assay buffer (50 mM Hepes, pH 7.4, 50 mM NaCl, 0.1 mM Na\textsubscript{2}VO\textsubscript{4}, 5 mM MgCl\textsubscript{2}, 5 mM MnCl\textsubscript{2}, 5 mM ATP) at room temperature for 2 h. Phosphorylated proteins were stored at -20°C.

Immunoprecipitations with GST-CD33 Cytoplasmic Tail Constructs—10 µg of GST fusion protein ± tyrosine phosphorylation were incubated with 1 ml of cell lysate (Triton-soluble fraction of 10\(^6\) unstimulated cells) at 4°C overnight. The GST fusion protein complexes were captured with 60 µl of 50% slurry of glutathione-Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4°C. The beads were washed three times with ice-cold lysis buffer, and bound proteins were eluted by boiling in SDS reducing sample buffer and resolved by 10% SDS-PAGE and immunoblotting.

Peptide Precipitation Analysis—Biotinylated CD33 phosphopeptides, CD33(336–345) (pY340) and CD33(353–363) (pY358), were produced by Alta Bioscience (Birmingham University, UK). pY340 is biotin-DEEL-XpY, and pY358 is biotin-DTSTEpYSEVRT, where pY is phosphotyrosine.

10 µg of peptide was incubated with 5 µg of GST alone, GST-N-SH2-C-SH2-SHP1, GST-N-SH2-SHP1, GST-C-SH2-SHP1, GST-N-SH2-C-SH2-SHP2, GST-N-SH2-SHP-2, or GST-C-SH2-SHP-2 in 1 ml of ice-cold Triton lysis buffer overnight at 4°C. The biotinylated peptides were captured by addition of 40 µl of UltraLink-Immobilon NeutrAvidin Plus beads (Pierce) for 1 h at 4°C. The beads were washed four times with lysis buffer, and bound proteins were eluted by boiling in SDS reducing sample buffer and resolved by 10% SDS-PAGE and immunoblotting.

Preparation of CD33 cDNA Constructs for Transfection of COS Cells—Site-directed mutagenesis by two-step polymerase chain reaction was used to generate full-length CD33 mutants: CD33R103A, CD33Y340A, CD33Y358A, and double mutant CD33Y340A/CD33Y358A. CD33 arginine 103-to-alanine mutation was introduced using the primer N-SH2-C-SH2-SHP2 (5’-GAGCTGCATGCGCCTCCTCATCCTACATCA TCACTACGTTGCTGCTGCTGCTGCTGAGGGC-3’), and RlH105A (5’-CCCTCGCGAAGAATGTAAGGCT-3’). Single and double tyrosine point mutations were introduced into CD33 wild type cDNA using primers tY340A, tY3540A, and tY358A described above. Wild type CD33 with the last 28 amino acids truncated (CD33a336–364) was generated by introducing a stop codon (primer r336–364: 5’-TCACTCATCATCATCCACAGT-3’) by polymerase chain reaction. All constructs including wild type CD33 were subcloned into...
expression vector pcDNA3 (Invitrogen). The sequences of all DNA constructs were verified by DNA sequence analysis.

Red Blood Cell Binding Assay—COS-1 cells were transiently transfected by electroporation with the different CD33 constructs and replated 24 h later at 2 × 10⁵ cells/well in six-well tissue culture plates (Falcon) in Dulbecco’s modified Eagle’s medium containing 0.5% fetal calf serum. Occasionally cells were incubated with 2 mM sodium butyrate overnight prior to the assays to enhance expression. Transfection efficiency was checked by flow cytometry prior to all assays. Binding assays with human red blood cells were performed 48–72 h after transfection as described previously (17) with sialidase pretreatment of COS cells. To quantify binding, the percentage of COS cell rosettes (defined as COS cell binding more than 20 red blood cells) was scored from counting at least 200 COS cells.

RESULTS

CD33 Is Tyrosine-phosphorylated and Recruits Both SHP-1 and SHP-2 after Pervanadate Treatment—Pervanadate, a protein-tyrosine phosphatase inhibitor, has previously been shown to induce tyrosine phosphorylation of proteins involved in proximal signal transduction pathways. We used pervanadate to determine whether CD33 is a target for tyrosine kinases and therefore could function as a tyrosine phosphorylated signaling molecule in myeloid cells. Pervanadate stimulated tyrosine phosphorylation of CD33 in various leukemic myeloid cell lines, including U937 (promonocytic), THP-1 (monocytic) (Fig. 1), and the more immature KG1 (CD34±, CD34−) and HL60 (myelomonocytic) cells (data not shown).

An additional tyrosine-phosphorylated protein, running at approximately 65 kDa, was co-immunoprecipitated with CD33. The two tyrosines in the cytoplasmic tail of CD33 form potential ITIM motifs, allowing the possible recruitment of the SH2 domain-containing cytoplasmic phosphatases SHP-1, SHP-2, or SHIP. The phosphoprotein co-immunoprecipitated with CD33 was too small for the inositol phosphatase SHIP (145 kDa) but could be SHP-1 (68 kDa) or the closely related SHP-2 (72 kDa). To determine whether CD33 indeed associates with either of these tyrosine phosphatases, CD33 immunoprecipitates from lysates of pervanadate-treated U937 and THP-1 cells were analyzed by immunoblotting (Fig. 2). In both myeloid cell lines, SHP-1 and SHP-2 were co-immunoprecipitated with tyrosyl-phosphorylated CD33.

Cell Surface Cross-linking of CD33 Also Results in Its Tyrosine Phosphorylation and Recruitment of Both SHP-1 and SHP-2—Having established that pervanadate stimulation results in tyrosine phosphorylation of CD33 and its specific interaction with SHP-1 and SHP-2, we next examined the effect of cross-linking CD33 on THP-1 cells. This induced rapid and transient tyrosine phosphorylation of CD33, apparent by 1 min (Fig. 3A) and decreasing to basal levels within 10 min (data not shown). As with pervanadate treatment, cross-linking resulted in recruitment of both SHP-1 and SHP-2 by the phosphorylated CD33.

CD22 (Siglec-2) acts as an inhibitory receptor in B cell signaling via its recruitment of SHP-1 (2, 3). From the above results, CD33 might have a similar role in myeloid cells. CD22 becomes tyrosine-phosphorylated and associates with SHP-1 following BCR engagement. In myeloid cells, aggregation of Fc receptors for IgG (FcγR) activates signal transduction pathways through tyrosine phosphorylation of various cellular proteins (6, 7). This results in the triggering of multiple myeloid effector functions. Since CD33 might be a potential target for tyrosine kinase activity stimulated by FcγRIIA (CD32) on THP-1 cells to determine whether this gave rise to CD33 association with CD32 and/or CD33 tyrosine phosphorylation (Fig. 3B). Although CD32 itself became tyrosine-phosphorylated along with a number of other proteins, CD33 immunoprecipitated from these cell lysates was not phosphorylated and neither CD33 nor SHP-1/SHP-2 were detected in the CD32 complex. Similarly, cross-linking FcγRI had no effect (data not shown).

Other myeloid cell stimuli, including cytokines (CSF-1, GM-CSF), chemotactic peptide (fMLP), or phorbol esters, also failed to induce CD33 tyrosine phosphorylation. Therefore, in view of the results from antibody cross-linking, physiological activation of CD33 signaling is most likely brought about by aggregation of CD33 by as yet unidentified ligand(s).

CD33 Is Associated with Protein-tyrosine Phosphatase Activity—Having shown that cross-linking CD33 results in association of SHP-1 and SHP-2 with tyrosine-phosphorylated CD33, we wanted to determine whether these recruited phosphatases were active and, if so, whether CD33 itself might be a target substrate. Proteins co-immunoprecipitated with CD33 from lysates of cells stimulated by CD33 cross-linking were incubated at 37 °C in phosphatase assay buffer. CD33 tyrosine phospho-
CD33 Tyrosine Phosphorylation Is Inhibited by a Src Family Kinase-specific Inhibitor—Src family kinases are involved in many leukocyte signal transduction pathways and therefore might be responsible for CD33 tyrosine phosphorylation. To test this, we examined whether the Src family-specific tyrosine kinase inhibitor PP2 (18) could inhibit phosphorylation of CD33 induced by cross-linking (Fig. 5). Genistein, a broad range tyrosine kinase inhibitor, and piceatannol, the Syk family-specific tyrosine kinase inhibitor, were used in comparison. Tyrosine phosphorylation of CD33 was significantly reduced in the presence of PP2 at 5 μM, and virtually abolished with PP2 at 50 μM, unlike piceatannol at the same concentration. Genistein also reduced CD33 phosphorylation, but was much less potent than PP2. The specific inhibition of CD33 phosphorylation by PP2 suggests that the Src family kinases are strong candidates for mediating tyrosine phosphorylation of CD33.

The First Tyrosine Phosphorylation Site of CD33 Is Dominant in Binding to SHP-1 and SHP-2—to determine which tyrosine phosphorylation binding site(s) on CD33 are required for SHP-1 and SHP-2 association, GST-CD33 cytoplasmic tail fusion proteins were made with one or both of the tyrosines mutated (CD33Y340A, CD33Y358A, CD33Y340A/Y358A) and phosphorylated with Src kinase. As expected, with the loss of both tyrosines, CD33 failed to bind SHP-1 and SHP-2 from THP-1 lysates. Interestingly, mutation of the first tyrosine (Y340A) significantly reduced the amounts of both SHP-1 and SHP-2 bound while mutation of the second tyrosine (Y358A) had no effect, with levels of SHP-1 and SHP-2 binding comparable to wild-type (Fig. 6). These results are in agreement with what would be predicted from the sequences of the two phosphotyrosine motifs. The first motif (LXYXXLXX) conforms exactly with the ITIM consensus sequence, (I/V/LX)(Y/F)(X)X, and therefore would be expected to constitute a higher affinity binding site for SHP-1/SHP-2 than the second motif (TXYXXLXX), which only partially fulfills the consensus criteria.

Differential Binding of CD33 Phosphopeptides to the Individual SH2 Domains of SHP-1 and SHP-2—in order to dissect the binding orientation of the SH2 domains of SHP-1 and SHP-2 to...
the phosphorylated tyrosines of CD33, recombinant GST fusion proteins expressing individual SH2 domains of SHP-1 and SHP-2 were prepared and incubated with biotinylated phosphopeptides corresponding to CD33(335–345) (pY340) and CD33(353–363) (pY358).

While both peptides associated with GST-N-SH2-C-SH2-SHP-1, and GST-C-SH2-SHP-1, the GST protein containing the single NH₂-terminal SH2 domain of SHP-1 bound only to the CD33 peptide encompassing Tyr-340 (Fig. 7A). Thus, the N-SH2 domain of SHP-1 displays much greater binding specificity than the C-SH2 domain.

In the case of SHP-2, only the CD33 peptide encompassing Tyr-340 bound significantly to the GST protein containing the single NH₂-terminal SH2 domain of SHP-2, as well as to the tandem SH2 domain-containing protein (Fig. 7B). Surprisingly, both peptides bound very weakly, if at all, to the COOH-terminal SH2 domain of SHP-2. Since removal of Tyr-340 from the CD33 tail did not entirely abolish its association with SHP-2 in cells (Fig. 6), this suggests that there is a weak but significant interaction of Tyr-358 with SHP-2, more readily detected with the cytoplasmic tail constructs than with the simple peptide structures.

Binding the NH₂-terminal SH2 domain of SHP-1 and SHP-2 is believed to be essential for activation of these phosphatases (19). Hence the selective interaction of tyrosine 340 with the N-SH2 domains of both SHP-1 and SHP-2 is likely to be critical for CD33 mediated stimulation of SHP-1 and SHP-2 protein-tyrosine phosphatase activity.

**CD33 Transiently Expressed in COS Cells Is Phosphorylated and Recruits SHP-2**—In order to analyze the effects of mutating the CD33 tyrosine phosphorylation sites in vivo, COS cells transiently expressing either wild type CD33 or constructs with single or double tyrosine point mutations were treated with pervanadate. Pervanadate stimulated strong phosphorylation of CD33 expressed in COS cells (running at approximately 62 kDa, probably due to glycosylation differences) (Fig. 8). Although predictably weaker, phosphorylation of the CD33 constructs containing a single tyrosine, CD33Y340A and CD33Y358A, was also observed. As expected, with loss of both tyrosines, CD33Y340/358A was not phosphorylated. COS cells express substantial amounts of SHP-2 but very little SHP-1. SHP-2 (and tiny amounts of SHP-1) were co-immunoprecipitated with CD33Y358A, as well as with wild type CD33. Loss of Tyr-340 dramatically reduced the recruitment of SHP-2 (and SHP-1) by CD33, reflecting the results with the GST-CD33 constructs (Fig. 6).

**Mutation of CD33 Tyrosine 340 Increases CD33-mediated Red Blood Cell Binding**—CD33, like all other characterized members of the Siglec subgroup of the Ig superfamily, is able to mediate sialic acid-dependent binding of human red blood cells as well as of other specific cell populations (9). We therefore used red blood cell binding to CD33 transiently expressed in COS cells as a convenient model to examine CD33 tyrosine phosphorylation in CD33-mediated sialic acid-dependent cell interactions. Tyrosine phosphorylation of CD33 was not detected following the 20–30-min incubation required for red cell binding to the transfected COS cells. This is presumably due to the rapid and transient kinetics of CD33 phosphorylation.

The importance of the CD33 tyrosine phosphorylation sites in red cell binding was determined by an alternative approach. We analyzed the effects of loss or mutation of Tyr-340 and/or Tyr-358 on adhesion. As a control for sialic acid-dependent binding the arginine residue in the F strand of the NH₂-terminal V-set domain of CD33 was mutated (CD33R103A). This arginine is conserved in all siglecs and has been shown to be essential for sialic acid binding by site directed mutagenesis studies of other siglecs and x-ray crystallography of sialoadhesin (20–22). As expected, wild type CD33 binding of RBC was completely abolished by this mutation. Interestingly, truncation of the last 28 amino acids of the cytoplasmic tail...
48–72 h after transient transfection, 10⁷ COS cells expressing CD33wt, phosphorylated with 1 mM pervanadate for 15 min at 37 °C. CD33Y340A, CD33Y358A, or CD33Y340/358A were either treated with 1 mM pervanadate for 15 min at 37 °C (+) or left unstimulated (−). Lysates were immunoprecipitated (IP) with either mouse IgG1 (mIgG1) or anti-CD33, 1C7/1 (CD33), both antibodies pre-coupled to protein G-Sepharose. Immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by immunoblotting with anti-phosphotyrosine (PY20), anti-CD33, anti-SHP-1 or anti-SHP-2 (C-18). It should be noted that due to the tiny amounts of SHP-1 in COS cells, a extended exposure of the anti-SHP-1 blot is shown (10 min rather than 30 s).

FIG. 8. Tyrosine phosphorylation of CD33 transiently expressed in COS cells, and recruitment of SHP-1 and SHP-2. 48–72 h after transient transfection, 10⁷ COS cells expressing CD33wt, CD33Y340A, CD33Y358A, or CD33Y340/358A were either treated with 1 mM pervanadate for 15 min at 37 °C (+) or left unstimulated (−). Lysates were immunoprecipitated (IP) with either mouse IgG1 (mIgG1) or anti-CD33, 1C7/1 (CD33), both antibodies pre-coupled to protein G-Sepharose. Immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by immunoblotting with anti-phosphotyrosine (PY20), anti-CD33, anti-SHP-1 or anti-SHP-2 (C-18). It should be noted that due to the tiny amounts of SHP-1 in COS cells, a extended exposure of the anti-SHP-1 blot is shown (10 min rather than 30 s).

In order to dissect this further, we examined red blood cell binding to full-length CD33 containing single tyrosine mutations (CD33Y340A and CD33Y358A). Mutation of tyrosine 358 alone resulted in wild type binding levels. However, mutation of tyrosine 340 alone increased binding to the levels observed with CD33Δ336–364 and CD33Y340A/Y358A (Fig. 9). Therefore, the ITIM-like motifs appear to have a negative regulatory role in CD33-mediated sialic acid-dependent cell interactions.

Our previous data suggest that the first cytoplasmic tyrosine (Tyr-340/Tyr-358), increased red blood cell binding to about 150% of the wild type level. A similar result was observed when both tyrosines were mutated in full-length CD33 (CD33Y340A/Y358A) (Fig. 9). Therefore, the ITIM-like motifs appear to have a negative regulatory role in CD33-mediated sialic acid-dependent cell interactions.

Association of CD33 with SHP-1 and SHP-2

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Our previous data suggest that the first cytoplasmic tyrosine (Tyr-340) of CD33 is critical for recruiting SHP-2 and SHP-1 both in vitro and in vivo. These findings show that mutation of this same tyrosine significantly increases CD33-mediated red blood cell adhesion. Therefore, it is tempting to postulate that the observed increase in red blood cell binding results from a reduction in CD33 recruitment of SHP-2 and/or SHP-1.

FIG. 9. Loss of the first CD33 phosphotyrosine motif increases CD33 binding of red blood cells. 48–72 h after transient transfection, human red blood cells were added to sialidase-pretreated COS cells, expressing CD33wt, CD33-R103A, CD33Δ336–364, CD33-Y340A, CD33-Y358A, or CD33-Y340/358A, and allowed to bind for 30 min at 37 °C. After washing, cells were fixed and the percentage of COS cells with red blood cell rosettes (defined as COS cell binding more than 20 red blood cells) was scored by counting at least 200 COS cells/well. All constructs were expressed at equivalent levels as determined by flow cytometry analysis prior to assays (data not shown). Results shown are mean ± S.D. of triplicate wells from one representative experiment of at least three performed.

Pretreatment of wild type CD33 transfected COS cells with the Src family kinase specific inhibitor (PP2), which inhibited phosphorylation of CD33 in THP-1 cells (Fig. 5), resulted in increased red blood cell binding (110–150% of wild type binding levels; data not shown). The inhibitor had no such effect on binding of red blood cells by COS cells expressing CD33 with both tyrosines mutated (CD33Y340/358A). This further suggests that there is a direct link between phosphorylation of CD33 and its ability to bind sialic acid-bearing ligands.

DISCUSSION

Recent rapid progress in the characterization of an increasing number of ITIM–bearing receptors has emphasized their important regulatory role in various signal transduction pathways and subsequent cellular responses. Critical to the function of all these molecules is the recruitment of SH2-containing phosphatases by their phosphorylated ITIMs. This report shows that tyrosine phosphorylation of the myeloid-specific receptor CD33 triggers recruitment of SHP-1 and SHP-2 in vivo and in vitro. We therefore propose that CD33 might be considered as a new member of the superfamily of ITIM-bearing coreceptors, potentially forming with CD22 a distinct sialic acid-binding Ig-related lectin (siglec) subgroup.

Tyrosine phosphorylation of CD33 could be stimulated both by its aggregation on the cell surface and by pervanadate, but not by ligation of the Fcγ receptors CD32 or CD64. Although the effects of other myeloid immune receptors, for example CD89 (IgA receptor), have yet to be tested, these initial results suggest that CD33 is not directly “activated” by immune receptor engagement and therefore unlikely to have an equivalent function to CD22. The latter conclusion is further supported by the finding that CD33 can associate with SHP-1 in combination with SHP-2, while CD22 binds SHP-1 only (2, 3). Recruitment of both SHP-1 and SHP-2 has previously been demonstrated for other ITIM-bearing receptors (7, 23–27); however, in many cases this has been based on in vitro studies, often using ITIM peptides only. In view of the possible discrepancies in vivo and in vitro binding capacities, we used a combination of approaches. We have shown CD33 association with SHP-1 and SHP-2 both in vivo and in vitro.

How CD33 regulates myeloid cell signaling through its association with SHP-1 and SHP-2 has yet to be determined. Many ITIM-bearing molecules act as inhibitory co-receptors; for example, KIRs suppress activation of NK cytotoxic function (8), while both CD22 and FcγRIIB down-modulate BCR-mediated...
signaling (6). However, both SHP-1 and SHP-2 could potentially act as negative or positive regulators depending on the phosphatase target substrates. In addition, as SHP-1 and SHP-2 are themselves tyrosine-phosphorylated when associated with CD33, they might also act as adaptor/docking molecules. Since CD33 is dephosphorylated in vitro by the recruited tyrosine phosphatases, it may also be an in vivo substrate and hence autoregulated by SHP-1 and/or SHP-2, as has been shown for CD72/SHP-1 (28) and SIRPα1/SHP-2 (23).

Although no other signaling intermediates appeared to associate with CD33 (data not shown), phosphorylation of CD33 was specifically inhibited by a Src family tyrosine kinase inhibitor. Neither of the two phosphotyrosine motifs of CD33 conform to the preferred binding sequence of the SH2 domain of the SRC family kinases, and CD33 lacks any proline-rich region for SH3 domain binding. Therefore, it seems unlikely that active SRC-family kinases actually form a stable association with CD33. CD33 may be phosphorylated by activated aggregated kinases in close proximity, or during transient, unstable interactions. Precisely which SRC family members are able to phosphorylate CD33 and how the kinase(s) is itself activated remains to be determined.

Selective recruitment of SHP-1/SHP-2 and SHIP by ITIM-bearing receptors is achieved through the binding site preferences of the component protein modules of these phosphatases and, possibly, by differential cell expression. There are two ITIM motifs in the cytoplasmic tail of CD33 suitable for the binding of the SH2 domains of SHP-1 and SHP-2. The preferred binding motif of the SH2 domains of these two phosphatases is the consensus sequence (I/V/LxYxxVLV). The SH2 domains of SHP-1 and SHP-2 are not notable in their requirement for a hydrophobic residue at the pY-2 position (5, 29). A highly conserved arginine residue at the a2 position of most SH2 domains is replaced with glycine in the SH2 domains of SHP-1 and SHP-2. This forms a gap in the SH2 domain that is filled by the side chain of the pY-2 residue of the target peptide (29). The first tyrosine phosphorylation site of CD33 (LXY\textsuperscript{346}XXL), which conforms exactly to the consensus motif, was predictably dominant in binding to SHP-1 and SHP-2. Furthermore, the NH\textsubscript{2}-terminal SH2 domain of SHP-1 was only able to bind to this first motif, whereas the COOH-terminal SH2 domain was also able to bind the second, less well conserved ITIM motif (TXY\textsuperscript{355}XXV). Therefore, the NH\textsubscript{2}-terminal SH2 domain of SHP-1 exhibits more selective binding than its COOH-terminal SH2 counterpart. This is not unexpected since the engagement of the NH\textsubscript{2}-terminal SH2 domain of SHP-1 by the first ITIM motif of CD33 is required to convert SHP-1 to its catalytically active conformation (19, 30). However, COOH-terminal SH2 domain binding is also likely to be important, as the association of the tandem SH2-domains of SHP-1 with the two phosphotyrosines of CD33, either in \textit{cis} or \textit{trans}, would be expected to confer the greatest specificity and affinity, resulting in optimal signaling (30). In contrast, while the NH\textsubscript{2}-terminal SH2 domain of SHP-2 exhibits the same preference for Tyr-340, the COOH-terminal SH2 domain binds both ITIMs very poorly. If association of tandem SH2 domains is indeed important, SHP-1 should show a higher affinity for CD33 than SHP-2, possibly biasing signaling through CD33 in favor of SHP-1. More detailed examination of the ITIM-SH2 domain binding could help understand how receptors distinguish between SHP-1 and SHP-2.

We have demonstrated that CD33 can recruit SHP-1 and SHP-2 through specific binding to its ITIM motifs and thus could mediate downstream signaling events associated with cell activation. It has previously been shown that CD33, like other members of the siglec family, can mediate sialic acid-dependent binding of specific cell populations as a recombinant protein (9). This suggests that CD33 on myeloid cells participates in cellular interactions, binding specific as yet unidentified sialic acid-bearing ligand(s) either in \textit{cis} on the same cell surface or in \textit{trans} on opposing cells. The adhesion assay using CD33 transfected COS cells and human red blood cells was used as a convenient model for these postulated interactions. Significantly, CD33-mediated cell adhesion was up-regulated by deletion or mutation of the tyrosine (Tyr-340) contained within its first ITIM. Since this ITIM is dominant in SHP-1 and SHP-2 binding both in \textit{vitro} and in \textit{vivo}, the observed increase in red blood cell adhesion could be correlated with a reduction in SHP-1 and/or SHP-2 recruitment. Hence, it is tempting to speculate that the role of CD33 as a sialic acid-dependent cell interaction molecule is regulated inside-out by its capacity to transmit signals through SHP-1 and SHP-2. CD33 phosphorylation could be induced by ligand occupancy and subsequent clustering, as mimicked by antibody cross-linking. The recruited tyrosine phosphatase might then trigger downstream signaling events that limit further CD33 interactions and potentiate other changes in myeloid cell function. An important step in testing this model is the identification of CD33 ligand(s).

Recently, other proteins sharing high sequence homology to CD33 have been identified, indicating the existence of a subgroup of CD33-like molecules within the siglec family. A placent-specific molecule composed of three Ig-like domains (CD33L) shares 70% identity with CD33 (15). Siglec-5, another myeloid-restricted membrane glycoprotein, also exhibits a high degree of sequence homology with CD33. Interestingly, not only does Siglec-5 appear at a later stage of myeloid differentiation than CD33, it is also retained at relatively high levels on neutrophils (17). Like CD33, the cytoplasmic tails of both of these molecules contain two potential phosphotyrosine binding motifs, but with increased spacing between the tyrosines. Studying these receptors would yield important information on the molecular and spatial requirements for selective recruitment of SH2-containing phosphatases. In addition, further insight into the regulatory role of CD33 may be gained since these molecules, although expressed in distinct cell subsets, are likely to have similar signaling functions.

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